Desulfurispora thermophila gen. nov., sp. nov., a thermophilic, spore-forming sulfate-reducer isolated from a sulfidogenic fluidized-bed reactor

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A thermophilic, Gram-positive, endospore-forming, sulfate-reducing bacterium was isolated from a sulfidogenic fluidized-bed reactor treating acidic metal- and sulfate-containing water. The strain, designated RA50E1^T, was rod-shaped and motile. The strain grew at 40–67 °C (optimum growth at 59–61 °C) and pH 6.4–7.9 (optimum growth at pH 7.0–7.3). The strain tolerated up to 1 % NaCl. Sulfate, sulfite, thiosulfate and elemental sulfur were used as electron acceptors, but not nitrate, nitrite or iron(III). Electron donors utilized were H₂/CO₂ (80 : 20, v/v), alcohols, various carboxylic acids and some sugars. Fermentative growth occurred on lactate and pyruvate. The cell wall contained meso-diaminopimelic acid and the major respiratory isoprenoid quinone was menaquinone MK-7. Major whole-cell fatty acids were iso-C_{15:0} and iso-C_{17:0}. Strain RA50E1^T was distantly related to representatives of the genera *Desulfotomaculum*, *Pelotomaculum*, *Sporotomaculum* and *Cryptanaerobacter*. On the basis of 16S rRNA gene sequence data, the strain cannot be assigned to any known genus. Based on the phenotypic and phylogenetic features of strain RA50E1^T, it is proposed that the strain represents a novel species in a new genus, for which the name *Desulfurispora thermophila* gen. nov., sp. nov. is proposed. The type strain of *Desulfurispora thermophila* is RA50E1^T (= DSM 16022^T = JCM 14018^T).

The previously described thermophilic sulfate-reducing bacteria (SRB) have been classified within the genera *Desulfotomaculum* (Campbell & Postgate, 1965), *Thermodesulfobacterium* (Zeikus *et al.*, 1983), *Thermodesulfovibrio* (Henry *et al.*, 1994), *Thermodesulforhabdus* (Beeder *et al.*, 1995), *Thermodesulfobium* (Mori *et al.*, 2003), *Thermodesulfatator* (Moussard *et al.*, 2004) and *Desulfovibrio* (Kaksonen *et al.*, 2007). Spore-forming SRB include members of the genera *Desulfotomaculum* (Campbell & Postgate, 1965), *Desulfosporosinus* (Stackebrandt *et al.*, 1997), '*Desulfosporomusa*' (Sass *et al.*, 2004) and *Desulfovibrio* (Kaksonen *et al.*, 2007). Recently, SRB have been isolated from sulfidogenic fluidized-bed reactors (FBR) treating acidic metal-containing wastewater (Kaksonen *et al.*, 2004c). This paper describes one of the isolates, a novel thermophilic spore-forming SRB, strain RA50E1^T.

Strain RA50E1^T was isolated from a sulfidogenic laboratory-scale FBR after over 600 days of continuous flow operation

Abbreviations: DMA, dimethyl acetal; FBR, fluidized-bed reactor; SRB, sulfate-reducing bacteria.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RA50E1^T is AY548776.

at 35 °C (Kaksonen *et al.*, 2004c). The FBR had been inoculated with enrichment cultures originating from methanogenic granular sludge (Neson Oy, Jokioinen, Finland) and sediments from Outokumpu's Pyhäsalmi mine, Finland (Kaksonen *et al.*, 2003b). The performance and microbiology of the FBR has been described previously (Kaksonen *et al.*, 2003a, b, 2004a, b, c). The isolation of strain RA50E1^T was performed at 50 °C using modified Postgate growth medium (pH 7.0–7.5) (Kaksonen *et al.*, 2004c) and anaerobic roll-tubes solidified with 1.5 % agar. For chemotaxonomic analysis and DNA isolation, the strain was cultured at 50 °C in modified DSM medium 641 containing lactate as electron donor. The medium was supplemented with 1 ml selenate-tungstate solution (DSM medium 385) l⁻¹ and sodium dithionate (25 mg l⁻¹) was used as the reducing agent instead of Na₂S.

