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ORIGINAL PAPER

Desulfosporosinus acidiphilus sp. nov.: a moderately acidophilic sulfate-reducing bacterium isolated from acid mining drainage sediments

New taxa: *Firmicutes* (Class Clostridia, Order *Clostridiales*, Family *Peptococcaceae*)

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Abstract An obligately anaerobic, spore-forming, acidophilic sulfate-reducing bacterium, strain SJ4^T, was isolated from an acid mining effluent decantation pond sediment sample (pH around 3.0). Cells were Gram negative, non-motile, curved rods occurring singly. Strain SJ4^T grew at pH 3.6–5.5 with an optimum at pH 5.2. Strain SJ4^T utilized H_2, lactate, pyruvate, glycerol, glucose, and fructose as electron donors. Lactate and glucose were weakly used. Sulfate was used as electron acceptors, but not sulfite, elemental sulfur, arsenate (V), and fumarate. The G + C content of genomic DNA was 42.3 mol% (HPLC). 16S rRNA gene sequence analysis indicated that strain SJ4^T belonged to the genus *Desulfosporosinus* within the family *Peptococcaceae* in the phylum *Firmicutes*. The level of 16S rRNA gene sequence similarity with other *Desulfosporosinus* species was 94.7–96.2%, *D. orientis* DSM 765^T (similarity of 96.2%) and *D. auripigmenti* DSM 13351^T (similarity of 95%) being its closest relatives. DNA–DNA relatedness values with *D. orientis* and *D. auripigmenti* were 16.5 and 31.8%, respectively. On the basis of phenotypic, phylogenetic, and genetic characteristics, strain SJ4^T represents a novel species within the genus *Desulfosporosinus*, for which the name *Desulfosporosinus acidiphilus* sp. nov. is proposed. The type strain is SJ4^T (=DSM 22704^T = JCM 16185^T).

Keywords Acid mining drainage · Acidophilic · Sulfate reduction

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The GenBank accession number for the 16S rRNA gene sequence of SJ4^T is FJ951625.

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Introduction

Bioremediation of acidic wastewaters from mining industries is a worldwide environmental concern. Acid mining drainage (AMD) waters are often highly acidic (pH < 4) and contain elevated concentrations of sulfate and dissolved metals. Remediation of mine waters can be achieved by continuous application of limestone or similar alkaline materials to neutralize acidic waters and precipitate metals, but generating large volumes of toxic sludge that still contain metals.

An alternative process is the selective precipitation of metals by biologically produced sulfides. Therefore, the development of microbial technologies by using sulfate-reducing bacteria (SRB) able to precipitate a wide spectrum of heavy metals and metalloid contaminants as sulfide minerals offer interesting potentials. While bioremediation of acidic, metal-contaminated waters using SRB has been successfully demonstrated in pilot-scale and full-scale operations during the past decade, the sensitivity of SRB to acidity limits their use in AMD remediation. Most known SRB have pH optimum for growth around 7 and are inhibited at pH values below 6 (Widdel 1988; Fauque and Ollivier 2004). As a consequence, a neutralization step is necessary before AMD effluents are submitted to the SRB treatment.

In recent years, sulfate reduction activity has been reported in natural or engineered low pH ecosystems

(Gyure et al. 1990; Kolmert and Johnson 2001; Koschorreck et al. 2003). Some SRB were isolated from acidic mine streams and lakes, but appeared neutrophilic and were not active below pH 5 (Kusel et al. 2001; Tuttle et al. 1969; Lee et al. 2009). The situation regarding the existence of acidophilic SRB is currently not clear. Pure cultures isolated from mixed cultures capable of reducing sulfate at pH 3 were not capable of reducing sulfate below pH 5.5 (Tuttle et al. 1969). Also, Gyure et al. (1990) reported that primary enrichments of SRB could be maintained and transferred at pH 3.8, but pure cultures growing at pH 3–5 were not obtained. It was therefore suggested that the SRB present in the acid environments occurred in microenvironments at higher pH in the sediment or around wood or other suspended particles (Tuttle et al. 1969; Gyure et al. 1990). Nevertheless, Sen and Johnson (1999) reported *Desulfosporosinus*-like Gram-positive bacteria able to grow at low pH values. It is only recently that Johnson et al. (2009) reported on SRB growing at pH 3.0 (strain CL4), while Karnachuk et al. (2009) reported on the isolation of a *Desulfosporosinus* sp. strain DB from gold mine tailing sediments with extremely low pH and high concentration of dissolved metals. However, these microorganisms have not been characterized so far. These authors attribute the reason for the success in obtaining cultures of acidophilic SRB to the use of non-acidic organic substrates (glycerol, methanol) in enrichment cultures.

