Halosarcina pallida gen. nov., sp. nov., a halophilic archaeon from a low-salt, sulfide-rich spring

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A novel halophilic archaeon, strain BZ256^T, was isolated from Zoddletone Spring, a sulfide- and sulfur-rich spring in south-western Oklahoma, USA. Cells were non-motile, non-flagellated cocci that divided along two axes, resulting in the formation of sarcina-like clusters. Strain BZ256^T grew at salt concentrations ranging from 1.3 to 4.3 M NaCl, with optimum growth at approximately 3.4 M, and required at least 1 mM Mg²⁺ for growth. The pH range for growth was 5.0 to at least 8.5, and the temperature range for growth was 25–45 °C. The two diether phospholipids that are typical of members of the order *Halobacteriales*, namely phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, were present in strain BZ256^T, as were two glycolipids chromatographically identical to S-DGD-1 and DGD-1. The 16S rRNA gene sequence of strain BZ256^T showed 96.8% similarity to that of the type strain of *Halogeometricum borinquense*, the closest recognized species within the order *Halobacteriales*. The DNA G+C content of strain BZ256^T was 65.4 mol%. Microscopic, physiological, biochemical and phylogenetic comparisons between strain BZ256^T and recognized genera of extremely halophilic archaea suggest that this strain represents a member of a novel genus and species within the family *Halobacteriaceae*, for which the name *Halosarcina pallida* gen. nov., sp. nov. is proposed. The type strain of *Halosarcina pallida* is BZ256^T (=KCTC 4017^T =JCM 14848^T).

Members of the family *Halobacteriaceae*, the single family recognized within the order *Halobacteriales*, have long been identified as the most abundant micro-organisms in hypersaline environments (Oren, 1994). At the time of writing, the family *Halobacteriaceae* comprised 25 recognized genera and 101 recognized species. Recently, the extent of the family has been undergoing rapid expansion, with the description (as of June 2007) of five new genera and 20 novel species since 2006. This expansion has been due not only to the identification of novel taxa isolated from well-known hypersaline environments, such as the genera *Halovivax*, *Halostagnicola*, *Haloplanus* and *Haloquadratum* (Castillo *et al.*, 2006a, b; Bardavid *et al.*, 2007; Burns *et al.*, 2007), but also to the recognition that members of the *Halobacteriales* can grow within saline

microniches in non-saline environments at relatively low salt concentrations (Elshahed *et al.*, 2004a; Savage *et al.*, 2007). In addition, recent studies have reported the isolation of novel halobacterial strains (Purdy *et al.*, 2004; Fukushima *et al.*, 2007) or the detection of novel *Halobacteriales*-affiliated 16S rRNA gene sequences from moderate- to low-salinity systems (Munson *et al.*, 1997; Takai *et al.*, 2001; Walsh *et al.*, 2005). These studies have shown that the order *Halobacteriales* is more diverse than previously believed. In the present study, we report on the isolation and characterization of strain BZ256^T from a sulfur- and sulfide-rich spring in south-western Oklahoma, USA. We suggest that this strain represents a novel species of a new genus within the order *Halobacteriales*.

The location and geochemical characteristics of the spring, as well as its archaeal diversity and the rationale for encountering halophilic archaea in this seemingly low-salt environment, have been described by Elshahed *et al.* (2004b) and Savage *et al.* (2007). Sampling and isolation procedures were as described in detail by Savage *et al.* (2007). Briefly, samples were plated on halophilic medium (HMD) containing the

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BZ256^T is EF055454.

A figure showing the results of lipid composition analysis of strain BZ256^T by TLC is available as supplementary material with the online version of this paper.

following (per litre distilled water): 20 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5 g K_2SO_4 , 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 g NH_4Cl , 0.05 g KH_2PO_4 , 0.5 g carbon source and 20 g agar; the pH was adjusted to 7.0–7.2 with NaOH (Savage *et al.*, 2007). The HMD included one of 12 different complex or defined substrates (glucose, glycerol, tryptone, tryptose, peptone, nutrient broth, citrate, sodium benzoate, cysteine, Casamino acids, yeast extract or glutamate) at three different NaCl concentrations (180, 250 or 300 g l^{-1}). Ampicillin and kanamycin (50 $\mu\text{g ml}^{-1}$) were added to select against halotolerant and halophilic bacteria. The plates were incubated at 37 °C under a 60 W light bulb placed approximately 30 cm above the plates until colonies appeared. We subsequently determined that cell growth and pigment formation in strain BZ256^T occurred when grown in the absence of light. To ensure purity, single colonies of the isolate were restreaked twice onto HMD plates.

