Thermodesulfovibrio aggregans sp. nov. and
Thermodesulfovibrio thiophilus sp. nov., anaerobic,
thermophilic, sulfate-reducing bacteria isolated
from thermophilic methanogenic sludge, and
emended description of the genus
Thermodesulfovibrio

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Four obligately anaerobic, thermophilic, sulfate-reducing bacterial strains, designated TGE-P1^T, TDV^T, TGL-LS1 and TSL-P1, were isolated from thermophilic (operated at 55 °C) methanogenic sludges from waste and wastewater treatment. The optimum temperature for growth of all the strains was in the range 55–60 °C. The four strains grew by reduction of sulfate with a limited range of electron donors, such as hydrogen, formate, pyruvate and lactate. In co-culture with the hydrogenotrophic methanogen *Methanothermobacter thermautotrophicus* ΔH^T, strains TGE-P1^T, TGL-LS1 and TSL-P1 were able to utilize lactate syntrophically for growth. The DNA G + C contents of all the strains were in the range 34–35 mol%. The major cellular fatty acids of the strains were iso-C_{17:0}, iso-C_{16:0}, C_{16:0} and anteiso-C_{15:0}. Phylogenetic analyses based on 16S rRNA gene sequences revealed that the strains belong to the *Thermodesulfovibrio* clade of the phylum 'Nitrospirae'. On the basis of their physiological, chemotaxonomic and genetic properties, strains TGL-LS1 (=JCM 13214) and TSL-P1 (=JCM 13215) were classified as strains of *Thermodesulfovibrio islandicus*. Two novel species of the genus *Thermodesulfovibrio* are proposed to accommodate the other two isolates: *Thermodesulfovibrio aggregans* sp. nov. (type strain TGE-P1^T =JCM 13213^T =DSM 17283^T) and *Thermodesulfovibrio thiophilus* sp. nov. (type strain TDV^T =JCM 13216^T =DSM 17215^T). To examine the ecological aspects of *Thermodesulfovibrio*-type cells in the sludge from which the strains were originally isolated, an oligonucleotide probe targeting 16S rRNA of all *Thermodesulfovibrio* species was designed and

Abbreviations: CLSM, confocal laser scanning microscope; FISH, fluorescence *in situ* hybridization; NTA, nitrilotriacetate; UASB, upflow anaerobic sludge blanket.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TGE-P1^T, TDV^T, TGL-LS1 and TSL-P1 and *Thermodesulfovibrio yellowstonii* DSM 11347^T are AB021302, AB231857, AB021303, AB021304 and AB231858, respectively.

Photomicrographs of the strains isolated in this study, graphs showing lactate degradation by strain TGE-P1^T in the presence and absence of *Methanothermobacter thermautotrophicus*, *in situ* hybridization of sections from thermophilic granules viewed by CLSM and probe/target sequences of TDV1015 are available as supplementary material with the online version of this paper.

applied to thin sections of thermophilic sludge granules. Fluorescence *in situ* hybridization using the probe revealed rod- or vibrio-shaped cells as a significant population within the sludge, indicating their important role in the original ecosystem.

The genus *Thermodesulfovibrio* was described as a group of obligately anaerobic, curved rod-shaped, thermophilic bacteria that reduce sulfate and other sulfur compounds (Garrity & Holt, 2001; Henry *et al.*, 1994). The genus belongs to a deeply branched lineage of descent in the domain *Bacteria*, forming the bacterial phylum 'Nitrospirae' (Garrity & Holt, 2001). To date, there are two species of the genus with validly published names: *Thermodesulfovibrio yellowstonii* and *Thermodesulfovibrio islandicus* (Henry *et al.*, 1994; Sonne-Hansen & Ahring, 1999). In addition, '*Thermodesulfovibrio hydrogeniphilus*' has been proposed very recently as a novel species of the genus (Haouari *et al.*, 2008), but the name has not yet been validly published. All three species were isolated from environments associated with freshwater hydrothermal sites; it was suggested that they perform oxidation of hydrogen and other organic matter through the reduction of sulfate in their original ecosystems (Henry *et al.*, 1994).

