Sulfurospirillum cavolei sp. nov., a facultatively anaerobic sulfur-reducing bacterium isolated from an underground crude oil storage cavity

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A novel facultatively anaerobic sulfur-reducing bacterium, designated strain Phe91^T, was isolated from petroleum-contaminated groundwater in an underground crude oil storage cavity at Kuji in Iwate, Japan. Cells of strain Phe91^T were slightly curved rods with single polar flagella. Optimum growth was observed at pH 7.0 and 30 °C. The novel strain utilized elemental sulfur, thiosulfate, sulfite, dithionite, arsenate, nitrate and DMSO as electron acceptors with lactate as an energy and carbon source, but nitrite was not utilized. Microaerophilic growth was also observed. Fumarate, pyruvate, lactate, malate, succinate, hydrogen (with acetate as a carbon source) and formate (with acetate) could serve as electron donors. Fumarate, pyruvate and malate were fermented. The DNA G + C content was 42.7 mol%. On the basis of 16S rRNA gene sequence phylogeny, strain Phe91^T was affiliated with the genus *Sulfurospirillum* in the class *Epsilonproteobacteria* and was most closely related to *Sulfurospirillum deleyianum* (sequence similarity 97 %). However, the DNA–DNA hybridization value between strain Phe91^T and *S. deleyianum* was only 14 %. Based on the physiological and phylogenetic data, Phe91^T should be classified as a representative of a novel species in the genus *Sulfurospirillum*; the name *Sulfurospirillum cavolei* sp. nov. is proposed, with Phe91^T (=JCM 13918^T = DSM 18149^T) as the type strain.

Our previous study detected a number of novel 16S rRNA gene sequences of uncultured bacteria from oil-contaminated groundwater in an underground oil storage cavity at Kuji in Iwate, Japan (Watanabe *et al.*, 2000). Among them, several sequences, particularly those affiliated to the class *Epsilonproteobacteria*, were distantly related to cultured strains and their physiology is of microbiological interest. One such sequence, represented by clone 1018, was most closely related to *Sulfurospirillum deleyianum* (97 % 16S rRNA gene sequence similarity).

At present, the genus *Sulfurospirillum* includes six species with validly published names: *S. deleyianum* (the type species), a sulfur-reducing bacterium isolated from anoxic mud of a forest pond (Schumacher *et al.*, 1992); *Sulfurospirillum multivorans*, isolated from activated sludge and able to reduce tetrachloroethene to *cis*-dichloroethene (Scholz-Muramatsu *et al.*, 1995); *Sulfurospirillum arcachonense*, a microaerophilic sulfur-reducing bacterium isolated from oxidized marine surface sediment (Finster *et al.*, 1997); *Sulfurospirillum barnesii*, isolated from

selenium-contaminated freshwater marsh and able to reduce selenate to elemental selenium (Stolz *et al.*, 1999); *Sulfurospirillum arsenophilum*, isolated from arsenic-contaminated freshwater sediments and able to reduce arsenate to arsenite (Stolz *et al.*, 1999) and *Sulfurospirillum halorespirans*, isolated from anaerobic soil polluted with chlorinated aliphatic compounds and able to reduce tetrachloroethene to *cis*-dichloroethene (Luijten *et al.*, 2003). One further species, '*Sulfurospirillum carboxydovorans*', isolated from marine methane seep and able to couple the oxidation of CO to the reduction of elemental sulfur, DMSO and thiosulfate (Jensen & Finster, 2005), has also been reported recently. In the present study, strain Phe91^T, which is phylogenetically identical to environmental clone 1018, was isolated and characterized. Physiological and taxonomic data indicated that this strain represents a novel species in the genus *Sulfurospirillum*.

A bicarbonate-buffered inorganic medium was used for enrichment cultivation. This medium contained (l⁻¹): 0.2 g KH₂PO₄, 0.2 g NH₄Cl, 0.1 g CaCl₂·2H₂O, 0.4 g MgCl₂·6H₂O and 0.2 g NaNO₃. After the medium was autoclaved, the following sterile solutions were added to 1 l of the medium under an N₂/CO₂ (80 : 20, v/v) atmosphere:

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Phe91^T is AB246781.

