NOTE***Desulfovibrio aespoeensis* sp. nov., a mesophilic sulfate-reducing bacterium from deep groundwater at Äspö hard rock laboratory, Sweden**

Mehrdad Motamedi and Karsten Pedersen

Author for correspondence: Mehrdad Motamedi. Tel: +46 31 773 2556. Fax: +46 31 773 2599.
e-mail: Mehrdad.Motamedi@gmm.gu.se

Department of General
and Marine Microbiology,
Institute of Cell and
Molecular Biology,
Göteborg University,
Box 462, S-405 30
Göteborg, Sweden

A sulfate-reducing bacterium, strain Aspo-2, was isolated from granitic groundwater sampled at a depth of 600 m. This and other strains of SRB frequently occur in the deep granitic rock aquifers studied. On the basis of its morphological, physiological and genotypical properties, and its unique habitat, we propose strain Aspo-2 as a new species of the genus *Desulfovibrio*, *Desulfovibrio aespoeensis* (DSM 10631^T).

Keywords: *Desulfovibrio aespoeensis* sp. nov., sulfate-reducing bacteria, groundwater

Over the last decade, interest in deep subsurface microbiology has increased markedly. Until the middle of this century, it was assumed that micro-organisms could only live in shallow aquifers. Bacteria have now been found in deep subterranean environments, deep-sea thermal vents and at the bottom of oil wells (12, 15). In recent years, interest has increased considerably in the area of deep hard rock microbiology. One reason is that deep (500 m) fractured rock environments are suggested as possible repository sites for disposal of nuclear fuel waste material; of particular interest is how subterranean micro-organisms affect the performance of future nuclear fuel waste repositories (16, 18). The relevance of different microbial activities possibly influencing repository barriers can only be evaluated with knowledge of the properties and ecology of micro-organisms that may inhabit a repository and its surroundings. In particular, an understanding of the ecology of sulfate-reducing bacteria (SRB) in subterranean environments is essential for evaluation of the adverse effects associated with their hydrogen sulfide production, which ultimately may corrode the metal canisters used for the disposal of nuclear fuel waste material.

SRB are a unique physiological group known for their strictly anaerobic nature and their ability to use elemental sulfur, sulfate or other oxidized sulfur

compounds as electron acceptor during anaerobic respiration (1, 2, 5, 25). SRB are harmful in industry because of their primary role in the anaerobic corrosion of iron in pipelines, heating systems and other structures (8, 24).

As part of an on-going study to investigate the microbial ecology of subterranean micro-organisms (16, 20), SRB have been isolated from granitic groundwater of south-eastern Sweden, where the Äspö hard rock laboratory (HRL) has been constructed to study the function of underground repositories for long-lived nuclear fuel waste. Eleven boreholes at Äspö HRL were sampled and screened for the presence of SRB (11) using repeated enrichments with water samples inoculated in a brackish medium containing lactate and sodium sulfate as the substrate and electron acceptor, respectively (26), and incubated for 2 weeks at room temperature. The growth of SRB was confirmed by blackening of the medium and production of sulfide. Repeated use of the agar roll-tube method (27) resulted in 16 pure cultures of SRB (11). The purity of these isolates was routinely checked by microscopy and inoculation on complex sulfate-free media. The 16S rRNA gene of the isolates were partially sequenced and compared with the sequences from the EMBL database (19). Seven isolates were found to be identical to *Desulfomicrobium baculatum*, seven isolates were distantly related to *Desulfovibrio salexigens* and two isolates were distantly related to *Desulfovibrio longreachii*. The strain Aspo-2 was isolated from borehole KAS03 at depth of 600 m. Also, six other strains with 100% 16S rRNA identity with strain Aspo-2 were

Abbreviation: SRB, sulfate-reducing bacteria.

The EMBL accession number for the 16S rRNA sequence of strain Aspo-2 is X95230.

Fig. 1. Phylogenetic dendrogram based on 16S rRNA sequence comparison, showing the positions of strain Aspo-2 within the radiation of members of sulfate-reducing bacteria and related taxa.

isolated from the depths of approximately 70 m (borehole KR0013, one isolate), 200 m (borehole HA 1327-B, two isolates) and 600 m (borehole KAS03, three isolates). Here we describe isolate Aspo-2, which has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) under the collection number DSM 10631, in detail.