In addition to oxidation with sulfate reduction, one of the other ecologically important physiological capabilities of some sulfate-reducing organisms is syntrophic degradation of organic compounds in the absence of sulfate (Bryant *et al.*, 1977). Without oxidized forms of sulfur compounds, some known sulfate-reducers can syntrophically degrade organic substances, such as lactate, ethanol and propionate, in close association with hydrogenotrophic methanogens via interspecies hydrogen (electron) transfer (Bryant *et al.*, 1977; Klemms *et al.*, 1985; Plugge *et al.*, 2002). The capability for syntrophic growth of members of the genus *Thermodesulfovibrio* has not been examined previously; hence, it remains unconfirmed. However, during a molecular survey of methanogenic sludge of a thermophilic (operated at 55 °C) upflow anaerobic sludge blanket (UASB) reactor fed with artificial organic wastewater, abundant 16S rRNA gene clones, accounting for 19% of the total prokaryotic clones examined, that were very closely related to members of the genus *Thermodesulfovibrio* were retrieved (Sekiguchi *et al.*, 1998). Because sulfate concentrations in the wastewater were insufficient for this abundance on the basis of dissimilatory sulfate reduction, it was presumed that the detected phylotypes degraded the organic substances either fermentatively or syntrophically in association with methanogens. Therefore, cultivation of such potentially facultatively syntrophic organisms was attempted.

Four strains (TGE-P1^T, TDV^T, TGL-LS1 and TSL-P1) belonging to the genus *Thermodesulfovibrio*, some of which were capable of degrading lactate in co-culture with hydrogenotrophic methanogens without sulfate, were isolated. A brief communication on the isolation of strains TGE-P1^T, TGL-LS1 and TSL-P1 has been published previously (Sekiguchi *et al.*, 2002). In addition to these

strains, strain TDV^T was newly isolated as a non-syntrophic member of the genus in this study. In this paper, detailed morphological, physiological and chemotaxonomic characteristics of the strains are reported and two novel species are proposed. In addition, an oligonucleotide probe specific for the 16S rRNA of members of the genus *Thermodesulfovibrio* was designed and used in the detection of members of the genus by fluorescence *in situ* hybridization (FISH) to determine their spatial distribution and abundance within the original sludge sample.

Strains TGE-P1^T and TGL-LS1 (=JCM 13214) were isolated from methanogenic sludge of a laboratory-scale, thermophilic (55 °C) UASB reactor treating artificial wastewater containing sucrose, fatty acids and peptone (Sekiguchi *et al.*, 1998). Strain TSL-P1 (=JCM 13215) was obtained from thermophilic (55 °C) anaerobic wastewater treatment sludge fed with pulp industry wastewater. Strain TDV^T was obtained from sludge of a full-scale anaerobic digester decomposing sewage sludge under thermophilic (53 °C) conditions (Osaka, Japan). *Thermodesulfovibrio yellowstonii* DSM 11347^T, *Thermodesulfovibrio islandicus* DSM 12570^T, *Thermodesulfovibrio commune* DSM 2178^T, *Thermodesulfovibrio thermophilum* DSM 1276^T, *Clostridium acetobutylicum* DSM 792^T, *Desulfotomaculum thermobenzoicum* subsp. *thermobenzoicum* DSM 6193^T, *Desulfotomaculum thermocisternum* DSM 10259^T, *Desulfovibrio vulgaris* DSM 644^T, *Desulfobulbus propionicus* DSM 6523 (=strain MUD) and *Methanothermobacter thermautotrophicus* DSM 1053^T (=ΔH^T) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). The basal medium used for enrichment, isolation and maintenance of strains TGE-P1^T, TDV^T, TGL-LS1 and TSL-P1 was prepared on the basis of that of Widdel & Pfennig (1981). The medium composition and cultivation have been described previously (Sekiguchi *et al.*, 2000). Primary enrichments were carried out anaerobically at 55 °C. A solid medium was prepared by adding purified agar (agar noble; Difco) to the medium as described above at a final concentration of 20 g l⁻¹. Methanogenic and various anaerobic strains were cultivated at either 37 or 55 °C using the same medium except that their substrates were supplemented in vials in accordance with the instructions of the DSMZ.

To determine the optimum pH for growth of the four isolates, the pH of the medium containing 20 mM lactate and 20 mM sulfate was adjusted to between pH 5.0 and 9.0 by adding HCl or NaOH under a 100% N₂ atmosphere. To evaluate the optimum temperature for growth, the isolates were cultivated anaerobically in lactate plus sulfate medium (pH_{25 °C} 7.0) at 20, 25, 37, 45, 50, 55, 60, 70 and 80 °C. For all cultivations, duplicate cultures (1% inoculum) were incubated and optical density (OD₄₀₀) was measured.

Growth and substrate utilization were examined in accordance with a previous report (Sekiguchi *et al.*, 2000). All cultures were incubated anaerobically at 55 °C, pH₂₅ °C 7.0 for over 4 weeks. Growth and substrate utilization of the strains were determined using potential carbon and energy sources by monitoring OD₄₀₀ increase and acetate production. In syntrophic growth/substrate utilization tests, cells of *Methanothermobacter thermautotrophicus* ΔH^T were added to the medium (2 % inoculum); growth and substrate utilization were examined by measuring OD₄₀₀ and methane production.