1 ml trace element solution SL-7 (DSM 1283 medium), 1 ml vitamin solution (DSM 148 medium), 1 ml vitamin B₁₂ solution (50 mg cyanocobalamine l⁻¹), 1 ml selenite-tungstate solution (Widdel & Bak, 1992), 10 ml NaHCO₃ solution (1.5 M) and 10 ml resazurin solution (0.2 g l⁻¹). Na₂S·9H₂O (2 mM) was added as a reducing agent. The medium (30 ml) was transferred into 50 ml vials sealed with Teflon-coated butyl rubber septa and crimped aluminium caps. The headspace consisted of N₂/CO₂ (80:20, v/v). Agar plates used for isolation of bacteria contained 1.5% Bactoagar (Difco) and a phosphate-buffered inorganic medium containing (l⁻¹): 0.53 g KH₂PO₄, 0.2 g NH₄Cl, 0.4 g MgCl₂·6H₂O and 0.85 g NaNO₃. The agar plate medium also contained (l⁻¹): 1 ml trace element solution SL-7, 1 ml vitamin solution, 1 ml vitamin B₁₂ solution, 1 ml selenite-tungstate solution, 10 ml CaCl₂·2H₂O solution (10 g l⁻¹), 10 ml K₂HPO₄ solution (106 g l⁻¹), 10 ml resazurin solution and 15 ml 4% TiCl₃ solution (a reducing agent; the 4% TiCl₃ solution was prepared by 5 × dilution of 20% TiCl₃ solution; Wako Chemicals). The pH was adjusted to 7.0 using a saturated Na₂CO₃ solution and the solution was autoclaved under an N₂ atmosphere. Agar plates were overlaid with 0.8% agarose containing the phosphate-buffered inorganic medium and 1 mM phenanthrene as described by Bogardt & Hemmingsen (1992). Agar plates were incubated under an oxygen-free N₂ atmosphere. Routine cultivation was conducted without shaking at 30 °C in a 50 ml vial under an oxygen-free N₂ atmosphere. A modified phosphate-buffered inorganic medium was used for routine cultivation. The medium contained (l⁻¹): 0.46 g NH₄H₂PO₄, 0.49 g MgSO₄·6H₂O, 1 ml trace element solution SL-7, 1 ml vitamin solution, 1 ml vitamin B₁₂ solution, 1 ml selenite-tungstate solution, 1 ml CaCl₂·2H₂O solution, 10 ml K₂HPO₄ solution and 1 ml resazurin solution. Pyruvate (10 mM) was used as a carbon source. Dithionite (1 mM) was used as a reducing agent unless otherwise stated; it could also serve as electron acceptor. For monitoring growth, cell concentrations were determined by direct counting of 4,6-diamidino-2-phenylindole-stained cells under an epifluorescence microscope as described previously (Kodama & Watanabe, 2003). Cells of Phe91^T were stored at -80 °C in the above-described liquid medium supplemented with 15% (v/v) glycerol.

Cell morphology was examined by transmission and scanning electron microscopy (Beveridge *et al.*, 1994). Motility was checked by phase-contrast microscopy. Gram staining was conducted according to standard procedures (Smibert & Krieg, 1994). Effects of temperature, pH and salinity (NaCl concentration) were examined using the phosphate-buffered inorganic medium (without NaNO₃) supplemented with pyruvate (5 mM) as a fermentation substrate. The optimum pH was determined using a buffer system as described by Gevertz *et al.* (2000). The DNA G + C content was determined by HPLC according to Katayama-Fujimura *et al.* (1984). DNA-DNA hybridization was carried out as described by Ezaki *et al.* (1989). Phylogenetic analysis based on 16S rRNA gene sequence analysis was conducted as

described previously (Watanabe *et al.*, 2000). CLUSTAL W version 1.7 (Thompson *et al.*, 1994) was used to align the sequences and the alignments were refined by visual inspection; secondary structures were considered for the refinement (Gutell, 1994). The phylogenetic dendrogram was constructed using the program NJPLOT in CLUSTAL W version 1.7.

For isolation of bacteria, 30 ml enrichment medium was inoculated with 3 ml oil-contaminated groundwater sampled in July 2002 from the TK101 underground crude oil storage cavity at Kuji. It was supplemented with 0.3 ml phenanthrene solution (10 000 mg phenanthrene per litre of 2,2,4,4,6,6,8,8-heptamethylnonane) and 2 mM nitrate and cultivated for 8 months at 25 °C without shaking. The resultant culture was spread on agar plates and incubated for 2 months. Colonies were analysed by direct PCR and sequencing of their 16S rRNA genes. The 16S rRNA gene sequence of one colony, designated Phe91^T, showed 100% similarity to that of the environmental clone 1018.

