Desulfosporosinus burensis sp. nov., a spore-forming, mesophilic, sulfate-reducing bacterium isolated from a deep clay environment

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A novel anaerobic, Gram-positive, spore-forming, curved rod-shaped, mesophilic and sulfate-reducing bacterium was isolated from pore water collected in a borehole at −490 m in Bure (France). This strain, designated BSREI1^T, grew at temperatures between 5 °C and 30 °C (optimum 25 °C) and at a pH between 6 and 8 (optimum 7). It did not require NaCl for growth, but tolerated it up to 1.5 % NaCl. Sulfate, thiosulfate and elemental sulfur were used as terminal electron acceptors. Strain BSREI1^T used crotonate, formate, lactate, pyruvate, fructose, glycerol and yeast extract as electron donors in the presence of sulfate. The sole quinone was MK-7. The G+C content of the genomic DNA was 43.3 mol%. Strain BSREI1^T had the type strains of *Desulfosporosinus lacus* (16S rRNA gene sequence similarity of 96.83 %), *Desulfosporosinus meridiei* (96.31 %) and *Desulfosporosinus hippei* (96.16 %) as its closest phylogenetic relatives. On the basis of phylogenetic and physiological properties, strain BSREI1^T is proposed as a representative of a novel species of the genus *Desulfosporosinus*, *Desulfosporosinus burensis* sp. nov.; the type strain is BSREI1^T (=DSM 24089^T=JCM 17380^T).

The genus *Desulfosporosinus* was proposed in 1997 to accommodate the species *Desulfotomaculum orientis* (Stackebrandt *et al.*, 1997). At the time of writing, it comprised seven species of obligately anaerobic, spore-forming bacteria with validly published names: *Desulfosporosinus orientis*, the type species of the genus *Desulfosporosinus*, (Stackebrandt *et al.*, 1997), *D. auripigmenti* (Stackebrandt *et al.*, 2003), *D. meridiei* (Robertson *et al.*, 2001), *D. lacus* (Ramamoorthy *et al.*, 2006) *D. hippei* (Vatsurina *et al.*, 2008) *D. youngiae* (Lee *et al.*, 2009) and *D. acidiphilus* (Alazard et *al.*, 2010). *Desulfosporosinus*-like organisms are widespread in different habitats. They occur in environments such as pristine aquifers, municipal drinking water, rice plant roots, permafrost and acid mining drainage sediments. Members of the genus also inhabit industrially impacted soil and sediments, coal-mining-impacted lakes and radionuclide-contaminated sediments (Vainshtein *et al.*, 2007).

Stackebrandt *et al.* (1997) distinguished this group from the genus *Desulfotomaculum* on the basis of phylogenetic evidence and their ability to grow under autotrophic conditions. However, the property of autotrophic growth (i.e. 50 % of the biomass derived from inorganic carbon) has not been demonstrated unequivocally for all the species. Vatsurina *et al.* (2008) recently reported *D. orientis* 343 as the type strain of a new species in the same genus, *D. hippei*, which does not oxidize H_2 with CO_2 or acetate. Here, we report another novel species of the genus *Desulfosporosinus* that is not able to grow lithotrophically and was isolated from a pore water sample collected through a borehole at −490 m in a clay sediment where H_2S was detected.

A pore water sample was taken from clay sediments at the Agence Nationale pour la Gestion des Déchets Radioactifs (ANDRA) Underground Research Laboratory (URL) at Bure (Meuse/Haute Marne, France). The sample (800 ml) was collected using a sterile anaerobic bottle attached to a drill head. Standard anaerobic culture techniques were used throughout this study (Hungate, 1969). The culture medium used for enrichment and isolation contained (g l^{-1}): yeast extract (0.1), KCl (0.1), KH_2PO_4 (0.6), K_2HPO_4 (0.6), $MgCl_2 . 6H_2O$ (0.3), $CaCl_2 . 2H_2O$ (0.05), cysteine . HCl (0.5), NH_4Cl (0.5), Widdel trace element solution (Widdel & Pfennig, 1981) (1 ml) and resazurin (1 ml), all under a stream of N_2 : CO_2 (80 : 20, v/v) gas. After sterilization, 10 %

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of BSREI1^T is JF810424.