Cell morphology was examined under a phase-contrast microscope (Olympus AX80T). Scanning electron microscopy was performed using a Hitachi S4500 as described previously (Sekiguchi *et al.*, 2000; Uemura & Harada, 1993). Gram-staining was performed by Hucker's method (Doetsch, 1981). Phase-contrast micrographs were obtained using wet mounts on agar-coated slides (Pfennig & Wagener, 1986) for exponential phase cultures. Short-chain fatty acids, methane, hydrogen and carbon dioxide were identified by GC (Sekiguchi *et al.*, 2000). Other compounds, such as lactate, were determined by HPLC as described previously (Imachi *et al.*, 2000). For fatty acid methyl ester analysis, fatty acids of cells were converted to methyl esters using HCl/methanol and were determined by GC-MS (Hitachi M7200A FC/3DQMS system) (Hanada *et al.*, 2002). For the analysis, cells were harvested from cultures grown on lactate plus sulfate medium at 55 °C.

DNA was extracted and purified in accordance with the method described previously (Kamagata & Mikami, 1991). DNA G+C content was determined by HPLC (Shimadzu LC-6A) with a UV detector (Shintani *et al.*, 2000). Genetic relatedness was investigated by slot-blot DNA-DNA hybridization with a DIG DNA Labelling and Detection kit (Roche). Target DNA (10–100 ng) denatured with 0.8 M NaOH was slotted onto a nylon membrane (Hybond-N; Amersham Pharmacia Biotech) and the labelled DNA was reassociated in DIG Easy Hyb (Roche). After incubation overnight at 40 °C, membranes were washed with SSC (0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1 % SDS. Hybridization signals were generated by NBT/BCIP solution included in the DIG detection kit (Roche) and measured by scanning colour intensities. Tests were performed at least in triplicate for each assay and self-hybridization of the probe with homologous target DNA was set to 100 %.

The genomic DNAs of the strains were recovered according to the method of Hiraishi (1992). 16S rRNA genes were amplified by PCR using *Taq* polymerase (Perkin Elmer) as described previously (Sekiguchi *et al.*, 2000). The PCR primers used in the amplification were the bacterial domain-universal primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3'; positions 8–27 in *Escherichia coli*) and the prokaryote-universal primer 1490R (5'-GGTTACCTTGTTACGACTT-3'; *E. coli* positions 1491–1509) (Weisburg *et al.*, 1991). The PCR product was directly sequenced on a Beckman CEQ8000 DNA sequencer using a CEQ DTC quick start

kit (Beckman Coulter). Sequence data were aligned in an ARB dataset using the ARB program package (Ludwig *et al.*, 2004) and the aligned data were corrected manually by using the editing tool in the package. Phylogenetic trees were constructed on the basis of 16S rRNA gene sequences by the neighbour-joining method (Saitou & Nei, 1987) with the ARB program package. To estimate the confidence of tree topologies, bootstrap resampling analysis (Felsenstein, 1985) for 1000 replicates was performed for the neighbour-joining and maximum-likelihood methods using the PAUP* 4.0 and TREEFINDER program packages, respectively.

Sludge samples collected from the thermophilic UASB reactor used for the isolation of the *Thermodesulfovibrio* strains were fixed and sectioned as described previously (Sekiguchi *et al.*, 1999; Yamada *et al.*, 2005). The following 16S rRNA-targeted oligonucleotide probes were used in this study: probe ARC915 specific to archaea (Stahl *et al.*, 1988) and probe TDV1015 (5'-CCCCTAAGGTCGTCCTT-3'; *E. coli* positions 1015–1029) specific to virtually all members of the genus *Thermodesulfovibrio* (designed in this study). The probes were labelled with either Cy3 or Cy5 for *in situ* hybridization. *In situ* hybridization for thin sections of granules was performed as described previously (Sekiguchi *et al.*, 1999). The hybridization stringency was adjusted by adding formamide to the hybridization buffer (35 % for ARC915, 15 % for TDV1015). The cells hybridized with the probes were observed under a confocal laser scanning microscope (CLSM; Olympus FLUOVIEW BX50).

Gently washed and homogenized sludge from a thermophilic (55 °C) UASB reactor was used for primary enrichments using 20 mM lactate (lactate culture) or 20 mM ethanol (ethanol culture) as the substrate (without sulfate). Growth and methane production were observed within a week after inoculation. The enrichments stably converted the respective substrates into acetate and methane over 10 successive transfers and the cultures seemed to contain several morphotypes comprising certain bacteria and *Methanothermobacter*-like F₄₂₀-autofluorescent rod-shaped cells. To isolate the bacteria responsible for syntrophic substrate degradation in the cultures, cultivation in pure culture with other substrates that could support growth in axenic culture (without methanogens) was attempted. Highly enriched cultures (after >10 successive transfers) were serially diluted and inoculated into media containing pyruvate or lactate plus sulfate; consequently, two vibrio-shaped strains were obtained from the two enrichments.

