Haladaptatus paucihalophilus gen. nov., sp. nov., a halophilic archaeon isolated from a low-salt, sulfide-rich spring

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Two novel strains of halophilic archaea, DX253^T and GY252, were isolated from Zodletone Spring, a low-salt, sulfide- and sulfur-rich spring in south-western Oklahoma, USA. The cells were cocci or coccobacilli and occurred singly or in pairs. The two strains grew in a wide range of salt concentrations (0.8–5.1 M) and required at least 5 mM Mg^{2+} for growth. The pH range for growth was 5–7.5 and the temperature range was 25–45 °C. In addition to having the capacity to grow at relatively low salt concentrations, cells remained viable in distilled water after prolonged incubation. The two diether phospholipids that are typical of members of the order *Halobacteriales*, phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, were present. Phosphatidylglycerol sulfate and two unidentified glycolipids were also detected. Each strain had two distinct 16S rRNA gene sequences that were only 89.5–90.8 % similar to sequences from the most closely related cultured and recognized species within the order *Halobacteriales*. The DNA G+C content of the type strain was found to be 60.5 mol%. The closest relatives were clones and uncharacterized isolates obtained from coastal salt-marsh sediments with salinities equivalent to that of seawater. The physiological, biochemical and phylogenetic differences between strains DX253^T and GY252 and other previously described genera of extremely halophilic archaea suggest that these novel strains represent a novel species and genus within the family *Halobacteriaceae*, for which the name *Haladaptatus paucihalophilus* gen. nov., sp. nov. is proposed. The type strain is DX253^T (=JCM 13897^T=DSM 18195^T=ATCC BAA-1313^T=KCTC 4006^T).

Members of the family *Halobacteriaceae*, domain *Archaea*, have long been known to inhabit hypersaline environments such as the Dead Sea, crystallizer ponds and salt lakes (Oren, 2000; Grant *et al.*, 2001). The family *Halobacteriaceae* was first described in 1974 to accommodate obligate halophilic micro-organisms that require at least 2.0 M NaCl for growth (Gibbons, 1974). Currently, the family includes 22 genera comprising micro-organisms that display a wide variety of morphologies including rods, cocci, squares, triangles and flattened discs (Grant *et al.*, 2001; Takashina *et al.*, 1990; Walsby, 1980; Oren, 2002; Burns *et al.*, 2004; Bolhuis *et al.*, 2004).

Despite the fact that members of the order *Halobacteriales* mainly inhabit environments of extreme salinity, where salt concentrations exceed 20 % (Oren, 1994), several reports have suggested the presence of extremely halophilic archaea in environments of moderate to low salinity. For example, Rodriguez-Valera *et al.* (1979) isolated an extremely halophilic coccus from ocean waters off the coast of Spain. Furthermore, with the advent of 16S rRNA gene sequence-based surveys, the presence of halophilic members of the *Archaea* in low-salt environments has been demonstrated. Studies by Munson *et al.* (1997) found that halophilic representatives of the *Archaea* were present in a coastal salt marsh in which the pore water salinity was approximately 0.8 M NaCl. Sequences affiliated with the order *Halobacteriales* were also reported in a survey of the archaeal diversity of a deep-sea hydrothermal vent (Takai *et al.*, 2001). Recently, extremely halophilic archaea

Abbreviations: PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulfate; TMAO, trimethylamine *N*-oxide.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain DX253^T are DQ344973 and DQ344974, and those for strain GY252 are DQ867122 and DQ867123.

Cell morphology and phospholipid/glycolipid patterns of strain DX253^T are shown in supplementary figures available in IJSEM Online.

probably representing several different genera were isolated from coastal salt-marsh sediments (Purdy *et al.*, 2004).

In addition, we have recently reported the presence, and examined the diversity of, halophilic members of the *Archaea* in Zodletone Spring, a sulfide- and sulfur-rich spring in south-western Oklahoma, USA, by using a combination of culture-independent and cultivation-based methods (Elshahed *et al.*, 2004a, b). In this study, we report on the isolation (from Zodletone Spring) and characterization of two novel halophilic strains that represent a novel species in a novel genus of the order *Halobacteriales*.