$NaHCO_3$ (0.15 ml), 1 M Na_2SO_4 (0.1 ml), 50 g yeast extract l^{-1} (0.1 ml), 1 M glucose (0.1 ml) and 1 M lactate (0.1 ml) were added per Hungate tube (5 ml). Strain purity was checked by phase-contrast microscopy. The culture medium was formulated after growth optimization. It contained (g l^{-1}): $(NH_4)_2SO_4$ (0.45), KH_2PO_4 (0.05), $MgSO_4.7H_2O$ (0.5), KCl (0.05), yeast extract (0.5), $Ca(NO_3)_2.4H_2O$ (0.014), Na_2SO_4 (3), cysteine.HCl (0.5), resazurin (1 ml) and Widdel trace element solution (1 ml), and pH was adjusted to 7.3. After autoclaving and before inoculation, it was supplemented with Balch's vitamin solution (0.05 ml) (Balch *et al.*, 1979), 10 % $NaHCO_3$ (0.15 ml) and 1 M fructose (0.1 ml).

Light-microscope examination was performed using a Nikon Eclipse 600 phase-contrast microscope. Gram reaction was obtained 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 analysed 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 medium for cultivation. Turbidity (580 nm) was used to assess growth. Temperature (5, 15, 20, 25, 30, 35, 45 °C), NaCl (0, 1, 2, 5, 10, 15, 20, 30 g l^{-1}) and pH (4–9) ranges and substrates were determined using the culture medium. For substrate tests, the final concentrations were 20 mM except for formate (40 mM), $H_2:CO_2$ (80:20, under 2 bars) and $H_2:CO_2$ (80:20, under 2 bars) with acetate (2 mM). The end products of sulfate respiration were determined by HPLC using an Aminex HPX-87H (Bio-Rad) column with 5 mM H_2SO_4 as mobile phase. The test for minimal medium was run in the culture medium using different concentrations of yeast extract (0, 0.1, 0.5 and 1 g l^{-1}) and Balch's solution vitamins (with or without) in the presence of fructose (20 mM). Electron acceptors (sulfate, thiosulfate, elemental sulfur, sulfite, fumarate), were supplied in the following, highly reduced medium (g l^{-1}): NH_4Cl (0.45), KCl (0.05), $MgCl_2$ (0.5), KH_2PO_4 (0.05), $CaCl_2$ (0.015), yeast extract (0.2), Widdel trace element solution (1 ml), cysteine.HCl (0.5) and resazurin (1 ml). For nitrate, nitrite, selenite (Na_2SeO3) and ferric iron [$Fe(OH)_3$], the previous medium was used, but cysteine.HCl and resazurin were omitted. The carbon source used for these tests was fructose (20 mM) and different electron acceptors were tested, with a final concentration of 20 mM, except for sulfite (2 mM), nitrite (2 mM), elemental sulfur (10 g l^{-1}), selenite (0.2 mM) and Fe^{3+} (900 mM). Sulfide was assayed photometrically as colloidal CuS using the method of Cord-Ruwisch (1985).

For the fermentative tests, the medium used was the highly reduced medium, and the carbon sources tested were crotonate, formate, lactate, pyruvate, fructose, glycerol and yeast extract. The culture medium supplemented with increasing concentrations of selenite (Na_2SeO_3): 0, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 10 or 20 mM was used to check the ability of the strain BSREI1^T to maintain or increase growth in the presence of selenium.

For 16S rRNA gene sequence analysis, PCR was performed directly on cells after 20 min at 80 °C, using the universal primers Fd1 and Rd1, and the sequence was determined and analysed (Maidak *et al.*, 2001; Weisburg *et al.*, 1991). The nearly complete sequence (1533 bp) of the 16S rRNA gene of BSREI1^T was aligned with closely related sequences from the GenBank database using MUSCLE (MUltiple Sequence Comparison by Log-Expectation) (Edgar, 2004). Nine aligned sequences were imported into the sequence editor BioEdit v 5.0.9 (Hall, 1999). The phylogenetic tree was constructed using various algorithms implemented in the TREECONW (Van de Peer & De Wachter, 1994) and PHYLIP (Felsenstein, 1990) software packages. A resulting phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987).