This paper reports the first characterization of a true acidophilic sulfate-reducing bacterium isolated from a sediment sample collected in a decantation pond receiving acid mining effluent.

Materials and methods

Source of the organism, media, isolation and growth conditions

A sediment sample was collected from an acid mining effluent decantation pond ($2.8 < pH > 3.0$) at the mining site of Chessy-Les-Mines (Beaujolais, France). Standard anaerobic culture techniques were used throughout this study (Balch et al. 1979; Hungate 1969). The culture medium described by Sen and Johnson (1999) with 10 mM glycerol as electron donor at pH 3.5 was used for enrichment and isolation of acidophilic SRB. The pH was adjusted to 3.5 with 2 M H_2SO_4 and the medium was boiled under a stream of O_2-free N_2 gas and cooled to room temperature. Five millilitre aliquots were dispensed into Hungate tubes and 20 mL in serum bottles, under a stream of N_2–CO_2 (80:20, v/v) gas and the sealed vessels were autoclaved for 20 min at 120°C. Serum bottles were inoculated with a moist sediment sample (around 1 g) and incubated for 3 weeks at 35°C. These cultures were subcultured into fresh medium. Isolation was performed by serial dilution up to 10^{-9} in the same medium supplemented with 2% low EEO agarose (Sigma A6013) using the roll tube method. SRB colonies were detected by the blackening of colonies as a result of the formation of black FeS at $pH > 4$. The culture was serially diluted tenfold in roll tubes and single colonies that developed were picked according to Hungate (1969) in an N_2 atmosphere. The process of serial dilution was repeated twice. The purity of strains was checked by phase-contrast microscopy.

The medium for cultivation and maintenance was formulated after growth optimization. It contained (basal medium), in g/L: $(NH_4)_2SO_4$, 0.45; KH_2PO_4, 0.05; $MgSO_4{\cdot}7H_2O$, 0.5; KCl, 0.05; yeast extract 0.5; $Ca(NO_3)_2{\cdot}4H_2O$, 0.014; KCl, 0.05; Na_2SO_4, 3; L-cysteine. HCl, 0.5; resazurin, 0.001; Widdel trace element solution (Widdel and Pfennig 1981), 1 mL/L. After autoclaving, the pH medium was 5.8. Before inoculation, it was supplemented with a buffer solution [Na_2HPO_4 (sol. at 50 g/L) 0.1/5 mL of medium; KH_2PO_4 (sol. at 250 g/L) 0.2/5 mL of medium], fructose (20 mM), Balch's vitamin solution (Balch et al. 1979), and 0.1/5 mL of medium. Na_2S was not added as reductant, because commercially available sodium sulfide contains impurities that have an inhibitory effect on the growth of Gram-positive spore-forming sulfate reducers (Spring and Rosenzweig 2006).

Morphological and physiological studies

Light-microscope examination was performed using a Nikon Eclipse 600 phase-contrast microscope. Gram reaction was performed by the Hucker staining method (Murray et al. 1994). Thin sections for electron microscopy were prepared as described by Fardeau et al. (1997). Photomicrographs were taken with a Hitachi model H600 electron microscope at an accelerating voltage of 75 kV. The presence of spores was analyzed by phase-contrast microscopic observations of young and old cultures and pasteurization tests performed at 80, 90 and 100°C for 10 and 20 min.

