Desulfotomaculum thermosubterraneum sp. nov., a thermophilic sulfate-reducer isolated from an underground mine located in a geothermally active area

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A thermophilic, Gram-positive, endospore-forming, sulfate-reducing bacterium was isolated from an underground mine in a geothermally active area in Japan. Cells of this strain, designated RL50JIII^T, were rod-shaped and motile. The temperature range for growth was 50–72 °C (optimum growth at 61–66 °C) and the pH range was 6.4–7.8 (optimum at pH 7.2–7.4). Strain RL50JIII^T tolerated up to 1.5 % NaCl, but optimum growth occurred in the presence of 0–1 % NaCl. Electron acceptors utilized were sulfate, sulfite, thiosulfate and elemental sulfur. Electron donors utilized were H₂ in the presence of CO₂, alanine, various carboxylic acids and alcohols. Fermentative growth occurred on lactate and pyruvate. The cell wall contained mesodiaminopimelic acid and the major respiratory isoprenoid quinone was menaquinone 7 (MK-7). Major whole-cell fatty acids were iso-C_{15:0}, iso-C_{17:0} DMA (dimethyl acetal), iso-C_{15:0} DMA and iso-C_{17:0}. Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed 98.7 % similarity with *Desulfotomaculum solfataricum* DSM 14956^T. However, DNA–DNA hybridization experiments with *Desulfotomaculum kuznetsovii*, *Desulfotomaculum luciae* and *D. solfataricum* and the G + C content of the DNA (54.4 mol%) allowed the differentiation of strain RL50JIII^T from the recognized species of the genus *Desulfotomaculum*. Strain RL50JIII^T therefore represents a novel species, for which the name *Desulfotomaculum thermosubterraneum* sp. nov. is proposed. The type strain is RL50JIII^T (= DSM 16057^T = JCM 13837^T).

Sulfate-reducing bacteria comprise a diverse group of prokaryotes that contribute to the biogeochemical cycling of sulfur in many environments. Most of the described thermophilic sulfate-reducers belong to the cluster of Gram-positive, spore-forming *Desulfotomaculum* species (Goorissen *et al.*, 2003). The genus *Desulfotomaculum* was first described by Campbell & Postgate (1965). At the time of writing, the genus comprises 15 thermophilic species: *D. alkaliphilum* (Pikuta *et al.*, 2000), *D. arcticum* (Vandieken *et al.*, 2006), *D. australicum* (Love *et al.*, 1993), *D. carboxydvorans* (Parshina *et al.*, 2005), *D. geothermicum* (Daumas *et al.*, 1988), *D. kuznetsovii* (Nazina *et al.*, 1989), *D. luciae* (Liu *et al.*, 1997), *D. nigrificans* (Werkman & Weaver, 1927; Campbell & Postgate, 1965), *D. putei* (Liu *et al.*, 1997),

D. salinum (Nazina *et al.*, 2005), *D. solfataricum* (Goorissen *et al.*, 2003), *D. thermoacetoxidans* (Min & Zinder, 1990), *D. thermobenzoicum* with two subspecies, namely subsp. *thermobenzoicum* and subsp. *thermosyntrophicum* (Tasaki *et al.*, 1991; Plugge *et al.*, 2002), *D. thermocisternum* (Nilsen *et al.*, 1996) and *D. thermosapovorans* (Fardeau *et al.*, 1995). Recently, we reported the enrichment and isolation of thermophilic sulfate-reducers from an underground mine in a geothermally active area in Japan (Kaksonen *et al.*, 2006). This paper describes a novel thermophilic, sulfate-reducing bacterium, strain RL50JIII^T, isolated from the mine.

Strain RL50JIII^T was isolated from a geothermal mine site 250 m below ground, from a black sediment layer beneath a thin red layer of ferric iron on a tunnel wall. Temperature at the site was 70–80 °C. For comparisons, reference of *D. kuznetsovii* DSM 6115^T, *D. luciae* DSM 12396^T and *D. solfataricum* DSM 14956^T, were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

Abbreviation: DMA, dimethyl acetal.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RL50JIII^T is DQ208688.

A phase-contrast micrograph of cells of strain RL50JIII^T is available as supplementary material in IJSEM Online.

Enrichment and isolation of strain RL50JIII^T were performed at 50 °C using modified Postgate growth medium (pH 7.0–7.5) (Kaksonen *et al.*, 2006) with lactate as the electron donor. Anaerobic roll-tubes solidified with 1.5 % agar were used for the isolation. For chemotaxonomic analysis and DNA isolation, the strain was cultured at 60 °C in modified DSM medium 641 containing lactate as the electron donor. The medium was supplemented with 1 ml selenate-tungstate solution l⁻¹ (DSM medium 385) and sodium dithionate (25 mg l⁻¹) was used as the reducing agent instead of Na₂S.

