Desulfotomaculum halophilum sp. nov., a halophilic sulfate-reducing bacterium isolated from oil production facilities

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A halophilic endospore-forming, sulfate-reducing bacterium was isolated from an oilfield brine in France. The strain, designated SEBR 3139, was composed of long, straight to curved rods. It grew in 1–14% NaCl with an optimum at 6%. On the basis of morphological, physiological and phylogenetical characteristics, strain SEBR 3139 should be classified in the genus Desulfotomaculum. However, it is sufficiently different from the hitherto described Desulfotomaculum species to be considered as a new species. Strain SEBR 3139 (= DSM 11559) represents the first moderate halophilic species of the genus Desulfotomaculum. The name Desulfotomaculum halophilum sp. nov. is proposed.

INTRODUCTION

Sulfate-reducing bacteria that form heat-resistant endospores are classified in the genus Desulfotomaculum (3). The genus currently includes thermophilic and mesophilic species, all of which are capable of using a large number of substrates (26). Most Desulfotomaculum species grow best in freshwater media and tolerate low salt concentrations. Desulfotomaculum geothermicum (7), isolated from saline geothermal ground water, was described as a halotolerant species of this genus. In addition, two other unnamed types, probably adapted to a saline environment, were isolated from an oilfield (6) and from marine sediments (13). In 1979, Nazina & Rozanova (20) described a subspecies of a thermophilic and slightly halophilic Desulfotomaculum from oil deposits. The strain named Desulfotomaculum nigrificans subsp. salinus was able to tolerate up to 4% NaCl.

In the course of a survey of different oilfields, we isolated several strains of sulfate-reducing bacteria (1, 18, 24, 25), including the mesophilic spore-forming strain designated SEBR 3139, an isolate from a French oilfield brine able to grow at salinities of about 14% NaCl. Strain SEBR 3139 represents the first halophilic spore-forming, sulfate-reducing bacterium described so far. Based on 16S rDNA sequence comparisons, strain SEBR 3139 was found to be phylogenetically related to Desulfotomaculum, but is sufficiently different from the recognized Desulfotomaculum species to be classified as a new species. We propose the name Desulfotomaculum halophilum sp. nov.

METHODS

Source of the isolate. Strain SEBR 3139 (T = type strain) was isolated from a French oil-producing well in the Paris Basin. Anoxic samples of a mixture of formation saline ground water and oil were aseptically collected at the wellhead. Sampling and primary cultures of SRB were done as previously described (1, 17). The in situ temperature of the fluid at the depth of 1820 m was 85 °C, and the pressure was 19.4 MPa. The fluid temperature at the wellhead was 60 °C. The total salinity of this formation water was 70 g l⁻¹ (55 g l⁻¹ NaCl). The salinity of the culture medium was adjusted according to the in situ conditions, but the incubation temperature was 35 °C.

Media and culture conditions. The strain was isolated using the streak plate method as described elsewhere (17). The strain was grown and maintained in anoxic medium containing (l⁻¹ distilled water): 3.0 g Na₂SO₄, 6.0 g CaCl₂, 2H₂O, 40 g NaCl, 20 g KCl, 0.3 g NH₄Cl, 0.2 g KH₂PO₄, 8.0 g
boiling, the medium was cooled under an N2/CO2 (90:10) gas mixture and the pH was adjusted to 7.2–7.3. The medium was dispensed into tubes for anaerobic cultures (Belleco) and plasma bottles (60 ml) containing 1–2 g iron powder (Prolabo) per 10 ml medium. Tubes and plasma bottles were then sealed with black butyl rubber stoppers, gassed with N2/CO2 (90:10), and autoclaved for 30 min at 105 °C. Only tubes with anoxic culture medium (as indicated by resazurine colour reduction from pink to colourless) were used.

The purity of the strain was checked by using both phase-contrast microscopy and growth tests (both aerobically and anaerobically) in sulfate-free TYG medium (2).

**Morphology.** The cell morphology was observed with a phase-contrast microscope (Olympus BH-2). Photomicrographs were obtained using the agar slide method (22). Negative staining of cells was achieved with 1% phosphotungstic acid and viewed with a transmission electron microscope. The fine structure of the cells was studied by transmission electron microscopy after fixation of a cell pellet with osmic acid and ultrathin sectioning of the cells according to Glazer et al. (12). Photomicrographs of the sections were obtained with a JEOL 1200 EX electron microscope.

**Metabolism and physiology.** The physiological and metabolic tests used to characterize the strains included the ability to use different carbon substrates and energy sources, a variety of electron acceptors, fermentative growth (Table 1), and growth tests at different temperature, pH and salinity; they have been described in a previous paper (17). Bacterial growth was estimated after two consecutive transfers in the same test culture medium, by (i) observing the

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**Fig. 1.** Phase-contrast photomicrograph of *D. halophilum* strain SEBR 3139T, grown with lactate as carbon and energy source. Bar, 10 μm. The various parts show different stages of cell sporulation.

