Desulfurispirillum indicum sp. nov., a selenate- and selenite-respiring bacterium isolated from an estuarine canal

Ines Rauschenbach,¹ Priya Narasingarao² and Max M. Häggblom¹

Correspondence

Max M. Häggblom

haggblom@AESOP.Rutgers.edu

¹Rutgers University, Department of Biochemistry and Microbiology, School of Environmental and Biological Sciences, 76 Lipman Drive, New Brunswick, NJ 08901, USA

²Marine Biology Research Division, Scripps Institution of Oceanography, University of California at San Diego, La Jolla, CA 92093, USA

Strain S5^T, a novel bacterium that was isolated for its capability to respire selenate to elemental selenium, is described. In addition to selenate respiration, it was also capable of dissimilatory selenite, arsenate and nitrate reduction with short-chain organic acids such as pyruvate, lactate and acetate as the carbon sources and electron donors. The isolate was unable to grow fermentatively. Strain S5^T was isolated from sediment of an estuarine canal in Chennai, India. Phylogenetic analysis of the 16S rRNA gene of this novel isolate revealed that it belonged to the family *Chrysiogenaceae* with sequence similarities of 92 and 98 %, respectively, with the type strains of *Chrysiogenes arsenatis* and *Desulfurispirillum alkaliphilum*, its closest known relatives. Strain S5^T and *D. alkaliphilum* were closely related in terms of their 16S rRNA gene phylogeny; however, they varied greatly in their genomic DNA G + C content (56 mol% versus 45 mol%) and cellular fatty acid compositions, as well as in many metabolic capabilities. Strain S5^T represents a novel species for which the name *Desulfurispirillum indicum* sp. nov. is proposed; the type strain is S5^T (=DSM 22839^T =ATCC BAA-1389^T).

Selenium (Se), a naturally occurring element, is essential for most organisms (Conde & Sanz Alaejos, 1997; Zannoni *et al.*, 2007). Although considered to be a trace element, its toxicity is highly dependent on its oxidation state and speciation. Se occurs in a variety of oxidation states, water-soluble selenate (SeO₄²⁻) and selenite (SeO₃²⁻), insoluble elemental selenium (Se⁰) and gaseous selenide. Even though redox transformations of selenium oxyanions can occur abiotically (Myneni *et al.*, 1997; Kessi & Hanselmann, 2004), the global selenium cycle is greatly influenced by micro-organisms (Stolz *et al.*, 2006). Various microbial species have been found to not only reduce selenate and/or selenite to elemental Se, but to also respire selenate oxyanions in order to gain cellular energy (Oremland *et al.*, 1989; Steinberg & Oremland, 1990; Narasingarao & Häggblom, 2007b; Stolz & Oremland, 1999; Stolz *et al.*, 2006). These selenium oxyanion-respiring micro-organisms display a broad phylogenetic diversity and can be found among the phyla 'Proteobacteria', 'Deferribacteres', 'Firmicutes' and 'Actinobacteria' (Narasingarao & Häggblom, 2006, 2007a, b; Switzer Blum *et al.*, 2001; von Wintzingerode *et al.*, 2001). The ability of bacteria to use selenate and selenite and other alternative terminal electron acceptors, such as arsenate,

nitrate, sulfate and/or iron, plays a major role in the oxidation of organic carbon in sediments and contributes to the biogeochemical cycling of these elements in nature. In this study, strain S5^T, a novel member of the family *Chrysiogenetes*, is described.

Strain S5^T is a strictly anaerobic, dissimilatory selenate- and selenite-respiring bacterium isolated from an enrichment culture of sediments collected in an estuarine channel in Chepauk in Chennai, located in southern India (Narasingarao & Häggblom, 2007a). Strain S5^T was isolated after sequential transfers into fresh anaerobic medium with selenate as electron acceptor and pyruvate as carbon source and electron donor. Purity of the culture was verified by microscopic analysis and by terminal restriction fragment length polymorphism analysis as described by Fennell *et al.* (2004). Strain S5^T was maintained in an anaerobic minimal salts medium (Fennell *et al.*, 2004) with 20 mM pyruvate as the carbon source and 10 mM selenate as the electron donor under a headspace of N₂.

