Desulfotomaculum alcoholivorax sp. nov., a moderately thermophilic, spore-forming, sulfate-reducer isolated from a fluidized-bed reactor treating acidic metal- and sulfate-containing wastewater

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A moderately thermophilic, Gram-positive, endospore-forming, sulfate-reducing bacterium was isolated from a fluidized-bed reactor treating acidic water containing metal and sulfate. The strain, designated RE35E1^T, was rod-shaped and motile. The temperature range for growth was 33–51 °C (optimum 44–46 °C) and the pH range was 6.0–7.5 (optimum pH 6.4–7.3). The strain grew optimally without additional NaCl. The electron acceptors were 10 mM sulfate, thiosulfate and elemental sulfur and 1 mM (but not 10 mM) sulfite. Various alcohols and carboxylic acids were utilized as electron donors. Fermentative growth occurred on pyruvate. The cell wall contained meso-diaminopimelic acid, and the major respiratory isoprenoid quinone was menaquinone MK-7. The major whole-cell fatty acids were iso-C_{15:0}, iso-C_{17:1ω10c} and iso-C_{17:0}. Strain RE35E1^T was related to representatives of the genera *Desulfotomaculum* and *Sporotomaculum*, the closest relatives being *Desulfotomaculum arcticum* DSM 17038^T (96.3 % 16S rRNA gene sequence similarity) and *Sporotomaculum hydroxybenzoicum* DSM 5475^T (92.0 % similarity). Strain RE35E1^T represents a novel species, for which the name *Desulfotomaculum alcoholivorax* sp. nov. is proposed. The type strain is RE35E1^T (=DSM 16058^T=JCM 14019^T).

The genus *Desulfotomaculum* constitutes a group of rod-shaped, endospore-forming, sulfate-reducing bacteria. The genus currently includes mesophilic, moderately thermophilic and thermophilic species (Werkman & Weaver, 1927; Campbell & Postgate, 1965; Vandieken *et al.*, 2006). The previously described mesophilic (optimal temperature 25–40 °C) *Desulfotomaculum* species are *D. ruminis* (Campbell & Postgate, 1965), *D. acetoxidans* (Widdel & Pfennig, 1977), *D. sapomandens* (Cord-Ruwisch & Garcia, 1985), *D. aeronauticum* (Hagenauer *et al.*, 1997), *D. halophilum* (Tardy-Jacquenod *et al.*, 1998) and *D. gibsoniae* (Kuever *et al.*, 1999). The thermophilic (optimal temperature 50–70 °C) species are *D. alkaliphilum* (Pikuta *et al.*, 2000), *D. australicum* (Love *et al.*, 1993), *D. carboxydovorans* (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. 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), *D. thermosapovorans* (Fardeau *et al.*, 1995) and *D. thermo-subterraneum* (Kaksonen *et al.*, 2006b). Additionally, the reclassification of '*Desulfotomaculum nigrificans* subsp. *salinus*' as a novel species ('*Desulfotomaculum salinum*') has been proposed (Nazina *et al.*, 2005). Currently, *Desulfotomaculum arcticum* is the only sulfate-reducing member of the genus that has an optimal temperature above 40 °C and below 50 °C (Vandieken *et al.*, 2006). Recently, we reported the isolation of sulfate-reducers from fluidized-bed reactors (FBRs) treating acidic metal- and sulfate-containing wastewater (Kaksonen *et al.*, 2004c). This paper describes one of those isolates, strain RE35E1^T, a novel, moderately thermophilic, spore-forming, sulfate-reducing bacterium.

Abbreviation: FBR, fluidized-bed reactor.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RE35E1^T is AY548778.

Strain RE35E1^T was isolated from a sulfidogenic laboratory-scale FBR after continuous-flow operation at 35 °C for more than 600 days (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 have been described previously (Kaksonen *et al.*, 2003a, b, 2004a, b, c). For comparison, *Desulfotomaculum arcticum* DSM 17038^T, obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), was used as a reference strain.