Characterization of strain BZ256^T was performed according to guidelines provided by Oren *et al.* (1997). Detailed protocols for the methodology of biochemical tests were obtained from Gerhardt *et al.* (1994), and NaCl was added as necessary. Physiological tests were conducted by using an HMD liquid or solid (2.0 % agar) medium with sucrose (0.5 g l^{-1}) as the carbon source, 180 g NaCl l^{-1} and 25 mM HEPES, unless otherwise stated. The minimum NaCl concentration necessary to stabilize cells in distilled water or HMD medium was tested as described by Savage *et al.* (2007). The NaCl concentrations tested ranged from 0 to 7 %. Growth rates at different pH, temperatures and Mg^{2+} and NaCl concentrations were determined by monitoring an increase in OD₆₀₀. The maximum pH tested was 8.5 due to the formation of a precipitate at higher pH values that interfered with optical density readings. The ability of strain BZ256^T to utilize DMSO (5.0 g l^{-1}), trimethylamine oxide (5.0 g l^{-1}), nitrate (30 mM), sulfate (30 mM), thiosulfate (30 mM) or elemental sulfur as a terminal electron acceptor and to ferment arginine (5.0 g l^{-1}) was tested in HMD prepared anaerobically in serum tubes according to the procedures of Bryant (1972) and Balch & Wolfe (1976). Sulfur was added in sublimated form suspended in an aqueous solution (Widdel, 2006). The sulfur tubes were emended with 0.02 % ferrous ammonium sulfate; a positive result was indicated by the formation of a black precipitate of ferrous sulfide. Acid production from carbohydrates was tested in unbuffered HMD medium and was determined by measuring the initial and final pH of the medium. A decrease of 1 pH unit or more was considered to indicate a positive acid production result.

Antibiotic sensitivity was determined in liquid HMD medium with antibiotic concentrations at 35 $\mu\text{g ml}^{-1}$ except for aphidicolin (30 $\mu\text{g ml}^{-1}$). Scanning electron microscopy was conducted at the University of Oklahoma's Samuel Roberts Noble Electron Microscopy Laboratory. Samples were prepared by fixing cells onto a poly(lysine)-coated coverslip with glutaraldehyde. Cells were then coated with gold/palladium and examined in a JSM-880 scanning electron microscope.

The DNA G+C content was determined by using the services of the German National Resource Centre for Biological Material (DSMZ). Membrane lipids were analysed by using two-dimensional TLC as described by Oren *et al.* (1996). The 16S rRNA gene was amplified by using primer pairs A1F (Tajima *et al.*, 2001) and UA1406R (Baker *et al.*, 2003) as described by Savage *et al.* (2007). The PCR product was then cloned by using the TOPO-TA cloning kit (Invitrogen). Ten clones were picked randomly and sequenced at the Oklahoma Medical Research Foundation (Oklahoma City, OK, USA), to determine whether strain BZ256^T possessed multiple distinct 16S rRNA gene sequences. 16S rRNA gene sequences were aligned via CLUSTAL_X (Thompson *et al.*, 1997), and distance trees were constructed with PAUP 4.01b10 (Sinauer Associates) by using the neighbour-joining algorithm and Jukes–Cantor corrections.

Strain BZ256^T was originally isolated from HMD plates containing 25 % NaCl and sodium benzoate as the carbon source. It formed pale-pink colonies that were punctiform, flat, circular and translucent. Colonies became larger and mucoid after prolonged incubation. Cells were non-motile cocci that developed in amorphous clumps. Electron microscopy revealed that cells divided along two axes, resulting in the formation of sarcina-like clusters during the exponential phase of growth (Fig. 1). During late-exponential and stationary phases of growth, cells were coccoid, occurring singly or in pairs. Wais (1985) described a similar cellular morphology in an uncharacterized halophilic archaeon isolated in Jamaica. Cells of strain BZ256^T stained Gram-negative (Dussault, 1955) and the presence of gas vesicles was not evident via light microscopy.

Fig. 1. Scanning electron micrographs of cells of strain BZ256^T showing the unique cell division pattern. Bars, 500 nm.