The location and geochemical properties of the spring have been described previously (Younger *et al.*, 1986; Senko *et al.*, 2004; Elshahed *et al.*, 2003). Although salinity measurements of the spring water did not exceed 0.2 M NaCl, concentrations of NaCl approached saturation in the top centimetre of soil along the bank of the spring, probably because of evaporative concentration (Elshahed *et al.*, 2004a). To isolate halophilic archaea, samples from the top 2.0 cm of the soil approximately 20.0 cm from the spring bank were collected (using a sterile spatula) into a sterile 50 ml conical tube. The tubes were immediately capped and kept on ice. Soil was inoculated into a liquid halophile-enrichment medium immediately upon return to the laboratory. The halophile medium (HMD) used for the isolation procedure was modified from Oren *et al.* (1997, 2000) and contained the following (g l^{-1}): $MgCl_2.6H_2O$ (20), K_2SO_4 (5), $CaCl_2.2H_2O$ (0.1), yeast extract (0.1), NH_4Cl (0.5), KH_2PO_4 (0.05), carbon source (0.5), agar (20) and NaCl (180, 250 or 300). The pH of the medium was adjusted to 7.0, and ampicillin and kanamycin were each added at a concentration of 50 μg ml^{-1} to suppress the growth of halotolerant members of the *Bacteria*. Soil samples were serially diluted and plated onto HMD plates containing one of the following carbon sources: glucose, glycerol, tryptone, tryptose, peptone, nutrient broth, citrate, sodium benzoate, cysteine, Casamino acids, yeast extract or sodium glutamate. The plates were incubated at 37 °C under a 60 W light bulb placed 30 cm above the plates, until colonies appeared. To ensure purity, a single colony of each strain was restreaked twice onto HMD plates.

Characterization was achieved by following the general guidelines presented by Oren *et al.* (1997) for describing novel taxa of the order *Halobacteriales*. Detailed protocols for the methodologies for the biochemical tests conducted were obtained from Gerhardt *et al.* (1994), and NaCl was added as necessary. The Gram reaction was determined by following the method outlined by Dussault (1955). Physiological tests were conducted using liquid or solid (2.0 % agar) HMD containing sucrose (0.5 g l^{-1}) as the carbon source, 180 g NaCl l^{-1} and 25 mM HEPES, unless stated otherwise. Liquid cultures were incubated at 37 °C on a shaking incubator at 200 r.p.m. Growth rates were determined by monitoring the increase in OD_{600}. Substrate utilization was tested by substituting various carbon sources into the HMD, as suggested by Oren *et al.* (1997). Acid production was tested in unbuffered HMD and was determined by measuring the initial and final pH of the medium. The culture was considered as positive for acid production if the pH decreased by at least 1 unit. The ability of strain DX253^T to use DMSO (5.0 g l^{-1}), trimethylamine *N*-oxide (TMAO; 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 procedures described by Bryant (1972) and Balch & Wolfe (1976). Sulfur was added as sublimed sulfur suspended in an aqueous solution (Widdel & Pfennig, 1999). The sulfur-containing tubes were amended with 0.02 % ferrous ammonium sulfate; a positive result was indicated by the formation of a black precipitate of ferrous sulfide.

The minimum salt concentration required to maintain cell stability was tested by inoculating washed cells into both low-salt HMD and a sterile saline solution containing 0, 5.0, 10.0, 20.0 or 30.0 g NaCl l^{-1}. HMD contained $MgCl_2$, which can help to stabilize cell walls at low salt concentrations (Grant *et al.*, 2001). Cells were then recovered by inoculation into standard HMD at different time intervals. In addition, all suspensions were checked microscopically for cell lysis.

Antibiotic sensitivity was determined by adding filter-sterilized antibiotic solutions to liquid HMD. The antibiotic concentrations were 35 μg ml^{-1} except in the case of aphidicolin, which was used at a concentration of 30 μg ml^{-1}. Scanning electron microscopy (JSM-880; JEOL) was conducted at the Electron Microscopy Laboratory of the University of Oklahoma. Phase-contrast micrographs were made using a Zeiss Axiovert 135TV microscope.

