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# THE ISOLATION AND CHARACTERIZATION OF NOVEL EXTREME HALOPHILIC ARCHAEA FROM A LOW-SALT, SULFIDE- AND SULFUR-RICH SPRING.

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# THE ISOLATION AND CHARACTERIZATION OF NOVEL EXTREME HALOPHILIC ARCHAEA FROM A LOW-SALT, SULFIDE- AND SULFUR-RICH SPRING.

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

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I dedicate this to my parents, Monty and Debbie Savage.
You honestly believed from the day that I was born that I could be anything that I wanted to be. Thank you for believing in me, and I hope that I have become a person that you can be proud of.

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# Preface

Zodletone spring was established as a National Science Foundation Microbial Observatory in 2003. One of the primary goals of the project was to describe the microbial community in two different locations; the source of the spring and the microbial mats. Upon analyzing the archaeal clone library we were surprised to find that 36% of the mat clone library and 4% of the source clone library had a phylogenetic affiliation with the extreme halophilic Archaea, family *Halobacteriaceae*. This community was not comprised of one particular type of halophilic archaeon, rather it was diverse community that contained several novel groups (1). This finding ultimately led to my work investigating the haloarchaeal community at Zodletone to gain insight into the properties of these novel groups that allow them to survive in a low-salt environment.

This dissertation contains five chapters and one appendix. Each of the first four chapters describes a novel halophilic archaeon that was isolated and characterized from Zodletone spring. Chapter five is a mini-review that reflects on the similarities between the extreme halophilic Archaea that have been found in different low-salt environments since our study began. The appendix contains results from a second project, and presents evidence of the biodegradation of low-molecular weight

hydrocarbons under sulfate-reducing conditions in Zodletone enrichment cultures.

Chapter one was written in the form recognized by the *International Journal of Systematic and Evolutionary Microbiology*. It was published in the November 2004 issue vol. 54 pp. 2275-2279. This manuscript characterized *Haloferax sulfurifontis* a novel species in a previously described genus (2). Dr. Mostafa Elshahed isolated the organism, and I did the subsequent characterization. Dr. Aharon Oren (The Hebrew University of Jerusalem) extracted and analyzed the membrane lipids, for this isolate and all of the following isolates presented. Dr. Ventosa (University of Seville) did the analysis of the G+C mol%. This isolate was of interest because it was capable of reducing sulfur under anaerobic conditions that could suggest that the organism plays a role in sulfur cycling at Zodletone. It could also grow at relatively low salt concentrations.

Chapter two was written according to the format described by the *International Journal of Systematic and Evolutionary Microbiology*. The manuscript was accepted for publication and published in January of 2007 vol. 57 pages 19-24. *Haladaptatus paucihalophilus* was established as both a novel genus and species within the family *Halobacteriaceae*. This organism displayed very unique characteristics including growth at relatively low-salt concentrations, the ability to

recover from suspension in low-salt solutions and it possessed two disparate 16S rRNA gene sequences (7).

Chapter three was also written according to guidelines provided by the *International Journal of Systematic and Evolutionary Microbiology*. The paper was accepted and published in the April 2008 issue, volume 58 pages 856-860. Electron Microscopy images taken at the Samual Roberts Noble Electron Microscopy Laboratory (University of Oklahoma) were selected for the cover image of this issue. *Halosarcina pallida* is a novel genus and species within the family *Halobacteriaceae*. It was characterized by its extremely pale pigment, its unique method of division and it's ability to withstand exposure to low-salt concentrations (8).

Chapter four is being prepared for submission to the *International Journal of Systematic and Evolutionary Microbiology*. *Haloenormitas infirmitas* is being proposed as a novel species and novel genus within the extreme halophilic Archaea. This is based on the low phylogenetic similarity to previously described genera, and biochemical and physiological characteristics. This isolate, much like the majority of the other halophilic Archaea from Zodletone, also has the ability to recover from exposure to low-salt concentrations.

Chapter five is a comprehensive minireview that is in preparation. This review encompasses the results from studies done on

the halophilic Archaea from Zodletone and those found in other low-salt environments. Several studies (4-6, 10) were finding the same groups of halophilic Archaea in low-salt environments, but they have not yet been detected in traditional hypersaline environments. Also, although they display most of the same general characteristics of the extreme halophilic Archaea, they have unique attributes that could be advantageous to surviving in low-salt systems. This review suggests that the defining characteristics of the extreme halophilic Archaea be modified to include a tolerance to low-salt conditions, and also encourages future groups to look for these organisms in ecosystem wide community studies.

The appendix is based on work we did that was a result of information from the original clone library studies. The source of Zodletone is contaminated with short-chain gaseous alkanes and community studies suggested that the resident microbial community was impacted by the presence of these alkanes. Several clones were related to uncultured bacterial clones from hydrocarbon contaminated areas or hydrocarbon degrading enrichments (3). Appendix A contains the work we did with the enrichment of organisms from the spring source that metabolized low-molecular weight alkanes. Propane and pentane were degraded under sulfate reducing, enrichments were successfully transferred and activity was maintained. Isotopic fractionation of the alkanes in active enrichments was evidence that microorganisms drove

the depletion. Isotope fractionation work was done by Jon Allen in Dr. R. Paul Philp's lab (University of Oklahoma, College of Earth and Energy). A key metabolite in propane degradation, propylsuccinic acid, also added evidence that microbial degradation was occurring. Dr. Lisa M. Gieg in Dr. Joseph M. Suflita's lab (University of Oklahoma) trained me in the process of metabolite extraction and identification. This work is to be continued with plans to isolate and characterize the alkane degrading organism(s) and analyzing the metagenome of the active enrichments.

All of the work was done at Zodletone Spring and was funded through the National Science Foundation Microbial Observatories Program (grant MCB-0240683). A description of the site and a breakdown of the results from the community studies that led to my work are included below.

**Site Description**

Zodletone spring is located in southwestern Oklahoma in Kiowa County. It is situated on the southern side of the Anadarko basin above the frontal fault zone of the Wichita Mountains. The spring is named because of its proximity to Zodletone Mountain. It has been suggested that it is fed from underneath the Anadarko Basin where a deep oil-field brine mixes with near-surface groundwater (9, 11). Water emerges from

the source flowing at a rate of approximately 8 l•min$^{-1}$. Along with the brine ejected from the source short chain gaseous alkanes (methane, ethane and propane) are being degassed and are bubbling up from the bed of the spring (3, 9).

The spring is a shallow anaerobic, mesophilic surficial spring. The unique geochemistry of the water lies in the high concentrations of dissolved sulfide (8-10 mM), these high sulfide concentrations help to maintain anoxic conditions throughout. The pH is neutral. Salt concentrations are approximately 0.2 M, sulfate concentrations are low (60 μM) in the last 5 m of the spring sulfate concentrations increase to 2.2 mM. There are trace amounts of fluorine, bromide, boron, strontium and barium (9).

The source is an area measuring 1 m$^2$, it is approximately 50 cm in depth with the bottom 15 cm covered by biomass and soft sediment. The walls are covered by purple and green photosynthetic bacteria and one side of the source is shaded by a crumbling concrete wall which is evidence of an attempt to develop the spring into a spa at one time (11). From the source, the spring flows 20 m before it confluences with Saddle Mountain Creek (3, 9, 11).

**Phylogenetic Analysis of Zodletone Spring**

The microbial community at Zodletone spring was analyzed at two different locations; the microbial mats and at the source of the

spring. DNA was extracted and amplified with both bacterial and archaeal specific primers. A total of 212 bacterial clones (96 of mat origin and 116 of source origin) were identified and 178 archaeal clones (65 of mat origin and 113 of source origin) (1, 3).

*Bacteria-Mat clone library*

Bacterial clones from the mat clone library fell into nine different bacterial lineages; *α-Proteobacteria*, *γ-Proteobacteria*, *δ-Proteobacteria*, *ε-Proteobacteria*, *Cyanobacteria*, *Chloroflexi*, *Firmicutes*, *Actinobacteria,* and *Verrumicrobia*. The mat clone library was dominated by three OTU's belonging to the *ε- Proteobacteria*, *Chloroflexi*, and *Cyanobacteria*. And a majority of the community belonged to groups composed exclusively of phototrophic organisms such as the *Cyanobacteria*, *Chromatium*, and members of the Group III *Chloroflexi*. Clones that belonged to lineages known to reduce sulfur and sulfate in the *δ-Proteobacteria* made up a smaller percentage of the clone library (3).

*Bacteria-Source clone library*

The bacterial source clone library belonged to eleven different bacterial divisions and candidate divisions. The *δ-Proteobacteria*, *γ-Proteobacteria*, along with clones which were not closely related to any known bacterial division dominated the clone library. Clones that were in non-proteobacterial, non-photosynthetic divisions comprised 40% of

the bacterial mat clone library, interestingly many of these clones were closely related to other clones from hydrocarbon- degrading enrichments or hydrocarbon-impacted sites. This indicates that the presence of short-chain gaseous alkanes at the spring source may play an integral role in shaping the community (3).

*Archaeal Community*

The archaeal mat and source clone libraries was dominated by the *Euryarchaeota* comprising 96% and 91% of the libraries respectively. Methanogens dominated the source and the mat clone libraries, the majority of these belonging to the order *Methanomicrobiales*, family *Methanocorpusculaceae*. Unaffiliated clones fell into four different groups called UAG1- UAG4. This group of unaffiliated clones made up 36% of the source clone library, some of which were related to other clones from hydrocarbon contaminated sites (1).

A surprising result was that 36% of the mat clone library and 4% of the source clone library grouped phylogenetically with the extreme halophilic Archaea, order *Halobacteriales*. The haloarchaeal clones were abundant in the mat clone library and they were not exclusive to one specific lineage, they were found to belong to five different groups. The majority of clones were not closely related to characterized genera within the family *Halobacteriaceae*. Those that did cluster with previously described genera were related to *Halogeometricum* and

*Natronomonas*. Clones from the source all fell within one group most closely related to the genus *Halococcus* (1).

Salinity measurements throughout the spring revealed consistent values ranging from 0.7- 1.0% salt. However, two cores that were collected along the stream bank, 5 and 30 cm from the spring revealed that salt concentrations increased (up to 25-30%) and moisture decreased in the top 1 cm of the soil, but salt concentrations decreased rapidly with depth (1). It is likely that the increase in salt concentrations in the uppermost layer of the soil is due to evapotranspiration and would fluctuate depending on precipitation conditions. This concentration of salt likely provides microenvironments that provide suitable conditions for the survival of thee extreme halophilic archaea (1).

The discovery of these extreme halophilic Archaea in a relatively low-salt environment combined with the diversity and novelty revealed in the culture independent analysis peaked interest in this particular group of organisms. In order to determine how these organisms were surviving under these seemingly sub-optimal conditions an effort was undertaken to isolate and characterize novel halophilic Archaea from Zodletone Spring. This effort resulted in the isolation and identification of several novel genera in the order *Halobacteriales*, these isolates have distinct characteristics that separate them from other halophilic Archaea

and provide evidence that they are specially adapted for survival in low-salt environments.

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## Abstract

The archaeal community of a low-salt, sulfur-rich spring in south-western Oklahoma, was analyzed at two different locations along the spring. Interestingly, 36% of the archaeal mat clone library and 4% of the source clone library were phylogenetically related to the extreme halophilic Archaea family *Halobacteriacea*. Geochemical analysis of the spring revealed low-salt concentrations throughout ranging from 0.7-1.0% (approximately 0.2 M) and extreme halophilic Archaea are defined by their requirement for high- salt concentrations (1.5 M). The haloarchaeal community was abundant, novel and diverse forming five different groups within the order *Halobacteriales*. Three novel genera and one novel species belonging within the family *Halobacteriacea* were successfully isolated from the spring. These include the following: *Haloferax sulfurifontis* (sp. nov.), *Haladaptatus paucihalophilus* (gen. nov., sp., nov.), *Halosarcina pallida* (gen., nov., sp., nov.), and *Haloenormitas infirmitas* (gen. nov., sp., nov). Each one of these organisms was shown to be extremely resistant to low-salt concentrations, could grow over a wide range of salt concentrations, and some of them grew at relatively low salt concentrations. This study reveals a novel group of halophilic Archaea that possess distinct characteristics that would be advantageous in low-salt environments. These halophilic Archaea have since been identified in several other low-salt environments further suggesting that they are uniquely adapted to these conditions.

# CHAPTER 1

## *Haloferax sulfurifontis* sp. nov., a halophilic archaeon isolated from a sulfide and sulfur-rich spring.


## Abstract

A pleomorphic halophilic archaeon (strain M6) was isolated from a sulfide and sulfur rich spring in south-western Oklahoma. It formed small (2-3 mm) salmon pink elevated colonies on agar medium. The strain grew in a wide range of NaCl concentrations (6% to saturation) and required at least 1 mM $Mg^{2+}$ for growth. Strain M6 is able to anaerobically reduce sulfur to sulfide. 16S rRNA sequence analysis indicated that it belongs to the family *Halobacteriaceae*, genus *Haloferax*, with similarity values ranging between 96.7-98.0% to other validly named members of the genus and 89% to *Halogeometricum borinquense*, its closest relative outside the genus *Haloferax*. Polar lipid analysis and the G+C content of the DNA further supported the placement of strain M6 in the genus *Haloferax*. DNA-DNA hybridization, as well as biochemical and physiological characterization allowed differentiation of strain M6 from other validly named members of the genus *Haloferax*, and we propose a new species, *Haloferax sulfurifontis*, to accommodate the strain. The type species is strain $M6^T$ (= JCM $12327^T$ = CCM $7217^T$ =DSM $16227^T$ =CIP $108334^T$).

Members of the family *Halobacteriaceae* are the dominant heterotrophic microorganisms in hypersaline ecosystems with NaCl concentrations greater than 25% such as saltern crystallizer ponds, the Dead Sea and other hypersaline lakes (7, 14). A shared character of all members of the order is the absolute requirement for high concentrations of NaCl. Currrently, 18 genera and 35 validly named species are recognized within the *Halobacteriaceae*.

The genus *Haloferax* was first described by Torreblanca *et al.* (24), and currently comprises five validly named species: *H. volcanii* (12), *H. denitrificans* (23), *H. gibbonsii* (10), *H. mediterranei* (18) and *H. alexandrinus* (1). A sixth species, *H. lucentensis*, has been described (9), but the name has not yet been validated. Members of the genus *Haloferax* are characterized by extreme pleomorphism and a relatively low salt requirement compared to other genera of the *Halobacteriaceae*.

In spite of the fact that members of the *Halobacteriaceae* are generally considered to have an absolute requirement for least 1.5 M NaCl for growth, several studies have demonstrated that members of the family could be recovered from a variety of low salt environments. Rodriguez-Valera *et al.* (19) isolated *Halococcus* species from sea water. *Natrialba* isolates have been recovered from low salt saltern ponds (11). In addition, several culture-independent analyses have detected 16S rRNA gene sequences suggestive of the presence of *Halobacteriaceae* species in other low-salt environments including coastal marshes (13), and hydrothermal black smoker chimney structures (20).

In the course of our studies of the microbial diversity in the sulfide- and sulfur-rich Zodletone spring (Oklahoma, USA), 16S rRNA genes were cloned from microbial mats (4). Clone libraries were observed to contain many clones related to the family *Halobacteriaceae*, even though the spring has a stream-water salinity of only 1%. Field measurements indicated the presence of relatively high salt habitats near the shallow stream. These habitats presumably provide a suitable envionment for members of the *Halobacteriaceae* to survive and grow (4). Eighteen halophilic strains were isolated from the microbial mats and mineral crusts that form near the stream using high-salt medium supplemented with antibiotics to suppress the growth of halophilic or halotolerant bacteria (4). Six of these strains were studied and a preliminary investigation (colony and cell morphnology, salt tolerance profile, lipid pattern and partial 16S rRNA gene sequence) suggested that all six isolates belong to a single species. In this paper, one of the isolates, strain $M6^T$, has been characterized in detail; it is proposed that this strain represents a novel species of the genus *Haloferax*.