With 20 mM pyruvate (without addition of sulfate), vibrio-shaped cells grew in cultures inoculated with the highly enriched ethanol culture. After three successive transfers into the pyruvate medium, roll-tube isolation was performed. White, very small, lens-shaped colonies, 0.1–0.2 mm in diameter were formed after 2 weeks of incubation. This step was repeated three times and the purified strain, designated TGE-P1^T, was obtained.

Similarly, in the presence of 20 mM lactate and 20 mM sulfate, slightly short, vibrio-shaped cells grew in cultures

inoculated with the highly enriched lactate culture. As observed with strain TGE-P1^T, white, small, lens-shaped colonies, 0.1–0.2 mm in diameter were formed within 2 weeks of incubation in roll-tube isolation, after three successive transfers in the lactate/sulfate medium. The isolation step was repeated three times and the purified strain, designated TGL-LS1, was obtained.

Strains TDV^T and TSL-P1 were obtained from enrichment cultures syntrophically degrading lactate under thermophilic (55 °C) conditions. These enrichment cultures were prepared from two types of thermophilic (53–55 °C) anaerobic wastewater treatment sludge and seemed to contain several morphotypes, as observed in enrichment cultures of strains TGE-P1^T and TGL-LS1. Strains TDV^T and TSL-P1 were isolated by roll-tube isolation from the enrichment cultures with pyruvate, similarly to strain TGE-P1^T. White, small, lens-shaped colonies, 0.1–0.2 mm in

diameter were formed within 2 weeks of incubation in roll-tube isolation.

Cells of isolates TGE-P1^T, TDV^T, TGL-LS1 and TSL-P1 were curved rods, 1–7 µm long and 0.3–0.4 µm wide (see Supplementary Fig. S1 available in IJSEM Online; Table 1). The length of the cells differed slightly between the strains (Table 1). Spore formation was not observed and Gram staining was negative for all the isolates. Motility was not observed in any isolate. Strain TGE-P1^T formed dense cell aggregates (flocs) in liquid medium, although the other strains showed dispersed cell growth in the same liquid medium.

Physiological properties of strain TGE-P1^T are shown in Table 1. Strain TGE-P1^T was an obligately anaerobic organism: no growth occurred in the presence of oxygen (20 %, v/v, in the gas phase). Yeast extract was not required, but it stimulated growth. Growth and substrate

Table 1. Characteristics of strains TGE-P1^T, TDV^T, TGL-LS1 and TSL-P1 and related *Thermodesulfovibrio* species

Strains: 1, *Thermodesulfovibrio aggregans* sp. nov. TGE-P1^T; 2, *Thermodesulfovibrio thiophilus* sp. nov. TDV^T; 3, *Thermodesulfovibrio islandicus* TGL-LS1; 4, *Thermodesulfovibrio islandicus* TSL-P1 (data in columns 1–4 were obtained in this study); 5, *Thermodesulfovibrio yellowstonii* YP87^T [most data obtained from Henry *et al.* (1994), but some data such as fatty acid composition and substrate range were additionally examined in this study for strain DSM 11347^T]; 6, *Thermodesulfovibrio islandicus* R1Ha3^T (data from Sonne-Hansen & Ahring, 1999); 7, '*Thermodesulfovibrio hydrogeniphilus*' Hbr5 (Haouari *et al.*, 2008). All strains give positive results for the utilization of pyruvate, hydrogen (plus acetate) and formate (plus acetate) as electron donors (and carbon source) in the presence of sulfate. All strains give negative results for the utilization of acetate and ethanol as electron donors. All strains are positive for fermentative growth with pyruvate, but negative for fermentative growth with lactate. All strains are positive for utilization of sulfate and thiosulfate as electron acceptors, but negative for elemental sulfur. –, Negative; +, positive; i, iso; ai, anteiso; ND, no data available.