The isolation of strain RE35E1^T was performed at 35 °C using ethanol-containing modified Postgate growth medium (pH 7.0–7.5) (Kaksonen *et al.*, 2004c) and anaerobic roll tubes solidified with 1.5 % agar. For DNA isolation and chemotaxonomic analyses (including whole-cell fatty acid determinations), the strain was cultured at 35 °C in modified DSM medium 641 containing pyruvate as an electron donor. The medium was supplemented with selenate/tungstate solution (DSM medium 385) at 1 ml l⁻¹; sodium dithionite (25 mg l⁻¹) was used as the reducing agent instead of Na₂S.

The isolate was examined using phase-contrast microscopy (Axioskop 2; Zeiss) and photomicrographs were obtained using the agar slide technique as described previously (Kaksonen *et al.*, 2004c). Cells were examined for flagella by using staining as described by Heimbrook *et al.* (1989) followed by light microscopy (Axiophot; 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 using both Gram staining and the KOH test (Griegersen, 1978).

The effects of temperature, pH and NaCl concentration on growth were determined as described previously (Kaksonen *et al.*, 2006a). The ability of the strain to utilize various electron donors (at 1–20 mM) was tested in a medium containing 20 mM sulfate (as Na₂SO₄). The medium was as the modified 641 medium described above, except that Na-thiosulfate and yeast extract were omitted and MgSO₄ was replaced with MgCl₆H₂O (1 g l⁻¹). The utilization of various electron acceptors (at 10 mM, and for sulfite also at 1 mM) was studied using ethanol (10 mM) as the electron donor. Amorphous Fe(III) oxyhydroxide was formed by neutralizing a FeCl₃ solution to pH 7 with NaOH. The cultures were incubated for 1–2 weeks. Electron-donor utilization was determined as bacterial growth (OD₆₆₀ in a Shimadzu UV-1601 spectrophotometer or an 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 (UV-1601; Shimadzu) with ferrozine

(Lovley & Phillips, 1986). Sulfate, sulfite, thiosulfate, nitrate and nitrite concentrations were determined by ion chromatography (DX-120; Dionex).

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 mass spectrometry (Kaksonen *et al.*, 2006b). Fatty acid 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 mixtures were separated in a gas chromatograph (5890 Series II; Hewlett Packard) as described previously (Kaksonen *et al.*, 2006b) and analysed using the Microbial Identification Standard software package, Sherlock (version 4.5) (Sasser, 1990).

The methods for the amplification and sequencing of the 16S rRNA gene were as described previously (Kaksonen *et al.*, 2004c). The DNA sequence chromatograms were analysed and single primer sequences were compiled using the BioEdit sequence-alignment editor (<http://www.ctu.edu.vn/~dvxe/Bioinformatic/Software/BioEdit.htm>). 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 RE35E1^T and related species was constructed using distance matrix and neighbour-joining methods in ARB. No correction factor was used for creating the tree. The robustness of the phylogeny was tested by bootstrap analysis with 1000 iterations. Genomic DNA for G+C content (mol%) determination was released by rupturing cells using a French pressure cell (Thermo Spectronic) and purified by chromatography 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 (mol%) of the resulting deoxyribonucleosides was

Fig. 1. Phase-contrast micrograph of strain RE35E1^T. Bar, 10 µm.

determined by using reversed-phase HPLC (Shimadzu) and was calculated from the ratio of deoxyguanosine and thymidine (Tamaoka & Komagata, 1984; Mesbah *et al.*, 1989).

The cells of strain RE35E1^T were straight rods 0.8–1.0 µm in diameter and 2–8 µm in length (Fig. 1). The strain formed spherical spores that were able to germinate after a heat shock. The spores were located centrally. The cells

Table 1. Characteristics of strain RE35E1^T compared with those of its closest relatives