Genomic DNA was extracted by phenol/chloroform extraction as described by Kerkhof & Ward (1993). The 16S rRNA gene was selectively amplified by PCR from the genomic DNA as described by Narasingarao & Häggblom (2006). PCR products were sequenced by Genewiz Inc, South Plainfield, NJ, USA. Sequence data were compiled

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain S5^T is DQ991965.

using Contig Express (Vector NTI Suite; Informax). The 16S rRNA gene sequences of related micro-organisms were identified by a BLAST search (Altschul *et al.*, 1997) and downloaded from GenBank. Phylogenetic and molecular evolutionary analyses of all sequences were conducted using MEGA4 (Tamura *et al.*, 2007), which utilizes CLUSTAL W (Chenna *et al.*, 2003) to align sequences. Neighbour-joining and maximum-parsimony phylogenetic trees with 1000 bootstrap replications were reconstructed using the program package MEGA4 with the maximum composite likelihood model for nucleotide substitutions. Comparative 16S rRNA gene sequence analysis placed strain S5^T within the family *Chrysiogenaceae*, which, at the time of writing, has only two known members. *Chrysiogenes arsenatis*, the first species to be identified, was isolated for its ability to respire arsenate to arsenite coupled to oxidation of acetate and other short-chain organic acids (Macy *et al.*, 1996). *Desulfurispirillum alkaliphilum*, the second organism, is capable of reducing elemental sulfur to sulfide and nitrate to ammonium (Sorokin *et al.*, 2007). The type strain of *D. alkaliphilum* only shared 91 % 16S rRNA gene similarity with that of *C. arsenatis* and so it was placed in a new genus, *Desulfurispirillum* (Sorokin *et al.*, 2007). The 16S rRNA gene sequence of strain S5^T shared similarities of 94 and 98 %, respectively, with those of the type strains of *C. arsenatis* and *D. alkaliphilum*. Phylogenetic analysis with unambiguously aligned 16S rRNA gene sequences of strain S5^T and other related strains (Fig. 1) indicated that strain S5^T belongs to the family *Chrysiogenaceae* and is most closely related to *D. alkaliphilum* and *C. arsenatis*. This was supported by high bootstrap values in both the neighbour-joining and maximum-parsimony analyses. All further physiological and molecular characterizations of strain S5^T were made in comparison to the two above-named organisms.

Strain S5^T was grown with selenate (10 mM) or nitrate (10 mM) as electron acceptors and pyruvate (20 mM) as carbon source to examine cell morphology via transmission electron microscopy (Narasimarao & Häggblom, 2006) and phase-contrast microscopy. Strain S5^T was a Gram-negative, slender, spiral-shaped bacterium, approximately 2–7 µm long and 0.10–0.15 µm in diameter (Table

1, Fig. 2). Cells grown in the presence of selenate produced a bright red precipitate that accumulated in the bottom of the culture tube. The precipitate was further evaluated and confirmed as elemental selenium by X-ray absorption near edge structure analysis (XANES; Narasingarao & Häggblom, 2007a). Strain S5^T grew in both liquid medium and soft agar shake tubes. It did not form distinct colonies when grown anaerobically in soft agar (0.4 % Noble agar; Difco) shake tubes with 10 mM selenate or 10 mM selenite. The agar first turned completely red and then black over a period of 14 days as the selenium oxyanions were reduced to elemental selenium.

Rapid motility was observed by microscopy; therefore, a flagella stain was applied to visualize the presence of flagella (Kodaka *et al.*, 1982). One drop of liquid culture of strain S5^T was applied to a clean glass slide and allowed to air dry completely. The flagella stain was then applied for 10 min before being washed off with deionized water. Bipolar flagella were observed that extended about 4–6 µm at either end of the cell and allowed for rapid movement of strain S5^T (Fig. 2b).