**Fig. 2.** Electron micrographs of *D. halophilum* strain SEBR 3139T. (a) Negative staining showing polar flagellum; bar, 500 nm; (b) thin section of a cell showing the Gram-positive cell wall; bar, 200 nm.
formation of black deposits in the tubes revealing sulfide production; (ii) observing bacterial division microscopically; and (iii) measuring end products from substrate utilization by HPLC as described by Cayol et al. (5).

G + C content of the DNA. The G + C content of strain SEBR 3139T DNA was determined by HPLC at the Identification Service of the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

16S rRNA sequence studies. Purification of genomic DNA, amplification and purification of the 16S rRNA gene (16S rDNA) from strain SEBR 3139T were by previously described techniques (16, 23). The purified PCR product was sequenced directly on an ABI automated DNA sequencer by using a Prism Dideoxy Terminator Cycle Sequencing Kit and protocols recommended by the manufacturer (Applied Biosystems). Using the sequence editor, ae2, the 16S rDNA sequence of the strain SEBR 3139T was aligned with sequences obtained from the Ribosomal RNA Database Project, version 5.0 (19), and from GenBank. Positions of sequence and alignment uncertainty were omitted from the analysis, and pairwise evolutionary distances of 820 unambiguous nucleotides from a total of 1526 nucleotides sequenced were computed. Phylogenetic analysis was performed using programs which form part of the PHYLIP package and include DNADIST (Jukes & Cantor option), NEIGHBOR-JOINING, transversion analysis and DNAPARS (11). Tree topology was re-examined using 100 bootstrapped data sets for which a script file with the following PHYLIP programs was used: SEQBOOT, DNADIST, FITCH and CONSENSE. Programs in the phylogenetic package MEGA (15) were also used. PHYLIP programs were run on a Sun SPARC work station and MEGA was run on a Compaq notebook (Contura model 410CX).

RESULTS

Cell morphology

The cells of strain SEBR 3139T are straight to curved rods, 0.5-5 μm wide and 3-6 μm long (Fig. 1). They are motile by a single polar flagellum (Fig. 2a). The formation of oval and terminal spores was observed. Gas vesicles were never observed. The vegetative cells stained Gram-negative, but electron microscopy demonstrated a typically Gram-positive cell wall structure (Fig. 2b).

Physiological properties

Strain SEBR 3139T grew optimally at 35°C, and slowly at 30 and 40°C. No growth occurred at or above 50°C, and at or below 20°C within 15 d incubation. The optimum pH was 7-3, the isolate grew slowly at 30 and 40°C. No growth occurred at or below 20°C within 15 d thereafter. Strain SEBR 3139T grew optimally at 35°C, and slowly at 30 and 40°C. No growth occurred at or above 50°C, and at or below 20°C within 15 d incubation. The optimum pH was 7-3, the isolate grew slowly at 30 and 40°C. No growth occurred at or below 20°C within 15 d thereafter.

An important characteristic of strain SEBR 3139T which discriminates it from other Desulfotomaculum strains is its halophily. The strain grew in the presence of 1-14% NaCl, with optimum growth at 4-6% NaCl; it was not able to grow without NaCl after two consecutive transfers.

Strain SEBR 3139T required yeast extract in the culture medium. Electron donors used for sulfate reduction included lactate, pyruvate, formate and ethanol. Lactate, pyruvate and ethanol were incompletely oxidized to acetate. Slight growth occurred in the presence of n-butyrate and malate. H₂ and formate were used as electron donors in the presence of acetate (Table 1). Pyruvate supported slow growth in the absence of sulfate, whereas lactate did not. In the presence of lactate, strain SEBR 3139T grew using sulfate, sulfite and thiosulfate as electron acceptors. Elemental sulfur, fumarate and nitrate were not used as electron acceptors (Table 1).