Strains: 1, RE35E1^T (this study); 2, *D. arcticum* DSM 17038^T (Vandieken *et al.*, 2006); *D. sapomandens* DSM 3223^T (Cord-Ruwisch & Garcia, 1985); 4, *D. thermosapovorans* DSM 6562^T (Fardeau *et al.*, 1995); 5, *D. gibsoniae* DSM 7213^T (Kuever *et al.*, 1999); 6, *D. geothermicum* DSM 3669^T (Daumas *et al.*, 1988). All of the organisms shared the following characteristics: spore formation, spherical central or subterminal spores, sulfate (10 mM) reduction and oxidation of formate (10 mM), butyrate (10 mM), hexanoate (2 mM) and ethanol (10 mM) as electron donors. RE35E1^T utilized pentanoate, pyruvate, crotonate, propanol and butanol (each at 10 mM), pentanol, 3-methylbutyrate and 4-methylbutyrate (each at 5 mM), phenylacetate (3 mM), octanoate (2 mM) and nonanoate (0.5 mM) as electron donors. RE35E1^T did not utilize nitrate or nitrite (each at 10 mM) as an electron acceptor; it did not utilize tartrate (10 mM), citrate, malonate, ribose, arabinose, xylose, glucose, mannose, galactose, *myo*-inositol, sucrose, lactose, maltose, cellobiose, trehalose or glutamate (each at 10 mM), or glycine or alanine (each at 5 mM) as electron donors. The concentrations apply only to this study. +, Positive; –, negative; NR, not reported.

Characteristic	1	2	3	4	5	6
Gram stain	+	–	–	NR	–	–
Motility	+	–	+	+	+	+
Temperature range (°C)	33–51	26–46.5	20–43	35–60	20–42	37–56
Optimal temperature (°C)	44–46	44	38	50	35–37	54
pH range	6.0–7.5	6.8–7.5	6.3–8.5	NR	6–8	6.0–8.0
Optimal pH	6.4–7.3	7.1–7.5	7.0	7.2–7.5	6.9–7.2	7.2–7.4
NaCl range (%)	0–0.25	0.05–4.5	0–0.5	0–3.5	0–3.5	0.2–5.0
Optimal salinity (NaCl, %)	0	1–1.5	NR	1.5	NR	2.4–3.4
DNA G + C content (mol%)	48	48.9	48	51.2	54.8	50.4
Electron acceptors						
Sulfite (10 or 1 mM)	–/+*	+	+	+	+	+
Thiosulfate (10 mM)	+	+	+	+	+	NR
Sulfur (10 mM)	+	–	+	–	–	–
Fe(III) (10 mM)	–	–	NR	NR	NR	NR
Electron donors						
H ₂ + CO ₂ (80 %/ 20 %)	–	+	NR	+	+	+
Acetate (10 mM)	–	–	+	–	–	–
Propionate (10 mM)	–	+	–	–	+	+
Heptanoate (10 mM)	+	+†	+	+	NR	–
Decanoate (1 mM)	–	+†	+	NR	+	–
Dodecanoate (1 mM)	–	–†	+	+	NR	NR
Tetradecanoate (1 mM)	+	+†	+	NR	–	NR
Hexadecanoate (1 mM)	+	+†	+	+	–	+
Isobutyrate (10 mM)	+	+†	NR	–	+	NR
2-Methylbutyrate (10 mM)	+	+†	–	NR	+	NR
Benzoate (5 mM)	–	–†	+	–	+	–
4-Hydroxybenzoate (2 mM)	–	–†	+	NR	+	NR
Lactate (20 mM)	–	+	–	+	–	+
Malate (10 mM)	–	+	+	+	+	NR
Fumarate (10 mM)	–	+	+	+	+	NR
Succinate (10 mM)	–	+	+	–	+	NR
Methanol (10 mM)	–	+	NR	+	–	–
Fructose (10 mM)	–	–	–	–	–	+
Fermentation of:						
Pyruvate (10 mM)	+	+	–	+	+	NR
Lactate (20 mM)	–	–	NR	+	NR	NR

*Slight production of H₂S was observed with 1 mM sulfite, but not with 10 mM.