Growth experiments were performed in duplicate, using Hungate tubes containing the optimized culture medium. Turbidity (580 nm) was used to assess growth. Temperature and NaCl ranges for growth were determined in the optimized growth medium at pH 5.2. The pH range of growth was determined by using the optimized basal medium buffered by Sörensen's citrate buffer I and II solutions (pH 2.2–4.8 and 5.0–6.8, respectively). The strain was subcultured at least once under the same experimental conditions prior to determination of growth rates. Substrates were tested at a final concentration of 20 mM in the SRB medium unless otherwise indicated. The end products

of sulfate respiration were determined by HPLC using an Aminex HPX-87H (Biorad) column with 5 mM H_2SO_4 as mobile phase. To test for electron acceptors, sulfate, sulfite, elemental sulfur, arsenate and nitrate were added to the medium at final concentrations of 20 mM, 2 mM, 0.1% (w/v), 1 mM and 10 mM, respectively. Sulfide was determined photometrically as colloidal CuS by using the method of Cord-Ruwisch (1985). The pH of the culture was increased to pH 10–11 to dissolve H_2S present in the gas phase. Desulfoviridin was determined as described by Postgate (1959).

The effect of pH on growth was determined at 35°C. The pH of basal optimized medium was adjusted to defined values with sterile 6 N solutions of HCl and KOH.

Phylogenetic and genomic analysis

Genomic DNA was extracted using the Wizard Genomic DNA Purification kit (Promega Corp. Madison, Win.), according to the instructions of the manufacturer. The 16S rRNA gene sequence of strain SJ4^T was selectively amplified by PCR by using the universal primers Rd1 and Fd1, and its sequence was determined and analyzed (Maidak et al. 2001; Weisburg et al. 1991). The nearly complete sequence (1,538 nt) of the 16S rDNA of SJ4^T was aligned with closely related sequences from GenBank database using programs provided by the Ribosomal Data Project II (Maidak et al. 2001). All the sequences were imported into the sequence editor BioEdit v 5.0.9 (Hall 1999). Positions of sequence with alignment uncertainties were omitted, and in total 1,330 positions of alignment were used in the analysis. Phylogenetic trees were constructed using various algorithms implemented in the TREECONW (van de Peer and De Wachter 1994) and PHYLIP (Felsenstein 1990) software packages. Pairwise evolutionary distances (expressed as estimated changes per 100 nucleotides) were computed by using Jukes and Cantor (1969) method. A resulting phylogenetic tree was constructed by the neighbor-joining method.

The G + C content of the DNA was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany. DNA was isolated and purified by chromatography on hydroxyapatite and its G + C content was determined by using HPLC as described by Mesbah et al. (1989). Non-methylated lambda DNA (Sigma) with a G + C content of 49.8 mol% was used as a standard.

DNA–DNA hybridization was performed at DSMZ as described by De Ley et al. (1970) with modifications reported by Escara and Hutton (1980) and Huß et al. (1983), using a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratory Inc., Oberlin, OH, USA). Renaturation rates were computed with the TRANSFER.BAS program (Jahnke 1992).

Cellular fatty acid analysis

The determination of fatty acids composition of strain SJ4^T was performed at the Identification Service of DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Fatty acid methyl esters (FAMEs) were extracted from fresh biomass and identified following the procedure recommended by Microbial Identification System (MIDI, Sherlock Microbial Identification System Version 4.0, MIS Operating Manual March 2001, Newark, Del.). The MIS system was used to compare the fatty acid methyl esters of strain SJ4^T with fatty acid patterns stored in MIDI fatty acid database.

Results and discussion

Morphology

Cells of strain SJ4^T were non-motile, slightly curved rods, 4–7 μm in length and 0.8–1.0 μm in width (Fig. 1a). Oval-shaped endospores in the subterminal position were occasionally formed, which caused swelling of the cell (Fig. 1b). Cells stained Gram negative. Electron microscopy of ultrathin sections of cells indicated the presence of a multilayered Gram-negative type cell envelope typically present in members of the genus *Desulfosporosinus* (Fig. 1c). Flagellation was not observed.