The 16S rRNA genes were amplified using primers A1F (5′-ATTCCGGTTGATCCTGC-3′) (Tajima *et al.*, 2001) and UA1406R (5′-ACGGGCGGTGWGTRCAA-3′) (Baker *et al.*, 2003). The PCR products were then cloned using a TOPO-TA cloning kit (Invitrogen). Twenty-eight clones of strain DX253^T and ten clones of strain GY252 were randomly picked and ten sequenced at the Oklahoma Medical Research Foundation (Oklahoma City, OK, USA). The 16S rRNA gene sequences were aligned using CLUSTAL_X (Thompson *et al.*, 1997) and distance trees were constructed with PAUP 4.01b10 (Sinauer Associates), using a neighbour-joining algorithm and Jukes–Cantor corrections. The G+C content (mol%) was determined using the services of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Membrane lipids were analysed using two-dimensional TLC, as described by Oren *et al.* (1996).

Two strains, DX253^T and GY252, were isolated independently from each other on HMD plates containing 25 % NaCl, on glucose and glycerol, respectively, after approximately 4 weeks incubation. Both strains were fully characterized.

The 16S rRNA gene sequence data (Fig. 1) together with the results from membrane-lipid analyses and physiological and biochemical tests suggested that the two strains represented the same species. Strain DX253^T was chosen to represent the type strain.

The cells of strain DX253^T stained Gram-negative and the colonies were small (0.2 mm), pink, translucent, convex and round with entire margins. The cells were non-motile and were generally coccoid, but sometimes appeared as short rods, especially in the early phases of growth. Phase-contrast and scanning electron microscopy of cells of strain DX253^T showed cocci occurring singly and in pairs (see Supplementary Fig. S1 available in IJSEM Online). The presence of gas vesicles was not evident under light microscopy.

Strain DX253^T grew in a wide range of salt concentrations from 0.8 to 5.1 M, with an optimum at 3.1 M NaCl. Cells did not immediately lyse when suspended in distilled water, and remained viable under these conditions for up to 2 weeks. The capacity of strain DX253^T to grow at relatively low salt concentrations and tolerate distilled water probably allows it to survive the fluctuating salt conditions encountered in Zodletone Spring. The concentration of NaCl on the banks of the spring depends greatly on temperature and rainfall patterns, so these locations may not always be hypersaline.

The detailed physiological and biochemical characteristics of strain DX253^T are listed in Table 1 and in the species description. In general, strain DX253^T is chemo-organotrophic, being capable of growing on a complex medium as