A wide range of electron donors and acceptors were used to characterize the metabolic capabilities of strain S5^T (Table 1). Selenium oxyanions, nitrate and arsenate were analysed using ion chromatography (DX 120; Dionex) as described previously (Knight *et al.*, 2002). The organic acids pyruvate, lactate and acetate were measured using HPLC (Shimadzu) equipped with a C₁₈ column (250 × 4.6 mm, 5 µm particle size, Spherclone; Phenomenex) as described by Narasingarao & Häggblom (2007a). Three consecutive transfers with no growth were scored as negative for substrate utilization.

In addition to respiring selenate to elemental selenium with pyruvate as the carbon source, strain S5^T also respired selenite, arsenate and nitrate (to ammonium). Pyruvate, acetate and lactate were its preferred carbon sources. Neither *C. arsenatis* nor *D. alkaliphilum* respired selenate and selenite and *D. alkaliphilum* did not respire arsenate (Table 1). In addition to pyruvate, lactate and acetate, both *C. arsenatis* and *D. alkaliphilum* were also able to use fumarate as a carbon source (Macy *et al.*, 1996; Sorokin

Fig. 1. Phylogenetic tree showing strain S5^T with closely related strains. The tree was reconstructed with aligned 16S rRNA gene sequences using the neighbour-joining method with global gap removal algorithm. The evolutionary distances were computed using the maximum composite likelihood method (1251 positions in analysis). Bootstrap values above 50 % are indicated. The 16S rRNA gene sequence of *Thermotoga maritima* FJSS3-B.1 was used as outgroup. Bar, 0.02 substitutions per nucleotide position.

Table 1. Differential physiological and metabolic characteristics of strain S5^T and closely related species

Taxa: 1, strain S5^T; 2, *Desulfurispirillum alkaliphilum* SR 1^T; 3, *Chrysiogenes arsenatis* BAL1^T (data from Macy *et al.*, 1996). All data are from this study, except where indicated. For strain S5^T, electron acceptors were tested with 20 mM pyruvate as electron donor and electron donors were tested with 10 mM nitrate as electron acceptor. +, Positive; –, negative; ND, not determined. All strains tested were Gram-negative and strictly anaerobic. All tested positive for reduction of nitrate and oxidation of the short-chain organic acids acetate, lactate and pyruvate. All strains were negative for reduction of oxygen, chlorate, citrate and fumarate. All strains were negative for reduction of Fe³⁺, sulfate and S₂O₃²⁻/SO₃²⁻ and oxidation of formate (data from Sorokin *et al.*, 2007).

Characteristics	1	2	3
Morphology	Spirillum	Spirillum	Vibrio
Motility	Highly motile	Highly motile	Motile
Cell size (µm):			
Length	2–7	2–5	1–2
Diameter	0.10–0.15	0.15–0.20	0.50–0.75
DNA G + C content (mol%)	56.1	44.8 ± 0.5*	49
Fermentation	–	+	–
Optimum growth temperature (°C)	28	30	25–30
Growth pH:			
Range	6.8–7.6	8.0–10.0	7.4–7.8
Optimum	7.4	9.0	ND
NaCl concentration (M):			
Range	0.10–0.75	0.1–2.5*	ND
Optimum	0.4	0.4	0.02†
Electron donors:			
Succinate	–	–	+
Fumarate	–	+	+
HS [–]	–	+*	ND
H ₂	–	+	–
Malate	–	–	+
Propionate	–	+	ND
Electron acceptors:			
Arsenate	+	–	+
Chromate	–	+	–
DMSO	–	–	ND
Nitrite	–	+	+
Selenite	+	–	–
Selenate	+	–	–
Sulfur	–	+	–

*Data from Sorokin *et al.* (2007).

