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Salinisphaera shabanensis gen. nov., sp. nov., a novel, moderately halophilic bacterium from the brine–seawater interface of the Shaban Deep, Red Sea

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Abstract A novel, moderately halophilic bacterium was isolated from the brine–seawater interface of the Shaban Deep, northern Red Sea. A polyphasic approach was used for the taxonomic characterization of this isolate, with the phenotypic and phylogenetic data clearly showing the distinctiveness of this bacterium. Cells of isolate *E1L3A* were Gram-negative, monotrichous cocci that showed a remarkable physiological flexibility, as could be seen by the quite broad growth ranges for oxygen, temperature, NaCl, and, to a smaller degree, pH. In addition, it was able to grow from atmospheric pressure up to 15 MPa, making it a piezotolerant bacterium. Phylogenetically, strain *E1L3A* represents a new, deeply branching lineage within the γ-Proteobacteria, as determined by 16S rRNA gene sequence analysis. No close relatives are known so far, with sequence similarity to other cultivated members of the γ-Proteobacteria being lower than 88%. The creation of the new genus *Salinisphaera* and the new species *Salinisphaera shabanensis* (DSM 14853; JCM 11575) for this new and highly versatile microorganism is therefore proposed.

Keywords Brine · Moderately halophilic · Piezotolerant · Red Sea · *Salinisphaera* · *Salinisphaera shabanensis* · Shaban Deep

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Introduction

Transition from continental to oceanic rift is currently occurring in the Red Sea. Data from the region suggest that initial emplacement of oceanic crust is punctiform and is progressing from south to north. This would justify the differential topography of the bottom of the Red Sea, with a fully formed rift valley in the southern area contrasting with only isolated deeps in the northernmost parts (Bonatti 1985). Some deeps are filled with highly saline brines as a result of leaching from subbottom Miocene evaporites (Manheim 1974; Zierenberg and Shanks 1986; Hartmann et al. 1998; Scholten et al. 1999).

The Shaban Deep (Shaban is the eighth month of the Muslim year) lies near 26°16' N, 35°21' E. The Deep was originally discovered in 1981 (Cruise Report Menor 1 1983; Cruise Report Menor 2 1984), but the northern and western basins were detected later (Pautot et al. 1984; Puchelt 1984). The Shaban Deep has an approximately rhombic shape and extends over an area of 10×6 km with a central ridge with two adjacent saddles, delineating four different basins (Pautot et al. 1984). The brine–seawater interface was found to occur at a water depth near 1,325 m, while an estimated maximum depth occurred at 1,540 m (southern basin). An average temperature of approximately 23 °C and pH values near 6.0 were registered in the upper brine of the Deep (Hartmann et al. 1998). All four basins are filled with H_2S-free anoxic brine waters with salinity values of 25.6–26.1%, close to saturation (Michaelis et al. 1990; Hartmann et al. 1998).

Despite the great microbiological potential of the brine pools of the Red Sea (Eder et al. 1999, 2001), they still remain a poorly studied environment, although some noteworthy exceptions do exist. Initial studies were performed on the Atlantis II and Discovery Deeps, but bacteria were only detected in the transition zone of the first Deep and in the sediments of the second, while the brines themselves were thought to be sterile (Watson and

Waterbury 1969). The existence of a halotolerant *Desulfovibrio* strain from the Atlantis II Deep brine–seawater interface was reported by Trüper (1969), but the strain was not fully described. About 20 years later, Fiala et al. (1990) isolated and characterized a new flexible Gram-negative bacterium, *Flexistipes sinusarabici*, from the Atlantis II Deep. Recently, novel 16S rRNA gene sequences were retrieved from the brine-sediment and the brine–seawater interface of Kebrit Deep, indicating that novel groups of Archaea and Bacteria might thrive in this extreme environment (Eder et al. 1999, 2001). Further work on samples from the brine–seawater interface of Kebrit Deep resulted in the isolation of two new strains of the genus *Halanaerobium*, which had been predicted by in situ phylogenetic analysis (Eder et al. 2001).

During ''Meteor'' cruise 44/3 in 1999, samples from the brine–seawater interface from the Shaban Deep were retrieved. Besides phylogenetic analysis (Eder et al. 2002), culture attempts were performed from sample ST-12 of the eastern basin, which resulted in the isolation of strain *E1L3A*. A polyphasic approach was used for the taxonomic characterization of this isolate, with the phenotypic and phylogenetic data clearly showing the distinctiveness of this bacterium. We therefore propose the creation of the new genus *Salinisphaera*, and the new species *Salinisphaera shabanensis* for this new, highly versatile bacterium.