The isolate was examined by phase-contrast microscopy (Axioskop 2; Zeiss) and photomicrographs were obtained using the agar slide technique as described by Kaksonen *et al.* (2004). Flagellum staining was performed as described by Heimbrook *et al.* (1989). Spore formation by the strain was examined microscopically and also by testing for growth after heat treatment (95 °C for 25 min). The Gram type of the cells was determined by both Gram staining and the KOH test (Gergersen, 1978).

The effects of temperature, pH and NaCl concentration on growth were determined as previously described (Kaksonen *et al.*, 2006). The ability of the strain to utilize various electron donors (1–20 mM) was tested in medium containing 20 mM sulfate. The utilization of various electron acceptors (10 mM) was studied using lactate (10 mM) as electron donor. Amorphous iron(III) oxyhydroxide was formed by neutralizing FeCl₃ solution to a pH of 7 with NaOH. The cultures were incubated for 1–2 weeks. Electron donor utilization was determined as bacterial growth (optical density at 660 nm, Shimadzu UV-1601 spectrophotometer, or Ultrospec II LKB Biochrom 4050 UV/visible spectrophotometer), hydrogen sulfide production or substrate conversion. Hydrogen sulfide production was determined spectrophotometrically and substrate conversion by GC as previously described (Kaksonen *et al.*, 2004). Ferrous iron was determined colorimetrically (UV-1601; Shimadzu) with ferrozine (Stookey, 1970). Concentrations of sulfate, sulfite, thiosulfate, nitrate and nitrite were determined by ion chromatography (DX-120; Dionex).

Cell wall preparations were obtained by boiling cells in 20 % (w/v) aqueous trichloroacetic acid solution for 20 min. Diaminopimelic acid isomers were detected in cell-wall hydrolysates (4 M HCl, 100 °C, 16 h) by TLC on cellulose sheets (Merck) using the solvent system of Rhuland *et al.* (1955). Respiratory isoprenoid quinones were extracted and analysed according to the methods described by Collins & Jones (1981), Monciardini *et al.* (2003) and Groth *et al.* (1996) using an HPLC apparatus (Shimadzu) fitted with a reversed-phase C18 column [150 mm × 4.6 mm (ID), 5 µm, porosity 90 Å; Vydac] with UV detection at 269 nm. The identity of the quinones was verified by GC-MS (GCMS-QP2000; Shimadzu) using direct injection with a temperature increase of 60–250 °C at 35 °C min⁻¹.

Fatty acid methyl esters of cellular fatty acids were obtained by saponification, methylation, extraction and base wash, as described by Kämpfer & Kroppenstedt (1996), Kroppenstedt (1985) and Miller (1982). The fatty acid methyl mixtures were separated by use of a gas chromatograph (5890 Series II; Hewlett Packard) equipped with a Hewlett Packard Ultra2 (cross-linked 5 % PH ME Siloxane) capillary column (25 m × 0.2 mm × 0.33 µm film thickness; HP Part No. 19091B - 102).

Methods for the amplification, sequencing and phylogenetic analysis of 16S rRNA genes were as described previously (Kaksonen *et al.*, 2006). Genomic DNA for G + C content determination and DNA–DNA hybridization experiments was released by rupturing cells using a French pressure cell (Thermo Spectronic) and then purified by chromatography on hydroxyapatite (Cashion *et al.*, 1977). DNA was hydrolysed with P1 nuclease and the nucleotides dephosphorylated with bovine alkaline phosphatase (Mesbah *et al.*, 1989). The G + C content of the resulting deoxyribonucleosides was determined by reversed-phase HPLC (Shimadzu) and calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) (Tamaoka & Komagata, 1984; Mesbah *et al.*, 1989). DNA–DNA hybridization experiments were performed at DSMZ according to the method of De Ley *et al.* (1970) with the modifications described by Huß *et al.* (1983), Escara & Hutton (1980) and Jahnke (1992) using a spectrophotometer (model 2600; Gilford) equipped with a thermoprogrammer and plotter (model 2527-R; Gilford).