Cells of strain Phe91^T were Gram-negative, slightly curved rods, 0.4–0.5 µm wide and 1.5–5.0 µm long (Fig. 1). They were motile by single polar flagella (Fig. 1). Growth of strain Phe91^T was observed between 20 and 40 °C, with optimum growth at 30 °C. Strain Phe91^T grew at pH 6.0–8.0, with optimum growth at pH 7.0. Growth was inhibited in the presence of 1% NaCl or more. Optimum growth was observed in the absence of NaCl. The DNA G + C content of strain Phe91^T was 42.7 mol%. A phylogenetic dendrogram based on 16S rRNA gene sequences (Fig. 2) shows the relationship between strain Phe91^T and other strains in the class *Epsilonproteobacteria*. This analysis showed that Phe91^T was affiliated with the genus *Sulfurospirillum* and most closely related to *S. deleyianum* (97% gene sequence similarity). The DNA-DNA hybridization value between strain Phe91^T and *S. deleyianum* was 14%.

Fig. 1. Transmission electron micrographs of cells of strain Phe91^T. Bar, 3 µm.

Fig. 2. Phylogenetic dendrogram based on 16S rRNA gene sequence comparison showing the position of strain Phe91^T and related genera within the class *Epsilon-proteobacteria*. *Desulfovibrio desulfuricans* subsp. *desulfuricans* was used as the out-group. Accession numbers of the sequences retrieved from the databases are given in parentheses. Numbers at the branch nodes are bootstrap values (per 100 trials); only values greater than 50 are shown. Bar, 0.02 substitutions per site.

Since the growth of cells of strain Phe91^T on phenanthrene plus nitrate was negligible, other available substrates for growth were examined. Strain Phe91^T utilized the following electron donors (in routine cultivation medium with 10 mM nitrate as the electron acceptor and 4% TiCl₃ solution as the reducing agent): lactate (10 mM), fumarate (10 mM), pyruvate (10 mM), succinate (10 mM) and malate (5 mM). Formate (10 mM) and hydrogen (10%, v/v, in the headspace) were also utilized as electron donors when acetate was present as a carbon source. The novel strain did not use acetate (10 mM), butyrate (10 mM), citrate (5 mM), crotonic acid (5 mM), propionate (10 mM), butanol (10 mM), ethanol (10 mM) or propanol (10 mM). Strain Phe91^T utilized the following electron acceptors [in routine cultivation medium with 5 mM lactate as the electron donor and titanium (III) citrate solution as a reducing agent; Jones & Pickard, 1980]: elemental sulfur (1%, w/v), thiosulfate (5 mM), sulfite (5 mM), dithionite (5 mM), DMSO (5 mM), nitrate (5 mM), arsenate (5 mM) and oxygen (1%, v/v, in the headspace). It did not utilize nitrite (5 mM), sulfate (5 mM), selenate (5 mM) or perchloroethylene (0.2 mM). Nitrite was the final product of nitrate reduction. The novel strain could not grow on any substrate when the headspace of a bottle was filled with air. Strain Phe91^T grew fermentatively on fumarate, pyruvate and malate, but did not grow on lactate, succinate, propionate, glucose, ethanol or butyrate (all substrates at 10 mM). The substrate-utilization pattern of strain Phe91^T was compared with those of other species of the genus *Sulfurospirillum* (Table 1). Analysis revealed that the pattern of physiological traits of strain Phe91^T differed from those of the other recognized species of the genus *Sulfurospirillum*.

On the basis of morphological and physiological properties, together with DNA–DNA hybridization and 16S rRNA gene sequence comparison, strain Phe91^T is proposed as a representative of a novel species of the genus *Sulfurospirillum*, *Sulfurospirillum cavolei* sp. nov.

Description of *Sulfurospirillum cavolei* sp. nov.

Sulfurospirillum cavolei (cav.o'le.i. L. neut. n. *cavum* cave, cavern; L. gen. neut. n. *olei* of/from oil; N.L. gen. n. *cavolei* of/from an oil cavern, as the organism was isolated from underground oil storage caverns).

