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 BSREI1T, 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 BSREI1T 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 BSREI1T had the type strains of Desulfosporosinus lacus (16S rRNA gene sequence similarity of 96.33%), Desulfosporosinus meridiei (96.31%) and Desulfosporosinus hippei (96.16%) as its closest phylogenetic relatives. On the basis of phylogenetic and physiological properties, strain BSREI1T is proposed as a representative of a novel species of the genus Desulfosporosinus, Desulfosporosinus burensis sp. nov.; the type strain is BSREI1T (=DSM 24089T=JCM 17380T).

The genus Desulfosporosinus was proposed in 1997 to accommodate the species Desulfitomaculum 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 Desulfitomaculum on the basis of phylogenetic evidence and their ability to grow at 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 H2 with CO2 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 H2S 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), KH2PO4 (0.6), K2HPO4 (0.6), MgCl2, 6H2O (0.3), CaCl2.2H2O (0.05), cysteine. HCl (0.5), NH4Cl (0.5), Widdel trace element solution (Widdel & Pfennig, 1981) (1 ml) and resazurin (1 ml), all under a stream of N2:CO2 (80:20, v/v) gas. After sterilization, 10%
NaHCO₃ (0.15 ml), 1 M Na₂SO₄ (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): (NH₄)₂SO₄ (0.45), KH₂PO₄ (0.05), MgSO₄·7H₂O (0.5), KCl (0.05), yeast extract (0.5), Ca(NO₃)₂·4H₂O (0.014), Na₂SO₄ (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% NaHCO₃ (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), H₂ : CO₂ (80 : 20, under 2 bars) and H₂ : CO₂ (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 H₂SO₄ as mobile phase. The test for minimal medium was checked by phase-contrast microscopy. The culture medium supplemented with Balch’s vitamin solution (0.05 ml) (Balch et al., 1979), 10% NaHCO₃ (0.15 ml) and 1 M fructose (0.1 ml).

For the fermentative tests, the medium used was the highly reduced medium, and the carbon sources tested were 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 (Na₂SeO₃): 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 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 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 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 x 6 multiecell 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 x 10 ml liquid culture (Sasser, 1990). Cultures of strain BSREI¹ 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, TSB40; GC model 6890N, Agilent Technologies) using GC analysis according to the MIDI Microbial Identification system (e.g. Method: TSB40). 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
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 BSREI1T was selected and used for further metabolic and physiological characterization.

The cells of strain BSREI1T 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 BSREI1T 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%. H2S production was also optimal at pH 7 (9 mM H2S produced after 15 days of incubation). Strain BSREI1T 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. Sulfitodigenic 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 BSREI1T 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 BSREI1T could use as electron donors crotonate, formate, lactate, pyruvate, fructose, glycerol and yeast extract with sulfate. Unlike D. lacus and D. meridei, strain BSREI1T 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 BSREI1T was able to use sulfate, thiosulfate and sulfate 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 BSREI1T. 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 BSREI1T stopped rapidly but even after 15 days of culture, the culture was still viable when reincubated in a standard culture medium.

The main fatty acids of strain BSREI1T were C₁₆:₁₀7c (39.9%), C₁₆:₀ (17.1%), C₁₆:₁₀9c (12.5%), C₁₈:₁₀7c (4.8%), Iso-C₁₇:₁₁ and/or anteiso-C₁₇:₁ B (4.2%), C₁₈:₁₀9c (3.3%), C₁₆:₁₀5c (2.7%), C₁₈:₀ (1.2%). MK-7 was the sole quinone found in the strain BSREI1T. 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 BSREI1T (1533 bp) revealed that it was affiliated to the genus Desulforosinus (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 BSREI1T, 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 BSREI1T 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 BSREI1T was 43.3 mol%, similar to the values quoted for other members of the genus Desulforosinus (41.6–46.9 mol%).