The isolation procedure was previously described in detail (4). Characterization of strain $M6^T$ followed the guidelines outlined by Oren *et al.* (16) for describing new species of the *Halobacteriaceae*. Physiological, biochemical, and nutritional tests were performed in halophilic medium adapted from Oren (14) and containing (g $l^{-1}$): NaCl (150), $MgCl_2.7H_2O$ (20), $K_2SO_4$ (5), $CaCl_2.2H_2O$ (0.1), yeast extract (5), pH 7.0. Cultures were incubated at $37^0$C with shaking (200 rpm) unless otherwise specified. Growth was monitored by

measuring the increase in $OD_{600}$. Growth was evaluated at NaCl concentrations between 0 and 37% (saturation), $Mg^{2+}$ concentrations between 0 and 200 mM, a temperature range of 4-60 °C, and a pH range of 3-11. Suitable organic buffers were included in pH range and optimum determination experiments (25 mM of MES, MOPS, HEPES, or TES) to prevent changes in pH due to acid production during growth. Substrate utilization was tested by lowering the yeast extract concentration to 0.1 g $l^{-1}$, including the substrate at a concentration of 0.5 g $l^{-1}$, and adding 25 mM MOPS as a buffer. Acid production from a variety of substrates was tested in unbuffered HM medium, with 5 g $l^{-1}$substrate and phenol red (0.004 g $l^{-1}$) as an indicator. Tests for the ability of strain M6 to grow utilizing $S^0$, $S_2O_3^{2-}$, polysulfide, $SO_4^{2-}$, $NO_3^-$, DMSO, and TMAO as electron acceptors, as well as its ability to ferment arginine, were performed in halophilic medium, prepared anaerobically in Balch tubes (2, 3). $SO_4^{2-}$ and $S_2O_3^{2-}$ reduction was followed using a Dionex ion chromatography system. Biochemical tests performed followed the methods outlined in Gerhardt *et al* (6). *Haloferax volcanii* DSM $33755^T$ was used as a control in all tests. 16S rDNA was amplified using the primers Arch21f and Arch1492r (17), cloned using a TOPO-TA cloning kit (Invitrogen), and sequenced at the Oklahoma Medical Research Foundation Core Sequencing Facility. Sequence alignment was performed using CLUSTAL_X (21). Phylogenetic analysis included evaluation of the evolutionary distance with a neighbor-joining algorithm and Jukes-Cantor corrections using PAUP 4.01b10 (Sinauer Associates). Samples

were fixed for electron microscopy on a poly(lysine)-coated cover slip using glutaraldehyde, coated with gold/palladium, and examined using a JSM-880 scanning electron microscope. G+C content of the total cellular DNA and DNA-DNA hybridization were determined according to methods outlined earlier (9).

Strain M6 formed extremely small (0.8-1 mm) salmon pink transparent and elevated punctiform colonies with undulate margins. Cells were extremely pleomorphic,showed motility, and stained Gram negative. Rods, irregular cocci, and flattened disc shapes were observed in the phase contrast microscopy and in the scanning electron microscope. Rod shaped cells (single or in pairs) were observed more frequently during the exponential growth phase, whereas irregularly shaped cocci were common during the stationary phase or in growth as colonies on agar plates (Fig. 2). Strain M6 grew in HM medium within a wide range of NaCl concentrations, from 60 g $l^{-1}$ to saturation, with an optimum at 125-150 g $l^{-1}$. It required at least 1 mM $Mg^{2+}$ and grows best at 30 mM $Mg^{2+}$ and higher. Cells lysed in distilled water, as well as in NaCl concentrations below 30 g $l^{-1}$, but they retained their viability for prolonged incubations in NaCl solutions of 40 g $l^{-1}$ and above (4). Strain M6 did not grow anaerobically on nitrate, sulfate, thiosulfate, DMSO, or TMAO as electron acceptors, nor was it able to ferment arginine. Strain M6 is, however, capable of reducing elemental sulfur to sulfide (4). Control experiments with *H. volcanii* indicated that this species could also reduce elemental sulfur to sulfide, albeit at a much slower rate (50 μM sulfide formed after in 3 months, compared to 0.4 mM in 2 weeks for

strain M6). These results suggest that strain M6 may be capable to survive in the anaerobic conditions in Zodletone spring by reducing elemental sulfur to sulfide, and that elemental sulfur reduction could be a common capability within the *Halobacteriaceae*, as suggested by Grant & Ross (8) and Tindall & Truper (22).

Detailed results of nutrition experiments, antibiotic sensitivity and the physiological description are given in the species description. In general, strain M6$^T$ was similar to other members of the genus *Haloferax* in being oxidase- and catalase- positive and able to grow on a single carbon source. It is also similar in that all species are unable to grow anaerobically on DMSO or TMAO and they are unable to ferment arginine or to decarboxylate lysine and ornithine. Differences between M6$^T$ and other members of the genus *Haloferax* are highlighted in Table 1.

16S rRNA gene sequence analysis indicated that strain M6$^T$ clusters within the genus *Haloferax* (Fig. 1). Sequence similarity calculations using a neighbor-joining analysis indicated that strain M6 has a sequence similarity ranging between 96.7 (*Haloferax mediterranei*) to 98.0 (*Haloferax lucentensis*) similarity to sequences of other members of the genus *Haloferax.* The closest relative of strain M6 was "*Haloferax* sp." strain D1227, isolated from soil contaminated with highly saline oil brine (5). The closest relative to strain M6$^T$ outside of the genus *Haloferax* was *Halogeometricum borinquense*, with a sequence similarity of only 89 %. Phospholipid analysis indicated the presence

of S-DGD-1 but absence of phosphatidylglycerosulfate, a pattern characteristic of members of the genus *Haloferax* (15). The DNA G+C content of strain M6$^T$ is 60.5 mol%, a value within the designated range of the genus (7). Results of DNA-DNA hybridization experiments (Table 1) show hybridization values between strain M6$^T$ and other species of the genus *Haloferax* ranging between 1 and 24 %, thus indicating that this strain is clearly a novel species of the genus *Haloferax.*

The physiological, biochemical, and genetic tests described above suggest that strain M6 is a member of the genus *Haloferax.* Differences outlined in Table 1, as well as 16S and DNA-DNA hybridization data justify the placement of strain M6 within a new species of the genus *Haloferax*, for which the name *Haloferax sulfurifontis* is proposed.

**Description of *Haloferax sulfurifontis* sp. nov.**

*Haloferax sulfurifontis* (sul.fu.ri.fon′tis. L. masc. n. *fons*, *fontis* spring; L. neut. n. *sulfur* sulfur; N.L. gen. n. *sulfurifontis* of a sulfurous spring).

Cells are extremely pleomorphic, occurring mainly as irregular shaped cocci 1-1.5 μm in diameter, especially during the stationary phase and as rods (1.5-1.7 x 0.5-0.6 μm), especially during the exponential growth phase. Occurs mostly in as single cells, sometimes in pairs and clusters. Cells stain negative in the Gram reaction. Colonies on agar medium with 150 g $l^{-1}$ NaCl are small (0.8-1 mm), salmon pink, transparent, and elevated with undulate margin. Halophilic; cells

lyse immediately in distilled water, within 24 h at 10-20 g $l^{-1}$ NaCl, NS 72 h at 30 g $l^{-1}$ NaCl. Cells survive prolonged incubation in 40-50 g $l^{-1}$ NaCl. Grows at a wide range of NaCl concentrations (60 g $l^{-1}$ to saturation), requires at least 1 mM $Mg^{2+}$ for growth, and grows best at 30 mM Mg and above. Incapable of utilizing $SO_4^{2-}$, $S_2O_3^{2-}$, $NO_3^-$, DMSO, or TMAO as electron acceptors, capable of reducing elemental sulfur to sulfide under strictly anaerobic conditions. Chemoorganotrophic, grows on complex medium with yeast extract, casamino acids, and peptone as carbon sources. Capable of growing in defined media. The following substrates could be used as a carbon source: acetate, arabinose, benzoate, citrate, fructose, fumarate, galactose, glucose, L-glutamate, malate, succinate, sucrose, and xylose. Compounds not used as carbon source included alanine, aspartate, arginine, glycerol, glycine, lactose, mannitol, sorbitol, ribose, and starch. Acid is produced in unbuffered medium from the following compounds: glycerol, sucrose, malate, acetate, sorbitol, yeast extract, lactose, fructose, glucose, xylose, maltose, mannitol. Acid is not produced from galactose and from starch. Catalase and oxidase positive, reduces thiosulfate aerobically to sulfide, incapable of aerobic nitrate reduction and nitrite is not formed from nitrate. Indole is formed from tryptophan. Gelatin is hydrolyzed; starch, casein and urea are not hydrolyzed. Produces polyhydroxyalkanoates. Phosphatase and β-galactosidase tests are negative. Ornithine and lysine are not decarboxylated. Resistant to ampicillin, erythromycin, chloramphenicol, carbenicillin, gentamycin, ceftriaxone, ciprofloxacin, doxycycline, cefaclor,

kanamycin, nalidixic acid, oxytetracycline, penicillin G, rifampicin, and bacitracin up to 100 μg/ml. Sensitive to aphidicolin, anisomycin, and novobiocin, and to high concentrations of bacitracin. The major polar lipids in the membrane are the diphytanyl ether derivatives of phosphatidylglycerol, the methyl ester of phosphatidylglycerophosphate, and S-DGD-1 as the main glycolipid. Phosphatidylglycerosulfate is absent. DNA G+C content is XXX mol%.

The type strain M6$^T$ (=JCM 12327$^T$ =CCM 7217$^T$ =DSM 16227$^T$ =CIP 108334$^T$). The DNA G+C content of M6$^T$ is 60.5 mol%. Isolated from the Zodletone spring in south-western Oklahoma, USA.

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Table 1. Characteristics distinguishing strain M6$^T$ from other validly described members of the genus *Haloferax*. Data compiled from Asker & Ohta (1), Mullakhanbhai & Larsen (12), Rodriquez-Valera et al., (18), Tomlinson et al., (23), Juez et al., (10), Gutierrez et al., (9), Oren (15), and Grant et al., (7). +, positive; -, negative; ND, not determined.

| Characteristic | Strain M6 | *H. volcanii* | *H. gibbonsii* | *H. denitrificans* | *H. medeterranii* | *H. alexandrinus* | *H. lucentensis* |
|---|---|---|---|---|---|---|---|
| Temperature optimum ($^0$C) | 32-37 | 40 | 35-40 | 50 | 40 | 37 | 37 |
| Temperatue range ($^0$C) | 18-50 | N.D. | 25-55 | 30-55 | 20-55 | 20-55 | 10-45 |
| NaCl range (M) | 1-5.2 | 1-4.5 | 1.5-5.2 | 1.5-4.5 | 1.3-4.7 | 1.8-5.1 | 1.8-5.1 |
| NaCl optimum (M) | 2.1-2.6 | 1.7-2.5 | 2.5-4.3 | 2-3 | 2.9 | 4.3 | 4.3 |
| Cell stability (M NaCl) | 0.5 | 0.5 | 0.5-0.7 | 1.5 | 0.5 | 1.7 | ND |
| Motility | + | - | - | - | + | - | + |
| pH optimum | 6.4-6.8 | 7 | 6.5-7 | 6-7 | 6.5 | 7.2 | 7.5 |
| Gelatin hydrolysis | + | - | + | + | + | + | - |
| Starch hydrolysis | - | - | - | - | + | - | - |
| Anaerobic nitrate reduction | - | - | - | + | + | - | - |
| Tween 80 hydrolysis | + | - | + | - | + | + | ND |
| Indole production | + | + | + | - | + | + (check) | + |
| $H_2S$ production from thiosulfate | + | + | + | + | - | + | + |
| Mol% G+C | | 63.4 | 61.8 | 64.2 | 59.1-62.2 | 59.5 | 64.5 |
| Casein hydrolysis | - | - | + | - | + | - | - |
| Resistance to Rifampicin | | - | - | - | - | + | ND |

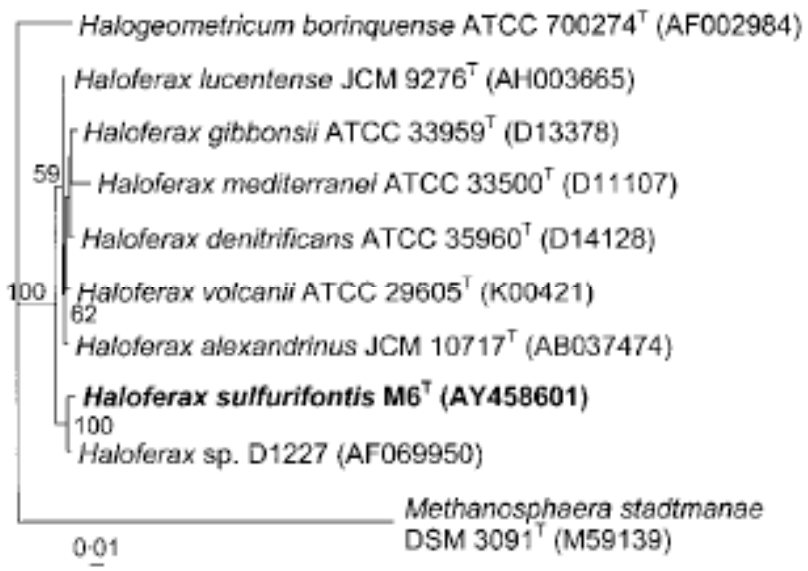


**Figure 1.** Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between isolate M6T, other members of the genus Haloferax and other selected genera of the family *Halobacteriaceae*. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 50% bootstrap support. Bar= 0.01 substitutions per site.

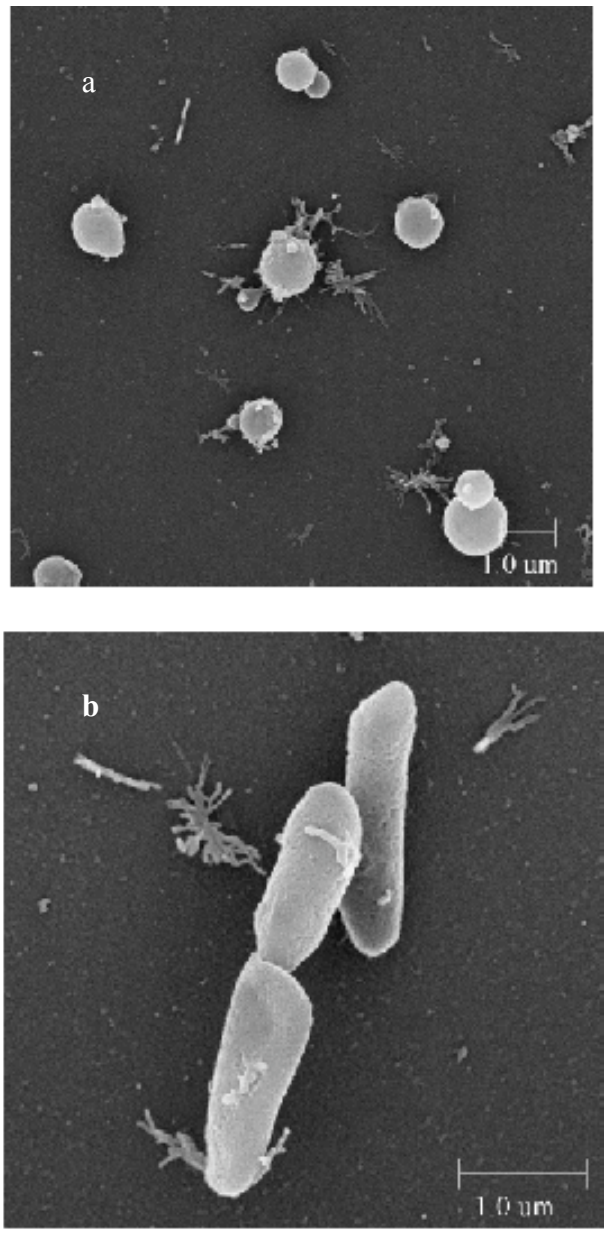


**Figure 2.** Scanning electron micrograph of (a), irregular cells observed in liquid culture of strain M6 during the stationary growth phase and (b), rod-shaped cells of strain M6 during the exponential growth phase.