Characteristic	1	2	3	4	5	6	7
Motility	–	–	–	–	+	+	+
Cell width (µm)	0.3–0.4	0.3–0.4	0.3–0.4	0.3–0.4	0.3	0.4	0.5
Cell length (µm)	2–7	2–3	2–4	1–4	1.5	1.7	2
Growth temperature (°C)							
Range	45–70	45–60	45–60	45–60	40–70	45–70	50–70
Optimum	60	55	55	55	65	65	65
pH for growth							
Range	6.0–8.5	6.0–8.5	6.0–8.0	6.0–8.5	5.5–8.5	ND	6.5–8.5
Optimum	6.5–7.0	7.0–7.5	7.0–7.5	6.5	6.8–7.0	ND	7.1
Major cellular fatty acids	i-C _{17:0} , i-C _{16:0} , ai-C _{15:0}	i-C _{17:0} , C _{16:0} , i-C _{16:0}	i-C _{17:0} , i-C _{16:0} , ai-C _{15:0}	i-C _{17:0} , i-C _{16:0} , ai-C _{15:0}	i-C _{16:0} , i-C _{17:0} , ai-C _{15:0}	ND	ND
DNA G + C content (mol%)	35.2	34.0	34.4	34.5	29.5	38	36.1
Utilization of lactate:							
With sulfate	+	+	+	+	+	+	–
In the presence of methanogens	+	–	+	+	+	ND	ND
Utilization of external electron acceptors							
Sulfite	–	+	–	–	+	–	+
Nitrate	–	–	–	–	–	+	–
Fe(III) NTA	+	+	+	+	+	ND	ND
Isolation source	Thermophilic anaerobic sludge	Thermophilic anaerobic sludge	Thermophilic anaerobic sludge	Thermophilic anaerobic sludge	Hydrothermal vent water	Slightly alkaline thermal spring	Terrestrial thermal spring

utilization were observed with the following substrates under optimum conditions (60 °C, pH 7.0) with sulfate (20 mM) as electron acceptor: hydrogen (1 atm), formate, L-lactate and pyruvate (all at 20 mM). Lactate was oxidized incompletely to acetate. Ethanol (20 mM) did not support growth of the strain in the presence of sulfate, although the strain was isolated from an ethanol enrichment culture. Growth with hydrogen and formate was dependent on the presence of acetate (5 mM) as the carbon source. The doubling time was approximately 1 day when grown on lactate and sulfate medium under optimum conditions (60 °C, pH 7.0). No growth was observed with the following compounds in the presence of sulfate (20 mM): malate, fumarate, succinate, methanol, 1-propanol, 1-butanol (all at 20 mM), benzoate (5 mM), phenol (1 mM), acetate, propionate and butyrate (all at 20 mM). Fermentative growth occurred only in the presence of pyruvate. The following substances did not support fermentative growth: Casamino acids (0.1 %), yeast extract (0.1 %), crotonate, betaine, glucose, ribose, xylose, glycerol, malate, fumarate and lactate (all at 20 mM).

Strain TGE-P1^T grew in co-culture with the hydrogenotrophic methanogen *Methanothermobacter thermautotrophicus* ΔH^T in the presence of lactate. As shown in Supplementary Fig. S2a, half of the 20 mM DL-lactate was degraded and transformed into acetate and methane within 2 weeks of incubation in co-culture with hydrogenotrophic methanogens (electron recovery 97 %). The strain could degrade the same amount of L-lactate completely to acetate in co-culture; hence, it was suggested that strain TGE-P1^T is unable to degrade the enantiomer D-lactate. In pure culture, acetate and hydrogen production from lactate occurred, but very slowly (Supplementary Fig. S2b). Ethanol did not support growth of the strain co-cultured with methanogens.

The following compounds were tested as electron acceptors using lactate medium: 20 mM sulfate, 20 mM nitrate, 2 mM sulfite, 20 mM thiosulfate and 5 mM ferric nitrilotriacetate [Fe(III) NTA]. Sulfate, thiosulfate and Fe(III) NTA supported growth of strain TGE-P1^T.

The same tests conducted on strain TGE-P1^T were also performed on the other three strains (TDV^T, TGL-LS1 and TSL-P1) and the physiological differences between the four strains and other *Thermodesulfovibrio* species are summarized in Table 1 (mainly differential data are listed). The doubling times for strains TDV^T, TGL-LS1 and TSL-P1 on lactate/sulfate medium under optimum conditions (55 °C, pH 7.0) were similar to that of strain TGE-P1^T.

Strain TDV^T used sulfite as an electron acceptor for growth in addition to the same external electron acceptors used by the other three strains (Table 1). Strain TDV^T co-cultured with hydrogenotrophic methanogens in the absence of sulfate did not form methane from lactate nor did it grow, unlike the other strains, indicating that this strain cannot degrade lactate syntrophically, although it was isolated from the enrichment culture capable of methanogenic lactate degradation.