†Calculated from Macy *et al.* (1989).

et al., 2007). In addition, *D. alkaliphilum* was also capable of using HS[–], H₂ and propionate as electron donors and carbon sources (Table 1) (Sorokin *et al.*, 2007).

To determine optimum pH, strain S5^T was grown with 10 mM nitrate as electron acceptor and 20 mM pyruvate as carbon source in anaerobic growth medium. The pH of the medium was adjusted with HCl and NaOH (pH 5–9). Strain S5^T grew at pH 6.8–7.6, with optimum growth at pH 7.4, in contrast to *D. alkaliphilum*, which grew at pH 8.0–10.0 (Table 1). Growth of strain S5^T at different NaCl concentrations (0–2.5 M) and temperatures (4–37 °C) was determined by growing cells in anaerobic

medium (pH 7.4) with 10 mM nitrate and 20 mM pyruvate. The isolate grew between 20 and 37 °C, with optimum growth at 28 °C. It grew in NaCl concentrations of 0.10–0.75 M, with optimum growth at 0.40 M NaCl.

The genomic DNA G+C content of strain S5^T was determined using a modified method of Mesbah *et al.* (1989). A Synergy 4U Fusion-RP 80A C18 reverse-phase column (Phenomenex) was used in an Agilent HPLC 1100 system. The mobile phase consisted of two eluents, 20 mM ammonium acetate (pH 4.5, eluent A) and acetonitrile (eluent B), at a flow rate of 1 ml min^{–1}. A gradient of eluent A from 95 to 60 % was established over a period of

Fig. 2. Cell morphology of strain S5^T. (a) Transmission electron micrographs of 2-week-old cultures with intracellular deposits of elemental selenium granules; bar, 0.5 µm. (b) Phase-contrast microphotograph of cells grown with nitrate (10 mM) and pyruvate (20 mM) and stained with flagella stain; bar, 5 µm.

10 min. Nucleosides were detected at a wavelength of 260 nm. Salmon sperm DNA was used for calibration with *Sedimenticola selenatireducens* AK4OH1^T as control. The G+C content of strain S5^T was 56 mol%, which differed greatly from those of *C. arsenatis* (49 mol%) and *D. alkaliphilum* (44.8 mol%), thus supporting the classification of strain S5^T as a representative of a novel species of *Desulfurispirillum* (Table 1).

The fatty acid content of strain S5^T was analysed using cells grown at 28 °C with 20 mM pyruvate and 10 mM nitrate. *D. alkaliphilum* was grown at 28 °C with 20 mM acetate as electron donor and 10 mM nitrate as electron acceptor (pH 9). Cellular fatty acids were methylated and analysed as described previously (Narasimharao & Häggblom, 2006) using an HP 5890 series II GC (Hewlett Packard) with the Sherlock Microbial Identification system (MIDI). The identities of fatty acid methyl esters detected by MIDI were further confirmed by GC-MS using an Agilent GC Series 6890 GC-MS. The two most common fatty acids found in strain S5^T were C_{18:1}ω7c (38.5%) and C_{16:0} (20.6%). Overall, unsaturated fatty acids, such as C_{18:1}ω7c, C_{16:1}ω7c and C_{16:1}ω5c made up about 56% of all fatty acids in strain S5^T. The predominant straight-chain saturated fatty acids were C_{12:0}, C_{16:0} and C_{18:0}, which made up about 25% of all fatty acids (Table 2). Strain S5^T also contained 3-hydroxy fatty acids, mainly C_{12:0} 3-OH and C_{12:1} 3-OH, which are indicative of fatty acids contained in the lipopolysaccharides of the outer

Table 2. Cellular fatty acid compositions (%) of strain S5^T and *Desulfurispirillum alkaliphilum* SR 1^T

Strains: 1, S5^T; 2, *Desulfurispirillum alkaliphilum* SR 1^T. Strain S5^T was grown at 28 °C with 20 mM pyruvate as electron donor and 10 mM nitrate as electron acceptor. *D. alkaliphilum* was grown at 28 °C with 20 mM acetate as electron donor and 10 mM nitrate as electron acceptor. ND, Not detected.