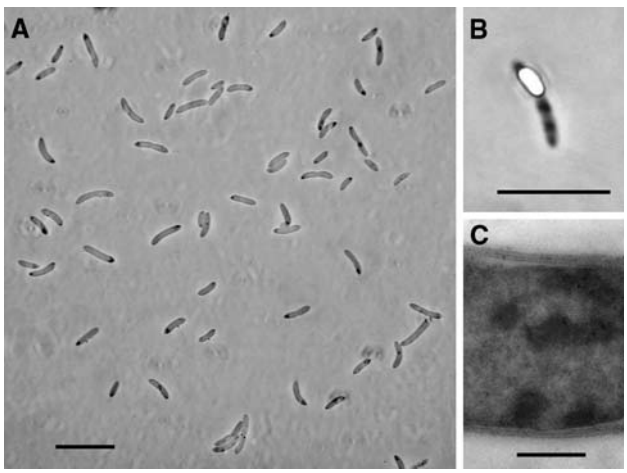

Fig. 1 Morphology of strain SJ4^T: **a** vegetative cells of a culture under phase-contrast microscope, *bar* 10 μm; **b** phase-contrast micrograph of a spore-forming cell of strain SJ4^T, *bar* 10 μm; **c** longitudinal section showing the multilayered cell wall structure, *bar* 0.5 μm

Growth parameters and metabolism

Strain SJ4^T was strictly anaerobic, growing optimally in basal medium containing glycerol or fructose as electron donors and sulfate as electron acceptor at 35°C (temperature growth range between 20 and 40°C). It grew in a pH range between 3.6 and 5.5 (changes in pH values were relatively low during incubation, usually 0.2 pH unit), with an optimum at pH 5.2 (Fig. 2). H_2S production was also optimal at pH 5.2 (9.1 mM H_2S produced after 10 days of incubation). In contrast, *D. orientis* and *D. auripigmenti*, as the closest phylogenetic relatives of strain SJ4^T, were neutrophilic, growing optimally at pH 6.2–7.0 and 6.4–7.0, respectively. The pH dependence of growth indicates therefore that strain SJ4^T is an acidophilic microorganism unable to grow at pH 7.0.

The substrates tested as possible energy and carbon sources in the presence of sulfate as electron acceptor are listed in Table 1. Strain SJ4^T differed from *D. orientis* and *D. auripigmenti* in using a small range of substrates including hydrogen, lactate, glycerol, pyruvate, and fructose while reducing sulfate. It was autotrophic, oxidizing H_2 in the absence of acetate as carbon source. Glycerol and fructose were incompletely oxidized to acetate. Lactate and glucose were weakly used. Neither pyruvate nor fructose was fermented. No growth was observed on the following substrates with sulfate as the electron acceptor: acetate (10 mM), formate (80 mM), citrate, succinate, malate, fumarate, propionate, butyrate, benzoate, syringate, ethanol, methanol, 1-propanol, butanol, xylose, yeast extract (1 g/L), and alanine (10 mM). It used sulfate, but not sulfite, elemental sulfur, ferric iron, or fumarate, as electron donors. Arsenate (V) was not used as electron acceptor, but this latter compound induced sporulation.

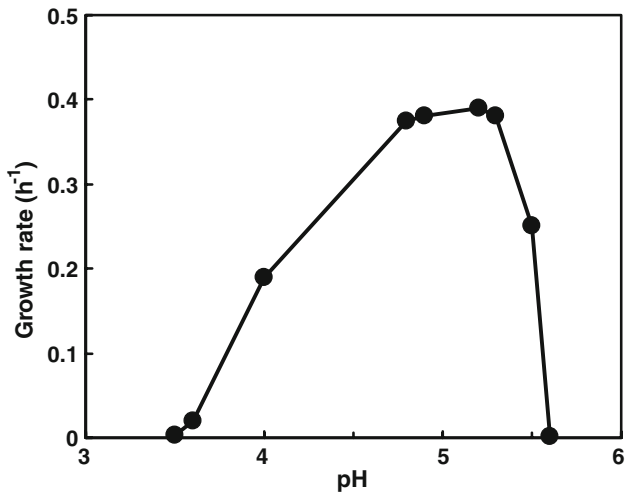

Fig. 2 Effect of pH on the specific growth rate of strain SJ4 (at 35°C). Final values were taken as the incubation pH