The DNA of strain Aspo-2 was extracted, and the 16S rRNA gene was amplified and sequenced as described elsewhere (19). An almost complete sequence consisting of 1502 bases of the amplified and cloned 16S rRNA gene of strain Aspo-2 was obtained using 11 primers. The sequence has 68 signatures which indicate that it is a member of the domain *Bacteria* and 28 signatures which show similarity with the delta subdivision of the *Proteobacteria* (28). Furthermore, the occurrence of distinctive features of *Desulfovibrio* signatures confirmed that strain Aspo-2 belongs to the genus *Desulfovibrio* (4). The complete 16S rDNA sequence of strain Aspo-2 was compared with sequences of various members of the bacterial phylum obtained from the EMBL database using the FASTA program in the GCG package (Genetics Computer Group, WI, USA). The isolate was found to be distantly related to an unnamed *Desulfovibrio* sp. (EMBL accession no. K42995; A. P. Teske and others, unpublished) and *Desulfovibrio* strain *Pib* (EMBL accession no. U33316; D. Krekeler and others, un-

Fig. 2. Scanning electron micrograph of negatively stained strain Aspo-2. Bar, 2 µm.

published) isolated from a hypersaline cyanobacterial mat, with an overall identity of 91.2% with both strains. The third closest species was *Desulfovibrio salexigens* with an identity of 90.3%. The nucleotide sequence was also compared with sequences representative of *Bacteria* and major groups of SRB using the PILEUP program in the GCG package. The positions at which any sequences had unknown or ambiguous nucleotides were omitted and pair-wise evolutionary distances were calculated for the remaining 1320 bases using DNADIST program [Jukes–Cantor method (10)]. A phylogenetic dendrogram (Fig. 1) was constructed from distance data using the Fitch–Margoliash method (6). The dendrogram was drawn using the DRAWTREE program. These programs are available in the PHYLIP package (7).

Determination of desulfovibrin and DNA base composition (using thermal melting point profile) was done as described previously (9, 22). The isolate was desulfovibrin-positive and the DNA G+C content was 61 ± 0.5 mol%. Cells of the strain Aspo-2 were vibrioid and motile. The cells were approximately 0.5 µm in diameter and 1.7–2.5 µm long and occurred singly and in chains when lactate was used as the substrate (Fig. 2). The Gram reaction was negative, electron microscopy of sectioned cells revealed a typical Gram-negative cell wall and spores were never observed. Circular, entire to erose, yellowish, raised and rough colonies were produced on plates with 1.5% agar and lactate as the substrate. Strain Aspo-2 grew optimally between 25 and 30 °C. No growth was observed at temperatures above 35 °C or below 4 °C. The optimum pH for growth was 7.5. It grew in medium containing up to 30 g NaCl l⁻¹. Optimum growth occurred at 7 g NaCl l⁻¹.

Substrate utilization was determined in duplicate tests by using the basal growth medium (26) supplied with 25 mM sodium sulfate as the electron acceptor and with different substrates at final concentrations between 5 and 57 mM depending on the substrate used. Production of acetate was determined using an acetic acid kit (Boehringer Mannheim). For cultivation with H_2 as the electron donor, duplicate basal medium was used supplied with 25 mM sodium sulfate with and without 4 mM acetate and a mixture of H_2/CO_2 (90/10, v/v) with an overpressure of 50–66 kPa (0.5 atm) in the headspace corresponding to two-thirds of the culture volume. To determine the reduction of different electron acceptors by strain Aspo-2, the duplicate basal medium was added with 57 mM sterile sodium lactate and supplied with 10–70 mM of the electron acceptors considered (sterile, anaerobic from stock solutions). Growth was determined by acridine orange direct count (14) and by following production of sulfide (3). In the presence of sulfate, isolate Aspo-2 oxidized lactate (57 mM) incompletely to acetate. Pyruvate (11 mM) was fermented in a sulfate-free medium. No growth was observed on the following substrates with sulfate as the electron acceptor: formate (20 mM), methanol (25 mM), fumarate (20 mM), malate (20 mM), 2-propanol (10 mM), isobutanol (10 mM), alanine (10 mM), butyrate (10 mM), phenol (1.25 mM), benzoate (5 mM), palmitate (1 mM), acetate (20 mM) and ethanol (20 mM). Hydrogen served as energy source in the presence of sulfate with 4 mM acetate as a carbon source. Thiosulfate and sulfur were used as electron acceptors in the presence of lactate. Nitrate and iron(III) hydroxide could not serve as electron acceptors in a medium supplied with lactate. Fermentation of succinate and fumarate on sulfate-free medium was not observed.