Cells of strain RL50JIII^T were straight or slightly curved rods, 0.8–1.0 µm in diameter and 3–10 µm in length (see Supplementary Fig. S1 in IJSEM Online). The strain formed spherical spores, which were located centrally or subterminally. The spores were able to germinate after a heat shock at 95 °C for 25 min. Sporulation caused swelling of the cells, giving them a lemon-shaped appearance. The cells were motile with two or more flagella and Gram-positive as determined by both Gram-staining and the KOH test. The temperature, pH and NaCl ranges for growth of strain RL50JIII^T are given in Table 1. The temperature at the sampling point was 70–80 °C, which is at or above the upper temperature limit of growth of strain RL50JIII^T. It is possible that the strain was present in the habitat as spores. Strain RL50JIII^T was able to use sulfate, sulfite, thiosulfate and elemental sulfur as electron acceptors. Electron donors utilized by the strain included H₂ in the presence of CO₂, alanine and various carboxylic acids, or their sodium salts, and alcohols (Table 1). A number of electron donors (e.g. propionate, butyrate, pentanoate, pyruvate, butanol, ethanol, lactate, isobutyrate, 2-methyl butyrate and succinate) were oxidized to acetate, whereas no acetate accumulated during the oxidation of others (e.g. alanine, hexadecanoate and nonanoate). The strain fermented lactate and pyruvate.

The cell wall of strain RL50JIII^T contained mesodiaminopimelic acid as the diagnostic diamino acid and the major respiratory isoprenoid quinone was menaquinone 7 (MK-7). The whole-cell fatty acid compositions of strain

Table 1. Characteristics of strain RL50JIII^T compared with those of its closest relatives in the genus *Desulfotomaculum*

Taxa: 1, strain RL50JIII^T; 2, *D. solfataricum* (data from Goorissen *et al.*, 2003); 3, *D. kuznetsovii* (Nazina *et al.*, 1989); 4, *D. luciae* (Liu *et al.*, 1997); 5, *D. thermobenzoicum* subsp. *thermobenzoicum* (Tasaki *et al.*, 1991); 6, *D. thermoacetoxidans* (Min & Zinder, 1990); 7, *D. geothermicum* (Daumas *et al.*, 1988); 8, *D. thermocisternum* (Nilsen *et al.*, 1996); 9, *D. australicum* (Love *et al.*, 1993); 10, '*D. salinum*' (Nazina *et al.*, 2005). All taxa shared the following characteristics: rod-shaped cells, spore formation, sulfate (10 mM) reduction, utilization of H₂/CO₂ (80:20, v/v) as sole electron and carbon source, and oxidation of lactate (20 mM). Strain RL50JIII^T did not utilize nitrite or iron(III) as electron acceptors (each 10 mM), or tartrate, glutamate, mannose, galactose, sucrose, cellobiose, *myo*-inositol, lactose or trehalose (each 10 mM), phenylacetate (3 mM), 4-hydroxybenzoate (2 mM), tetradecanoate or dodecanoate (each 1 mM) as electron donors. The concentrations for electron donors, electron acceptors and fermentation given in the table apply only to the present study. +, Positive; –, negative; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8	9	10
Motility	+	–	+	+	+	+	+	+	+	+
Gram stain	+	–	–	+ / –	+	–	–	NR	–	–
Gram reaction	+	+*	+*	+*	+	NR	NR	NR†	NR†	NR†
Temperature range (°C)	50–72	48–65	50–85	50–70	40–70	45–65	37–56	41–75	40–74	45–75
Optimal temperature (°C)	61–66	60	60–65	NR	62	55–60	54	62	68	60–65
pH range	6.4–7.8	6.4–7.9	NR	6.3–7.8	6–8	6.0–7.5	6–8	6.2–8.9	5.5–8.5	6.0–8.5
Optimal pH	7.2–7.4	7.3	7.0–7.2	7.0–7.9‡	7.2	6.5	7.2–7.4	6.7	7–7.4	7.0
NaCl range (%)	0–1.5	0–1.5	0–3	<2	0	0–1.5	0.2–5	0–4.7	>0	0–6
Optimal salinity (NaCl %)	0–1	0	0	<1‡	0	0	2.4–3.4	0.3–1.2	NR	1
DNA G+C content (mol%)	54.5	48.3	49	51.4	52.8	49.7	50.4	56–57	48.1	50.8
Electron acceptors:										
Nitrate (10 mM)	–	–	–	–*	+	–	–	–	NR	–
Sulfite (10 mM)	+	+	+	–	+	–	+	+	NR	+
Sulfur (10 mM)	+	+*	– / +*	+*	–	+	–	–	NR	+
Thiosulfate (10 mM)	+	+	+	+	+	+	NR	+	NR	+
Electron donors:										
Acetate (10 mM)	–	+	+	–	–	+	–	–	+	+
Alanine (5 mM)	+	–*	–	NR	NR	+	NR	NR	NR	NR
Benzoate (5 mM)	–	–	–	NR	+	–	–	–	+	–
Butanol (10 mM)	+	+	+	NR	+	+	NR	+	NR	+
Butyrate (10 mM)	+	+	+	NR	+	+	+	+	–	+
Crotonate (10 mM)	+	+*	+*	NR	+	NR	NR	NR	NR	NR
Decanoate (1 mM)	+	+*	+*	NR	NR	NR	–	+	NR	NR
Ethanol (10 mM)	+	+	+	+	+	–	+	+	+	+
Formate (10 mM)	+	+	+	+	+	+	+	–	–	+
Fructose (10 mM)	–	+	–	NR	–	NR	+	–	NR	–
Fumarate (10 mM)	+	+	+	NR	+	NR	NR	NR	–	+
Glucose (10 mM)	–	+	–	–	–	–	NR	NR	–	–
Heptanoate (10 mM)	+	+*	+*	NR	NR	NR	–	+	NR	NR
Hexadecanoate (1 mM)	+	+*	+	NR	NR	–	+	+	+	+
Hexanoate (2 mM)	+	+*	+	NR	+	NR	+	+	NR	+
Malate (10 mM)	+	+*	+	–	+	+	NR	NR	–	+
Methanol (10 mM)	–	+	+	–	–	NR	–	–	–	+
Nonanoate (0.5 mM)	+	+*	+*	NR	NR	NR	+	+	NR	NR
Octanoate (2 mM)	+	+*	+	NR	NR	NR	+	+	NR	NR
Pentanoate (10 mM)	+	+*	+	NR	+	+	NR	+	NR	+
Pentanol (5 mM)	+	+*	+*	NR	NR	+	NR	NR	NR	NR
Propanol (10 mM)	+	+	+	NR	+	+	NR	+	NR	+
Propionate (10 mM)	+	+	+	NR	+	+	+	+	–	+
Pyruvate (10 mM)	+	+*	+	+	+	+	NR	+	+	+