# CHAPTER 2

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


## Abstract

A novel halophilic archaeon, Strain DX253$^T$ was isolated from Zodletone spring, a low-salt, sulfide and sulfur-rich spring in Southwestern Oklahoma, USA. Cells were cocci or coccobacilli and occurred as singles or in pairs. Strain DX253$^T$ grew at a wide range of salt concentrations, 5-30% (w/v), with an optimum occurring around 18%, and required at least 10 mM $Mg^{2+}$ for growth. The pH range for growth of strain DX253$^T$ was 5 to 7.5, and the temperature range was 25-45 °C. In addition to its ability to grow at relatively low salt concentrations, strain DX253$^T$ remained viable in distilled water after prolonged incubation. The two diether phospolipids that are typical of the order *Halobacteriales*, phosphatidylglycerol (PG) and phosphatidylglycerol phosphate methyl ester (Me-PGP) were present. Phosphatidyl glycerol sulfate (PGS), as well as two yet unidentified glycolipids were also detected. Strain DX253$^T$ had two distinct 16S rRNA gene sequences that were 95.8% similar to each other, and both sequences were only 89.5-90.8% similar to the closest related cultured and named species within the order *Halobacteriales.* The mol% G+C content is 60.5. The closest relatives of strain DX253$^T$ are clones and uncharacterized isolates obtained from coastal salt marsh sediments with an equivalent of

seawater salinity. Physiological, biochemical and phylogenetic differences between strain DX253$^T$ and other previously described genera of the extreme halophilic *Archaea* suggest that this strain represents the first species in a new genus within the family *Halobacteriaceae*, for which we propose the name *Haladaptatus paucihalophilus*. The type strain is DX253$^T$ (=JCM 13897$^T$ =DSM 18195$^T$ =ATCC BAA-1313$^T$ = KTCC 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 (16, 22). The family *Halobacteriaceae* was first described in 1974 to accommodate obligate halophilic microorganisms that require at least 2.0 M NaCl for growth (14). Currently, the family includes 22 genera that display a wide variety of morphologies including rods, cocci, squares, triangles and flattened disks (4, 7, 16, 20, 30, 33).

Despite the fact that members of the order *Halobacteriales* mainly inhabit environments of extreme salinity where salt concentrations exceed 20% (21), several reports have suggested the presence of extreme halophilic *Archaea* in moderate to low salinity environments. For example, Rodriguez-Valera *et al.* (26) isolated an extremely halophilic coccus from ocean waters off the coast of Spain. With the advent of 16S rRNA gene-based surveys, the presence of halophilic *Archaea* in low salt environments has been further demonstrated. Studies by Munson *et al*. (18) found that halophilic archaeal representatives were present in a coastal salt marsh where pore water salinity was approximately 0.8 M NaCl. Members of the order *Halobacteriales* were also reported in a survey of the archaeal diversity in a deep-sea hydrothermal vent (29). Recently, extremely halophilic *Archaea* likely representing several different genera were isolated from coastal salt marsh sediments (25).

In addition, we have recently reported on the presence, and examined the diversity of halophilic *Archaea* in Zodletone spring, a sulfide and sulfur-rich spring in Southwestern Oklahoma, USA, using a combination of culture-independent and cultivation methods (10, 11). In this study, we further report on the isolation and characterization of strain DX253$^T$, a novel halophilic archaeon isolated from Zodletone Spring, as the first species in a novel genus of the order *Halobacteriales* for which we propose the name *Haladaptatus paucihalophilus*.

The location and geochemical properties of the spring have been described previously (12, 27, 36). Although salinity measurements in the spring water do not exceed 0.2 M NaCl, concentrations of NaCl approached saturation in the top 0-1 cm of soil along the bank of the spring, likely due to evaporative concentration (10). For isolation of halophilic *Archaea*, samples from the top 2.0 cm of 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 directly upon returning to the laboratory. The halophile medium (HMD) used for the isolation procedure was modified from (10, 22, 24) and contained the following: (g $l^{-1}$): $MgCl_2$•6 $H_2O$ (20), $K_2SO_4$ (5), $CaCl_2$•2 $H_2O$ (0.1), Yeast Extract (0.1), $NH_4Cl$ (0.5), $KH_2PO_4$ (0.05), carbon source (0.5), agar (20), and either 180, 250 or 300 g $l^{-1}$ of NaCl. The pH of the medium was adjusted to 7.0, and ampicillin and kanamycin were added at a concentration of 50 μg $ml^{-1}$ to suppress the growth of halotolerant *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, benzoate, cysteine, casamino acids, yeast extract or glutamate. The plates were incubated at 37°C under a 60-Watt light bulb placed 12 inches above the plates until colonies appeared. To ensure purity, a single colony of each strain was re-streaked twice onto HMD plates.

Characterization followed the general guidelines for describing new taxa in the order *Halobacteriales* presented by (24). Detailed protocols for the methodology of biochemical tests conducted were obtained from Gerhardt (13), and NaCl was added as necessary. Gram reaction was determined following the method outlined by Dussault (9). Physiological tests were conducted using a HMD liquid or solid (2.0% agar) medium with sucrose (0.5 g $l^{-1}$) as the carbon source, 180 g $l^{-1}$ NaCl and 25 mM HEPES, unless otherwise stated. Liquid cultures were incubated at 37°C on a shaking incubator at 200 rpm. Growth rates were determined by monitoring the increase in optical density at $\lambda$=600 nm. Substrate utilization was tested by substituting various carbon sources into the HMD medium as suggested by Oren *et al.* (24). Acid production was tested in HMD un-buffered medium and was determined by measuring the initial and final pH of the medium. The culture was considered positive for acid production if the pH decreased by at least one unit**.** The ability of strain DX253$^T$ to use DMSO (5.0 g $l^{-1}$), 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 previously described procedures (3, 6). Sulfur was added as sublimed sulfur suspended in an aqueous solution (34). The sulfur 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 a low salt HMD medium and a sterile saline solution with 0, 5.0, 10.0, 20.0 or 30.0 g $l^{-1}$ NaCl. The HMD medium contained $MgCl_2$ which could help stabilize cell walls at lower salt concentrations (16). 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 medium. The antibiotic concentrations were 35 μg $ml^{-1}$ except for aphidicolin (30 μg $ml^{-1}$). Scanning electron microscopy was conducted at the University of Oklahoma's Samuel Roberts Noble Electron Microscopy Laboratory. Cells were viewed using a JSM-880 Scanning Electron Microscope.

The 16S rRNA genes were amplified using A1F (5'-ATTCCGGTTGATCCTGC-3') (28) and UA1406R (5'-ACGGGCGGTGWGTRCAA-3') (2) primers. The PCR product was then cloned using the TOPO-TA cloning kit (Invitrogen Corp., Carlsbad, CA).

Twenty-eight clones were randomly picked and sequenced at the Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, USA. 16S rRNA gene sequences were aligned using CLUSTAL_X (31), and distance trees were constructed with PAUP 4.01b10 (Sinauer Associates, Sunderland, MA) using a neighbor-joining algorithm and Jukes-Cantor corrections. G+C mol% was determined using the services of the German National Resource Centre for Biological Material (DSMZ). Membrane lipids were analyzed using two-dimensional thin layer chromatography as described by Oren *et al*. (23).

Two strains, DX253$^T$ and GY252, were isolated independently from each other on HMD plates with 25% NaCl on glucose and glycerol respectively, after approximately four weeks of incubation. Both strains were fully characterized. 16S rRNA sequence data (Fig. 1), membrane lipid analysis, physiological and biochemical tests suggested that both strains were of the same species. Strain DX253$^T$ was chosen to represent the type strain.

Strain DX253$^T$ cells stained Gram-negative and colonies were small (0.2 mm), pink, translucent, convex and circular with an entire margin. The cells are non-motile generally coccoid or may appear as short rods especially in early growth phases. Cocci occur singularly or in pairs (Fig. 2). The presence of gas vesicles was not evident through light microscopy.

Strain DX253$^T$ grew at a wide range of salt concentrations from (0.8-4.3 M), with an optimum at 3.1 M NaCl. Cells did not immediately lyse when suspended in distilled $H_2O$ and retained viability under these conditions for up to

2 weeks. The ability of strain DX253$^T$ to grow at relatively low salt concentrations along with its tolerance to distilled water, likely allows these *Archaea* to survive the fluctuating salt conditions encountered in Zodletone Spring. The development of concentrated NaCl on the banks of the spring depends widely on temperature and rainfall patterns, and therefore these locations may not always remain hypersaline.

Detailed physiological and biochemical characteristics of Strain DX253$^T$ are listed in table 1, as well as the species description. In general, strain DX253$^T$ is chemoorganotrophic, capable of growing on a complex medium as well as on a single carbon source. It produced acid when growing on carbohydrates. No growth was detected when grown anaerobically with TMAO, DMSO, sulfate, thiosulfate, nitrate or sulfur as a terminal electron acceptor.

The 16S rRNA sequences of strain DX253$^T$ was 89.5-90.8% similar to the closest described species in the family *Halobacteriaceae, Halalkalicoccus tibetensis*. Strains DX253$^T$ had two distinct 16S rRNA gene sequences that were 95.8% similar to each other (Fig. 1). A majority of the differences between these two sequences were found between base pairs 1-200 and 400-800. Despite very low similarity to validly described members of the extremely halophilic *Archaea* strain DX253$^T$ matched closely (99.2%) to uncharacterized isolates retrieved from an estuarine ecosystem, and these isolates also contained two divergent 16S rRNA gene sequences (25) (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 sequences with a high similarity to strain DX253$^T$ were encountered in a low-salinity ecosystem provides further evidence that the strains described here are adapted to relatively low-salt systems or fluctuating salt concentrations. Whether or not members of this group are entirely absent from truly hypersaline environments cannot be stated unequivocally.

The presence of multiple heterogeneous 16S genes is not unprecedented among prokaryotes in general (1), and halophilic *Archaea* in particular (1, 8, 16, 19). Within the order *Halobacteriales*, several members of the genus *Haloarcula (Har. marismortui, Har. quadrata, and Har. vallismortis)*, *Halosimplex carlsbadense*, and *Natrinema* sp. strain XA3-1 have been shown to contain at least two divergent 16S rRNA genes. Differences between the heterogenous 16S genes within the order *Halobacteriales* ranges from approximately 5.0-6.8% (1, 5, 19, 32), similar to the 4.2% difference seen in strain DX253$^T$ . In *Haloarcula marismortui,* both 16S rRNA genes are transcribed during growth, and it has been proposed that the presence of heterogeneous 16S rRNA operons may aid in tolerating environmental stresses (8). The fact that strains DX253$^T$, GY252 and the closely related isolates from salt-marsh sediments in Essex, UK all have heterogenous 16S rRNA gene sequences indicates that it might be characteristic of this novel genus, it is unclear if this feature confers any competitive advantage to the microorganisms in a low-salt environment**.**

Strain DX253$^T$ contained the phospholipids, phosphatidyl glycerol (PG), phosphatidyl glycerol phosphate methyl ester (Me-PGP) and phosphatidyl glycerol sulfate (PGS). The presence of PGS within this group helps to differentiate this isolate from the other neutrophilic genera of halophilic *Archaea* such as *Haloferax*, *Natrialba*, *Halobaculum*, *Halococcus*, and *Halogeometricum* that do not contain PGS (16, 20). Analysis of the glycolipids using thin layer chromatography revealed that strain DX253$^T$ contained at least two glycolipids that have yet to be identified. Thin layer chromatography plates showing the pattern of glycolipids and phospholipids for Strain DX253$^T$ (Fig. 3).

This study provides evidence that strain DX253$^T$ is a member of the extreme halophilic *Archaea*, order *Halobacteriales*, family *Halobacteriaceae*. Lipid data combined with biochemical and physiological characteristics differentiate strain DX253$^T$ from other previously described members of this family. The low percentage similarity of the 16S rRNA gene sequence to other genera within the order *Halobacteriales* further justifies the claim that this is the first species in a novel genus within this family, for which we propose the name *Haladaptatus paucihalophilus*.

**Description of *Haladaptatus* gen. nov.**

(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 in singles or in pairs. Colonies are pink pigmented. Members of the genus *Haladaptatus* have at least two heterogenous 16S rRNA sequences. Cells contain PG, Me- PGP and PGS. Two unidentified glycolipids are present. Chemoorganotrophic, growing on a wide range of substrates including single and complex carbon sources. Produces acid from carbohydrates. Hydrolyses starch, gelatin, casein and Tween 80. Grows at 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 gentamycin. Isolated from a low-salt, high sulfide spring. Survives low-salt concentrations and can recover after prolonged exposure to less than 0.2 M NaCl. The mol% G+C content of the type strain is 60.5%. The type species of this genus is *Haladaptatus paucihalophilus*. Recommended three-letter abbreviation: *Hap.*

**Description of *Haladaptatus paucihalophilus*** sp. nov.

(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)

Cells are cocci or coccobacilli occurring in singles and pairs. Cells are approximately 1.2 μm in diameter. Doubling time is approximately 12- 13 hours. Non-motile and staining Gram-negative. Colonies are small (0.2 mm), pink, translucent, round, convex with an entire margin. PG, Me-PGP and PGS

are present. Possesses two unidentified glycolipids. Sensitive to novobiocin, bacitracin, anisomycin and aphidicolin. Partially sensitive to rifampicin and trimethoprim. Resistant to erythromycin, penicillin, ampicillin, chloramphenicol, neomycin, nalidixic acid and gentamycin. Grows from 0.8-5.1 M NaCl, with optimal growth at 2.6-3.1 M NaCl. Optimal temperature for growth is 25-30°C, with a range of 25-45°C. Growth requires a minimum of 5 mM $Mg^{2+}$. Grows at pH values ranging from 5.0-7.5, with an optimum of 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, esculin, salicin, trehalose, sucrose, fructose, xylose, dextrose, 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 produced from tryptophan. Does not reduce nitrate under aerobic conditions. Hydrolyzes starch, casein, gelatin and Tween 80. G+C mol% of the type strain is 60.5%. The type strain is $DX253^T$ (=JCM $13897^T$ =DSM $18195^T$ =ATCC BAA-$1313^T$

=KCTC 4006$^{T}$) and was isolated from Zodletone Spring in Southwestern Oklahoma, USA.