The G+C content of the DNA was determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. DNA–DNA hybridization between strain BSREI1^T and *D. lacus* was performed at the DSMZ. The following method was used: cells are disrupted by using a French pressure cell (Thermo Spectronic) and the DNA in the crude lysate is purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization is carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huss *et al.* (1983) using a Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian).

The fatty acid composition of the strain was determined at the Identification Service of DSMZ using 2 × 10 ml liquid culture (Sasser, 1990). Cultures of strain BSREI1^T were stopped at the end of exponential phase and sent to DSMZ for fatty acid analysis. Fatty acids were extracted using the method of Miller (1982), with the modifications of Kuykendall *et al.* (1988), and the profile of cellular fatty acids was analysed by GC using the Microbial Identification System (MIDI, Sherlock version 6.1; database, TSBA40; GC model 6890N, Agilent Technologies) using GC analysis according to the MIDI Microbial Identification system (e.g. Method: TSBA40). Respiratory quinones were analysed by the Identification Service and Dr Brian Tindall, DSMZ, Braunschweig, Germany. Cytochromes were analysed using Postgate's method (Postgate, 1959) and the following method was used: Respiratory lipoquinones are extracted from 100 mg of freeze-dried cell material using the two stage method described by Tindall (1990a, b). Respiratory quinones are extracted using methanol:hexane (Tindall, 1990a, b), followed by phase separation into hexane. Respiratory

lipoquinones are separated into their different classes (menaquinones, ubiquinones, etc.) by TLC on silica gel (Macherey–Nagel Art. No. 805 023), using hexane : *tert*-butylmethylether (9 : 1, v/v) as solvent. UV absorbing bands corresponding to the different quinone classes (e.g. menaquinones or ubiquinones) were removed from the plate and further analysed by HPLC. This step was carried out on a LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (Macherey–Nagel, 2 mm × 125 mm, 3 μm, RP18) using methanol : heptane (9 : 1, v/v) as the eluant. Respiratory lipoquinones are detected at 269 nm.

A 0.5 ml aliquot of sample from pore water collected in a borehole at −490 m was inoculated in Hungate tubes containing 5 ml culture medium. The tubes were then incubated at 20 °C. To obtain pure cultures, the enrichment was subcultured several times under the same growth conditions before isolation. For isolation, the culture was serially diluted tenfold in roll tubes (Miller & Wolin, 1974) containing the same culture medium and supplemented with agar (2 % w/v); several colonies developed after incubation at 20 °C and were harvested separately. Colonies were transparent and circular with diameters ranging from 1.0 mm to 2.0 mm after 1 month incubation at 25 °C. The process of serial dilution was repeated several times until the isolates were deemed anoxic. Several strains were isolated; they were similar in morphology and phylogeny, with the same metabolic profile for fructose. A strain designated BSREI1^T was selected and used for further metabolic and physiological characterization.

The cells of strain BSREI1^T were non-motile, curved rods 5.0–7.0 μm long and 0.1 μm wide, and stained Gram-positive. Oval-shaped endospores in the subterminal position were occasionally formed, which caused swelling of the cell. Strain BSREI1^T was anaerobic, growing optimally in medium for cultivation and maintenance containing fructose as electron donor and sulfate as electron acceptor at 25 °C (temperature growth range 5–30 °C). It grew in the pH range 6–8 with an optimum at pH 7 and in salinity between 0 and 1.5 %. H_2S production was also optimal at pH 7 (9 mM H_2S produced after 15 days of incubation). Strain BSREI1^T coupled growth to sulfate respiration over the range 5–30 °C, with an optimum between 25 °C and 30 °C. Under optimal growth conditions, the growth rate was 0.095 h^{-1}. All temperature tests were conducted at pH 7. pH optima were determined at 25 °C. Sulfidogenic growth was confined to the pH range 6–8. The substrates tested as possible energy and carbon sources in the presence of sulfate as electron acceptor are listed in Table 1.