16S rRNA gene sequences analysis

Table 1. Electron donors and acceptors tested for the growth of Desulfotomaculum halophilum strain SEBR 3139T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Result</th>
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<tbody>
<tr>
<td>Growth (with 20 mM sulfate) on:</td>
<td></td>
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<tr>
<td>H₂ + CO₂ (0.1 MPa)</td>
<td>(+)</td>
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<tr>
<td>H₂ + acetate (0.1 MPa, 10 mM)</td>
<td>+</td>
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<tr>
<td>Lactate (10 mM)</td>
<td>+</td>
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<tr>
<td>Pyruvate (10 mM)</td>
<td>+</td>
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<tr>
<td>Malate (10 mM)</td>
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<tr>
<td>Formate + acetate (10 mM)</td>
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<tr>
<td>Acetate (10 mM)</td>
<td>-</td>
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<tr>
<td>Ethanol (10 mM)</td>
<td>+</td>
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<tr>
<td>Butanol (10 mM)</td>
<td>(+)</td>
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<tr>
<td>Crude oil†</td>
<td>-</td>
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<tr>
<td>Growth (without sulfate) on:</td>
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<tr>
<td>Pyruvate (10 mM)</td>
<td>(+)</td>
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<tr>
<td>Lactate (10 mM)</td>
<td>-</td>
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<tr>
<td>Electron acceptors (with lactate):</td>
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<tr>
<td>Sulfate (20 mM)</td>
<td>+</td>
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<tr>
<td>Sulfit (10 mM)</td>
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<td>Thiosulfate (10 mM)</td>
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<td>Sulfur</td>
<td>-</td>
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<td>Fumarate (10 mM)</td>
<td>-</td>
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<td>Nitrate (10 mM)</td>
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*In presence of yeast extract (1 g l⁻¹).
†0.5 ml per 10 ml mineral medium.

Table 1. Electron donors and acceptors tested for the growth of Desulfotomaculum halophilum strain SEBR 3139T

Growth was checked after 7 d incubation. – No growth; + good growth; (+) slight growth. The following substrates were tested in the presence of sulfate, but were not utilized (mM): fumarate (10), succinate (10), propionate (10), glycerol (10), n-butyrate (10), isobutyrate (5), valerate (5), crotonate (2), octanoate (2), nonanoate (2), palmitate (5), tartrate (2), benzoate (5), 1,4,5-trimethoxybenzoate (25), citrate (5), 2-oxoglutarate (5), gluconate (10), glutarate (5), gallate (5), glycollate (2), thioglycollate (2), thioacetamide (2), methanol (10), 2-propanol (10), acetone (5), glucose (5), fructose (5), catechol (0-5), alanine (5), lysine (10), methionine (10), cysteine (5), glutamate (5), aspartate (5), glycine betaine (5), glycine (5), indole (0-25), phenol (0-5), nicotinate (2).
bering according to Winker & Woese (27) was aligned with representatives of the various phyla of the domain Bacteria, and phylogenetic analysis was performed. Several data sets which included different representatives from the various phyla of domain Bacteria were also created and in all cases, strain SEBR 3139T was consistently placed as a member of the genus Desulfotomaculum in the sub-branch containing Gram-positive bacteria with a G+C content less than 55 mol% (10, 16, 23). The evolutionary distances separating strain SEBR 3139T and its relatives, and the dendrogram derived from these distances are depicted in Table 2 and Fig. 3, respectively. The G+C content of the total DNA of strain 3139T was calculated to be 56.3 ± 0.4 mol%, and that of the 16S rRNA gene was 53 mol%. Transversion analysis did not affect the relationship of strain 3139T with its nearest relatives.

DISCUSSION

Strain SEBR 3139T was isolated from oilfield brines with total salinity of 70 g l⁻¹ NaCl), shows that it is well adapted to its environment. However, the isolate is unable to grow at the in situ temperature, and is probably unable to exhibit sulfate reduction activity in the oil production facilities. Nevertheless, the presence of heat-resistant endospores would allow strain SEBR 3139T to survive for a long time in oil reservoirs with temperatures higher than the maximum growth temperature of the organism.

On the basis of phenotypic characteristics (dissimilatory sulfate reduction to sulfide, Gram-positive cell wall structure and formation of endospores) strain SEBR 3139T can be considered as a member of the genus Desulfotomaculum (4). Among the mesophilic species of this genus, SEBR 3139T was morphologically and metabolically close to Desulfotomaculum orientis (3, 14). However, it differed from D. orientis by its inability either to oxidize 3,4,5-trimethoxybenzoate or to ferment lactate. Moreover, the halophilic character of the isolate adapted to high salt concentrations (up to 14%) has never been observed in the genus Desulfotomaculum. Only one strain representing a subspecies, D. nigrificans subsp. salinus, has been
Desulfotomaculum halophilum sp. nov.

Desulfotomaculum halophilum (ha.lo'phi.lum. Gr. n. halos salt; Gr. adj. philos loving; N.L. adj. halophilum salt-loving).

Strictly anaerobic. Reduces sulfate, sulfite and thiosulfate with production of sulfide. Elemental sulfur, fumarate and nitrate are not used as electron acceptors. H₂, formate, lactate, pyruvate, malate, ethanol and n-butanol are oxidized. Pyruvate is fermented. Yeast extract is required as growth factor. The G+C content of the DNA is 56·3 ± 0·4 mol%. The GenBank/EMBL accession number for the 16S rRNA sequence is U88891. Habitat: production fluid from an oil-producing well in France. The type strain is SEBR 3139 (= DSM 11559).

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REFERENCES