The isolate was examined by phase-contrast microscopy (Zeiss Axioskop 2) and photomicrographs were obtained using the agar slide technique as described previously (Kaksonen *et al.*, 2004c). Flagellum staining was performed as described by Heimbrook *et al.* (1989) and cells were examined for flagella using an Axiophot light microscope (Zeiss). Spore formation by the strain was determined

microscopically and by testing for growth after heat treatment (80 °C for 20 min). The Gram type of the cells was determined by both Gram staining and the KOH test (Gregersen, 1978).

The effects of temperature, pH and NaCl concentration on growth were determined as described previously using lactate as electron donor in modified DSM medium 641 (Kaksonen *et al.*, 2006a). The ability of the strain to utilize various electron donors (1–20 mM) was tested in a 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 pH 7 with NaOH. The cultures were incubated for 1–2 weeks. Electron donor utilization was assessed by bacterial growth (OD 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 was determined by GC as described previously (Kaksonen *et al.*, 2004c). Ferrous iron was determined colorimetrically (Shimadzu UV-1601) with ferrozine (Stookey, 1970). Concentrations of sulfate, sulfite, thiosulfate, nitrate and nitrite were determined by ion chromatography (Dionex DX-120).

Diaminopimelic acid isomers were detected in cell-wall hydrolysates by TLC as described previously (Rhuland *et al.*, 1955; Kaksonen *et al.*, 2006b). 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) by using HPLC and electron impact MS (Kaksonen *et al.*, 2006b). Methyl esters of cellular fatty acids were obtained by saponification, methylation, extraction and base wash, as described previously (Kämpfer & Kroppenstedt, 1996; Kroppenstedt, 1985; Miller, 1982). The fatty acid methyl ester mixtures were separated by GC (Hewlett Packard 5890 Series II) as described previously (Kaksonen *et al.*, 2006b).

For 16S rRNA gene sequencing, cells were pelleted from 1.5 ml liquid culture at 10 000 g for 1 min, washed with PBS (7.2 mM Na₂HPO₄, 2.8 mM NaH₂PO₄, 130 mM NaCl, pH 7.2) and resuspended in 50 µl PBS. 16S rRNA genes were amplified by direct lysis PCR using primers 27F and 1492R (Kaksonen *et al.*, 2004c). The reaction mixtures contained 25 µl HotStarTaq master mix (Qiagen), 1 µM each of both primers, 1 µl cell suspension and 23 µl sterile distilled water. Thermal cycling was carried out with a Minicycler (MJ Research) as follows: initial denaturation and activation of HotStarTaq at 95 °C for 15 min followed by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 50 °C for 2 min and primer extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. PCR products were purified with a QIAquick PCR Purification kit (Qiagen) and visualized by gel electrophoresis using ethidium-bromide-stained (0.2 mg l⁻¹) 1% agarose gels.

The 16S rRNA genes were sequenced using primers 27F, 518R, 704F, 787R, 1100R and 1241F (Kaksonen *et al.*, 2004c) in separate sequencing reactions. Sequencing was performed using the ABI PRISM Big-Dye Terminator Ready Reaction kit on an ABI 373 automated sequencer or on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) operated by the Australian Neuromuscular Research Institute (ANRI) or Royal Perth Hospital DNA sequencing service. The DNA sequence chromatograms were analysed and single primer sequences were compiled using the BioEdit sequence alignment editor (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Phylogenetic affiliations of the sequences were estimated initially using the program BLAST (<http://www.ncbi.nlm.nih.gov/blast/>) (Altschul *et al.*, 1990). A phylogenetic tree of the 16S rRNA gene sequence of strain RA50E1^T (1545 bp between *Escherichia coli* positions 28 and 1491) and related species was constructed using distance matrix and neighbour-joining methods in ARB. The robustness of the phylogeny was tested by bootstrap analysis with 1000 iterations.