According to *Bergey's Manual of Systematic Bacteriology* (27), considering the key to the genera of SRB, isolate Aspo-2 belongs to genus *Desulfovibrio*, because a positive desulfovibrin test and morphological characters such as motility and vibrioid shape distinguish most *Desulfovibrio* from *Desulfobacter*, *Desulfococcus*, *Desulfosarcina*, *Desulfomicrobium* and *Desulfobulbus*. Strain Aspo-2 and the closest described and accepted species in the 16S rRNA gene database, *D. salexigens*, differ phenotypically in several aspects. Strain Aspo-2 is a halotolerant micro-organism that grows with up to 30 g NaCl l⁻¹ and optimum growth was observed in the presence of 7 g NaCl l⁻¹. In contrast, *D. salexigens* is a moderately halophilic micro-organism that requires 20 g NaCl l⁻¹ for growth (26). In addition to the sequence differences, strain Aspo-2 differed from all other *Desulfovibrio* species [except *D. longus* (13)] by its inability to utilize ethanol. The G+C content of strain Aspo-2 (61 ± 0.5 mol%) falls within the genus *Desulfovibrio* (47–65 mol%) (26), but differs markedly from *D. salexigens* (49 mol%). In contrast to *D. salexigens*, which can grow on pyruvate only in the presence of sulfate (23), strain Aspo-2 grows on pyruvate without any ad-

ditional electron acceptor. Growth of strain Aspo-2 on a medium containing elemental sulfur as electron acceptor and lactate as the carbon and energy source was observed and confirmed by acridine orange direct count and production of sulfide.

Aspo-2 was isolated from depth at Äspö HRL in south-eastern Sweden together with two other SRB, a *Desulfomicrobium baculatum* and a *Desulfovibrio* sp. strain (11, 19). The number of viable SRB in the boreholes KR0013 (70 m), HA1327-B (130 m) and KAS03 (600 m) where strain Aspo-2 was found, was > 5, 130 and 1390 cells ml⁻¹, respectively. In these boreholes, pH varied between 7.1 and 7.5, and the sulfate concentration varied between 1.45 and 2.12 mM. The amount of dissolved organic carbon decreased with depth from 15 mg l⁻¹ at 70 m to 1.0 mg l⁻¹ at 600 m, and salinity was approximately 1% in all three boreholes. The temperature increased with depth from 9 °C at 70 m to approximately 20 °C at 600 m. Overall, the deep groundwater environment from which Aspo-2 was isolated shows potential for growth of SRB. The amounts of organic carbon and sulfate are sufficient to support the growth of SRB. Moreover, the pH and salinity observed are almost optimal for growth of strain Aspo-2, and its ability to grow at a low temperature (≥ 4 °C) means temperature is not a limiting factor for *in situ* activity. 16S rRNA gene diversity analysis of attached and unattached groundwater bacteria in boreholes at Äspö HRL (70–190 m depth) revealed sequences related to SRB in several boreholes as previously described (19). Recently, 16S rRNA sequences that are similar to Aspo-2 have been found in Äspö HRL at 400 m depth [EMBL accession nos Z69309 and Z69325 (21)]. Geological, hydrological, stable isotope and groundwater chemistry data indicate that sulfate reduction was on-going in the sites from which Aspo-2 was isolated before the tunnel was constructed (17). Altogether, these results suggest that the deep granitic aquifers of Äspö are a natural habitat for SRB communities, of which Aspo-2 is a member. A potential for microbially induced corrosion of nuclear waste storage canisters is consequently possible and must be evaluated further.

In conclusion, on the basis of habitat, physiological and phylogenetic differences compared with other members of genus *Desulfovibrio*, strain Aspo-2 is not a member of any previously described *Desulfovibrio* species. We propose, therefore, that this organism belongs to a new species, *Desulfovibrio aespoeensis*, with deep granitic groundwater as its major habitat.

Description of *Desulfovibrio aespoeensis* sp. nov.

Desulfovibrio aespoeensis (ae.spoe.en'sis. M. L. adj. *aespoensis* pertaining to Äspö, south-eastern Sweden).

Vibrioid-shaped cells are 0.5 µm in diameter and 1.7–2.5 µm long and occur singly. Cells are motile and

contain desulfovibrin. Spores are never produced. In the presence of sulfate, lactate is utilized as the sole electron donor and carbon source. Lactate is incompletely oxidized to acetate. It grows lithoheterotrophically on H_2 and acetate (as the carbon source). Pyruvate is fermented in the absence of sulfate. The following substrates are not used in the presence or absence of sulfate: formate, succinate, methanol, fumarate, malate, 2-propanol, isobutanol, alanine, butyrate, phenol, benzoate, palmitate, ethanol and acetate. Electron acceptors include sulfate, thiosulfate and sulfur. Nitrate and iron(III) hydroxide are not used as electron acceptors. Addition of NaCl is not required, but up to 30 g NaCl l^{-1} is tolerated, and optimum growth occurs in the presence of 7 g NaCl l^{-1} . The temperature range for growth is 4–35 °C, the optimum being 25–30 °C. The optimum pH for growth is 7.5. The G+C content of DNA is 61 ± 0.5 mol % (as determined by thermal melting point profile). The EMBL accession number for the 16S rRNA sequence of strain Asp-2 is X95230. *D. aespoensis* was isolated from deep subterranean granitic groundwater (600 m) of south-eastern Sweden. The type strain is Asp-2 (= DSM 10631).

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