Materials and methods

Sampling

The brine–seawater interface of the east basin of the Shaban Deep, Red Sea, was sampled during R.V. ''Meteor'' cruise M 44/3 in 1999 using a rosette water sampler equipped with 12 Niskin bottles (10 l) and a CTD unit for monitoring salinity, temperature, transmission, oxygen, and pressure (Hydro-Bios Apparatebau, Kiel-Holtenau, Germany). Sample ST-12 (station no. 197a; 26°14.00' N, 35°22.71' E) was taken from the east basin of the Shaban Deep at a depth of 1,331 m. An in situ temperature of 23 °C and hydrostatic pressure of 13.38 MPa were recorded. The sample presented a salinity range between 21.2% and 23% (over the 1-m length of the Niskin bottle) as measured with a hand refractometer (Atago, Tokyo, Japan). A pH of 6.5 was determined with Neutralit (Merck Eurolab, Darmstadt, Germany). Oxygen in the sample was reduced with sodium dithionite (about 0.1 μM). The sample was transported to the laboratory by air at 4 °C and afterwards was stored at 4 °C.

Light and electron microscopy

Light microscopy, electron microscopy, and photography were carried out as described previously (Huber et al. 1998).

Growth conditions

Cells were routinely grown on modified SD medium (Eder 2000) , which was designated as SD0 medium and contained (per liter): NaCl, 100 g; $MgSO_4 \cdot 7H_2O$, 3.45 g; $MgCl_2 \cdot 6H_2O$, 3.0 g; NH_4Cl, 0.25 g; $CaCl_2 \cdot 2H_2O$, 0.14 g; $CaSO_4 \cdot 2H_2O$, 0.25 g; KH_2PO_4,0.14 g; $NaHCO_3$, 1.00 g; mineral solution (Balch et al. 1979), 1.0 ml. The pH was adjusted to 6.0 with HCl. Portions (10 ml) of the medium were dispensed into 28-ml serum tubes, which were stoppered, sealed, and pressurized (300 kPa N_2). Before inoculation, tubes were supplemented with O_2 and yeast extract (Becton Dickinson, Le Pont de Claix, France) to a final concentration of 1% (v/v) and 0.01%, (w/v), respectively, and with 1.0 ml of a vitamin solution (Balch and Wolfe 1976). SD0 solid medium was obtained by addition of 2% agar (w/v; final concentration) before autoclaving. Anaerobic growth was tested using SD0 medium, supplemented with different carbohydrates or different electron acceptors to a final concentration of 1% (w/v), and reduced with 0.25% H_2S (v/v; final concentration).

Morphological, physiological, and biochemical studies

All investigations were performed at 30 °C, pH 6.0, and 10% NaCl unless otherwise stated. The main characteristics of taxonomical significance were considered to be those employed by Ventosa et al. (1998) for distinction of aerobic or facultatively anaerobic Gram-negative, moderately halophilic bacteria. Colony morphology and the presence of cytochrome oxidase and catalase were determined after growth on SD0 agar as described by Smibert and Krieg (1981). Growth at different temperatures, pH values, and NaCl concentrations was determined in SD0 medium. Cells were grown in high-pressure chambers (Huber et al. 1994) at 30 °C using a wide range of pressures and NaCl concentrations. The ability to hydrolyze gelatin, casein, starch, esculin, Tween 80, and DNA and the presence of phosphatase and H_2S production were assessed after growth on SD0 agar as described by Hudson et al. (1986) and Smibert and Krieg (1981). Acid production from D(–) arabinose, D(+) glucose, lactose, trehalose, and D(–) mannitol was assessed with standard SD0 medium supplemented with bromocresol purple (0.017%; w/v) prior to autoclaving. Filter-sterilized solutions of the selected carbohydrates were added before inoculation at a final concentration of 1% (w/v).

NMR analysis of compatible solutes

For determination of the compatible solute composition and content in *E1L3A*, the isolate was grown aerobically in SD0 medium with 0.1% yeast extract at different NaCl concentrations, harvested, and freeze-dried. The cells were suspended in H_2O and disrupted first by ultrasonication and then by passing through a French pressure cell at 3.3 MPa (three times). Small aliquots of each lysate (50 μl) were heated to 80 °C for 5 min, and protein concentrations were determined using the Bradford assay. Extraction of intracellular solutes was performed by boiling the French press lysates in 80% ethanol for 10 min. The extraction was repeated twice and the combined extracts were evaporated to dryness under vacuum; the residue was dissolved in a mixture of water and chloroform (2:1; v/v) to remove lipid components. The aqueous fraction was dried in a speed-vac and dissolved in ^2H_2O. Samples were analyzed by ^1H- and ^{13}C-NMR spectroscopy on a Bruker DRX500 (Bruker, Rheinstetten, Germany) spectrometer as previously described (Silva et al 1999).