Fatty acid	1	2
C _{12:0}	1.4 ± 0.8	3.2 ± 0.3
C _{12:1} 3-OH	4.1 ± 1.8	1.7 ± 0.1
C _{12:0} 3-OH	1.4 ± 0.7	0.4 ± 0.1
C _{14:0}	ND	0.7 ± 0.4
iso-C _{15:0} 3-OH	0.5 ± 0.7	ND
C _{15:0} 2-OH	0.5 ± 0.7	ND
C _{16:1} ω9c	1.1 ± 0.2	1.3 ± 0.2
C _{16:1} ω7c	6.7 ± 0.8	4.8 ± 0.4
C _{16:1} ω5c	4.3 ± 0.6	3.5 ± 0.2
C _{16:0}	20.6 ± 3.6	14.9 ± 0.9
iso-C _{17:1} ω5c	ND	8.9 ± 0.6
C _{17:1} ω8c	0.9 ± 0.4	ND
C _{17:1} ω6c	2.1 ± 0.1	1.4 ± 0.2
iso-C _{17:0} 3-OH	7.8 ± 1.1	ND
C _{18:1} ω9c	ND	0.6 ± 0.1
C _{18:1} ω7c	38.5 ± 10.1	39.6 ± 3.3
C _{18:1} ω5c	2.9 ± 0.7	2.1 ± 0.3
C _{18:0}	3.2 ± 1.5	3.1 ± 0.4
iso-C _{18:1} H	1.0 ± 0	ND
iso-C _{18:1} ω8c H	3.9 ± 1.6	ND

membranes of Gram-negative bacteria. A fair amount of iso-C_{17:0} 3-OH (7.8%) was also detected. In contrast, the fatty acid composition of its closest relative, *D. alkaliphilum*, grown under as identical conditions as possible, was distinctly different (Table 2). Overall, the fatty acid composition of strain S5^T was made up of 14.3% hydroxy fatty acids compared to only 2.1% detected in *D. alkaliphilum*. In strain S5^T and *D. alkaliphilum*, the most prevalent fatty acids were C_{18:1}ω7c and C_{16:0}; however, the abundance of C_{16:0} differed (15% in *D. alkaliphilum* vs 21% in strain S5^T). Straight-chain saturated fatty acids (C_{12:0}, C_{16:0}, C_{14:0} and C_{18:0}) made up 22% of all fatty acids. The fatty acid composition determined for *D. alkaliphilum* differed slightly from that reported previously by Sorokin *et al.* (2007), probably due to the different growth conditions.

Description of *Desulfurispirillum indicum* sp. nov.

Desulfurispirillum indicum (in'di.cum. L. neut. adj. *indicum* of India, Indian).

Gram-negative, highly motile, spiral-shaped bacterium, approximately 2–7 µm long and 0.10–0.15 µm in diameter. Strictly anaerobic. Non-fermenting. Respires selenate to elemental selenium coupled to utilization of short-chain organic acids. Capable of selenite, nitrate and arsenate respiration. Mesophilic, with a maximum growth temper-

ature of 37 °C (optimum at 28 °C). Tolerates NaCl concentrations of 0.10–0.75 M. The predominant cellular fatty acids are C_{12:1} 3-OH, C_{16:0}, C_{16:1} ω7c, iso-C_{17:0} 3-OH and C_{18:1} ω7c. Groups within the family *Chrysiogenaceae*.

The type strain is S5^T (=DSM 22839^T =ATCC BAA-1389^T), isolated for its ability to respire selenate to elemental selenium from an estuarine canal in Chepauk in Chennai, located in southern India. The genomic DNA G + C content of the type strain is 56 mol%.

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