Chemotaxonomy

Cellular fatty acid composition profiles of strain SJ4^T and its phylogenetic closest relatives are presented in Table 2. The qualitative composition was overall similar to those of others members of the genus *Desulfosporosinus*; however, there were quantitative differences, especially in the abundance of 16:1*cis9*, iso-15:0, and 14:0. Indeed, branched saturated fatty acid iso-15:0 (28%), absent in *D. orientis* and *D. auripigmenti*, was predominant in strain SJ4^T, whereas fatty acid C16:1*cis9*, predominant in *D. orientis* and *D. auripigmenti*, was only present in small quantities in strain SJ4^T.

Phylogeny and comparative genomics (16S rRNA gene sequence analysis)

Analysis of the almost complete sequence of the 16S rRNA gene of the strain SJ4^T revealed that it was affiliated to the genus *Desulfosporosinus*, family *Peptococcaceae* (order *Clostridiales*). The most closely related species were *D. orientis* and *D. auripigmenti* with an overall 16S rRNA gene similarity of 96.2 and 95.0%, respectively (Fig. 3). The cluster consisting of *Desulfosporosinus* species was strongly supported by maximum likelihood and by bootstrap analysis (distance matrix method). Tree was also constructed using the parsimony method, but displayed no significant differences in branching order from the neighbor-joining method.

The DNA G + C content of strain SJ4^T was 42.3 mol%, comparable to values quoted for others members of the genus *Desulfosporosinus* (41.6–46.9%). DNA–DNA hybridization experiments revealed that strain SJ4^T shows only 16.5% reassociation with *D. orientis* and 31.8% with *D. auripigmenti*, thus indicating that strain SJ4^T represents a distinct species within the genus *Desulfosporosinus*.

Taxonomic and environmental considerations

Representatives of the genus *Desulfosporosinus* are characterized by their ability to use hydrogen as electron donors and to form endospores. This genus currently comprises six validly described species, *D. orientis* (Stackebrandt et al. 1997), *D. auripgmenti* (Stackebrandt et al. 2003), *D. meridiei* (Robertson et al. 2001), *D. lacus* (Ramamoorthy et al. 2006), *D. hippei* (Vatsurina et al. 2008), and the recently described *D. youngiae* (Lee et al. 2009). The type species of the genus is *D. orientis*. They form a coherent phylogenetic group within the *Peptococcaceae*.

In the current study, an acidophilic SRB (strain SJ4^T) was isolated from an AMD stream, whereas its closest phylogenetic relatives, *D. auripigmenti* and *D. orientis*,

Table 1 Main characteristics differentiating strain SJ4^T from its closest phylogenetic relatives *Desulfosporosinus auripigmenti* and *D. orientis*

Characteristics	Strain SJ4^T	*Desulfosporosinus auripigmenti*[a] DSM 13351^T	*Desulfosporosinus orientis*[b] DSM 765^T
Morphology	Curved rods	Rods	Curved rods
Cell size (μm)	4–7 × 0.8–1	ND × 0.4	ND × 0.7–1
Gram	Negative	Positive	Negative
Endospore position	Subterminal	Subterminal	Subterminal
Motility	−	−	+
Temperature range (°C)	25–40	4–42	30–47
Temperature optimum (°C)	30	25–30	30–37
pH range	3.6–5.6	ND	5.6–7.4
pH optimum	5.2	6.4–7	6.4–7
NaCl range (%)	0–0.6	<4	<4.5
NaCl optimum (%)	1.5	25	8.8–14.6
Electron donors in presence of sulfate			
Lactate	(+)	+	+
Butyrate	−	+	+
Formate	−	+	+
Fumarate	−	−	+
Malate	−	+	−
Ethanol	−	+	+
Glycerol	+	+	−
Methanol	−	−	+
Fructose	+	−	−
Glucose	(+)	−	−
Yeast extract	−	+	+
H_2 (carbon source)	+ (CO_2)	+ (acetate)	+ (CO_2)
Electron acceptors*			
Sulfur	−	−	+
Arsenate	−	+	−
Fumarate	−	−	+
Fe(III)	−	−	+
Fermentation in the absence of sulfate			
Lactate	−	ND	+
Ethanol	−	ND	+
Methanol	−	ND	+
Pyruvate	−	ND	+
DNA G + C mol%	42.3	41.6	45.9