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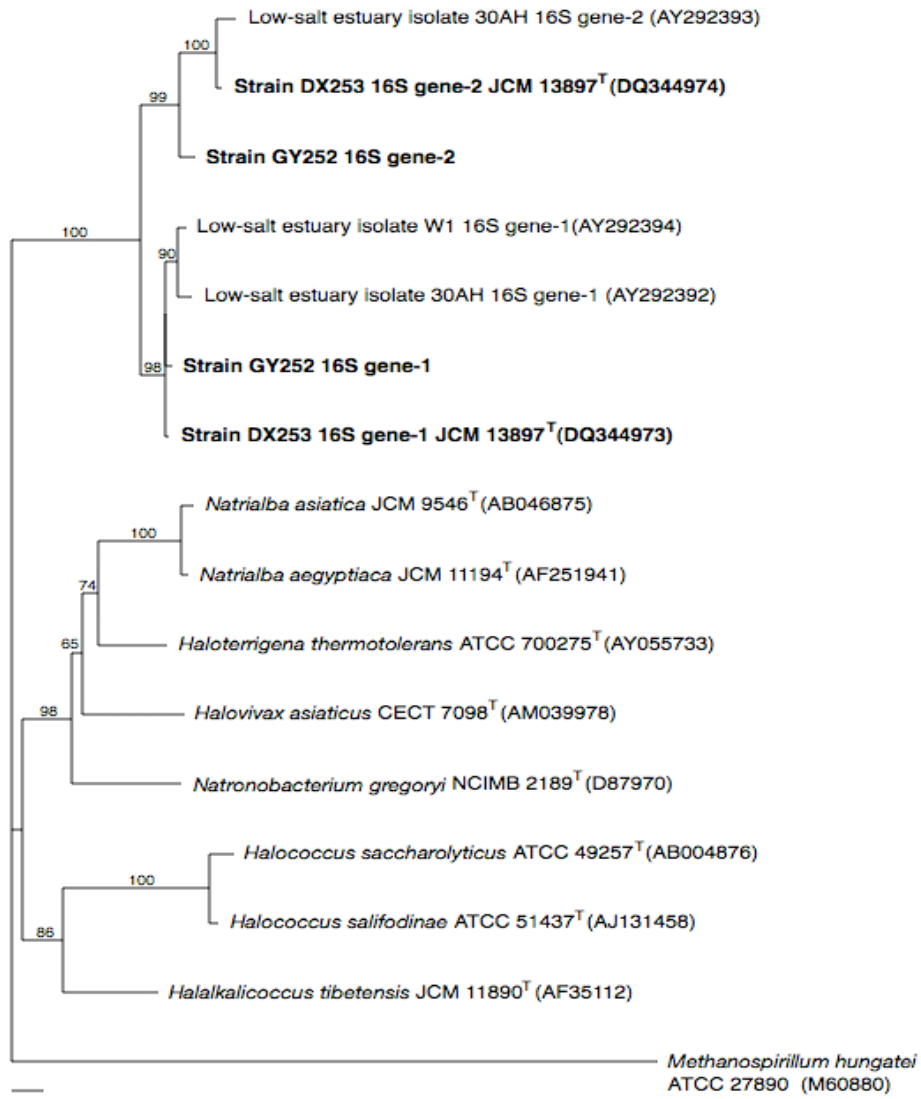


**Figure 1.** Distance dendrogram showing the relationship between the two 16S rRNA gene sequences of strain DX253$^T$ (DX253$^T$ 16S-1 and DX253$^T$ 16S-2) and strain GY252 (GY252 16S-1 and GY252 16S-2), and other close relatives within the family *Halobacteriaceae*. Sequences were retrieved from GenBank and the accession numbers are indicated by parentheses. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 50% bootstrap support. The bar is equivalent to 0.01 substitutions per site.

**Table 1.** Characteristics that distinguish strain DX253$^T$ from other closely related genera within the order *Halobacteriales*. 1,Strain DX253$^T$; 2, *Halalkilococcus;* 3*, Natronobacterium;* 4*, Halococcus;* 5*, Haloarcula;* 6*, Haloferax;* 7*, Natronococcus*. Data collected from, (11, 15-17, 22, 35). +, Positive; -, negative; v, results variable; NR, not reported

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Cell Shape | Coccus | Coccus | Rod | Coccus | Pleo-morphic | Pleo-morphic | Coccus |
| Cell Size (μm) | 1.0-1.5 | 1.0-1.5 | 0.5-1.0 x 2-15 | 0.8-1.5 | 0.2-2 x 0.5-5 | 0.4-3 x 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) | 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) | 30 | 40 | 37 | 30-40 | 35-55 | 32-50 | 35-45 |
| pH optimum | 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 $H_20$ | - | - | + | - | + | + | - |
| PGS | + | - | - | - | + | - | - |
| G+C of DNA (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: | | | | | | | |
| 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 |

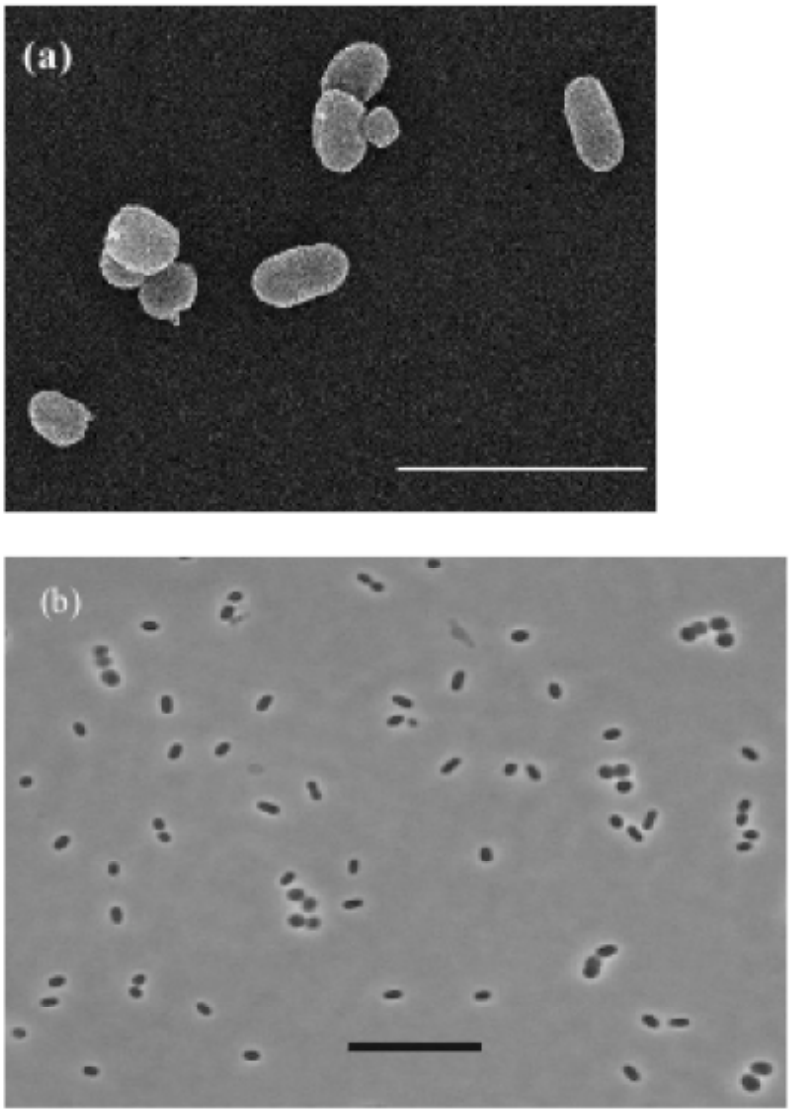


**Figure 2**. (a) Scanning electron micrograph and (b) light microscopy photomicrograph of cells of strain DX253T. Bars, 5 μm (a) and 10 μm (b).

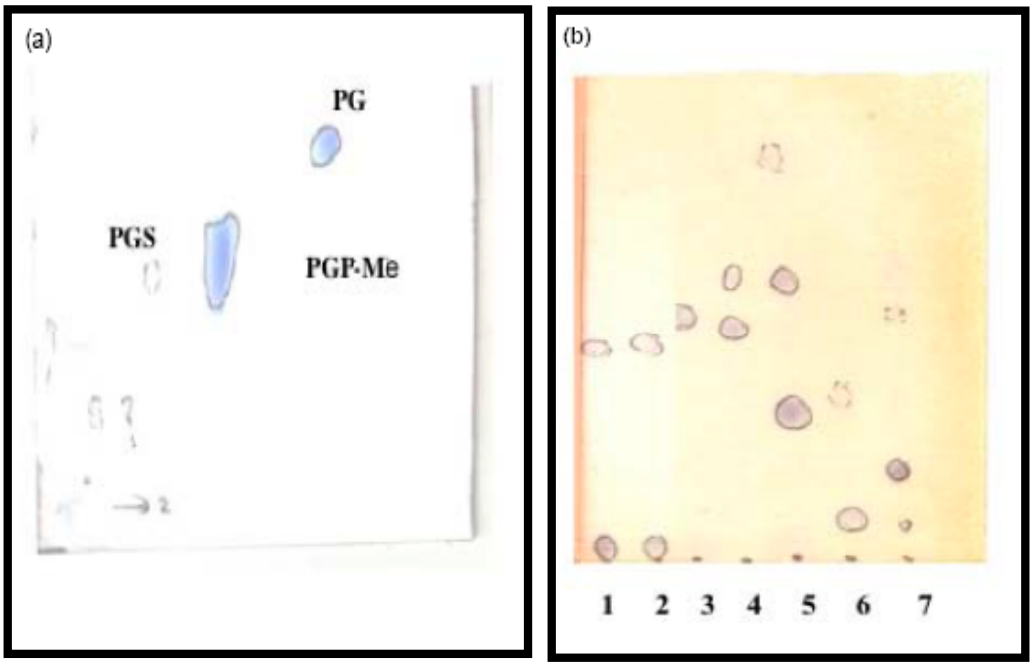


**Figure 3.** Analysis of the membrane lipids of strain DX253$^T$ by TLC. (a) Two-dimensional plate displaying the phospholipid pattern of strain DX253T confirming the presence of phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and phosphatidylglycerol sulfate (PGS). (b) One-dimensional plate comparing the glycolipid pattern of strain DX253T with those of other members of the family *Halobacteriaceae*. 1, Strain GY252; 2, strain DX253T; 3, *Halogeometricum borinquense* strain PR 3$^T$; 4, *Haloarcula marismortui*; 5, *Haloferax gibbonsii*; (6) *Natrialba asiatica*; 7, *Halobacterium salinarum* strain R1. The glycolipid pattern of strain DX253$^T$ is unlike previously identified glycolipid patterns.

# Chapter 3

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


## Abstract

A novel halophilic archaeon, Strain BZ256$^T$ was isolated from Zodletone spring, a sulfide and sulfur-rich spring in Southwestern Oklahoma, USA. Cells were cocci, non motile, non-flagellated, and divide along two axes resulting in the formation of sarcina-like clusters. Strain BZ256$^T$ grew at salt concentrations ranging from 1.3-4.3 M NaCl, with an optimum near 3.4 M and required at least 1 mM $Mg^{2+}$ for growth. The pH range for growth was 5.0 to at least 8.5, and the temperature range was 25-45 °C. The two diether phospholipids that are typical of the order *Halobacteriales*, phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester were present, as were two glycolipids chromatographically identical to S-DGD-1 and DGD-1. The small subunit rRNA sequence of strain BZ256$^T$ was 96.8% similar to *Halogeometricum borinquense*, the closest recognized species within the order *Halobacteriales.* The DNA G+C content of strain BZ256$^T$ is 65.4 mol%. Microscopic, physiological, biochemical and phylogenetic comparisons between strain BZ256$^T$ and other previously described genera of the extreme halophilic *Archaea* suggest that this strain is a member of a new genus within the family *Halobacteriaceae*, for which we propose the name *Halosarcina pallida*. The type strain 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 microorganisms in hypersaline environments (17). The family *Halobacteriaceae* currently has 25 recognized genera and 101 recognized species. Recently, the family has been undergoing rapid expansion, with the description of 5 new genera and 20 new species since 2006 (as of June 2007). This expansion is not only due to the identification of novel taxa isolated from traditional hypersaline environments such as *Halovivax, Halostagnicola, Haloplanus* and *Haloquadratum* (4-6, 8), but also due to the recognition that members of the *Halobacteriales* can grow within saline microniches in non-saline environments at relatively low salt concentrations (10, 22). Several recent studies reported the isolation of novel halobacterial strains (11, 21) or the detection of novel *Halobacteriales*-affiliated 16S rRNA gene sequences from moderate to low-salinity systems (16, 24, 27). 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$^T$ that represents a novel genus within the order *Halobacteriales* from a sulfur and sulfide-rich spring in southwestern Oklahoma, USA.

The location and geochemical characteristics of the spring, as well as archaeal diversity and rationale for encountering halophilic *Archaea* in this seemingly low-salt environment have previously been described (9, 22). Sampling and isolation procedures have been described in detail (22). Briefly, samples were plated on halophilic medium (HMD) containing the following (g $l^{-1}$): $MgCl_2 \bullet 6H_2O$

(20), $K_2SO_4$ (5), $CaCl_2 \bullet 2H_2O$ (0.1), $NH_4Cl$ (0.5), $KH_2PO_4$ (0.05), carbon source (0.5), agar (20) pH was adjusted using NaOH to 7.0-7.2, (22), 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, and 300 g $l^{-1}$). Ampicillin and kanamycin (50 μg $ml^{-1}$) were added to select against halotolerant and halophilic *Bacteria*. The plates were incubated at 37°C under a 60-Watt light bulb placed approximately 30 cm above the plates until colonies appeared. We subsequently determined that cell growth and pigment formation were normal for strain BZ256 when grown in the absence of light. To ensure purity, single colonies of the isolate were re-streaked twice onto HMD plates.

Characterization of strain $BZ256^T$ was performed according to guidelines provided by Oren *et al.* (20). Detailed protocols for the methodology of biochemical tests were obtained from Gerhardt (12), and NaCl was added as necessary. Physiological tests were conducted using a HMD liquid or solid (2.0% agar) medium with sucrose (0.5 g $l^{-1}$) as the carbon source, 180 g $l^{-1}$ NaCl and 25 mM HEPES, unless otherwise stated. The minimum concentration of NaCl necessary to stabilize cells of strain $BZ256^T$ was tested using a method described by Savage *et al.* (22) the NaCl concentrations tested ranged from 0-7%. Growth rates for pH, $Mg^{2+}$, temperature and NaCl ranges were determined by monitoring an increase in $OD_{600}$. The maximum pH tested was 8.5 due to the formation of a precipitate at higher pH's that interfered with optical density readings. The ability of strain $BZ256^T$ to use

DMSO (5.0 g $l^{-1}$), 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 previously described procedures (2, 3). Sulfur was added as sublimed sulfur suspended in an aqueous solution (28). The sulfur tubes were amended 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 HMD un-buffered medium and was determined by measuring the initial and final pH of the medium. A decrease of one pH unit or greater was considered positive for acid production**.**

Antibiotic sensitivity was determined in liquid HMD medium with antibiotic concentrations at 35 μg $ml^{-1}$ except for aphidicolin (30 μg $ml^{-1}$). 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 cover-slip with glutaraldehyde. The cells were then coated with gold/palladium and examined in a JSM-880 Scanning Electron Microscope.

G+C mol% was determined using the services of the German National Resource Centre for Biological Material (DSMZ). Membrane lipids were analyzed using two-dimensional thin layer chromatography as described by Oren *et al*. (18). The 16S rRNA gene was amplified using primer pairs A1F (23) and UA1406R (1) as described earlier (22). The PCR product was then cloned using the TOPO-TA cloning kit (Invitrogen Corp., Carlsbad, CA). Ten clones were randomly picked and

sequenced at the Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, USA to determine whether strain BZ256 possessed multiple distinct 16S rRNA gene sequences. 16S rRNA gene sequences were aligned using CLUSTAL_X (25), and distance trees were constructed with PAUP 4.01b10 (Sinauer Associates, Sunderland, MA) using a "neighbor-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 forms pale pink colonies that are punctiform, flat circular and translucent. Colonies become larger and mucoid after prolonged incubation. Cells are non-motile cocci and develop as amorphous clumps of cells. Electron microscopy revealed that the cells divide along two axes resulting in the formation of sarcina-like clusters during the exponential phase of growth (Fig. 1). Wais (26) described a similar cellular morphology in an uncharacterized halophilic archaeon isolated in Jamaica. During late exponential and stationary phases cells are coccoid occurring singly or in pairs. The cells stain Gram-negative and the presence of gas vesicles was not evident through light microscopy.