Strain BZ256^T was able to grow over a wide range of salt concentrations, 1.7–4.3 M, with optimum growth at 3.1 M NaCl. Cells lysed immediately when suspended in distilled water, and could not be recovered from sterile 1–3 % salt solutions. However, cells could be recovered after being suspended in sterile 4 % NaCl solution for 24 h. When suspended in HMD medium lacking NaCl, cells remained viable for up to 72 h. Detailed physiological and biochemical characteristics of strain BZ256^T are listed in Table 1, as well as in the species description. In general, strain BZ256^T was able to grow in defined as well as complex media; sucrose, glucose and glycerol as carbon sources yielded the best growth. Strain BZ256^T produced acid when grown on carbohydrates. No growth was detected when strain BZ256^T was grown anaerobically under any of the tested conditions. Strain BZ256^T produced indole from tryptophan and reduced nitrate aerobically, but did not produce amylase, caseinase or lipase. Strain BZ256^T was found to have a single 16S rRNA gene sequence that was 96.8 % similar to that of its closest relative, *Halogeometricum borinquense* PR 3^T (Montalvo-Rodriguez *et al.*, 1998) (Fig. 2). However, strain BZ256^T showed 99 % 16S rRNA gene sequence similarity to recently reported isolates obtained from a traditional Japanese saltern (Fukushima *et al.*, 2007), where salt concentrations oscillate seasonally between 3.0 g l⁻¹ and saturation. As Zodletone Spring, from which strain BZ256^T was isolated, also experiences wide seasonal and spatial variation in NaCl concentrations, it is plausible that members of this phylogenetic cluster are well adapted to survival in such environments.

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain BZ256^T and other close relatives within the family *Halobacteriaceae*. Sequences were retrieved from the GenBank database; accession numbers are given in parentheses. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 50 % bootstrap support. The sequence of *Methanospirillum hungatei* DSM 864^T was included as the outgroup. Bar, 0.01 substitutions per site.

Table 1. Differential characteristics between strain BZ256^T and closely related genera within the order *Halobacteriales*

Taxa: 1, strain BZ256^T; 2, *Halogeometricum*; 3, *Halobaculum*; 4, *Haloquadratum*; 5, *Halorubrum*; 6, *Haloferax*; 7, *Halococcus*. Data for reference genera are from Burns *et al.* (2007), Castillo *et al.* (2006c), Cui *et al.* (2006), Goh *et al.* (2006), Grant *et al.* (2001), Montalvo-Rodriguez *et al.* (1998), Oren *et al.* (1995) and Xu *et al.* (2007). +, Positive; –, negative; +/–, variable; NR, not reported.

Characteristic	1	2	3	4	5	6	7
Cell shape	Coccus	Pleomorphic	Rod	Squares	Pleomorphic/rods	Pleomorphic	Coccus
Gas vesicles	–	+	–	+	+/–	+/–	NR
Motility	–	+	–	–	+/–	+/–	–
Cell size (µm)	0.8–1.0	1–3 × 1–2	5–10 × 0.5–1.0	2.0 × 2.0	1–12 × 0.5–1.2	0.4–3 × 2–3	0.8–1.5
NaCl range (M)	1.3–4.3	1.4 (minimum)	1.0 (minimum)	2.4–6.2	1.5–5.2	1.0–5.2	2.1–5.2
NaCl optimum (M)	3.4	3.4–4.3	1.5–2.5	3.1	2.5–4.5	1.7–4.3	2.6–4.3
Temperature optimum (°C)	35–37	40	40	45	37–50	32–50	30–40
pH optimum	6.5	7	6–7	7.0	7.0–7.5/9.0–10.0	6.4–7.5	6.8–9.5
Lysis in distilled water	+	+	+	+	+	+	–
Presence of phosphatidyl glycerol sulfate	–	–	–	–	+/–	–	–
DNA G + C content (mol%)	65.4	59.1	70.0	46.9	62.7–71.2	59.1–64.5	59.5–66.0
Aerobic nitrate reduction	+	+	+	NR	+/–	+/–	+
Hydrolysis of:							
Starch	–	–	+	–	+/–	+/–	+/–
Gelatin	–	+	–	NR	+/–	+/–	+/–
Pigmentation	Pale pink	Pink	Orange–red	Pink	Orange–red	Red/pink	Red

Strain BZ256^T contained phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester as phospholipids but not phosphatidylglycerol sulfate. TLC analysis of the glycolipids revealed that strain BZ256^T contained two glycolipids that were chromatographically identical to S-DGD-1 and DGD-1 from *Haloferax* (see Supplementary Fig. S1 in IJSEM Online). One minor glycolipid had a similar migration pattern to unidentified glycolipids from *Haladaptatus paucihalophilus*, which was also isolated from Zodletone Spring (Supplementary Fig. S1). Whether this latter lipid is characteristic of halophilic archaea encountered in low-salt environments remains to be assessed.