Strain BSREI1^T needed no yeast extract or Balch's solution vitamin for growth, but the yields were significantly increased with 0.1 g yeast extract l^{-1}. Strain BSREI1^T could use as electron donors crotonate, formate, lactate, pyruvate, fructose, glycerol and yeast extract with sulfate. Unlike *D. lacus* and *D. meridiei*, strain BSREI1^T was unable to grow autotrophically. With sulfate as electron acceptor, no growth was observed on the following substrates: acetate, benzoate, butyrate, citrate, fumarate, malate, propionate, succinate, glucose, butanol, ethanol or methanol. Strain BSREI1^T was able to use sulfate, thiosulfate and sulfite as electron acceptor but not elemental sulfur, fumarate, nitrate, nitrite, selenite or Fe(III). Elemental sulfur and nitrite were both observed to be inhibitors for strain BSREI1^T. It tolerated selenite in the range 0.1–5.0 mM. Between 0.1 mM and 0.5 mM of selenite, growth was enhanced by a factor of 0.5 compared with the test without added selenite. At a concentration of 10 mM the growth of strain BSREI1^T stopped rapidly but even after 15 days of culture, the culture was still viable when reinoculated in a standard culture medium.

The main fatty acids of strain BSREI1^T were $C_{16:1}\omega7c$ (39.9 %), $C_{16:0}$ (17.1 %), $C_{16:1}\omega9c$ (12.5 %), $C_{18:1}\omega7c$ (4.8 %), Iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B (4.2 %), $C_{18:1}\omega9c$ (3.3 %), $C_{16:1}\omega5c$ (2.7 %), $C_{18:0}$ (1.2 %). MK-7 was the sole quinone found in the strain BSREI1^T. The analysis of cytochromes showed the presence of Sauret's band.

Analysis of the almost complete sequence of the 16S rRNA gene of the strain BSREI1^T (1533 bp) revealed that it was affiliated to the genus *Desulfosporosinus* (order *Clostridiales*, family *Peptococcaceae*). Positions of sequences with alignment uncertainties were omitted, and 1395 unambiguous aligned base-pairs were used in the analysis. The most closely related strain was the type strain of *D. lacus*. The phylogenetic relations between strain BSREI1^T, the closest phylogenetic relative strain (*D. lacus*) and the type strain of the type species of the genus (*D. orientis*) are shown in Fig. 1. The value of DNA–DNA hybridization was 29.6 % between BSREI1 and *D. lacus*. Strain BSREI1^T does not belong to the species *D. lacus* when the recommendations of a threshold value of 70 % DNA–DNA hybridization for the definition of bacterial species by the ad hoc committee (Wayne *et al.*, 1987) are considered. The DNA G+C content of strain BSREI1^T was 43.3 mol%, similar to the values quoted for other members of the genus *Desulfosporosinus* (41.6–46.9 mol%)

Strain BSREI1^T was isolated from pore water collected in a borehole at −490 m. Compared with other strains of the genus *Desulfosporosinus*, strain BSREI1^T is not able to grow autotrophically or to use S^0 as an electron acceptor, and has a single quinone.

One difference between strain BSREI1^T and strain *D. lacus* was that BSREI1^T stained Gram-positive like *D. auripigmenti* whereas *D. lacus* stained negative. The composition of quinones is clearly different: only MK-7 was detected for strain BSREI1^T whereas MK-7 and MK-5 were present in the ratio of 64 : 36 for *D. lacus*. Furthermore, unlike to *D. lacus*, strain BSREI1^T is not able to grow autotrophically, cannot use Fe(III) as electron acceptor and cannot ferment lactate. For electron donors,

Table 1. Main characteristics differentiating strain BSREI1^T from its closest phylogenetic relative *D. lacus* and the type species of the genus *D. orientis*

Strains: 1, BSREI1^T; 2, *D. lacus* DSM 15449^T [data from Ramamoorthy *et al.* (2006) and Lee *et al.* (2009)]; 3, *D. orientis* DSM 765^T [data from Ramamoorthy *et al.* (2006) and Alazard *et al.* (2010)]. +, Supported growth; −, did not support growth.