Strain BZ256$^T$ grows at a wide range of salt concentrations from 1.7- 4.3 M, with an optimum at 3.1 M NaCl. Cells immediately lysed when suspended in distilled water. When suspended in sterile 4% NaCl solution strain BZ256$^T$ could be recovered after 24 hours. However, cells remained viable when suspended in HMD medium with 0% NaCl for at least 72 hours. At lower NaCl concentrations the number of viable cells did decrease over time. 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$^T$ grows in defined as well as complex media, sucrose, glucose and glycerol are the carbon sources that yield the best growth. It produces acid when growing on carbohydrates. No growth was detected when grown anaerobically under any of the tested conditions. Strain BZ256$^T$ produces indole from tryptophan, reduces nitrate aerobically, but does not produce amylase, caseinase, and lipase. BZ256$^T$ was found to have a single 16S rRNA gene sequence that was 96.8% similar to the closest relative, *Halogeometricum borinquense* (15) (Fig. 2). Strain BZ256$^T$ is however, 99% similar to recently reported isolates obtained from a traditional Japanese saltern (11), where salt concentrations seasonally oscillate between 3.0 g $l^{-1}$ and saturation. Since Zodletone spring, from which strain BZ256$^T$ was isolated, also experiences wide seasonal and spatial variation in NaCl concentrations, then it is plausible that members of this group are well adapted to survival in such environments.

Strain BZ256$^T$ contains the phospholipids phosphatidyl glycerol and phosphatidyl glycerol phosphate methyl ester but does not contain phosphatidyl glycerol sulfate. Analysis of the glycolipids using thin layer chromatography revealed that strain BZ256$^T$ contains two glycolipids chromatographically identical to S-DGD-1 and DGD-1 of *Haloferax* (Fig. 3). One minor glycolipid has a similar migration pattern to unidentified glycolipids in *Haladaptatus paucihalophilus* which was also isolated from Zodletone spring (Fig. 3). Whether this lipid is characteristic of halophilic *Archaea* encountered in low salt environments remains to be assessed.

This study provides evidence that strain BZ256$^T$ is a member of the extreme halophilic *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) suggest that strain BZ256$^T$ could not be accommodated as a novel species within the genus *Halogeometricum*, its most closely related genus based on 16S rRNA phylogeny. Therefore, we propose that this strain constitutes a novel species in a novel genus within the family *Halobacteriaceae*, for which we propose the name *Halosarcina pallida*. The type strain is Strain BZ256$^T$

**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)
Non-motile. Gram-negative cocci. Cells form sarcina-like clusters during the exponential growth phase. Oxidase and catalase positive. Chemoorganotrophic, growing on a wide range of substrates including single and complex carbon sources. Produces acid from carbohydrates. Aerobically reduces nitrate to nitrite without the formation of gas. Lysis in distilled water. Cells contain phosphatidylglycerol and phosphatidylglycerol phosphate methyl ester, but phosphatidyl glycerol sulfate is absent. Two glycolipids are present that are chromatographically identical to S-DGD-1 and DGD-1. The DNA G+C content of strain BZ256$^T$ is 65.4 mol%. The type species is *Halosarcina pallida*. Recommended three-letter abbreviation: *Hsn.*

**Description of *Halosarcina pallida* sp. nov.** *pallida* (pal'li.da L. fem. adj. pallida pale)

Cells are non-motile single cocci, staining Gram-negative. Colonies are small (0.2 mm), pink, translucent, round, convex with an entire margin. Grows from 1.7-4.3 M NaCl, with optimal growth at 3.1 M NaCl. Optimal temperature for growth is 30°C, with a range of 25-45°C. Growth requires a minimum of 1 mM $Mg^{2+}$. Grows at pH values ranging from 5 to at least 8.5, with an optimum of 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 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, esculin and salicin. Substrates not utilized include L-Arginine, L-alanine, sodium citrate, xylose, mannitol, L-threonine, dulcitol, dextrin, L-methionine, 3,3,-dimethylglutaric acid and L-tyrosine. Produces acid when grown on sucrose, glucose, starch, fructose, lactose, galactose, sorbitol and glycerol. Does not produce acid when grown on L-arginine, L-alanine, L-glutamic acid, DL-aspartic acid, glycine, acetate, lactate, malic acid, fumaric acid, citrate, succinate and pyruvate. Able to utilize complex carbon sources such as yeast extract and casamino acids. Catalase and oxidase positive. Indole is produced from tryptophan. Reduces nitrate to nitrite without the formation of gas under aerobic conditions. Does not

hydrolyze starch, casein, gelatin and Tween 80. Phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester are present. Phosphatidyl glycerol sulfate is absent. Two glycolipids are present, chromatographically indentical to S-DGD-1 and DGD-1. Sensitive to novobiocin, bacitracin, anisomycin, rifampacin and aphidicolin. Resistant to erythromycin, penicillin, ampicillin, chloramphenicol, neomycin, nalidixic acid, kanamycin, gentamycin and trimethoprim. The DNA G+C content of strain BZ256$^T$ is 65.4 mol%. The type strain is BZ256$^T$ (=KCTC 4017$^T$ = JCM 14848$^T$) and was isolated from Zodletone Spring in Southwestern Oklahoma, USA.

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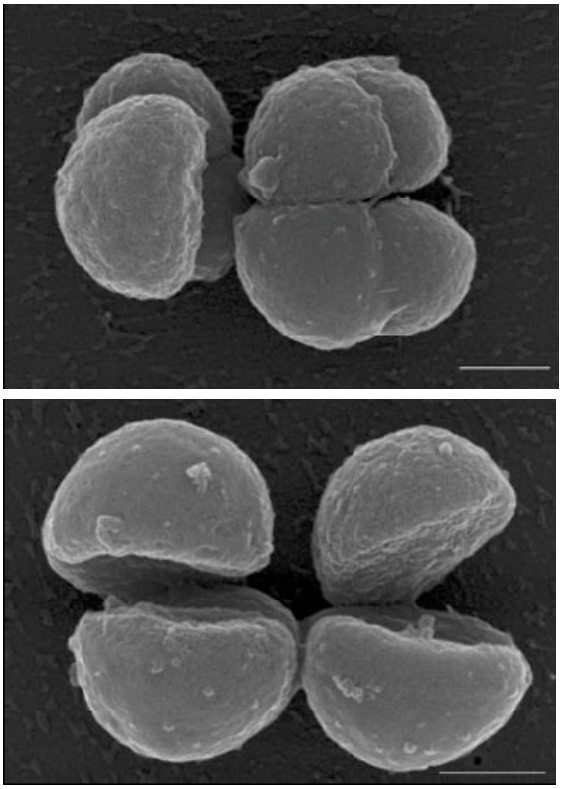

**Figure 1.** Scanning electron micrographs of strain BZ256$^{T}$ showing the unique division pattern of the isolate. Bar is equal to 500 nm.

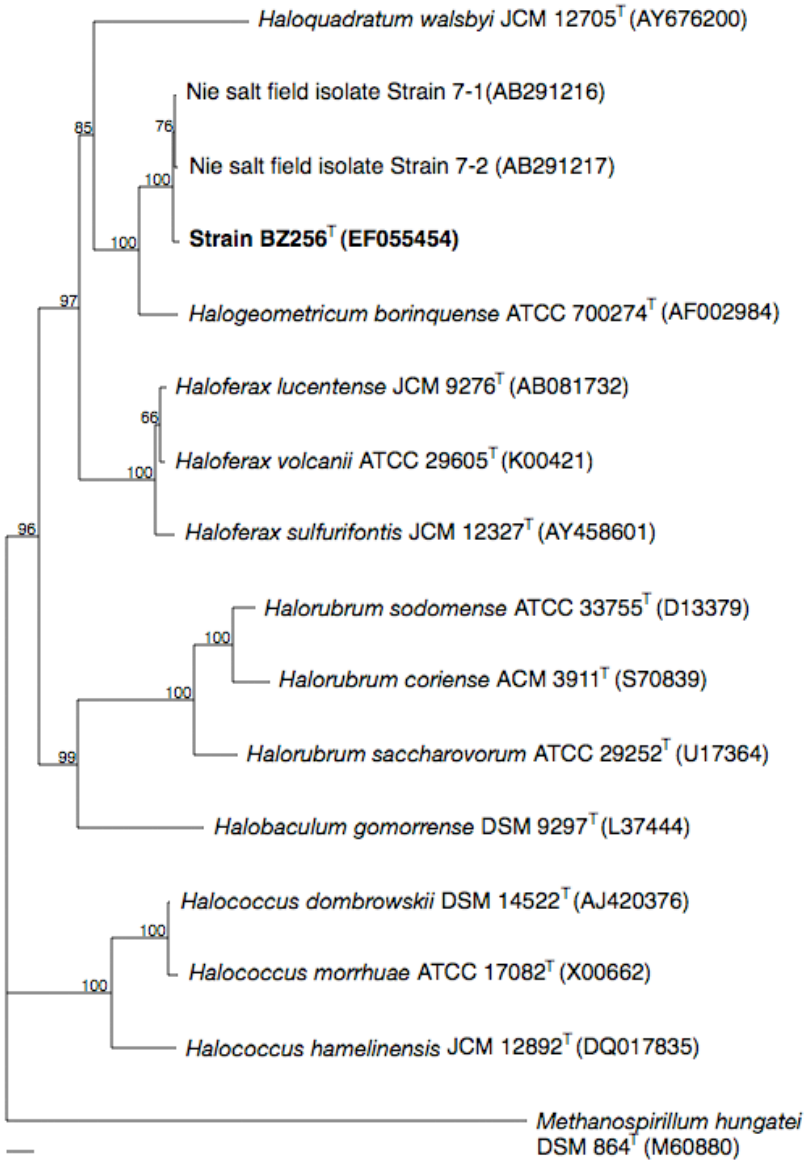


**Figure 2.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain BZ256$^T$and other close relatives. Sequences were retrieved from the GenBank database; accession numbers are given in parentheses. Bootstrap values (%) are based on 1000 replicates 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.

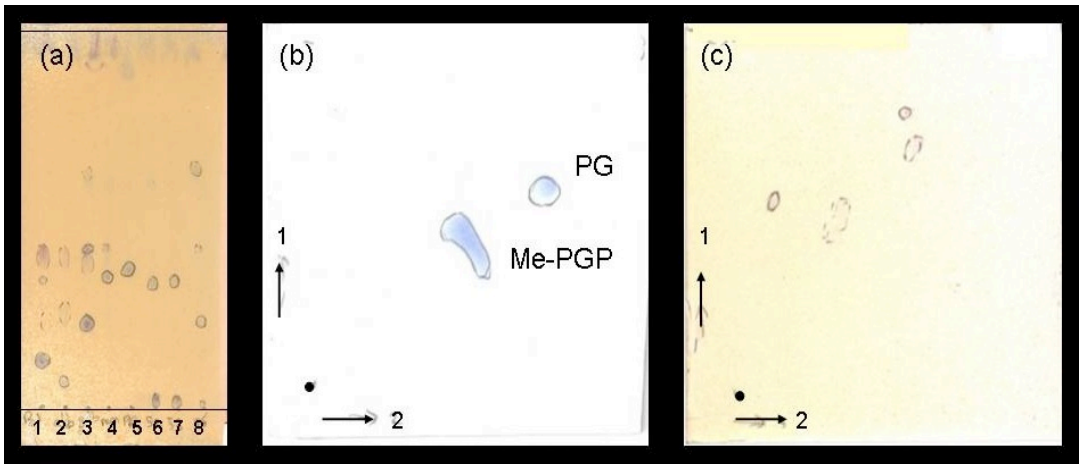


**Figure 3.** Analysis of the lipid composition of strain BZ256$^T$ using TLC. (a) One-dimensional TLC stained with orcinol reagent. Lanes: 1, Halobacterium salinarum R1; 2, Natrialba asiatica strain; 3, Haloferax gibbonsii strain; 4, Haloarcula marismortui strain; 5, Halogeometricum borinquense PR 3$^T$; 6 and 7, Haladaptatus paucihalophilus DX253$^T$; 8, strain BZ256$^T$. (b) Two-dimensional TLC stained for phospholipids, showing the presence of phosphatidylglycerol (PG) and phosphatidylglycerol phosphate methyl ester (Me-PGP) in strain BZ256T and the absence of phosphatidylglycerol sulfate. (c) Two-dimensional TLC stained with α-naphthol-sulfuric acid, showing the presence of two glycolipids in strain BZ256$^T$.

**Table 1.** Characteristics that distinguish strain BZ256$^T$ from other closely related genera within the order *Halobacteriales*. 1, Strain BZ256$^T$; 2, *Halogeometricum*; 3, *Halobaculum*; 4, *Haloquadratum*; 5, *Halorubrum*; 6, *Haloferax*; 7, *Halococcus*. Data derived from (4, 5, 7, 13-15, 19, 29). +, Positive; -, negative; +/-, results variable; NR, not reported. Phosphatidyl glycerol sulfate (PGS)

| Characteristic | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Cell Shape | Coccus | Pleomorphic | Rod | Squares | Pleomorphic/ Rods | Pleomorphic | Coccus |
| Gas Vesicles | - | + | - | + | +/- | +/- | NR |
| Cell Size (μm) | 0.8-1.0 | 1-3 x 1-2 | 5-10 x 0.5-1.0 | 2.0 x 2.0 | 1-12 x 0.5-1.2 | 0.4-3 x 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 |
| Temp 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 | + | + | + | + | + | + | - |
| PGS | - | - | - | - | +/- | - | - |
| G+C of DNA (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 | - | + | - | NR | +/- | +/- | +/- |
| Gelatin | | | | | | | |
| Pigmentation | Pale Pink | Pink | Orange-red | Pink | Orange-Red | Red/pink | Red |

# Chapter 4

# *Halenormitas recuperatonis* gen. nov., sp. nov., a halophilic archaeon from a low-salt, sulfide-rich spring


## Abstract

Strain GLYP1 a novel extreme halophilic archaeon was isolated from microbial mats present on the banks of Zodletone spring, a low-salt (0.2M), sulfide and sulfur-rich spring in southwestern Oklahoma. Cells of GLYP1$^T$ formed extremely small (1 mm) dark pink colonies on agar, stained gram negative and were non motile and pleomorphic. The range of NaCl concentrations that supported growth was 1.4- 5.1 M, the pH range for growth was 6.5-8.5, and the temperature range was 15-42°C. Cells remained viable in distilled water and could recover in high-salt halophilic medium even after one week. Phosphatidyl glycerol and phosphatidylglycerol phosphate methyl ester were detected, as well as phosphatidylglycerol sulfate. One glycolipid was detected and it spotted identically to S-DGD-1 from *Haloferax*. The 16S rRNA gene of strain GLYP1$^T$ had relatively low similarity (91.7%) to other characterized isolates within the *Halobacteriaceae,* but had much higher similarity (98.5 %) to uncharacterized isolates retrieved from salt-marsh sediments. The physiological, biochemical and phylogenetic differences between strain GLYP1$^T$ and other recognized genera within the *Halobacteriaceae* suggests that this strain

represents a novel species and genus within the family *Halobacteriaceae*, for which the name *Halenormitas recuperatonis* is proposed. The type strain is GLYP1$^{T}$.