Determination of mean base composition of DNA

The G+C content of the overall genome was determined by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, using the HPLC method.

DNA extraction, PCR, and sequencing

Extraction of nucleic acids, PCR reactions (primers: 9bF and 1512uR), and sequencing of the 16S rRNA gene (primers: 9 bF, 519 uF, 704bR, 1116bR and 1512uR) were carried out as described previously (Eder et al. 2001; Eder and Huber 2002).

Phylogenetic analysis

For the analysis, an alignment of about 11,000 homologues of full (and partial) primary sequences available in public databases (ARB project, Ludwig and Strunk, http://www.arb-home.de; Ludwig 1995) was used. The new 16S rRNA gene sequence (1,448 nucleotides) was fitted in the 16S rRNA tree by using the respective automated tools of the ARB software package (Ludwig and Strunk, http://www.arb-home.de). Distance matrix (Jukes and Cantor correction), maximum parsimony, and maximum likelihood (fastDNAml) methods were applied as implemented in the ARB software package (Page and Holmes 1998; Ludwig and Klenk 2001). Phylogenetic distances were determined by using Distance Matrix without applying a correction factor. The sequence alignment was checked manually and was also submitted to the CHECK_CHIMERA program of the Ribosomal Database Project (RDP) (Maidak et al. 2000) to detect the presence of a possible chimeric artifact.

Nucleotide sequence accession number

The 16S rRNA gene sequence of *E1L3A* was submitted to EMBL/DDBL/GenBank databases and has been assigned accession number AJ421425.

Results and discussion

Strain *E1L3A* was isolated from sample ST-12, collected from the brine–seawater interface of Shaban Deep during ''Meteor'' cruise 44/3 in 1999. Initial enrichment cultures were established in 28-ml serum tubes using 10 ml brine water as the basal medium. This medium was used under microaerophilic and anaerobic conditions (addition of 2.5% H_2S (v/v; final concentration) and was supplemented with various sterile organic and inorganic compounds (e.g., yeast extract, peptone, $NaNO_3$, Na_2SO_4). After 29 days' incubation at 25 °C, coccoid cells became visible in a medium with 1% O_2, 0.2% FeO(OH), and 0.01% of a mixture of equal parts of yeast extract, meat extract, peptone, and brain-heart infusion (C-Org). The organisms were transferred several times in the supplemented brine and then were successfully cultivated in SD medium containing 21.5% NaCl (w/v; Eder 2000). A pure culture was achieved using the ''optical tweezers'' method as previously described (Huber et al. 1995, 2000).

Cells of isolate *E1L3A* were Gram-negative cocci with a diameter of approximately 0.7–1.2 μm, although they appeared to pass through an intermediary stage before cell division, where they might be mistaken for short rods. Cells occurred singly or in pairs and were motile by a single flagellum (Fig. 1). After 5 days' incubation at 30 °C on SD0 agar, colonies were small (diameter approximately 0.15 mm), round, and brownish-yellow. Colonies showed a positive reaction for catalase and a negative reaction for phosphatase, were cytochrome oxidase positive, and produced no hydrogen sulfide. Also, no acid was formed from D(–) arabinose, D(+) glucose, lactose, trehalose, or D(–) mannitol. In addition, Tween 80, gelatin, casein, starch, esculin, and DNA were not hydrolyzed. The G+C content of *E1L3A* was shown to be 61.8 mol%.

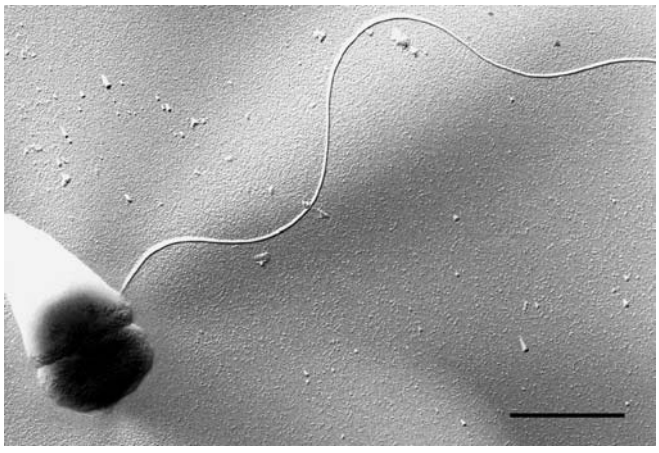

Fig. 1 Transmission electron micrograph of a dividing *Salinisphaera shabanensis* cell showing a flagellum. The cells were air dried and platinum shadowed. Bar, 1 μm