The DNA G + C contents (mean ± SD) of strains TGE-P1^T, TDV^T, TGL-LS1 and TSL-P1 were calculated to be 35.2 ± 0.3, 34.0 ± 0.1, 34.4 ± 0.2 and 34.5 ± 0.3 mol%, respectively. Fatty acid methyl ester analysis showed that the major fatty acids of the strains were iso-C_{17:0}, iso-C_{16:0}, C_{16:0} and anteiso-C_{15:0} (Table 2). For all the strains isolated, >1450 nt of the 16S rRNA genes were sequenced and used for phylogenetic analyses to determine their phylogenetic position in the domain *Bacteria*. Phylogenetic analyses based on gene sequences revealed that the strains belonged to the phylum 'Nitrospirae', in which their closest relatives were *Thermodesulfovibrio yellowstonii*, *Thermodesulfovibrio islandicus* and '*Thermodesulfovibrio hydrogeniphilus*' (Fig. 1). The 16S rRNA gene sequence similarities of strain TGE-P1^T with *Thermodesulfovibrio yellowstonii* DSM 11347^T, *Thermodesulfovibrio islandicus* R1Ha3^T and '*Thermodesulfovibrio hydrogeniphilus*' Hbr5 were 95.9, 95.8 and 95.9 %, respectively, indicating the novelty of strain TGE-P1^T at least at the species level (Stackebrandt & Goebel, 1994). The 16S rRNA gene sequence similarities of strain TDV^T with *Thermodesulfovibrio yellowstonii* DSM 11347^T, *Thermodesulfovibrio islandicus* R1Ha3^T and '*Thermodesulfovibrio hydrogeniphilus*' Hbr5 were 95.5, 95.5 and 93.7 %. Strain TDV^T was also relatively distant from strain TGE-P1^T (94.1 % similarity). On the basis of these observations, strains TGE-P1^T and TDV^T should be classified as a representative of novel species of the genus *Thermodesulfovibrio*. Two novel species of the genus *Thermodesulfovibrio* are therefore proposed: *Thermo-*

Table 2. Cellular fatty acid compositions of the strains isolated in this study and the type strains of *Thermodesulfovibrio yellowstonii* and *Thermodesulfobacterium commune*

Strains: 1, *Thermodesulfovibrio aggregans* sp. nov. TGE-P1^T; 2, *Thermodesulfovibrio thiophilus* sp. nov. TDV^T; 3, *Thermodesulfovibrio islandicus* TGL-LS1; 4, *Thermodesulfovibrio islandicus* TSL-P1; 5, *Thermodesulfovibrio yellowstonii* DSM 11347^T; 6, *Thermodesulfobacterium commune* DSM 2178^T. Data were obtained in this study, and are shown as percentages of the total fatty acids detected. ND, Not detected.

Fatty acid	Genus <i>Thermodesulfovibrio</i>					6
	1	2	3	4	5	
C _{14:0}	0.1	0.8	ND	ND	0.4	0.1
iso-C _{15:0}	0.5	ND	2.0	1.7	0.6	0.3
anteiso-C _{15:0}	5.8	13.4	8.3	7.8	8.1	0.2
C _{15:0}	0.0	0.8	ND	ND	0.1	ND
iso-C _{16:0}	20.5	11.9	25.0	37.1	57.6	13.1
C _{16:0}	2.4	17.3	2.0	1.4	5.9	3.4
Branched C _{17:0}	3.0	2.4	12.7	6.7	1.1	5.7
iso-C _{17:0}	63.4	49.4	48.1	43.2	25.4	46.1
C _{17:0}	ND	1.6	ND	ND	0.1	0.1
iso-C _{18:0}	4.1	1.4	1.9	1.9	0.4	5.0
C _{18:0}	0.2	1.0	ND	0.2	0.3	26.0

Fig. 1. Phylogenetic positions of strains TGE-P1^T, TDV^T, TGL-LS1 and TSL-P1 among deeply branched groups of the phylum 'Nitrospirae'. The 16S rRNA gene sequences obtained and reference sequences were aligned using the ARB program. The phylogenetic tree was constructed by the neighbour-joining method. The 16S rRNA gene sequences of *Chloroflexus aurantiacus* J-10-fl^T (GenBank accession no. D38365), *Oscillochloris trichoides* DG-6^T (AF093427) and *Roseiflexus castenholzii* HLO8^T (AB041226) were used to root the tree (not shown). Bootstrap resampling analysis of 1000 replicates was performed to estimate the confidence of the tree topologies. Branching points with supported probabilities above 95 % by all the analyses (estimated using neighbour-joining and maximum-likelihood methods) are indicated by solid circles, whereas nodes with open circles indicate >75 % bootstrap probability support by the two analyses. Nodes with open squares indicate >50 % probability support by both the analyses. Nodes without symbols were not highly resolved (<50 %) as specific groups in the analyses. Bar, 0.05 changes per sequence position. Clones TUG4 and TUG5 (GenBank accession nos AB011332 and AB011333, respectively), which were retrieved frequently as *Thermodesulfovibrio*-type clones from the original sludge sample of strains TGE-P1^T and TGL-LS1 (Sekiguchi *et al.*, 1998), are not shown in the tree because these sequences are less than 1000 bp.

desulfovibrio aggregans sp. nov., with strain TGE-P1^T as the type strain, and *Thermodesulfovibrio thiophilus* sp. nov., with strain TDV^T as the type strain.