Halophilic Archaea are characterized by their requirement for high salt and their ability to inhabit hypersaline environments where salt concentrations can range from 2.6 M to greater than 5.1 M (Grant *et al.*, 2001; Oren, 1999; Oren, 2000). Members of this family are the dominant heterotrophs inhabiting hypersaline environments e.g. The Dead Sea, the Great Salt Lake, solar salterns and alkaline soda lakes (Grant *et al.*, 2001; Oren, 2000). Surprisingly, however, recent molecular surveys of the archaeal communities in environments that have relatively low-salt concentrations have identified 16S rRNA gene sequences that belong to the *Halobacteriaceae*. This observation has subsequently been followed by isolation of novel members belonging to this family, typically in high salt, antibiotic supplemented media (Elshahed *et al.*, 2004b; Fukushima *et al.*, 2007; Purdy *et al.*, 2004; Savage *et al.*, 2007; Savage *et al*., 2008). Examples of such environments in which halophilic *Archaea* were detected using culture independent and/or culture dependant approaches include: a deep-sea hydrothermal vent (Takai *et al*., 2001), coastal salt marsh sediments in Essex, UK (Munson *et al.*, 1997; Purdy *et al.*, 2004), sea-water samples (Rodriguez-Valera *et al*., 1979), a Japanese style saltern (Fukushima *et al*., 2007) and, Zodletone spring, a low-salt sulfur rich spring in southwestern, Oklahoma, USA (Elshahed *et al*., 2004a; Elshahed *et al.*, 2004b; Savage *et al*., 2007; Savage *et al*., 2008). It has been proposed that evapotranspiration results in the concentration of salt that in turn provides a suitable environment for these extreme halophiles to survive. Characterization of isolates from these environments revealed potentially advantageous phenotypes that could aid in their survival in these low-salt

environments, notably the ability of the isolates to recover from prolonged exposure to low-salt or even salt free solutions. Several isolates can also grow at lower salt concentrations than previously identified haloarchaea (Elshahed et al., 2004b; Purdy et al., 2004; Savage et al., 2007). The survival mechanism of these extreme halophiles when salt concentrations are low (less than 0.5 M NaCl) has not yet been investigated.

This study reports on the isolation and characterization of strain GLYP1$^T$ from Zodletone spring: a low-salt (0.2M), sulfide and sulfur-rich spring, located in southwestern Oklahoma, USA. The location, geochemistry, and microbial diversity within the spring had been described in previous publications (Elshahed et al., 2004a; Elshahed et al., 2003; Senko *et al.*, 2004). Strain GLYP1$^T$ was identified during a mass isolation effort in which mat samples from the spring banks were diluted into a liquid high-salt plus antibiotic medium. The samples were then plated on the same medium with the addition of agar. The medium used to enrich for halophilic Archaea (HMD) was modified from (Oren *et al*., 1997) and was composed of the following (g $l^{-1}$) $MgCl_2 \bullet 6H_2O$ (20), $K_2SO_4$ (5), $CaCl_2 \bullet 2H_2O$ (0.1), yeast extract (0.1), $NH_4Cl$ (0.5), $KH_2PO_4$ (0.05), carbon substrate (0.5), agar (20) and NaCl (180, 250 or 300). The pH of the medium was adjusted using NaOH to approximately 7.0, ampicillin and kanamycin (50 μg $ml^{-1}$) were added to select against halotolerant bacteria. Twelve different carbon substrates were used (glucose, glycerol, tryptone, tryptose, peptone, nutrient broth, citrate, sodium benzoate,

cysteine, casamino acids, yeast extract or sodium glutamate). Strain GLYP1$^T$ was isolated from solid HMD medium supplemented with glycerol.

Characterization of strain GLYP1$^T$ followed the guidelines for characterization of novel taxa within the order *Halobacteriales* (Oren *et al*., 1997). Biochemical tests were performed according to Gerhardt *et al*., (1994), and NaCl was added to the media recipes as required. Gram staining was performed according to the modification of Dussault (Dussault, 1955). The physiological tests were conducted using liquid or solid (20.0 g $l^{-1}$ agar) HMD containing casamino acids (0.5 g $l^{-1}$) as the carbon source, 180 g $NaCl^{-1}$ and 25 mM HEPES, unless otherwise stated. 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 $OD_{600}$. Acid production was tested in unbuffered HMD and was determined by measuring the initial and final pH of the medium. The culture was considered positive for acid production if the pH decreased by more than one pH unit. The ability of strain GLYP1$^T$ to grow anaerobically using nitrate (30 mM), sulfate (30 mM) or sulfur (5.0 g $l^{-1}$) as well as the ability to ferment arginine (5.0 g $l^{-1}$) was tested in HMD prepared anaerobically in serum tubes according to strict anaerobic techniques described by Bryant (Bryant, 1972) and Balch & Wolfe (Balch and Wolfe, 1976). 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 range and optimal growth as a function of NaCl concentration, $MgCl_2$ concentration, pH and temperature was measured by measuring an increase in optical

density $OD_{600}$. Antibiotic sensitivity was determined by adding filter-sterilized antibiotics to liquid HMD. Antibiotic concentration for aphidicolin was 30 μg $ml^{-1}$ for all other antibiotics the concentrations were 35 μg $ml^{-1}$.

Recovery of cells from low-salt concentrations was tested as previously described Savage *et al.* (2007). Briefly, cells were innoculated into HMD with decreased salt concentrations (0, 0.2 and 0.3 M NaCl) and saline solutions with the same low-salt concentrations (0, 0.2 and 0.3 M NaCl). Cells grown under standard conditions were washed in equivalent low-salt solutions and then inoculated into the low-salt medium or saline solutions. Recovery was determined by removing samples at various time points and inoculating into standard HMD liquid medium and diluting to extinction, and the results were compared to recovery of cultures from solutions were salt concentrations were kept at 3.1M NaCl. Cells were recovered at 0, 24, 48 and 162 hours.

Primers used for amplification of the 16S rRNA gene were forward primer A1F (5'-ATTCCGGTTGATCCTGC-3') (Tajima *et al*., 2001) and reverse primer UA1406R (5'-ACGGGCGGTGWAA-3') (Baker et. al., 2003). The 16S rRNA genes of both strains were sequenced at the Oklahoma Medical Research Foundation (Oklahoma City, OK, USA). Phylogenetic trees were constructed by aligning sequences using CLUSTAL_X (Thompson *et al*., 1997) and distance trees were constructed using PAUP 4.01b10 (Sinauer Associates), using the neighbor-joining algorithm with Jukes-Cantor corrections. G+C content was determined using the Deutsche Sammlung von Mikroorganismen and Zellkulturen (Braunschweig, Germany)

commercially available service. Phospholipid and glycolipid analysis was done using two-dimensional thin layer chromatography (Oren *et al.*, 1996).

Cells of strain GLYP1$^T$ stained gram negative, were non-motile and pleomorphic (Fig. 1). Strain GLYP1$^T$ formed small colonies (approximately 1 mm in diameter) that were rough, irregular, opaque, raised with an entire margin.

Physiological characterization of strain GLYP1$^T$ revealed that optimal growth occurred between 3.1-3.4 M NaCl, at 30°C and a pH of 7.5. Ranges for growth of strain GLYP1$^T$ were 1.4-5.1 M NaCl, a temperature range of 15-42°C, a pH of 6.5-9.0. Cells did not lyse in distilled water, and maintained viability at low-salt concentrations (0, 0.2 and 0.3M NaCl) with approximately 10% of the cells recovering from distilled water after one week. The ability of this isolate to maintain viability at such low salt concentrations could prove advantageous in low-salt environments where precipitation may dilute hypersaline niches that develop due to evaporation.

Details of physiological and biochemical characteristics of strain GLYP1$^T$ can be found in Table 1 and in the species description. Strain GLYP1$^T$ is capable of growth in complex medium as well as on single carbon sources. Strain GLYP1$^T$ had a doubling time of approximately 13 hours at 3.1 M NaCl growing on glycerol ($OD_{600}$=0.700). However, when strain GLYP1$^T$ was grown under the same conditions on solid media growth was poor with slight growth appearing in the first quadrant only after several days. Acid was produced when grown on carbohydrates.

Nitrate was reduced aerobically to nitrite. No growth was detected under anaerobic conditions with sulfur, nitrate or sulfate. Strain GLYP1$^T$ did not ferment arginine. 16S rRNA gene sequence analysis indicated that *Haloferax volcanii* is the most closely related species within the *Halobacteriaceae* with 91.7% similarity to strain GLYP1$^T$ (Fig. 2). Strain GLYP1$^T$ however, had a much higher similarity (98.5% similarity) to other uncharacterized isolates that originated from a salt marsh (Purdy *et al*., 2004) (Fig.2).

Strain GLYP1$^T$ contained the phospholipids phophatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and phosphatidylglycerol sulfate (PGS), which is not present in the genus *Haloferax* (Grant *et al*., 2001). Thin layer chromatography was also used to analyze the glycolipid composition that revealed the presence of one glycolipid chromatographically identical to S-DGD-1 (Fig. 3).

Strain GLYP1$^T$ represents an addition to the list of halophilic archaeal isolates that originated from low-salt or environments were salinity fluctuates. It has the ability to recover from suspension in low-salt solutions like several other extreme halophiles that were isolated from Zodletone spring. Strain GLYP1$^T$, however possesses unique physiological, biochemical, and phylogenetic characteristics that differentiates it from these other Zodletone isolates, as well as all other genera within the *Halobacteriaceae.* We propose the name *Halenormitas recuperatonis* gen. nov., sp. nov. to accommodate strain GLYP1$^T$.

## Description of *Halenormitas* gen. nov.

*Halenormitas* (Hal.a.enormitas Gr. n. *hals* salt; L. part. adj. *enormitas* irregular shape; a salt-requiring with irregular shape)

Gram-negative pleomorphic cells occurring singly. Colonies have a dark pink pigment. Cells contain PG, PGP-Me and PGS. The glycolipid is S-DGD-1. Chemo-organotrophic, growing on a wide range of substrates, including single and complex carbon sources. Produces acid from carbohydrates. Little to no growth in the presence of casein and Tween 80. No hydrolysis of starch. Gelatin was hydrolyzed. Requires at least 1.4 M NaCl to grow, but can remain viable in distilled water for up to one week. Sensitive to rifampacin, novobiocin, aphidicolin, bacitracin and anisomycin. Resistant to naladixic acid, neomycin, kanamycin, erythromycin, gentamicin, penicillin, chloramphenicol and ampicillin. The type species is *Halenormitas recuperatonis*. Recommended three-letter abbreviation: *Hrt*.

## Description of *Halenormitas recuperatonis* sp. nov.

*Halenormitas recuperatonis* (*recuperatonis* L. recovery)

Exhibits the following properties in conjunction with properties given in the genus description. The doubling time is approximately thirteen hours. Non-motile. Colonies are small approximately 1 mm in diameter, opaque, rough, irregular, raised with an entire margin. Grows in NaCl at 1.4-5.1 M; optimal growth at 3.1-3.4 M NaCl. Optimal temperature for growth is 30-37°C; temperature range for growth

was 15-42°C. At least 0.1 g $l^{-1}$ $MgCl_2 \bullet 6H_2O$ required for growth. Optimal pH is 7.0-7.5; pH range for growth 6.5-8.5. Does not grow anaerobically with $NO_3^-$, $SO_4^{2-}$, or elemental sulfur. Does not ferment arginine. Capable of using both single carbon substrates and complex carbon substrates. Utilizes peptone, tryptone, tryptose, yeast extract, glutamic acid, alanine, pyruvic acid, glycerol, mannitol, fructose, sucrose, xylose, maltose and dextrose. Does not utilize aspartic acid, arginine, glycine, lysine, acetate, citrate, fumarate, lactate, succinic acid, sorbitol, ribose, mannose, lactose and starch. Indole is produced from tryptophan. Reduces nitrate to nitrite under aerobic conditions, no gas production.

The DNA G+C content of the type strain is 64.1 mol%. The type strain, GLYP1[1] was isolated from microbial mats in Zodletone spring in south-western Oklahoma, USA.

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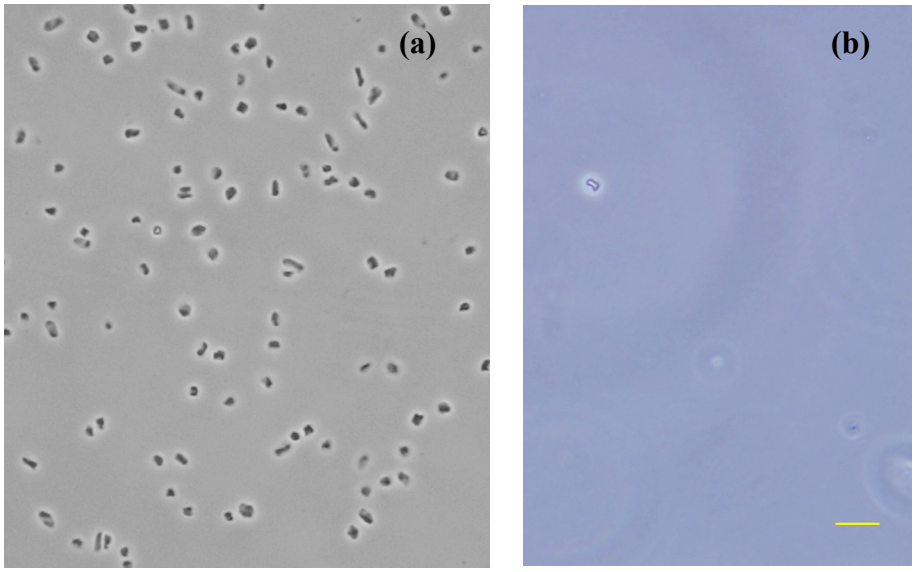


**Figure 1.** Light microscopy photomicrograph showing the pleomorphic nature of cells of strain GLYP1$^{T}$. Bar, 10 μm (b)

**Table 1.** Characteristics of strain GLYP1$^T$ compared to other closely related genera within the order *Halobacteriales*. 1, Strain GLYP1$^T$; 2, *Haloferax*; 3, *Halogeometricum*; 4, *Haloplanus*, 5, *Halobaculum*; and 6, *Haladaptatus.* Data collected from (Elevi Badavid *et al.*, 2007; Grant *et al.*, 2001; Oren *et al.*, 1995; Montalvo-Rodriguez *et al*., 1998; Savage *et al.*, 2007 and Xu *et al*., 2007). +, positive; -, negative; v, results variable.

| Character-istic | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Cell shape | Pleo-morphic | Pleo-morphic | Short and long rods, squares, triangles | Pleo-morphic | Rod | Coccus |
| NaCl range | 1.4-5.1 | 1.0-5.2 | 1.4 | 2.6-4.3 | 1.0* | 0.8-5.1 |
| NaCl optimum | 3.1-3.4 | 1.7-4.3 | 3.4-4.3 | 3.0 | 1.5-2.5 | 2.6-3.1 |
| Nitrite from Nitrate | + | v | + | + | + | - |
| Starch Hydrolysis | - | v | - | - | + | + |
| DNA G+C | 64.1 | 59.1-64.5 | 59.1 | 66.1-66.4 | 70.0 | 60.5 |
| Phospha-tidyl-glycerol sulfate | + | - | - | + | - | + |
| Polar Lipids | S-DGD-1 | DGD S-DGD | Un-identified | S-DGD-1 | S-DGD-1 | Un-identified |