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10
Succinate (10 mM)	+	+	+	NR	—	+	NR	NR	—	+
Fermentation:										
Lactate (20 mM)	+	+	+	+	+	—	NR	NR	+	—
Pyruvate (10 mM)	+	+	+	+	+	+	NR	+	+	+

*Determined in this study.

†Gram-positive cell wall based on microscopic examination.

‡Data obtained from Nazina *et al.* (2005).

RL50JIII^T and related species are presented in Table 2. Significant proportions of saturated iso-branched fatty acids were found in strain RL50JIII^T, the major components being iso-C_{15:0}, iso-C_{17:0} DMA (dimethyl acetal), iso-C_{15:0} DMA and iso-C_{17:0} (Table 2).

Table 2. Whole-cell fatty acid content of strain RL50JIII^T compared with reference species of the genus *Desulfotomaculum*

Taxa: 1, strain RL50JIII^T; 2, *D. solfataricum*; 3, *D. kuznetsovii*; 4, *D. luciae*. All data are from this study. Values shown are percentages of the total whole-cell fatty acids. —, Not present; ECL, equivalent chain length; DMA, dimethyl acetal; c, cyclopropane.

ECL	Fatty acid	1	2	3	4
12·62	iso-C _{13:0}	0·1	—	—	—
13·62	iso-C _{14:0}	—	0·1	0·2	0·1
14·00	C _{14:0}	1·1	2·5	2·4	0·8
14·48	C _{14:0} DMA	0·1	—	—	—
14·63	iso-C _{15:0}	49·6	43·2	36·5	39·3
14·71	anteiso-C _{15:0}	—	1·0	0·9	0·9
14·95	C _{16:0} aldehyde	0·3	0·8	1·0	0·4
15·00	C _{15:0}	—	2·5	2·5	0·6
15·11	iso-C _{15:0} DMA	10·6	1·0	0·7	1·6
15·48	C _{15:0} DMA	—	0·3	0·2	0·1
15·63	iso-C _{16:0}	—	1·0	1·9	1·0
15·82	C _{16:1} ω7c	0·1	—	—	0·2
15·91	C _{16:1} ω5c	—	—	—	—
16·00	C _{16:0}	4·3	15·6	19·9	10·5
16·47	C _{16:0} DMA	1·4	1·9	2·3	2·0
16·63	iso-C _{17:0}	8·9	17·6	17·4	23·6
16·72	anteiso-C _{17:0}	—	1·3	1·4	1·5
17·00	C _{17:0}	—	0·6	0·9	0·5
17·11	iso-C _{17:0} DMA	16·0	4·0	3·6	8·8
17·20	anteiso-C _{17:0} DMA	—	0·2	0·2	0·6
17·63	iso-C _{18:0}	—	—	0·2	—
17·72	C _{18:2} ω6,9c	0·4	0·2	0·2	0·4
17·77	C _{18:1} ω9c	1·5	0·5	0·8	1·5
17·82	C _{18:1} ω7c	—	—	—	0·2
18·00	C _{18:0}	0·2	0·2	0·7	1·2
18·64	iso-C _{19:0}	—	—	—	0·1
	Total unidentified	5·6	5·5	6·2	3·9