Strains TGL-LS1 and TSL-P1 were very closely related to the type strains of *Thermodesulfovibrio yellowstonii* and *Thermodesulfovibrio islandicus* (99.5 % 16S rRNA gene sequence similarity) and were closely related to each other (99.6 % similarity). Therefore, to compare the differences between the strains and related species at the species level, DNA–DNA hybridization tests of respective genomic DNAs were performed. Genomic DNAs from strains TGL-LS1 and TSL-P1 were extracted and labelled as the DNA probe and their relatedness with DNAs from strains TGE-P1^T, TDV^T, TGL-LS1 and TSL-P1, *Thermodesulfovibrio yellowstonii* DSM 11347^T and *Thermodesulfovibrio islandicus* DSM 12570^T was examined. Genomic DNA from strain TGL-LS1 had high DNA–DNA hybridization values with that of strain TSL-P1 (mean $\pm$ SD of 99.1 ± 6.9 %) and *Thermodesulfovibrio islandicus* DSM 12570^T (81.4 ± 9.0 %); DNA–DNA hybridization values with the other strains were lower than 60 %. Similarly, genomic DNA from strain TSL-P1 had a high DNA–DNA hybridization value with DNA from *Thermodesulfovibrio islandicus* DSM 12570^T (74.1 ± 6.8 %); the hybridization

values with the other strains were in the same ranges as values obtained with DNA from strain TGL-LS1. On the basis of the recommended cut-off point of 70 % DNA–DNA hybridization for bacterial species delineation (Wayne *et al.*, 1987), it is concluded that strains TGL-LS1 and TSL-P1 should be classified as strains of the species *Thermodesulfovibrio islandicus*.

On the basis of 16S rRNA gene sequence information, an oligonucleotide probe specific to 16S rRNA of virtually all members of the genus *Thermodesulfovibrio* was designed (probe TDV1015; see Supplementary Table S1). The probe was used successfully to detect whole cells of *Thermodesulfovibrio yellowstonii*, *Thermodesulfovibrio islandicus* and the isolates obtained in this study by FISH. None of the other bacterial strains listed above (seven species) reacted with the probe (data not shown), indicating its specificity. Thin sections of sludge granules obtained from the thermophilic UASB reactor (from which strains TGE-P1^T and TGL-LS1 were isolated) were prepared and subjected to FISH analysis using probe TDV1015. The probe detected a number of rod- or vibrio-shaped cells within the sludge granules (Supplementary Fig. S3); these cells were widely distributed within the outer and inner layers of the sludge granules.

This observation indicates that members of the genus *Thermodesulfovibrio* make up a significant population within the methanogenic thermophilic sludge. Indeed, *Thermodesulfovibrio*-type 16S rRNA gene clones [TUG4 (AB011332) and TUG5 (AB011333)] have frequently been retrieved from the same thermophilic sludge (Sekiguchi *et al.*, 1998).

In previous 16S rRNA gene cloning and FISH studies of the same sludge sample (Sekiguchi *et al.*, 1998, 1999), it was observed that the sludge possessed *Methanothrix* ('*Methanosaeta*')-type cells and *Methanobacterium*-type cells as the major archaeal populations. Under normally adjusted CLSM conditions, double-staining FISH with probes TDV1015 and ARC915 (Stahl *et al.*, 1988) of sections did not clearly show the close juxtaposition between *Thermodesulfovibrio*-type cells and archaeal cells (Supplementary Fig. S3a), because ARC915-positive, thick rod-shaped cells, morphologically similar to *Methanothrix*, fluoresced brightly with the ARC915 probe and consequently hid other weakly fluorescent archaeal cells (such as *Methanobacterium*-like cells) in the CLSM images. However, when the detection level of archaeal signals was increased when observing CLSM fields in order to visualize these weakly fluorescent archaeal cells, a number of slightly thin rod-shaped cells, resembling *Methanobacterium*, were observed as archaeal cells within the outer and middle layers of the sludge granules and some of these thin rod-shaped cells were found within microcolonies of TDV1015-positive cells (Supplementary Fig. S3b). Because the sludge had been used for the treatment of wastewater containing a limited amount of sulfate (160 mg sulfate l⁻¹, 3 % of the total chemical oxygen demand removed attributed to sulfate reduction) (Sekiguchi *et al.*, 1998), most of the *Thermodesulfovibrio*-type cells in the sludge were presumed to thrive without dissimilatory sulfate reduction. Based on these observations in association with the physiological traits of the *Thermodesulfovibrio* strains isolated in this study, a possible ecophysiological role of the *Thermodesulfovibrio* in the sludge could be consumption of lactate in syntrophic association with hydrogenotrophic methanogens. Lactate has been suggested to be one of the most important intermediate compounds in methanogenic degradation of carbohydrates in thermophilic anaerobic wastewater treatment process (Stams & Zehnder, 1990).