Genomic DNA for G+C content determination was released by rupturing cells using a French pressure cell (Thermo Spectronic) followed by chromatographic purification on hydroxyapatite (Cashion *et al.*, 1977). The DNA was hydrolysed with P1 nuclease and the nucleotides were 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 dG:dT ratio (Tamaoka & Komagata, 1984; Mesbah *et al.*, 1989).

Cells of strain RA50E1^T were straight or slightly curved rods, 0.6–1.0 µm in diameter and 2.0–9.0 µm in length (Fig. 1). The size of the cells was dependent on growth medium and temperature. The strain formed oval spores, which were able to germinate after a heat shock. Spores were located centrally or subterminally. Sporulation did not cause significant swelling of the cells. Cells were motile with two or more

Fig. 1. Phase-contrast micrograph of cells of strain RA50E1^T. Bar, 10 µm.

Table 1. Characteristics of strain RA50E1^T and some of its closest sulfate-reducing relatives

Strains: 1, RA50E1^T; 2, *Desulfotomaculum thermocisternum* ST90^T (unless indicated, data from Nilsen *et al.*, 1996); 3, *Pelotomaculum thermopropionicum* SI^T (Imachi *et al.*, 2002); 4, *Sporotomaculum hydroxybenzoicum* BT^T (Brauman *et al.*, 1998); 5, *Cryptanaerobacter phenolicus* LR7.2^T (Juteau *et al.*, 2005). NR, Not reported. All strains have rod-shaped cells and none reduce nitrate.

Characteristic	1	2	3	4	5
Motility	+	+	—	+	NR
Spores*	O, C/ST	S, C	S, C	S, C	NR
Gram stain/reaction	+	NR†	—†	+	NR†
DNA G + C content (mol%)	53.5	56–57	52.8	48	51
Menaquinones	MK-7	MK-7‡	MK-7, MK-7(H ₄)	NR	NR
Temperature range (°C)	40–67	41–75	45–65	24–37	NR
Optimal temperature (°C)	59–61	62	55	30	30–37
pH range	6.4–7.9	6.2–8.9	6.5–8.0	6.8–8.1	NR
Optimal pH	7.0–7.3	6.7	7.0	7.3–7.6	7.5–8.0
NaCl range (%)	0–1	0–4.7	0–0.4	0.2	NR
Optimal NaCl (%)	0	0.3–1.2	NR	NR	NR
Electron acceptors:					
Sulfate	+	+	—	—	—
Sulfite	+	+	—	—	—
Thiosulfate	+	+	—	—	—
Sulfur	+	—	—	NR	NR
Nitrite	—	NR	NR	NR	—
Fe(III)	—	NR	—	—	—

*O, Oval; S, spherical; C, central; ST, subterminal.

†Gram-positive type cell wall observed by transmission electron microscopy.

‡Data from Imachi *et al.* (2002).

flagella and Gram-positive as determined by both Gram staining and the KOH test. The temperature, pH and NaCl ranges for growth of strain RA50E1^T are shown in Table 1. The operating temperature of the FBR (35 °C) was lower than the minimum temperature required for growth of RA50E1^T. Therefore, the strain was probably not active in the FBR, but rather survived as spores in the biofilm.

RA50E1^T was able to use sulfate, sulfite, thiosulfate and elemental sulfur as electron acceptors, but not nitrate, nitrite or iron(III). Electron donors utilized by RA50E1^T included H₂/CO₂ (80:20, v/v), lactate (20 mM), formate, butyrate, isobutyrate, pentanoate, heptanoate, pyruvate, crotonate, mannose, glucose, fructose, *myo*-inositol, ethanol, propanol and butanol (each at 10 mM), pentanol and alanine (each at 5 mM), hexanoate and octanoate (each at 2 mM), decanoate, dodecanoate, tetradecanoate and hexadecanoate (each at 1 mM) and nonanoate (0.5 mM). A number of electron donors (e.g. lactate, pyruvate, hexanoate, fructose, *myo*-inositol, butanol and alanine) were oxidized incompletely to acetate, but no significant acetate production was observed during the sulfidogenic oxidation of octanoate and nonanoate. RA50E1^T did not utilize acetate, propionate, benzoate, malate, malonate, fumarate, succinate, tartrate, citrate, methanol, arabinose, xylose, sucrose, lactose, maltose, galactose, cellobiose, trehalose or glutamate (each at 10 mM),