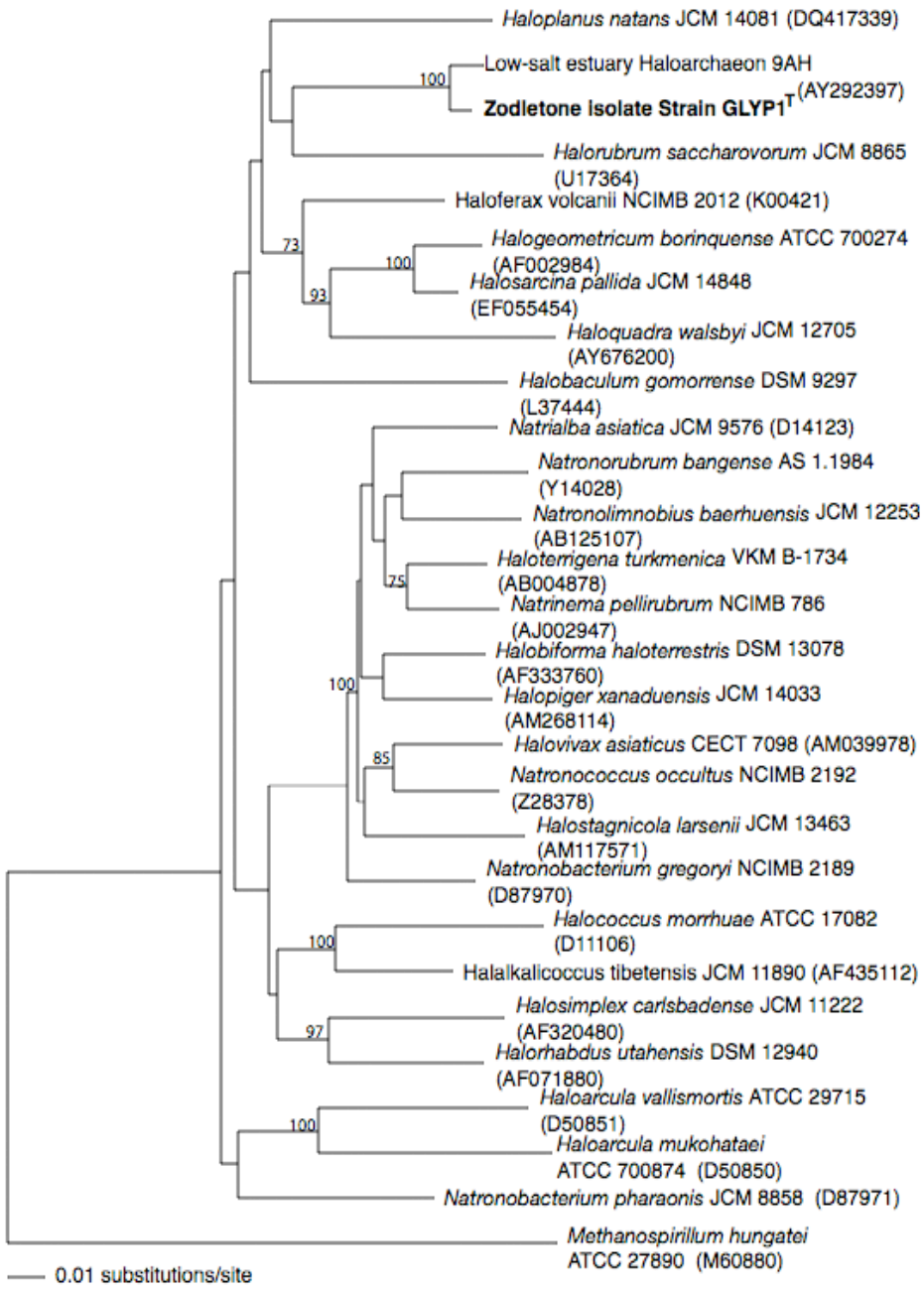


**Figure 2.** Distance dendogram showing the relationship between strain GLYP1$^{T}$ and the close relatives within the family *Halobacteriaceae*. Sequences were retrieved from GenBank and the accession numbers are indicated by parentheses. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 50% bootstrap support.

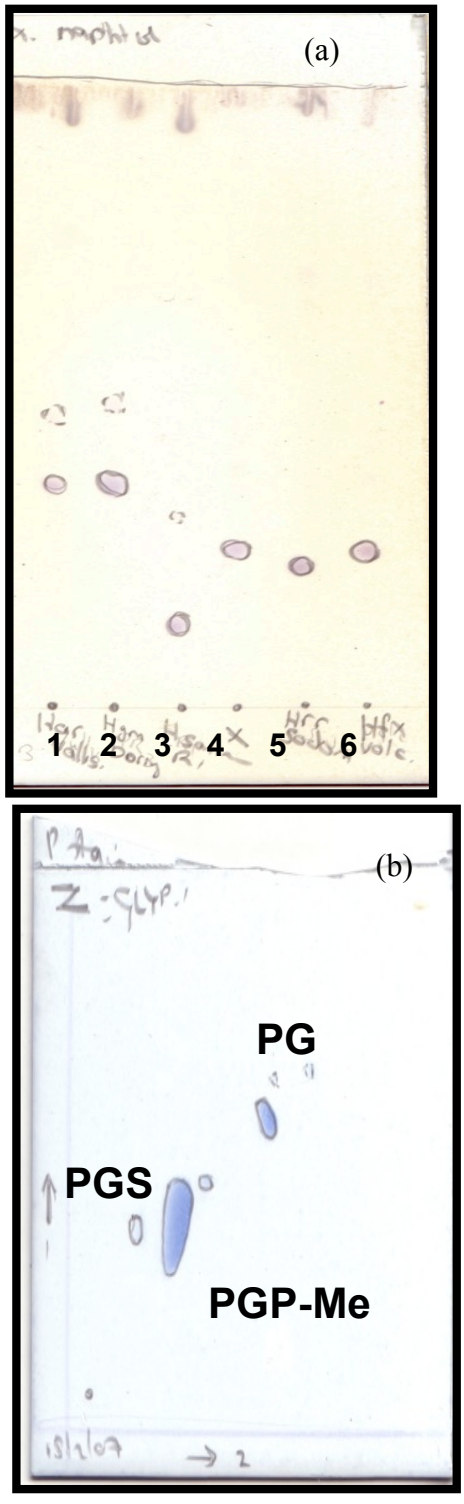


**Figure 3.** Analysis of the membrane lipids of strain GLYP1$^T$ by TLC. (a) One-dimensional plate comparing the glycolipid pattern of strain GLYP1$^T$ with those of other members of the family *Hlaobacteriaceae*. 1, *Haloarcula vallismortis*; 2, *Halogeometricum borinquense*; 3, *Halobacterium salinarum* strain R1; 4, Strain GLYP1$^T$; 5, *Halorubrum sodomense*; and 6, *Haloferax volcanii*. The glycolipid pattern of strain GLYP1$^T$ matches *Haloferax volcanii*. (b) Two-dimensional plate displaying the phospholipids pattern of strain GLYP1$^T$ confirming the presence of phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and phosphatidylglycerol sulfate (PGS).

# CHAPTER 5

## Pass The Salt: Halophilic Archaea found in Low-Salt Environments


## Abstract

Several culture dependent and independent studies have revealed the presence of extreme halophilic Archaea belonging to the order *Halobacteriales* in relatively low-salt environments. These halophilic Archaea appear to be abundant, diverse and novel in these environments. Furthermore, several of these organisms have been detected in multiple low-salt environments but have not been detected in hypersaline environments. This review reflects on the similarities of the organisms found in each of these environments and the characteristics they posses that facilitate their survival in seemingly sub-optimal conditions.


### Introduction

Halophilic and halotolerant organisms are found in all three domains of life. These organisms have been found to exist in environments with a wide range of salinities from freshwater systems to seawater to environments were salt is saturating (21, 22, 24). The extreme halophilic Archaea, belonging to the order *Halobacteriales*, consists exclusively of halophiles and are among the most halophilic organisms known. These organisms are known to be the dominant hetertrophs in hypersaline environments. Extreme halophilic Archaea are defined by their absolute requirement for salt, requiring 1.5 M NaCl to grow, growing optimally

between 3.5-4.5 M NaCl and often growing well under salt saturation (8, 11, 26, 28). In fact, interest in this group began early in the twentieth century because of their ability to contaminate fish and animal hides that had been salted to preserve them (28).

Thalassohaline environments are developed from the evaporation of seawater and athalassohaline environments develop from the evaporation of freshwater; both are hypersaline but differ in their ionic composition and both are found to host many halophilic Archaea (26). The Dead Sea, has been the source of several characterized isolates belonging to the halophilic Archaea (2). It has been determined that these organisms may be present in numbers as high as $1x10^7$ cells per ml in this particular environment (31). The Dead Sea has an average total dissolved salt content of 23.7%, chlorine is the dominant anion, and magnesium and sodium are the dominant cations. The temperature varies with location from approximately 21°C to 36°C and the pH is slightly acidic (Table 1) (20).

The Great Salt Lake has also been the source of several haloarchaeal isolates. The north arm of this lake is approximately 33% salt while the southern arm is slightly less saline at 12% salt. The Great Salt Lake is a much cooler environment than the Dead Sea with temperatures ranging from -5°C to 35°C. The dominant cation is sodium and the dominant anion is chloride, magnesium is much less abundant than it is at the Dead Sea. The Great Salt Lake is slightly more alkaline with the pH (Table 1) (32). Other environments that host a variety of halophilic

archaea include alkaline soda lakes s (i.e. Lake Magadii, China), solar salterns and salted animal hides (26, 28).

Halophilic Archaea do not possess peptidoglycan, and therefore are resistant to many bacterial specific antibiotics (3). The extreme halophilic Archaea do possess ether-linked lipids, typical of this domain. All halophilic Archaea posses the polar lipids phosphatidyl glycerol (PG) and phosphatidyl glycerolphosphate methyl-ester (PGP-Me). The presence of phosphatidylglycerol sulfate (PGS) is variable, but its presence or absence can be characteristic of a particular genera (12, 28). One of the striking features of the *Halobacteriales* is the unique cell morphologies, the typical bacterial shapes such as rods and cocci are present but triangles, squares and pleomorphic cell morphologies are also encountered (8, 40, 44). Noncoccoid shaped cells generally lyse in distilled water, due to their proteinaceous cell walls. The cocci shaped cells are generally more stable under low-salt conditions (8, 28, 44).

Halophilic Archaea can easily be identified by the presence of a characteristic red, pink or orange pigment that is a result of $C_{50}$ carotenoid pigments that are believed to aid in protecting the cell against sun damage (4). They also possess the retinal pigments bacteriorhodopsin, an outward light-driven proton pump (38) and halorhodopsin and inward light-driven chloride pump (13).

Considering that the extreme halophilic Archaea are defined by dependence on high salt concentrations for survival and the ability to dominate hypersaline environments, it was surprising to find representatives belonging to the order *Halobacteriales* in several different culture independent analyses of relatively low-

salt environments. There is now evidence, both through isolation and through clone library studies, that members of this family exist in many different types of environments, which are different from the Great Salt Lake and the Dead Sea (Table 1) (5-7, 17, 33, 35-37, 39, 45). The subject of this review is to report on the findings of these studies, and to reflect on both the phylogenetic similarities and the unique characteristics of the halophilic Archaea found in these environments.

## Studies in Low-Salt Environments

The first account of an extreme halophilic archaeon isolated from a low-salt environment was in 1979 (35). Strain F1-4 was obtained from seawater samples (0.6 M salt). Although originally identified as a bacterium the presence of a red pigment, the lack of peptidoglycan in the cell wall and the resistance to penicillin suggests that this was a member of the extreme halophilic Archaea. Optimal growth of strain F1-4 occurred at 4.3 M salt (35). This isolate was never identified phylogenetically, but was described as an extremely halophilic cocci that could survive, but not grow in low salt concentrations. It was suggested that this organism originated in Spanish salterns and was merely surviving in the seawater from which it was isolated (35).

More recently, the extensive use of culture independent analysis in various environments exposed the existence of these “extreme” halophilic Archaea in many different low-salt environments. Numerous studies not only detected the presence of haloarchaea, but also revealed that this group was abundant, diverse and novel. Some of these novel groups of halophilic archaea were detected in multiple low-salt

environments (Table 2) but have not been detected in or isolated from traditional hypersaline environments suggesting that these isolates may have adapted to survive under low-salt conditions.

A community study of Archaea in sediment samples from a coastal salt marsh in Essex, UK was one of the first studies to detect haloarchaea in a relatively low-salt environment (17). Salinity, at the site, ranged from 0.6-0.8 M. The pH ranged from 6.0-7.5. Of 133 archaeal clones analyzed, 23 of them were found to belong to the extreme halophilic Archaea, or 17.3%. These clones clustered into three groups (*Natronobacterium*-related, *Haloferax*-related and a novel group that was distantly related to the genus *Halobacterium*). It is interesting to note that *Haloferax* is a genus that is characterized by the ability to grow at lower salt concentrations than other halophilic Archaea (26). A follow-up study attempted to isolate members of the extreme halophilic Archaea from this environment (33). An analysis of 100 colonies using ERIC genomic fingerprinting also revealed three different groups within the haloarchaea. The study referred to these groups as HA group 1, HA group 2 and HA group 3. These three groups were not all representative of the groups found in the previous study. Cultured representatives have often been found to be different from microscopic identifications and fatty acid analysis in past studies (26).

HA group 1 was unique in that all of the isolates within this group contained two distinct 16S genes that were approximately 95% similar to each other, and they were 89% similar to sequences recovered from the first study at the site (Fig. 1). The

presence of multiple divergent 16S genes has been documented in other halophilic Archaea (8, 18, 43). This group grew at 2.5% NaCl (0.4 M), albeit slowly, on both solid and in liquid media this is the lowest documented salt concentration to support growth of an extremely halophilic Archaea. HA group 1 grew optimally at 1.7 M NaCl. HA group 2 was 94% similar to *Haloferax* and *Halogeometricum*. HA group 2 also grew at low salt concentrations, 3.5% NaCl (0.6 M) and grew optimally at 3.4 M NaCl. The third group (HA group 3) was 98% similar to environmental clone sequences from the same site (Fig. X) and 96% similar to *Haloferax*. This report demonstrated that several of these isolates could grow at lower salinities than previously identified halophilic Archaea. Regardless of their ability to grow at lower salt concentrations, they suggest that evaporation could lead to transient areas of high salinity that allows these halophilic Archaea to survive and grow in this environment (33).

A study that looked at the distribution of Archaea in a deep-sea hydrothermal vent discovered that 96% of the T-RFLP ribotypes from inside the root of the chimney belonged to the order *Halobacteriales* (39). All of these samples clustered within the previously defined genera *Haloarcula* (Fig. 1). The majority of members within this genus posses two disparate 16S rRNA genes (8, 18), and this could explain why the sequences formed two different clusters within this genus. In the same study they attempted to enumerate the halophilic Archaea through enrichment and MPN calculations, however, they were not successful in culturing any representative extreme halophilic Archaea (39).

The studies conducted at Zodletone spring, a sulfide-rich spring located in southwestern Oklahoma, USA, originally identified the presence of extreme halophilic Archaea through a culture independent analysis of the spring microbial mats and at the source of the spring (5). The salinity at this spring is approximately 0.2 M. It is a shallow anaerobic spring where sulfide concentrations range from 8-10 mM. The temperature is 22°C and the pH is 7.7 (Table 1) (46). Results from the clone libraries revealed that 36% of the mat archaeal community and 4% of the source community belonged to the order *Halobacteriales.* Phylogenetic analysis revealed the presence of five different groups (designated Groups I to V). The majority of clones fell within Groups I and II, these two groups were not affiliated with any previously described genera. Groups III and V were related to members of the genera *Halogeometricum* and *Natronomonas*. Group IV formed a branch related to the genus *Halococcus*, this group was composed exclusively of clones from the source of the spring (5). A more in depth salinity measurement was taken at the spring. This revealed the loss of moisture and the concentration of salt in the uppermost layers of sediment on the bank of the stream. It is assumed that evapotranspiration allows the concentration of salt resulting in micro-environments for the growth of these extreme halophilic Archaea, however precipitation events would cause rapid and likely severe fluctuations in salinity (5). From this same site three novel halophilic Archaea were successfully isolated and characterized (*Haloferax sulfurifontis* (6), *Haladaptatus paucihalophilus* (36) and *Halosarcina pallida* (37)). *Haloferax sulfurifonits* could anaerobically reduce sulfur, suggesting a

possible role in sulfur cycling at the spring and also exhibited growth at a wide range of salt concentrations (1.0-5.2 M). Cells did lyse in distilled water (6). *Haladaptatus* and *Halosarcina* were both established as novel genera with their only described species to date being isolates from this low-salt system. *Haladaptatus* was characterized by growth at relatively low salt concentrations (0.8 M) and the presence of two disparate 16S rRNA genes, this is in agreement with closely related isolates from the salt marsh (Fig. 1), having both two distinct 16S rRNA gene sequences and growth at low-salt concentrations, although growth at lower salt concentrations than 0.8 M were not observed for the Zodletone isolate. Perhaps the most striking characteristic of this isolate its ability to recover from salt-free solutions even after two weeks (36). *Halosarcina pallida* did not grow in salt concentrations as low as the other isolates from the spring but also displayed great resiliency when exposed to distilled water, recovering even after one week in salt-free solutions (37). *Halosarcina pallida* was also 95% similar to one of the clones retrieved in the initial survey of the system (Fig. 1). The ability to withstand exposure to sub-optimal salinity conditions could be advantageous in these relatively low-salt environments where hypersaline conditions depend on evaporation and precipitation could cause severe fluctuations in the salinity, allowing the haloarchaea to remain dormant until optimal salinity conditions were reestablished.