The G + C content of the genomic DNA of strain RL50JIII^T was 54·5 mol% (Table 1). A phylogenetic tree constructed on the basis of 16S rRNA gene sequences showed that the closest relative of strain RL50JIII^T was *D. solfataricum* DSM 14956^T (Fig. 1), with which it shared 98·7% sequence similarity in the ARB similarity matrix (which omits insertions and deletions from the pairwise comparison). Strain RL50JIII^T shared a sequence similarity of 96·2% with *D. kuznetsovii* DSM 6115^T in the ARB similarity matrix and of 94·2% with *D. luciae* DSM 12396^T. The 16S rRNA gene sequence of strain RL50JIII^T has a 104-bp insert, which is not present in *D. solfataricum* or *D. kuznetsovii*, but which shows 95·2% similarity to that in the 16S rRNA gene sequence of *D. luciae*. 16S rRNA gene sequence similarities of strain RL50JIII^T with the type strains of *D. solfataricum*, *D. kuznetsovii* and *D. luciae* as calculated with the BIOEDIT program (which includes insertions and deletions in the pairwise comparison) were 92, 90 and 94%, respectively. Levels of DNA–DNA relatedness between strain RL50JIII^T and *D. solfataricum* DSM 14956^T, *D. kuznetsovii* DSM 6115^T and *D. luciae* DSM 12396^T were 47·4, 59·5 and 47·8%, respectively, which justifies a novel species designation for strain RL50JIII^T (Wayne *et al.*, 1987).

Strain RL50JIII^T exhibits many features typical of the genus *Desulfotomaculum*, such as rod-shaped, Gram-positive, motile, spore-forming cells and MK-7 as the major menaquinone. Moreover, the strain is phylogenetically closely related to members of this genus. However, strain RL50JIII^T differs from its closest relatives based on analysis of genomic DNA, higher G + C content (Table 1), the ability or inability to use certain electron acceptors and donors (Table 1), and a higher proportion of iso-C_{15:0} DMA and iso-C_{17:0} DMA and a lower proportion of C_{16:0} and iso-C_{17:0} in the whole-cell fatty acids (Table 2). Based on these phenotypic and genomic differences, strain RL50JIII^T can be assigned as the type strain of a novel species of the genus *Desulfotomaculum*, for which the name *Desulfotomaculum thermosubterraneum* sp. nov. is proposed.

Description of *Desulfotomaculum thermosubterraneum* sp. nov.

Desulfotomaculum thermosubterraneum (ther.mo.sub.ter.ra'ne.um. Gr. adj. *thermos* hot; L. adj. neut. *subterraneum*

Fig. 1. Phylogenetic tree generated using the distance matrix and neighbour-joining methods based on the 16S rRNA gene sequences of strain RL50JIII^T (1505 bp between *Escherichia coli* positions 69 and 1409) and related taxa. *Archaeoglobus veneficus* (Y10011) was used as the outgroup (not shown). Numbers at nodes represent bootstrap percentages based on 1000 samplings. Bar, 0.05 changes per nucleotide position.

subterranean, underground, below the Earth's surface; *thermosubterraneum* thermophilic inhabitant of the Earth's subsurface).

Cells are motile, Gram-positive, spore-forming rods (0.8–1.0 × 3–10 µm). Growth occurs at 50–72 °C (optimum 61–66 °C), pH 6.4–7.8 (optimum pH 7.2–7.4) and NaCl concentration of 0–1.5 % (optimum 0–1 % NaCl). Sulfate, sulfite, thiosulfate and elemental sulfur are used as electron acceptors. The following substrates are used as electron donors in the presence of sulfate: H₂ in the presence of CO₂, alanine, various carboxylic acids and alcohols (see Table 1). Fermentative growth occurs on lactate and pyruvate. The cell wall contains mesodiaminopimelic acid as the diagnostic diamino acid. The major isoprenoid quinone is MK-7. Major whole-cell fatty acids are iso-C_{15:0}, iso-C_{17:0} DMA, iso-C_{15:0} DMA and iso-C_{17:0}. The G+C content of the DNA is 54.4 mol%.

The type strain, RL50JIII^T (= DSM 16057^T = JCM 13837^T), was isolated from a geothermally active underground mine in Japan.

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