†Determined in this study.

were motile, having two or more flagella, and were Gram-positive (as determined from both Gram staining and the KOH test). The temperature, pH and NaCl (%) ranges for growth of strain RE35E1^T are as shown in Table 1. Strain RE35E1^T was able to use the following electron acceptors: sulfate, thiosulfate and elemental sulfur (each at 10 mM) and 1 mM (but not 10 mM) sulfite. RE35E1^T utilized various alcohols and carboxylic acids or their sodium salts, but not lactate, for example, as electron donors (Table 1). The strain fermented pyruvate.

The cell wall of strain RE35E1^T contained *meso*-diaminopimelic acid and the major respiratory isoprenoid quinone was menaquinone MK-7. The whole-cell fatty acid content of strain RE35E1^T is listed in Table 2. Significant proportions of iso-branched saturated fatty acids were found, the major fatty acids being iso-C_{15:0}, iso-C_{17:1}ω10*c* and iso-C_{17:0} (Table 2). The G+C content of the total DNA of strain RE35E1^T was 48 mol% (Table 1). Strain RE35E1^T was found to be related to representatives of the genera *Desulfotomaculum* and *Sporotomaculum* (Fig. 2), the closest relatives within these genera being *D. arcticum* DSM 17038^T (96.3% 16S rRNA gene sequence similarity) and *Sporotomaculum hydroxybenzoicum* DSM 5475^T (92.0% similarity). The 16S rRNA gene of RE35E1^T does not contain any long insertions that do not appear in the genes of the closest relatives.

Strain RE35E1^T clustered phylogenetically with members of the genera *Desulfotomaculum* and *Sporotomaculum*. The strain also exhibits many features typical of these two genera, such as rod-shaped, Gram-positive, spore-forming cells and the presence of MK-7 as the major menaquinone. Strain RE35E1^T and the members of the genus *Desulfotomaculum* are able to perform dissimilatory sulfate reduction, unlike the members of the genus *Sporotomaculum*. RE35E1^T differs from its closest relative, *D. arcticum* DSM 17038^T, in being able to reduce S⁰ and unable to oxidize propionate, decanoate, lactate, malate, fumarate, succinate or methanol. Moreover, 10 mM sulfite was not reduced by RE35E1^T. On the basis of the phenotypic and genomic differences described here, strain RE35E1^T can be assigned to a novel species of the genus *Desulfotomaculum*, for which the name *Desulfotomaculum alcoholivorax* sp. nov. is proposed.

Description of *Desulfotomaculum alcoholivorax* sp. nov.

Desulfotomaculum alcoholivorax (al.co.ho.li.vo'rax. N.L. n. *alcohol* alcohol; L. adj. *vorax* voracious; N.L. neut. adj. *alcoholivorax* alcohol-devouring).

Cells are motile, Gram-positive, spore-forming rods (0.8–1.0 × 2–8 µm). Growth occurs at 33–51 °C (optimum 44–46 °C), pH 6.0–7.5 (optimum pH 6.4–7.3) and NaCl concentrations of 0–0.25% (optimum 0%). Sulfate, thiosulfate, elemental sulfur and sulfite are used as electron acceptors. Alcohols and carboxylic acids (Table 1) are used

Table 2. Whole-cell fatty acid content of strain RE35E1^T in comparison with some reference strains

Strains: 1, RE35E1^T (this study); 2, *D. arcticum* DSM 17038^T (this study); 3, *D. sapomandens* DSM 3223^T; 4, *D. thermosapovorans* DSM 6562^T; 5, *D. gibsoniae* DSM 7213^T; 6, *D. geothermicum* DSM 3669^T (Kuever *et al.*, 1999). ECL, Equivalent chain-length; DMA, dimethyl acetal; c, cyclopropane; ND, not detected.