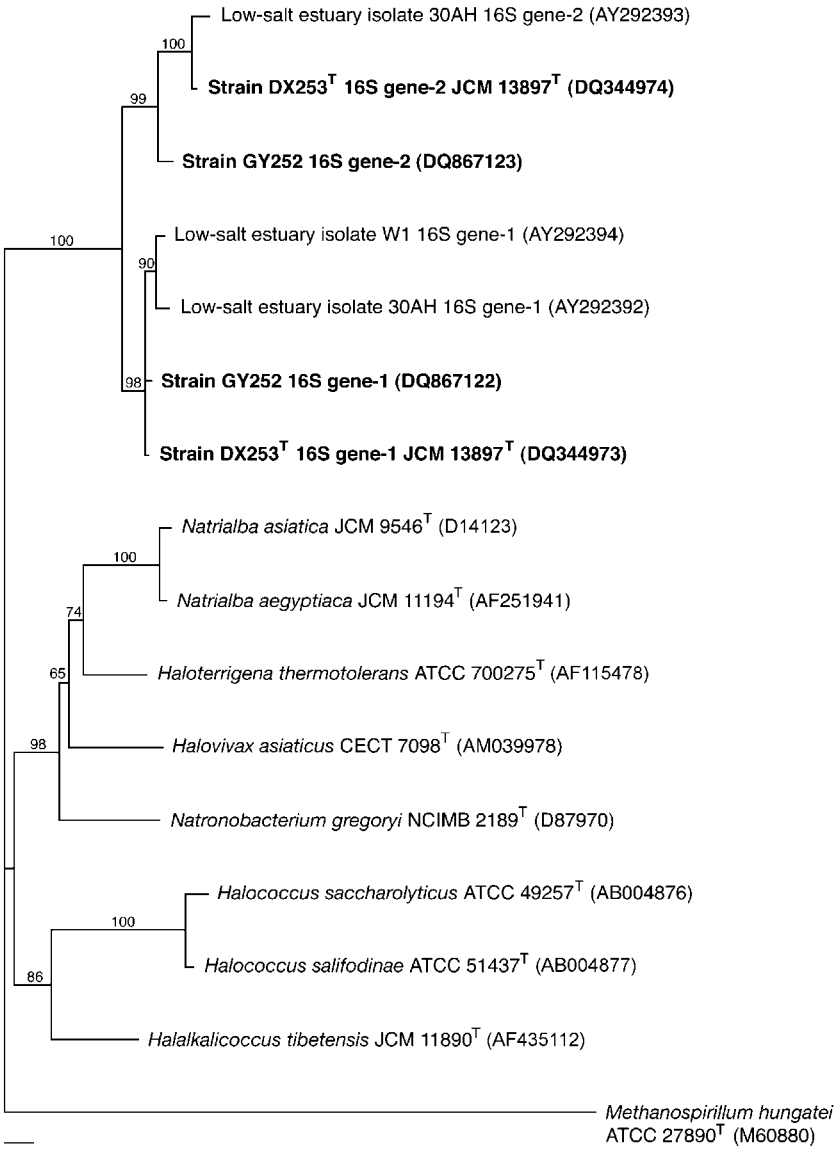

Fig. 1. Distance dendrogram showing the relationships between the two 16S rRNA gene sequences of strain DX253^T (DX253^T 16S-1 and DX253^T 16S-2), the two 16S rRNA gene sequences of strain GY252 (GY252 16S-1 and GY252 16S-2) and the 16S rRNA gene sequences of close relatives within the family *Halobacteriaceae*. Sequences were retrieved from GenBank; accession numbers are indicated in parentheses. Bootstrap values, expressed as percentages of 1000 replicates, are shown for branches with more than 50% bootstrap support. Bar, 0.01 substitutions per site.

Table 1. Characteristics that distinguish strains DX253^T and GY252 from closely related genera within the order *Halobacteriales*

Taxa: 1, *Haladaptatus paucihalophilus* gen. nov., sp. nov. (strains DX253^T and GY252); 2, *Halalkalicoccus*; 3, *Natronobacterium*; 4, *Halococcus*; 5, *Haloarcula*; 6, *Haloferax*; 7, *Natronococcus*. Data were derived from Oren (2000), Grant *et al.* (2001), Gutierrez *et al.* (2002), Elshahed *et al.* (2004b), Xue *et al.* (2005) and Goh *et al.* (2006). +, Positive; −, negative; V, variable; NR, not reported.

Characteristic	1	2	3	4	5	6	7
Cell shape	Coccus	Coccus	Rod	Coccus	Pleomorphic	Pleomorphic	Coccus
Cell size (μm)	1.0–1.5	1.0–1.5	0.5–1.0 × 2–15	0.8–1.5	0.2–2 × 0.5–5	0.4–3 × 2–3	1.0–2.0
NaCl range (M)	0.8–5.1	1.4–5.2	2.0–5.2	2.1–5.2	1.7–5.2	1.0–5.2	1.4–5.2
NaCl optimum (M)	2.6–3.1*	3.4	3.0	2.6–4.3	2.5–4.3	1.7–4.3	2.5–3.6
Temp. optimum (°C)	25–30*	40	37	30–40	35–55	32–50	35–45
pH optimum	6.0–6.5*	9.5–10.0	9.5	6.8–9.5	6.5–7.5	6.4–7.5	9.0–10.0
Lysis in distilled water	−	−	+	−	+	+	−
PGS	+	−	−	−	+	−	−
DNA G+C content (mol%)	60.5	61.5	65.0	59.5–66	60.1–65	59.1–64.5	63.5–64.0
Aerobic nitrate reduction	−	+	−	+	+/NR	V	+
Hydrolysis of:							
Starch	+	−	−	V	V	V	V
Casein	+	−	NR	−	−/NR	V	NR
Tween 80	+	−	NR	V	V	V	NR
Pigmentation	Pink	Orange	Red	Red	Red	Red/pink	Red