ND not determined

+, supported growth; −, did not support growth; (+), weak growth

* with glycerol as electron donor

[a] Data from Stackebrandt et al. 2003

[b] Data from Ramamoorthy et al. 2006

have been isolated from freshwater sediment taken from Upper Mystic Lake in Woburn, MA, USA (Stackebrandt et al. 2003) and from soil from Singapore (Campbell and Postgate 1965), respectively. Physiological and molecular characterization indicated that strain SJ4^T was able to grow at pH 4.5 in the medium described by Sen and Johnson (1999), indicating that such bacterium has the potential for the bioremediation of acidic waters. This is particularly true for new applications in remediating metal-rich wastewaters and recovering metals from them.

Here, we confirm with the isolation of strain SJ4 that non-polar substrates (ethanol, glycerol, or methanol) are

Table 2 Percentage of individual fatty acids of the total whole cell fatty acids obtained from strain SJ4^T and closely related *Desulfosporosinus* species

Retention time	Fatty acid	Strain SJ4^T	*Desulfosporosinus auripigmenti*[a] DSM 13351^T	*Desulfosporosinus orientis*[b] DSM 765^T
7.24	14:0	15.7	2.2	4.0
8.20	iso-15:0	28.1	–	–
8.34	anteiso-15:0	2.4	–	–
8.44	15:2	2.8	1.9	2.8
8.70	16:0 ald	4.9	1.5	3
10.07	16:1 *cis*7	1.8	31.6	25.5
10.14	16:1 *cis*9	3.6	14.2	17.4
10.44	16:0	18.7	1.7	1.8
10.94	16:1 *cis*9 dma	3.0	13.4	8.7
11.26	16:0 dma	10.8		
12.36	Un 17.103 17:0 i dma	1.9		
11.97	17:0 cyc	4.2	0.6	–
14.33	18:1 *cis*9 dma	1.8	4.4	1.9