Strain BSREI1T was isolated from pore water collected in a borehole at ~490 m. Compared with other strains of the genus Desulforosinus, strain BSREI1T is not able to grow autotrophically or to use S⁰ as an electron acceptor, and has a single quinone.

One difference between strain BSREI1T and strain D. lacus was that BSREI1T 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 BSREI1T whereas MK-7 and MK-5 were present in the ratio of 64:36 for D. lacus. Furthermore, unlike to D. lacus, strain BSREI1T is not able to grow autotrophically, cannot use Fe(III) as electron acceptor and cannot ferment lactate. For electron donors,
strain BSREI\textsuperscript{T} cannot use ethanol and methanol in presence of sulfate, unlike \textit{D. lacus}. Finally, based on the phenotypic, phylogenetic and genetic differences observed between strain BSREI\textsuperscript{T} and other members of the genus \textit{Desulfosporosinus} (see Table 1), we identify this strain as a representative of a novel species in this genus, and propose the name \textit{Desulfosporosinus burensis} sp. nov.

**Description of \textit{Desulfosporosinus burensis} sp. nov.**

\textit{Desulfosporosinus burensis} (bu.ren’sis. N.L. masc. adj. burensis belonging to Bure, the area where the strain was isolated).

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**Table 1.** Main characteristics differentiating strain BSREI\textsuperscript{T} from its closest phylogenetic relative \textit{D. lacus} and the type species of the genus \textit{D. orientis}

<table>
<thead>
<tr>
<th>Character</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell diameter ((\mu)m)</td>
<td>0.9–1.1</td>
<td>0.5–0.7</td>
<td>0.7–1.0</td>
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<td>Gram reaction</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
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<tr>
<td>Endospore position</td>
<td>Subterminal</td>
<td>Subterminal</td>
<td>Subterminal</td>
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<tr>
<td>Motility</td>
<td>Variable</td>
<td>Variable</td>
<td>Motile</td>
</tr>
<tr>
<td>Temperature range ((^\circ)C)</td>
<td>5–30</td>
<td>4–32</td>
<td>30–47</td>
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<tr>
<td>Temperature optimum ((^\circ)C)</td>
<td>25</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>pH range</td>
<td>6–8</td>
<td>6.5–7.5</td>
<td>5.6–7.4</td>
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<tr>
<td>pH optimum</td>
<td>7</td>
<td>7</td>
<td>6.4–7.0</td>
</tr>
<tr>
<td>Electron donor in presence of sulfate:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>H(_2) (carbon source)</td>
<td>– (with CO(_2))</td>
<td>+ (with CO(_2))</td>
<td>+ (with CO(_2))</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Ethanol</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Glycerol</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Methanol</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Electron acceptors:*</td>
<td></td>
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<tr>
<td>Sulfite</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Sulfur</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fermentative growth on lactate</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>43.3</td>
<td>42.7</td>
<td>46.9</td>
</tr>
</tbody>
</table>

\(\ast\) With fructose as electron donor for strain BSREI\textsuperscript{T} and lactate as electron donor for the other strains.

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A Gram-negative, curved rod. The cells are 5–7 \(\mu\)m long and 1.0 \(\mu\)m 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 \(^\circ\)C with an optimum at 25 \(^\circ\)C. The upper limit for salt tolerance is 15 g NaCl l\(^{-1}\). Sulfate is reduced to H\(_2\)S 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\textsuperscript{T} to the genus \textit{Desulfosporosinus}. Bar, 2\% estimated sequence divergence.
methane. Sulfate, thiosulfate and sulfate are used as electron acceptors, but not elemental sulfur, fumarate, nitrate, nitrite or Fe(III). The major fatty acids are: C₁₆:1(7°), C₁₆:0 and C₁₆:1(9°). It contains a single quinone with a side chain of seven isoprene units (MK-7).

The type strain is BSREI 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%.

**Acknowledgements**

We thank the French Institution Agence Nationale pour la Gestion des Déchets Radioactifs (ANDRA) who provided financial assistance to realize this work.

**References**