*Optimum NaCl, pH and temperature for growth were 3.1 M, pH 6.5 and 30 °C for strain DX253^T and 2.6 M, pH 6.0 and 25 °C for strain GY252.

well as on a single carbon source. It produced acid when growing on carbohydrates. No growth was detected when the strain was grown anaerobically with TMAO, DMSO, sulfate, thiosulfate, nitrate or sulfur as a terminal electron acceptor.

Strains DX253^T and GY252 each possessed two distinct 16S rRNA gene sequences that were, respectively, 95.8 and 97.7 % similar to each other (Fig. 1). Both of the 16S rRNA gene sequences of strain DX253^T were 89.5–90.8 % similar to the sequence of *Halalkalicoccus tibetensis*, which is the most closely related *Halobacteriaceae* species with a validly published name. Most of the differences between these two sequences were found between base pairs 1–200 and 400–800. Despite showing very low levels of sequence similarity to recognized members of the extremely halophilic *Archaea*, strain DX253^T closely matched (99.2 %) uncharacterized isolates (retrieved from an estuarine ecosystem) each of which also contained two divergent 16S rRNA gene sequences (Purdy *et al.*, 2004) (Fig. 1). The absence of similar 16S rRNA gene sequences in clone or isolate data obtained from traditional hypersaline environments, combined with the fact that the only 16S rRNA gene sequences with high levels of similarity to those of strain DX253^T were encountered in a low-salinity ecosystem, provides further evidence that the novel strains described here are adapted to relatively low-salt systems or fluctuating salt concentrations. Whether members of this group are entirely absent from truly hypersaline environments cannot be stated unequivocally.

The presence of multiple heterogeneous 16S rRNA genes is not unprecedented among prokaryotes in general (Acinas *et al.*, 2004) and among halophilic archaea in particular (Mylvaganam & Dennis, 1992; Dennis *et al.*, 1998; Grant *et al.*, 2001; Acinas *et al.*, 2004). Within the order *Halobacteriales*, several members of the genus *Haloarcula* (*Haloarcula marismortui*, *Haloarcula quadrata* and *Haloarcula vallismortis*), *Halosimplex carlsbadense* and *Natrinema* sp. strain XA3-1 have each been shown to contain at least two divergent 16S rRNA genes. Differences between the heterogeneous 16S rRNA genes within the order *Halobacteriales* range from approximately 5.0 to 6.8 % (Mylvaganam & Dennis, 1992; Vreeland *et al.*, 2002; Boucher *et al.*, 2004; Acinas *et al.*, 2004), which is similar to the 4.2 % difference seen in strain DX253^T. In *Haloarcula marismortui*, both of the 16S rRNA genes are transcribed during growth, and it has been proposed that the presence of heterogeneous 16S rRNA operons may help in the toleration of environmental stresses (Dennis *et al.*, 1998). The fact that strain DX253^T, strain GY252 and their closest relatives (isolates from salt-marsh sediments in Essex, UK) all have heterogeneous 16S rRNA gene sequences indicates that this feature might be characteristic of a novel genus, but it is unclear whether this characteristic confers any competitive advantage upon micro-organisms inhabiting a low-salt environment.

Strain DX253^T contained the phospholipids phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and phosphatidylglycerol sulfate (PGS). The

presence of PGS within this group helps to differentiate this isolate from the other neutrophilic genera of halophilic archaea that do not contain PGS, such as *Haloferax*, *Natrialba*, *Halobaculum*, *Halococcus* and *Halogeometricum* (Grant *et al.*, 2001; Oren, 2002). Analysis of the glycolipids using TLC revealed that strain DX253^T contains at least two glycolipids that have yet to be identified (see Supplementary Fig. S2 available in IJSEM Online).