Character	1	2	3
Cell diameter (μm)	0.9–1.1	0.5–0.7	0.7–1.0
Gram reaction	Positive	Negative	Negative
Endospore position	Subterminal	Subterminal	Subterminal
Motility	Variable	Variable	Motile
Temperature range (°C)	5–30	4–32	30–47
Temperature optimum (°C)	25	30	35
pH range	6–8	6.5–7.5	5.6–7.4
pH optimum	7	7	6.4–7.0
Electron donor in presence of sulfate:			
H_2 (carbon source)	− (with CO_2)	+ (with CO_2)	+ (with CO_2)
Fructose	+	−	−
Ethanol	−	+	+
Glycerol	+	+	−
Methanol	−	+	+
Electron acceptors:*			
Sulfite	+	+	+
Sulfur	−	−	+
Fe(III)	−	+	+
Fermentative growth on lactate	−	+	+
DNA G+C content (mol%)	43.3	42.7	46.9

* With fructose as electron donor for strain BSREI1^T and lactate as electron donor for the other strains

strain BSREI1^T cannot use ethanol and methanol in presence of sulfate, unlike *D. lacus*. Finally, based on the phenotypic, phylogenetic and genetic differences observed between strain BSREI1^T and other members of the genus *Desulfosporosinus* (see Table 1), we identify this strain as a representative of a novel species in this genus, and propose the name *Desulfosporosinus burensis* sp. nov.

Description of *Desulfosporosinus burensis* sp. nov.

Desulfosporosinus burensis (bu.ren′sis. N.L. masc. adj. *burensis* belonging to Bure, the area where the strain was isolated).

A Gram-negative, curved rod. The cells are 5–7 μm long and 1.0 μm wide. They are non-motile. Endospores, subterminal and oval in shape, are weakly produced and swell the cells. The pH range for growth is 6–8, with an optimum at pH 7. The temperature range for growth is 5–30 °C with an optimum at 25 °C. The upper limit for salt tolerance is 15 g NaCl l^{-1}. Sulfate is reduced to H_2S in the presence of crotonate, formate, lactate, pyruvate, fructose, glycerol and yeast extract. Organic substrates are incompletely oxidized to acetate except for crotonate (acetate and traces of formate), formate (H_2/CO_2 and traces of acetate) and yeast extract (traces of acetate and propionate). The following substrates are not used as electron donors: acetate, benzoate, butyrate, citrate, fumarate, malate, propionate, succinate, glucose, butanol, ethanol and

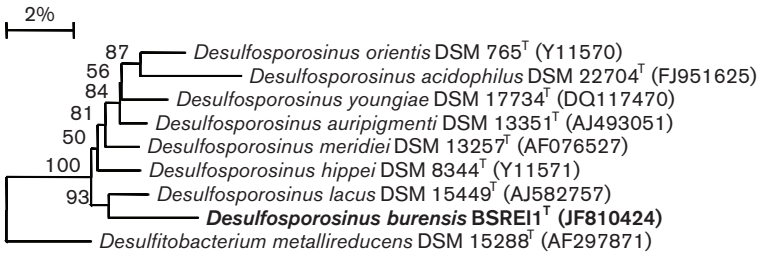

Fig. 1. Phylogenetic tree based on an alignment of 1395 unambiguous base-pairs of 16S rRNA gene sequences, showing the affiliation of strain BSREI 1^T to the genus *Desulfosporosinus*. Bar, 2% estimated sequence divergence.

methanol. Sulfate, thiosulfate and sulfite are used as electron acceptors, but not elemental sulfur, fumarate, nitrate, nitrite or Fe(III). The major fatty acids are: $C_{16:1}\omega7c$, $C_{16:0}$ and $C_{16:1}\omega9c$. It contains a single quinone with a side chain of seven isoprene units (MK-7).

The type strain is BSREI1^T (=DSM 24089^T=JCM 17380^T), isolated from pore water collected in a borehole at −490 m in Bure (France) in a clay sediment. The DNA G+C content of the type strain is 43.3 mol%.

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