This study provides evidence that strain BZ256^T is an extremely halophilic member of the *Archaea*, order *Halobacteriales*, family *Halobacteriaceae*. Salient characteristics of strain BZ256^T (cell morphology and division pattern, lack of motility, glycolipids, ability to tolerate low-salt conditions) suggested that it could not be accommodated as representing a novel species within the genus *Halogeometricum*, its most closely related genus based on 16S rRNA gene sequence phylogeny. Therefore, we propose that this strain constitutes a novel species of a new genus within the family *Halobacteriaceae*, for which the name *Halosarcina pallida* gen. nov., sp. nov. is proposed.

Description of *Halosarcina* gen. nov.

Halosarcina [Ha.lo.sar.ci'na. Gr. n. *hals*, *halos* salt; L. fem. n. *sarcina* a package; N.L. fem. n. *Halosarcina* a salt (-loving) package].

Cells are non-motile, Gram-negative cocci. Cells form sarcina-like clusters during the exponential growth phase and occur singly or in pairs in late exponential and stationary phases. Oxidase- and catalase-positive. Chemo-organotrophic, growing on a wide range of substrates, including single and complex carbon sources. Produce acid from carbohydrates. Reduce nitrate to nitrite aerobically without the formation of gas. Lyse in distilled water. Cells contain phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, but phosphatidylglycerol sulfate is absent. Two glycolipids are present that are chromatographically identical to S-DGD-1 and DGD-1. The type species is *Halosarcina pallida*. Recommended three-letter abbreviation: *Hsn*.

Description of *Halosarcina pallida* sp. nov.

Halosarcina pallida (pal'li.da. L. fem. adj. *pallida* pale).

Has the following characteristics in addition to those given for the genus. Colonies are small (0.2 mm), pink, translucent, round and convex with an entire margin. Grows at 1.7–4.3 M NaCl, with optimal growth at 3.1 M NaCl. Grows at 25–45 °C, with optimal growth at 30 °C. Requires a minimum of 1 mM Mg²⁺ for growth. Grows at pH values ranging from 5 to at least 8.5, with optimum growth at pH 6.5. Does not grow anaerobically with nitrate, sulfate, elemental sulfur, thiosulfate, DMSO or trimethylamine

oxide. Does not ferment arginine. Capable of using single-carbon substrates. Utilizes acetate, lactate, malic acid, fumaric acid, sucrose, L-glutamic acid, glucose, fructose, succinate, lactose, DL-aspartic acid, pyruvate, glycine, galactose, sorbitol, glycerol, starch, L-histidine, trehalose, DL-norleucine, D-glucuronic acid, DL-phenylalanine, aesculin and salicin, but not L-arginine, L-alanine, sodium citrate, xylose, mannitol, L-threonine, dulcitol, dextrin, L-methionine, 3,3-dimethylglutaric acid or L-tyrosine. Produces acid when grown on sucrose, glucose, starch, fructose, lactose, galactose, sorbitol and glycerol, but not L-arginine, L-alanine, L-glutamic acid, DL-aspartic acid, glycine, acetate, lactate, malic acid, fumaric acid, citrate, succinate or pyruvate. Able to utilize complex carbon sources such as yeast extract and Casamino acids. Indole is produced from tryptophan. Does not hydrolyse starch, casein, gelatin or Tween 80. Sensitive to novobiocin, bacitracin, anisomycin, rifampacin and aphidicolin. Resistant to erythromycin, penicillin, ampicillin, chloramphenicol, neomycin, nalidixic acid, kanamycin, gentamicin and trimethoprim. The DNA G + C content of the type strain is 65.4 mol%.

The type strain, BZ256^T (=KCTC 4017^T =JCM 14848^T), was isolated from Zodletone Spring in south-western Oklahoma, USA.

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