[a] Data from Stackebrandt et al. 2003

[b] Data from Ramamoorthy et al. 2006

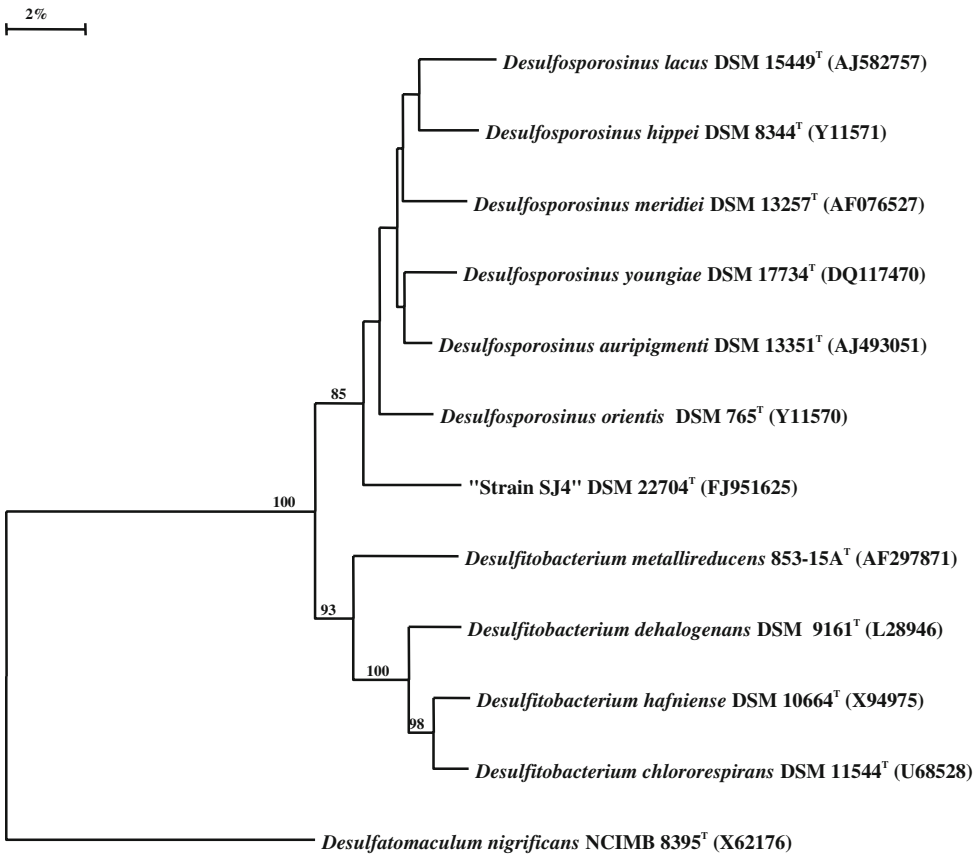

Fig. 3 Phylogenetic dendrogram based on 16S rRNA gene sequence comparison indicating the position of strain SJ4^T within the genus *Desulfosporosinus*. *Desulfotomaculum nigrificans* was taken as the outgroup. *Bar* corresponds to 5 nucleotide substitutions per 100 nucleotides. Bootstrap values, expressed as a percentage of 100 replications, are shown at branching points. Only values above 90% were considered to be significant and therefore reported

preferably used by acidophilic SRB in microbial niches that they inhabit. Indeed, the oxidation of such substrates by these SRB appeared common because of the high toxicity of acidic substrates, such as lactate, in their undissociated form even at relatively low concentration (Norris and Ingledew 1992).

Finally, based on the phenotypic, phylogenetic, and genetic differences observed between strain SJ4^T and other

members of the genus *Desulfosporosinus*, we propose to assign it to a novel species within this genus, *Desulfosporosinus acidiphilus* sp. nov.

Description of *Desulfosporosinus acidiphilus* sp. nov.

Desulfosporosinus acidiphilus (a.ci.di.phi'lus. N.L. n. *acidum* (from L. adj. *acidus*, sour), an acid; N.L. adj. *philus–a-um* (from Gr. adj. *philos ê–on*), friend, loving; N.L. masc. adj. *acidiphilus*, acid-loving).

These are Gram-variable curved rods with multilayered cell wall. Cells are 0.8–1.0 μm in width and 4.0–7.0 μm in length. They are non-motile and occur generally singly. Endospores are sometimes produced; they are subterminal and oval in shape and swell the cells. The pH range for growth is from pH 3.6–5.5, with an optimum at pH 5.2. The temperature range for growth is 25–40°C, with an optimum at 30°C. The upper limit for salt tolerance is 6 g/L NaCl. Sulfate is reduced to sulfide in the presence of H_2, lactate, pyruvate, glycerol, glucose, and fructose. Organic substrates are incompletely oxidized to acetate. Arsenic (V) is not used as acceptor electrons. The following substrates are not used as electron donors: formate, acetate, succinate, malate, fumarate, citrate, ethanol, methanol, propionate, butyrate, benzoate, syringate, and yeast extract. The predominant whole cell fatty acids are iso-15:0, 16:0, 14:0, 16:0 dma, 16:0 ald, and 18:1 cis9. The G + C content of the DNA is 42.3 mol%. Phylogenetically, it is a member of the order *Clostridiales* within the *Firmicutes*. The type strain *Desulfosporosinus acidiphilus*, strain SJ4^T (=DSM 22704^T = JCM 16185^T) was isolated from a sediment sample taken in a decantation pond receiving acid mining effluent (pH around 3.0) at Chessy-les-Mines (France).

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