The NaCl range for growth was quite broad, with growth capability between 1% and 28% NaCl and optimal growth at 10% NaCl. No growth was observed at 0% and 29% NaCl. The ability to grow over such a wide salinity range is not uncommon, but such a broad range is unusual for the aerobic or facultatively anaerobic Gram-negative, moderately halophilic bacteria outside the family *Halomonodaceae* (Ventosa et al. 1998). *E1L3A* grew best between 30 °C and 37 °C, with growth occurring between 5 °C and 42 °C but not at 50 °C. Growth was detected between pH 4.0 and 8.0, with optimal growth between 6.5 and 7.5. No growth occurred at pH 3.0. Growth at higher pH values was not assessed because of the formation of precipitates of unknown chemical composition in the medium. Growth between 0% and 20% O_2 took place, with optimal growth between 10% and 20% O_2. Growth also occurred under strictly anaerobic conditions; however, the final cell densities were much lower (up to about 10^7 cells/ml). Anaerobic growth appeared to rely on complex organic components (e.g., yeast extract) as a substrate for growth. Growth on anaerobic SD0 medium supplemented with D(–) arabinose, D(+) glucose, lactose, trehalose, D(–) mannitol, NO_3^-, SO_3^-, SO_4^{2-}, $S_2O_3^{2-}$, or Fe^{3+} failed to induce higher cell densities. The addition of sulfur even had an inhibitory effect on the growth of *E1L3A*.

The first studies focusing on the joint effect of high-pressure (5, 15, and 20 MPa) and high-salt stress conditions on the growth behavior of any moderately halophilic microorganism were performed. Cells of *E1L3A* grew in media containing 5%, 10%, or 20% NaCl at hydrostatic pressures up to 15 MPa, but no growth occurred at 20 Mpa. Highest cell concentrations were observed at the optimal salt concentration of 10% NaCl. At all three salt concentrations, the final cell densities decreased with increasing pressure (e.g.,

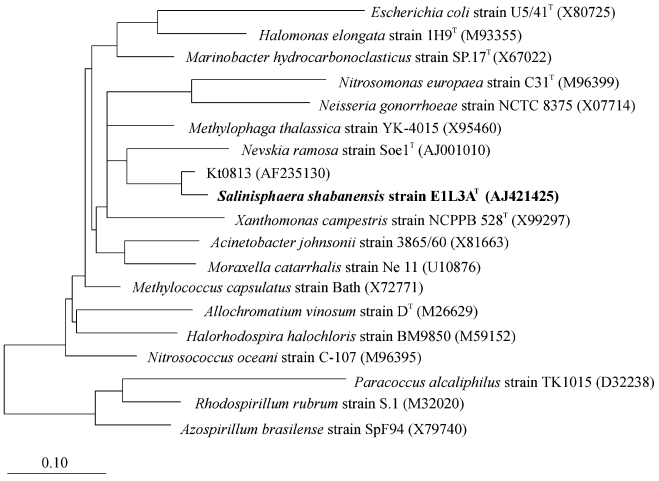

Fig. 2 16S rRNA-based phylogenetic tree showing the position of *Salinisphaera shabanensis* within the γ-Proteobacteria. The tree topology is based on the ARB database of 11,000 sequence entries and was reconstructed using the ARB parsimony tool. A filter defining positions that share identical residues in at least 50% of all included sequences from the β- and γ-Proteobacteria was used for reconstructing the tree. Multi furcations indicate that a (statistically) significant relative branching order could not be determined or is not supported by other treeing methods. Reference sequences of the β- and γ-Proteobacteria were chosen to represent a broad phylogenetic diversity, while members *of the α-Proteobacteria we*re used as outgroup. Accession numbers for the sequences are indicated. The scale bar represents 0.10 fixed mutations per nucleotide position

10^8 cells/ml [100 kPa], 5×10^7 cells/ml [5 Mpa], and 10^7 cells/ml [15 MPa] at 10% NaCl), defining isolate *E1L3A* as a piezotolerant bacterium (Yayanos 1995).