This study provides evidence that strains DX253^T and GY252 are members of the extremely halophilic *Archaea*, order *Halobacteriales*, family *Halobacteriaceae*. Lipid data combined with biochemical and physiological characteristics serve to differentiate these strains from other previously described members of this family. The low levels of 16S rRNA gene sequence similarity with respect to other genera within the order *Halobacteriales* further justify the claim that these isolates represent a novel species in a novel genus within this family, for which the name *Haladaptatus paucihalophilus* gen. nov., sp. nov. is proposed.

Description of *Haladaptatus* gen. nov.

Haladaptatus (Hal.a.dap.ta′tus. Gr. n. *hals* salt; L. part. adj. *adaptatus* adapted to a thing; N.L. masc. n. *Haladaptatus* a bacterium adapted to salt).

Gram-negative cocci or coccobacilli occurring singly or in pairs. Colonies are pink-pigmented. Possess at least two heterogeneous 16S rRNA gene sequences. Cells contain PG, PGP-Me and PGS. Two unidentified glycolipids are present. Chemo-organotrophic, growing on a wide range of substrates, including single and complex carbon sources. Produce acid from carbohydrates. Hydrolyse starch, gelatin, casein and Tween 80. Grow in a wide range of NaCl concentrations. Sensitive to novobiocin, bacitracin, anisomycin and aphidicolin. Partially sensitive to rifampicin and trimethoprim. Resistant to erythromycin, penicillin, ampicillin, chloramphenicol, neomycin, nalidixic acid and gentamicin. Survive at low salt concentrations and can recover after prolonged exposure to less than 0.2 M NaCl. The type species is *Haladaptatus paucihalophilus*. Recommended three-letter abbreviation: *Hap*.

Description of *Haladaptatus paucihalophilus* sp. nov.

Haladaptatus paucihalophilus (pau.ci.ha.lo′phi.lus. L. adj. *paucus* little; Gr. n. *hals* salt; Gr. adj. *philos* loving; N.L. masc. adj. *paucihalophilus* low-salt loving).

Exhibits the following properties in addition to those given in the genus description. Cells are approximately 1.2 μm in diameter. Doubling time is approximately 12–13 h. Non-motile. Colonies are small (0.2 mm), translucent, round and convex with entire margins. Grows in NaCl at 0.8–5.1 M; optimum growth is at 2.6–3.1 M NaCl. Optimal temperature for growth is 25–30 °C (range, 25–45 °C). A minimum of 5 mM Mg^{2+} is required for growth. Grows at pH 5.0–7.5, with an optimum at pH 6.0–6.5. Does not grow anaerobically with NO_3^-, SO_4^{2-}, elemental sulfur, $S_2O_3^{2-}$, DMSO or TMAO. Does not ferment arginine. Capable of using single-carbon substrates. Utilizes glutamic acid, histidine, norleucine, phenylalanine, D-glucuronic acid, dextrin, aesculin, salicin, trehalose, sucrose, fructose, xylose, glucose, starch, galactose, acetate, lactate, malate, fumarate, citrate, pyruvate, mannitol and glycerol. Threonine, methionine, tyrosine, arginine, alanine, aspartic acid, glycine, lactose, succinate, sorbitol, dulcitol and 3,3-dimethylglutaric acid are not utilized as carbon sources. Produces acid when grown on sucrose, xylose, glucose, starch, fructose, galactose, mannitol and glycerol. Able to utilize complex carbon sources such as yeast extract and Casamino acids. Catalase- and oxidase-positive. Indole is produced from tryptophan. Does not reduce nitrate under aerobic conditions.

The DNA G+C content of the type strain is 60.5 mol%. The type strain, DX253^T (=JCM 13897^T=DSM 18195^T=ATCC BAA-1313^T=KCTC 4006^T), was isolated from Zodletone Spring in south-western Oklahoma, USA.

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