Description of *Thermodesulfovibrio aggregans* sp. nov.

Thermodesulfovibrio aggregans (ag'gre.gans. L. part. adj. *aggregans* aggregating, aggregate-forming).

Curved rods or vibrios, 0.3–0.4 µm long and 2.0–7.0 µm wide. Non-motile. Gram-reaction-negative. Obligate anaerobe. Growth occurs between 45 and 70 °C, with optimum at 60 °C. The pH range is 6.0–8.5. Optimum growth occurs at pH 6.5–7.0. With sulfate as electron acceptor, the electron donors are lactate, hydrogen (in the presence of acetate), formate (in the presence of acetate) and pyruvate. Organic substrates are oxidized incompletely

to acetate. Malate, fumarate, succinate, methanol, 1-propanol, 1-butanol, benzoate, phenol, acetate, propionate and butyrate are not used as electron donors. With lactate as electron donor, the electron acceptors are sulfate, thiosulfate and Fe(III) NTA. Hydrogenotrophic methanogens can also be used as the electron-accepting system. Nitrate, sulfite, fumarate and sulfur are not used. Fermentative growth occurs with pyruvate. Casamino acids, crotonate, betaine, glucose, ribose, xylose, glycerol, malate, fumarate, succinate, lactate, yeast extract and tryptone do not support fermentative growth.

The type strain is TGE-PI^T (=JCM 13213^T =DSM 17283^T), isolated from thermophilic sludge granules treating organic wastewater. The genomic DNA G+C content of the type strain is 35.2 mol%.

Description of *Thermodesulfovibrio thiophilus* sp. nov.

Thermodesulfovibrio thiophilus (thi.o'phi.us. Gr. n. *theion* sulfur; Gr. adj. *philos* loving; N.L. masc. adj. *thiophilus* sulfur-loving).

Curved rods or vibrios, 0.3–0.4 µm long and 2.0–3.0 µm wide. Non-motile. Gram-reaction-negative. Obligate anaerobe. Growth occurs between 45 and 60 °C, with optimum at 55 °C. The pH range is 6.0–8.5. Optimum growth occurs at pH 7.0–7.5. With sulfate as electron acceptor, the electron donors are lactate, hydrogen (in the presence of acetate), formate (in the presence of acetate) and pyruvate. Organic substrates are oxidized incompletely to acetate. Malate, fumarate, succinate, methanol, 1-propanol, 1-butanol, benzoate, phenol, acetate, propionate and butyrate are not used as electron donors. With lactate as electron donor, the electron acceptors are sulfate, sulfite, thiosulfate and Fe(III) NTA. Hydrogenotrophic methanogens can also be used as the electron-accepting system. Fumarate, nitrate and sulfur are not used. Fermentative growth occurs with pyruvate. Casamino acids, crotonate, betaine, glucose, ribose, xylose, glycerol, malate, fumarate, succinate, lactate, yeast extract and tryptone do not support fermentative growth.

The type strain is TDV^T (=JCM 13216^T =DSM 17215^T), isolated from thermophilic sewage sludge decomposing organic solid wastes. The genomic DNA G+C content of the type strain is 34.0 mol%.

Emended description of *Thermodesulfovibrio* Henry *et al.* 1994

In addition to the criteria given by Henry *et al.* (1994), the genus circumscription is emended as follows. All species may use Fe(III) NTA as the electron acceptor for growth; some species use nitrate as well. Some species can also use hydrogenotrophic methanogens as the electron-accepting system. The main fatty acids are iso-C_{17:0}, iso-C_{16:0}, C_{16:0} and anteiso-C_{15:0}. The genomic DNA G+C content is

29–38 mol%. The phylogenetic position is in a lineage of the phylum 'Nitrospirae'.

Acknowledgements

We thank Xian-Ying Meng of the National Institute of Advanced Industrial Science and Technology (AIST) for transmission and scanning electron microscopy.

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