glycine (5 mM) or 4-hydroxybenzoate (2 mM) as electron donors. RA50E1^T fermented lactate and pyruvate.

The cell wall of strain RA50E1^T contained *meso*-diaminopimelic acid and the major respiratory isoprenoid quinone was menaquinone MK-7. The whole-cell fatty acid composition of strain RA50E1^T is shown in Table 2. Significant proportions of iso-branched saturated fatty acids were found, the major fatty acids being iso-C_{15:0} and iso-C_{17:0} (Table 2). Strain RA50E1^T contained larger amounts of iso-C_{17:0} and less C_{16:0} fatty acid than representatives of related genera (Table 2). Additionally, RA50E1^T contained iso-C_{15:1}, cyclopropane fatty acid (C_{17:0} cyc) and dimethyl acetals (DMA; iso-C_{15:0} DMA, C_{16:0} DMA and iso-C_{17:0} DMA), which were not detected in the selected representatives of related genera (Table 2). The G + C content of the total DNA of strain RA50E1^T was 53.5 mol% (Table 1).

The 16S rRNA gene of strain RA50E1^T contained several regions of variable length that did not align well with sequences of related species and probably represent inserts. Based on 16S rRNA gene sequences, strain RA50E1^T was distantly related to representatives of the genera *Desulfotomaculum*, *Pelotomaculum*, *Sporotomaculum* and *Cryptanaerobacter* (Fig. 2). The closest relatives within these genera were *Desulfotomaculum thermocisternum* ST90^T (89.3 % 16S

Table 2. Whole-cell fatty acid compositions (%) of strain RA50E1^T and related type strains

Strains: 1, RA50E1^T; 2, *Desulfotomaculum thermocisternum* DSM 10259^T (Imachi *et al.*, 2002); 3, *Pelotomaculum thermopropionicum* SI^T (Imachi *et al.*, 2002); 4, *Cryptanaerobacter phenolicus* LR7.2^T (Juteau *et al.*, 2005). DMA, Dimethyl acetal; c, *cis* isomer; cyc, cyclopropane.

Fatty acid	1	2	3	4
C _{14:0}	2.0	6.2	3.2	5.8
iso-C _{15:1}	4.1			
iso-C _{15:0}	54.4	63.3	76.4	13.7
anteiso-C _{15:0}		3.9		18.9
C _{15:0}		3.9		3.5
iso-C _{15:0} DMA	2.5			
iso-C _{16:0}		2.4		7.5
C _{16:1} ω7c	1.0			
C _{16:1} ω9c				13.7*
C _{16:0}	3.2	10.4	10.7	12.5
C _{16:0} DMA	2.0			
iso-C _{17:0}	8.2		2.9	5.7
Branched C _{17:0}		2.4		
anteiso-C _{17:0}				7.3
C _{17:0} cyc	3.8			
iso-C _{17:0} DMA	2.9			
C _{18:1} ω9c	1.2			4.6
C _{18:0}	0.3	2.4	6.7	4.2

*Present as methyl ester (7.8%) and dimethyl aldehyde (5.9%) in the analysis.