Within the Shaban Deep, the optimal growth conditions of *E1L3A* appear to point to a preference for the brine–seawater interface, which is in agreement with the origin of the sample. However, the physiological properties of this microorganism might allow it to thrive in a broader area, including the water overlying the brine, and could therefore constitute an advantage in such a harsh environment. In this context, it is noteworthy that a closely related 16S rRNA gene sequence was identified from a surface-water sample, collected at a 1-m depth in the North Sea (Fig. 2; Eilers et al. 2000).

The finding that *E1L3A* accumulates increasing amounts of organic compatible solutes at increasing NaCl concentrations shows that this organism has adopted a strategy of osmoadaptation that enables a high degree of adaptability to changes in salinity (Table 1). Ectoine and betaine, the most common compatible solutes found in mesophilic halophiles, are used by *E1L3A* to cope with high salinity in the medium. Especially impressive are the intracellular concentrations estimated for these osmolytes at 25% NaCl (above 4 M). Glycerol does not seem to play a role in osmoadaptation, since it was found only at low salinity; however, to our knowledge, this is the first report on accumulation of this compound in prokaryotes.

Table 1 Composition and content of organic solutes in *Salinisphaera shabanensis*, grown aerobically at 33 °C and at different NaCl concentrations

Solute	Concentration (μmol/mg protein)		
	5% NaCl	10% NaCl	25% NaCl [a]
Ectoine	0.2	1.6	16
Betaine	1.0	3.5	28
Glycerol	2.2	–	–

[a]Cells grown at 30 °C

E1L3A showed a remarkable flexibility, as can be seen by the quite broad growth ranges for temperature, NaCl concentration, and , to a smaller degree, pH. The isolate is a facultative anaerobe that is able to grow at anaerobic, microaerophilic, and oxygen-saturated conditions. It can grow at atmospheric pressure and also under hydrostatic pressure of 15 MPa. In agreement with these various capabilities is the outstanding phylogenetic position of *E1L3A*. Based on phylogenetic analysis, *E1L3A* represents a new, deeply branching lineage within the γ-Proteobacteria. There are no close cultivated relatives known so far, with sequence similarity to other members of the γ-Proteobacteria being lower than 88% (Fig. 2). Therefore, due to the significant phenotypic and phylogenetic differences between *E1L3A* and the previously described bacteria, we propose the creation of the new genus *Salinisphaera* and the new species *Salinisphaera shabanensis*.

Description of *Salinisphaera* Antunes, Eder, Fareleira, Santos, and Huber gen. nov.

Salinisphaera (Sa.li.ni.sphae'ra, L. adj. *salinus*, saline; M.L. fem. n. *sphaera*, a sphere; *Salinisphaera*, coccoid microorganism, capable of growth at high salt). Gram-negative, monotrichous cocci, occurring singly or in pairs. Facultatively anaerobic. Catalase and oxidase positive. Moderately halophilic and piezotolerant. Phylogenetically, the genus *Salinisphaera* represents a new, deeply branching lineage within the γ-Proteobacteria, as determined by 16S rRNA gene sequence analysis. The G+C content of the DNA of the type species is 61.8 mol%, as determined by the HPLC method. The type species is *Salinisphaera shabanensis*.

Description of *Salinisphaera shabanensis* Antunes, Eder, Fareleira, Santos, and Huber sp. nov.

Salinisphaera shabanensis (sha.ba.nen'sis, M.L. adj. *shabanensis*, from Shaban, referring to Shaban Deep, the place of isolation). Cells are Gram-negative monotrichous cocci 0.7–1.2 μm in diameter, occurring singly or in pairs. Colonies are small, round, and brownish-yellow. Facultatively anaerobic. Catalase and

oxidase positive. Moderately halophilic and piezotolerant. Growth occurs between 1% and 28% NaCl; optimal growth at 10%; no growth at 0% or 29% NaCl. Growth occurs from 5 °C to 42 °C; optimal temperature is between 30 °C and 37 °C; the pH range for growth is between 4.0 and 8.0; optimum pH is 6.5 to 7.5. Acid is not produced from D(–) arabinose, D(+) glucose, lactose, trehalose, or D(–) mannitol. Tween 80, gelatin, casein, starch, esculin, and DNA are not hydrolyzed. Phosphatase negative; hydrogen sulfide is not produced. The G+C content of the DNA of strain *E1L3A* is 61.8 mol%, as determined by the HPLC method. The organism was isolated from the brine–seawater interface of the Shaban Deep, Red Sea. Strain *E1L3A* has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany as strain DSM 14853 and in the Japan Collection of Microorganisms as strain JCM 11575.

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