Gram-negative. Cells are slightly curved rods (0.4–0.5 × 1.5–5.0 µm). Motile by single polar flagella. Growth is observed only at low NaCl concentrations (below 1%). Optimum growth occurs at 30 °C (temperature range 20–40 °C) and pH 7.0 (pH range 6.0–8.0). Elemental sulfur, thiosulfate, sulfite, dithionite, DMSO, nitrate and arsenate can serve as electron acceptors. Lactate, fumarate, pyruvate, succinate and malate can be used as electron donors. Hydrogen and formate can serve as electron donors when acetate is used as the carbon source. Nitrate is reduced to nitrite. Microaerophilic growth (1% oxygen in the vapour phase) is observed. Can grow fermentatively on fumarate, pyruvate and malate. The DNA G+C content of the type strain is 42.7 mol%.

The type strain, Phe91^T (=JCM 13918^T=DSM 18149^T), was isolated from petroleum-contaminated groundwater in an underground crude oil storage cavity in Kuji, Iwate, Japan.

Table 1. Characteristics that enable strain Phe91^T to be differentiated from other *Sulfurospirillum* species

Strains: 1, Phe91^T (this study); 2, *S. deleyianum* DSM 6946^T (Schumacher *et al.*, 1992; Stolz *et al.*, 1999; Luijten *et al.*, 2004); 3, *S. multivorans* DSM 12446^T (Scholz-Muramatsu *et al.*, 1995; Holliger *et al.*, 1998; Luijten *et al.*, 2003); 4, *S. arcachonense* DSM 9755^T (Finster *et al.*, 1997; Jensen & Finster, 2005); 5 and 6, *S. barnesii* DSM 10660^T and *S. arsenophilum* DSM 10659^T, respectively (Stolz *et al.*, 1999; Luijten *et al.*, 2003, 2004); 7, *S. halorespirans* DSM 13726^T (Luijten *et al.*, 2003); 8, '*S. carboxydovorans*' DSM 16295 (Jensen & Finster, 2005). +, Positive; −, negative; ND, not determined. All strains are able to utilize the following as electron donors: formate (+ acetate), hydrogen (+ acetate) and pyruvate. All strains are positive for the fermentation of fumarate.

Characteristic	1	2	3	4	5	6	7	8
Morphology	Slightly curved rods	Curved spiral rods	Curved/helical rods	Vibrioid to spirillum	Vibrioid to spirillum	Vibrioid to spirillum	Slightly curved rods	Vibrioid to spirillum
DNA G+C content (mol%)	42.7	40.6	41.5	32	40.8	40.9	41.8	30.5
16S rRNA gene similarity to strain Phe91 ^T (%)	100	97.0	96.1	93.6	95.1	95.9	96.0	92.9
Temperature range/optimum (°C)	20–40/30	20–36/ND	ND/30	8–30/26	ND/33	ND/20	ND/25–30	9.9–31.9/30.2
pH range/optimum	6.0–8.0/7.0	ND/7.0–7.1	ND/7.0–7.5	6.1–8.2/7.0–7.4	ND/7.5	ND/7.5	ND/ND	5.9–8.5/7.3
NaCl range/optimum (%)	<1.0/0	<0.2/ND	ND/ND	0.6–4.0/1.2–2.0	ND/0.05	ND/0.1	ND/ND	1.0–5.5/2.0
Electron acceptors:								
Arsenate	+	+	+	−	+	+	+	+
Dithionite	+	ND	ND	ND	ND	ND	ND	ND
DMSO	+	+	ND	−	−	ND	ND	+
Elemental sulfur	+	+	ND	+	+	+	+	+
Fe(III)	ND	−	ND	−	+	−	ND	−
Nitrate	+ (→NO ₂)	+ (→NH ₄)	+ (→NO ₂)	−	+ (→NH ₄)	+ (→NH ₄)	+ (→NH ₄)	+
Nitrite	−	+	−	−	+	+	+	+
Oxygen (microaerobic)	+	+	ND	+	+	+	+	+
Perchloroethylene	−	ND	+	ND	−	ND	+	+
Selenate	−	−	+	−	+	+	+	+
Sulfite	+	+	ND	−	−	ND	−	+
Thiosulfate	+	+	ND	−	+	+	−	+
Trimethylamine N-oxide	ND	+	ND	−	+	ND	ND	ND
Electron donors:								
CO	ND	ND	ND	−	ND	ND	ND	+
Fumarate	+	+	+	+	+	+	ND	+
Lactate	+	−	+	+	+	+	+	+
Fermentation of:								
Malate	+	+	ND	−	ND	ND	ND	+
Pyruvate	+	ND	+	−	ND	ND	+	−

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