Fig. 2. Phylogenetic tree generated using distance matrix and neighbour-joining methods based on the 16S rRNA gene sequences of strain RA50E1^T and related taxa. *Archaeoglobus veneficus* SNP6^T (GenBank accession no. Y10011) was used as outgroup (not shown). Numbers at nodes represent bootstrap percentages based on 1000 samplings. Bar, 0.05 changes per nucleotide position.

rRNA gene similarity in pairwise comparison using BioEdit), obligately syntrophic *Pelotomaculum schinkii* HH^T (88.7%), *Sporotomaculum hydroxybenzoicum* DSM 5475^T (85.1%) and *Cryptanaerobacter phenolicus* LR7.2^T (84.4%).

Strain RA50E1^T exhibited many features that are typical of members of the genera *Desulfotomaculum* and *Pelotomaculum*, such as rod-shaped, Gram-positive, spore-forming cells and MK-7 as the major menaquinone (Table 1). Members of the genus *Pelotomaculum* do not reduce sulfate, whereas the genus *Desulfotomaculum* harbours a collection of SRB that vary significantly with respect to their phylogeny and physiological capacities, such as complete against incomplete oxidation of substrates. Based on the phylogenetic tree (Fig. 2), there are species of the genus *Desulfotomaculum* that seem to be more divergent from each other than from the novel isolate or from representatives of the genera *Pelotomaculum*, *Sporotomaculum* and *Cryptanaerobacter*. Therefore, the genus *Desulfotomaculum* rather represents a ‘super-genus’ consisting of several ‘sub-genera’. Considering 16S rRNA gene sequencing, the genus *Desulfotomaculum* could be reclassified into several genera. On the basis of 16S rRNA gene sequence data, strain RA50E1^T cannot be assigned to any genus with a validly published name. Based on its phylogenetic and phenotypic distinctiveness, it is proposed that strain RA50E1^T represents a novel species in a new genus, for which the name *Desulfurispora thermophila* gen. nov., sp. nov. is proposed.

Description of *Desulfurispora* gen. nov.

Desulfurispora (De.sul.fu.ri.spo’ra. L. pref. *de* from; L. n. *sulfur* sulfur; Gr. fem. n. *spora* a seed and, in biology, a spore; N.L. fem. n. *Desulfurispora* a spore-forming organism that reduces sulfur compounds).

Cells are rod-shaped, spore-forming, Gram-positive, strictly anaerobic and thermophilic. Sulfate and other sulfur compounds are used as electron acceptors. Optimal growth occurs at neutral pH. The cell wall contains *meso*-diaminopimelic acid and MK-7 is the major menaquinone. Major cellular fatty acids are iso-C_{15:0} and iso-C_{17:0}. Phylogenetic position based on 16S rRNA gene sequencing is in the Gram-positive bacteria. The type species is *Desulfurispora thermophila*.

Description of *Desulfurispora thermophila* sp. nov.

Desulfurispora thermophila (ther.mo.phi’la. Gr. fem. n. *thermê* heat; Gr. adj. *philos* loving; N.L. fem. adj. *thermophila* heat-loving).

In addition to the properties given in the description of the genus, the following properties are observed. Cells are motile and 0.6–1.0 × 2.0–9.0 µm. Growth occurs at 40–67 °C (optimum 59–61 °C), pH 6.4–7.9 (optimum pH 7.0–7.3) and NaCl concentration 0–1 % (optimum 0 % NaCl). Sulfate, sulfite, thiosulfate and elemental sulfur are used as electron acceptors, but nitrate, nitrite and iron(III) are not.

Electron donors include H₂/CO₂ (80:20, v/v), lactate, formate, butyrate, isobutyrate, pentanoate, heptanoate, pyruvate, crotonate, mannose, glucose, fructose, *myo*-inositol, ethanol, propanol, butanol, pentanol, alanine, hexanoate, octanoate, decanoate, dodecanoate, tetradecanoate, hexadecanoate and nonanoate. Fermentative growth occurs on lactate and pyruvate.

The type strain is RA50E1^T (= DSM 16022^T = JCM 14018^T), isolated from a sulfidogenic FBR treating acidic metal-containing wastewater. The genomic DNA G + C content of the type strain is 53.5 mol% (as determined by HPLC).

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