ECL	Fatty acid	Content (%) in strains:					
		1	2	3	4	5	6
11	C _{11:0}	ND	ND	ND	0.5	1.9	0.9
13.62	iso-C _{14:0}	ND	ND	ND	1.4	1.6	ND
14	C _{14:0}	1.3	1.0	1.0	2.1	2.6	7.9
14.41	iso-C _{15:1}	ND	3.9	0.5	1.0	ND	6.5
14.48	C _{14:0} DMA	0.1	ND	ND	ND	ND	ND
14.63	iso-C _{15:0}	40.0	37.5	2.0	23.1	5.4	32.8
14.71	anteiso-C _{15:0}	0.4	0.4	ND	ND	ND	1.4
14.79	C _{15:1} ω8 <i>c</i>	0.3	ND	0.6	ND	2.7	ND
14.95	C _{16:0} aldehyde	ND	ND	ND	0.6	2.2	ND
15	C _{15:0}	1.3	0.6	0.8	1.4	ND	1.5
15.11	iso-C _{15:0} DMA	0.7	0.7	ND	1.2	ND	2.2
15.48	C _{15:0} DMA	ND	ND	5.4	ND	1.7	ND
15.63	iso-C _{16:0}	0.7	1.1	3.5	2.6	ND	1.6
15.77	C _{16:1} ω9 <i>c</i>	ND	ND	6.5	0.7	3.3	ND
15.82	C _{16:1} ω7 <i>c</i>	ND	2.0	21.3	3.6	15.1	4.0
15.91	C _{16:1} ω5 <i>c</i>	ND	ND	6.2	ND	3.6	ND
16	C _{16:0}	3.9	5.8	10.2	15.7	9.6	10.1
16.29	C _{16:1} ω7 <i>c</i> DMA	0.2	0.7	4.2	1.1	4.6	ND
16.42	iso-C _{17:1} ω10 <i>c</i>	28.9	22.7	4.6	5.0	4.8	7.2
16.48	C _{16:0} DMA	ND	1.7	1.8	3.9	6.7	5.0
16.63	iso-C _{17:0}	9.4	5.7	0.7	8.9	ND	2.4
16.72	anteiso-C _{17:0}	0.2	ND	ND	ND	ND	ND
16.79	C _{17:1} ω8 <i>c</i>	0.3	ND	1.0	ND	ND	ND
16.86	C _{17:1} ω6 <i>c</i>	ND	ND	1.9	ND	ND	ND
16.88	C _{17:0} c	5.3	9.9	ND	5.8	2.7	3.7
17	C _{17:0}	ND	ND	0.5	1.4	ND	ND
17.11	iso-C _{17:0} DMA	0.9	1.1	ND	7.5	ND	ND
17.47	C _{17:0} DMA	ND	ND	1.7	ND	ND	ND
17.72	C _{18:2} ω6,9 <i>c</i>	0.2	0.6	ND	ND	ND	ND
17.77	C _{18:1} ω9 <i>c</i>	1.0	2.2	3.4	ND	2.0	ND
17.82	C _{18:1} ω7 <i>c</i>	0.2	ND	6.9	0.9	3.3	ND
17.92	C _{18:1} ω5 <i>c</i>	ND	ND	2.5	ND	2.8	ND
18	C _{18:0}	0.4	2.6	4.4	3.2	5.6	ND
18.29	C _{18:1} ω7 <i>c</i> DMA	ND	ND	1.4	ND	1.5	ND
	Total unidentified	4.2	4.4	1.7	8.5	19.2	13.0

as electron donors in the presence of sulfate. Fermentative growth occurs on pyruvate. The cell wall contains *meso*-diaminopimelic acid. The major isoprenoid quinone is MK-7. The major whole-cell fatty acids are iso-C_{15:0}, iso-C_{17:0}ω10*c* and iso-C_{17:0}. The DNA G+C content is 48 mol%.

The type strain, RE35E1^T (=DSM 16058^T=JCM 14019^T), was isolated from a sulfidogenic FBR treating acidic metal- and sulfate-containing wastewater.

Fig. 2. Phylogenetic tree, generated using distance-matrix and neighbour-joining methods, based on 16S rRNA gene sequences of strain RE35E1^T (1429 bp between *Escherichia coli* positions 28 and 1459) and related taxa. *Desulfotomaculum dehalogenans* DSM 9161^T (L28946) was used as outgroup (not shown). Numbers at nodes represent bootstrap percentages (based on 1000 samplings). Bar, 0.05 nucleotide changes per nucleotide.

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