The last study was an attempt to determine if halophilic Archaea were really thriving in any natural Japanese environments (7). They isolated halophilic Archaea from a salt-field that is operated seasonally. Salt is produced in the summer when

seawater is spread over wet sand, and evaporation leads to concentration of salt. However, when the field is not used for salt production in the winter, the field is often covered in snow affecting the salinity of the site (41). Ten strains of halophilic Archaea were isolated from this site and their tolerance to low salt concentrations was tested. The ten strains fell into three groups *Haladaptatus*, *Halococcus* and *Halogeometricum* (Fig. 1). The *Haladaptatus* strains were found to be extremely tolerant to low salt concentrations remaining viable in a 0.5% Seawater medium (SW) after nine days, similar to the characterized isolate (36). However the *Halogeometricum* related strains (which also showed high similarity to *Halosarcina*) died instantly upon suspension in 3% and 0.5% SW. The *Halococcus* related strains survived five days at 3% SW but only survived one day at 0.5% SW (7). This study did not report any information on the salt range that supported growth for these particular strains.

These are not environments that would be considered ideal ecosystems for the proliferation of extreme halophilic Archaea, a comparison of the water chemistry of Zodletone Spring to traditional hypersaline environments (Table 1) shows that the water chemistries are very different. Likewise it seems that the halophilic Archaea that have been isolated from these environments possess unique characteristics that separate them from traditional extreme halophilic Archaea. It was suggested for each of these environments that evaporation lead to the concentration of salts that provide optimal environments for extreme halophilic Archaea. How do these isolates survive in environments during precipitation events? To answer this question we must look

at the unique characteristics of these isolates. Specifically the ability to tolerate and recover from low-salt conditions after extended periods of time. The ability to grow at low-salt concentrations is an obvious advantage. It is also interesting to note that several of the isolates possessed multiple divergent 16S rRNA genes which may or may not provide some sort of adaptive advantage (36).

## Discussion

As previously mentioned, the domain Bacteria also contains halophilic and halotolerant organisms, however, these two groups inhabit different environments with little overlap between the two (10, 25, 42). A competition experiment using a continuous culture system revealed that under high salt conditions extreme halophilic Archaea dominated halophilic Bacteria (42). The difference in the niches occupied by halophilic Bacteria and halophilic Archaea is undoubtedly due to the differences in the mechanisms used to adapt to osmotic pressure.

Halophilic Archaea accumulate molar amounts of potassium ions inside the cell to counter high salt concentrations inside the cell (21). The intracellular proteins and enzymes of these organisms are subject to extremely high concentrations of salt. Normally high salt concentrations have a devastating affect on proteins causing them to denature (21, 23, 29). Halophilic Archaea have specialized proteins, that not only can tolerate high salt conditions, but actually require the presence of high salt to function (14). The adaptation of these enzymes was identified as the accumulation of a relatively high number of acidic amino acids. Proteins of halophilic Archaea have

been compared to similar proteins from non-halophilic organisms and been found to be more acidic (14). Ultimately the possession of these acidic proteins, while allowing halophilic Archaea to adapt to high salt conditions, also confines them to these same environments.

An alternative method of osmoadaptation used by the majority of halophilic Bacteria is the accumulation low molecular weight organic compounds collectively called compatible solutes (21). These compatible solutes are accumulated inside the cell, either they are transported across the membrane from the surrounding environment or they synthesized inside *de novo*. Compatible solutes protect proteins from dehydration and effectively counter high external salt concentrations (21, 34). The benefit of this particular osmoadaptive method is that it, unlike the accumulation of potassium ions, does not increase the ionic concentration of the intracellular environment. The consequence of this is the energetic expense associated with either the uptake or generation of these compatible solutes. This mode of osmoadapatation allows organisms to tolerate lower-salt conditions, and fluctuating salt concentrations because proteins can function in the presence of compatible solutes without having any special adaptations (21). The difference in these methods of osmotic adaptation is reflected in the different salt ranges that support growth of these organisms (Fig. 2).

There is a discrepancy between the halophilic Archaea that are recovered from typical hypersaline environments such as the Dead Sea, and the Great Salt Lake and those recovered from these environments where “hypersaline” conditions are

seemingly transitory. If these isolates, from low-salt systems, are indeed subjected to fluctuating salt conditions it seems likely that they would use a different osmoadaptive mechanism than other extreme halophilic Archaea, a method that would allow them to deal with fluctuating salt conditions. Perhaps these halophilic Archaea use a method of osmoadaptation that is more closely related to methods used by halophilic Bacteria or a combination of these methods that allows them to thrive at high salt concentrations and at the same time tolerate fluctuating salt conditions. *Salinibacter ruber* is an example of a halophilic bacterium that is restricted to high-salt concentrations, and it was shown that this organism accumulates potassium under high salt conditions resulting in a high-salt requirement for growth (Fig. 2) (30). Employing a mechanism such as the uptake or generation of osmotic solutes would be more energy intensive making them unable to compete with the extreme halophilic Archaea in hypersaline environments but allow them to survive the fluctuating salt concentrations of their respective environments.

Certainly to answer these questions much more work needs to be done, and an exhaustive search for their presence in hypersaline environments would also be beneficial. If these halophilic Archaea accumulate potassium ions, generate compatible solutes or utilize an entirely different method, yet undiscovered, is certainly of interest for future study. Undoubtedly the discovery of halophilic Archaea in low-salt environments, and their subsequent isolation and characterization has changed the definition of the extreme halophilic Archaea. Halophilic Archaea are now known to grow in salt concentrations as low as 0.4 M

(33), some are extremely tolerant to low-salt conditions (7, 33, 36, 37) and perhaps most importantly these organisms are not restricted to hypersaline systems. Much insight into this particular group of organisms can be gained through their continued isolation and characterization. Probing for these organisms in other environments, quantification and spatial identification in their natural environment and sequencing the genome of unique isolates would also provide insight into their roles in these environments.

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**Table 1.** A comparison of the water chemistry between environments from which extreme halophilic Archaea have been recovered. Data collected from the following sources (20, 32, 46).

| Total Dissolved Salts | Environment | | | |
|---|---|---|---|---|
| | Zodletone Spring | Great Salt Lake | | Dead Sea |
| | | North Arm | South Arm | |
| **Mg ($g\ l^{-1}$)** | 0.14 | 11.1 | 4.2 | 36.2 |
| **Cl ($g\ l^{-1}$)** | 8.88 | 181.0 | 65.3 | 196.9 |
| **Na ($g\ l^{-1}$)** | 2.55 | 35.9 | 105.4 | 38.5 |
| **pH** | 7.7 | 7.7 | 8.2 | 6.3 |
| **Temperature (°C)** | 22 | -5 to 35 | | 21 to 36 |

**Table 2.** The distribution of extreme halophilic Archaea uncultured clones and isolates in low-salt environments. -, not detected.

| Location | Clone/ isolate | *Halococcus* | *Haloferax* | *Hal-adaptatus* | *Halosarcina* | *Haloarcula* | *Halosarcina* | Reference |
|---|---|---|---|---|---|---|---|---|
| **Salt Marsh Essex, UK** | Clone | - | 2MT310, 2MT103, 2C82 | - | - | - | - | (17) |
| | Isolate | - | 8AHG | 30AH | - | - | 10AH | (33) |
| **Low-salt, sulfur spring Oklahoma, USA** | Clone | ZAR 47 ZAR 48 ZAR 49 | - | - | ZAR25 | - | - | (5) |
| | Isolate | - | Strain M6 | Strain DX253 | Strain BZ256 | - | Strain GLYP1 | (6, 36, 37) |
| **Black Smoker Chimney** | Clone | - | - | - | - | pPACMAK pPACMAS pPACMAU pPACMAT | - | (39) |
| **Salt field Nie, Japan** | Isolate | Nie 14-1 | - | Nie 11-1 Nie 14-2 | Nie 7-2 | - | - | (7) |

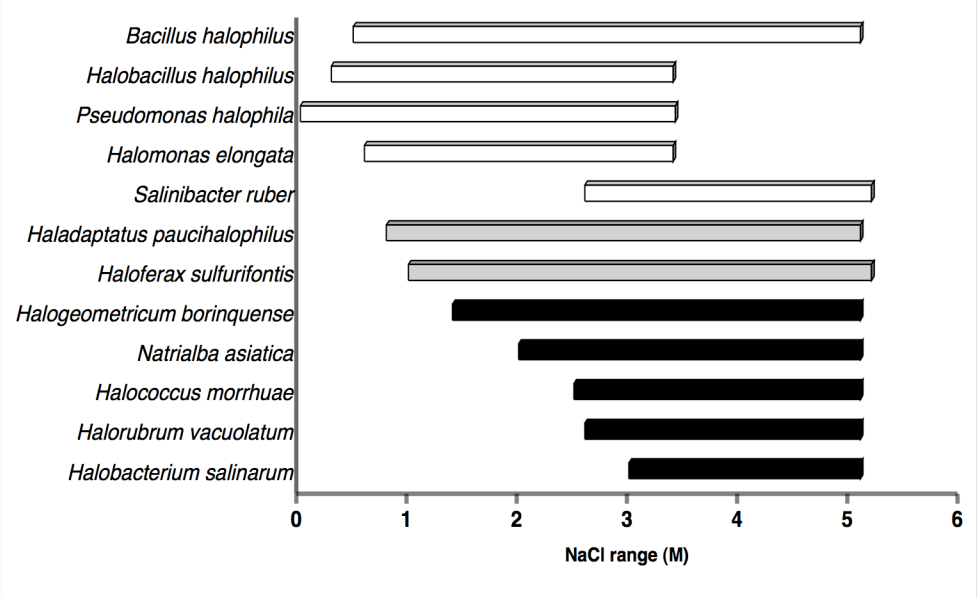


**Figure 1**. Relative salt ranges of halophilic bacteria versus halophilic Archaea Halophilic Bacteria (white-shaded bars); Halophilic Archaea (Black-shaded bars); Halophilic Archaea isolated from Zodletone spring (Grey-shaded bars). Data collected from (1, 6, 9, 15, 16, 19, 27, 36, 42)

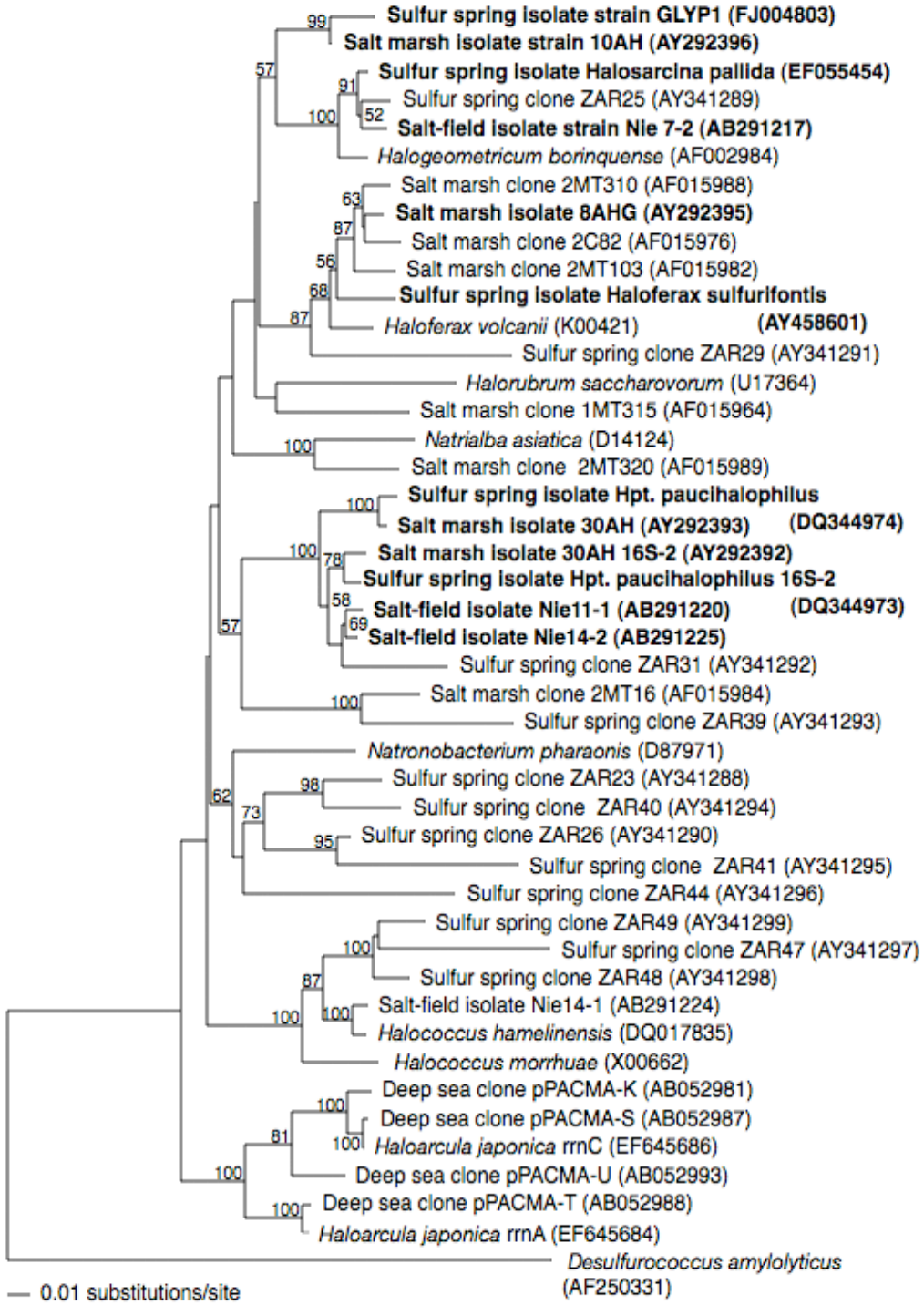


**Figure 2.** Uncultured archaeal clones and archaeal isolates (in bold) from low-salt environments.

# Appendix A: Evidence of Biodegradation of Low-Molecular Weight Alkanes Under Sulfate Reducing Conditions


## Abstract

Zodletone spring in Southwestern Oklahoma is a mesophilic spring characterized by high dissolved sulfide and sulfur concentrations through its course, and high concentrations of short chain gaseous alkanes (methane, ethane, and propane), especially at the source of the spring. 16S rRNA gene based analysis of the spring source sediments suggested hydrocarbons influenced the microbial community. To investigate the ability of the native microbiota to metabolize the abundant short chain hydrocarbons ($C_2$-$C_6$), enrichments were set up using source sediments and spring groundwater under sulfate-reducing conditions. Substrate loss and sulfate-reduction were monitored periodically and propane and pentane were observed to be degraded to non-detectable levels. These enrichments were then subcultured and the majority of propane 77.6 (+/- 20.1)% and pentane 60.3 (+/- 19.4)% were degraded after 485 and 207 days of incubation, respectively. Substrate loss in the subcultures was coupled to a loss of sulfate in stiochiometric excess in active enrichments. Carbon isotope analysis of residual propane of active enrichment showed that propane became enriched in $^{13}C$ by 6.7 (±2.0) per mil after 660 days of incubation. Propylsuccinic acid, a fumarate addition product and likely metabolic intermediate in alkane degradation, was detected in active propane degrading enrichments and not in unamended controls. Additionally alkyl succinate synthase was detected (Amy Callaghan personal communication). This work provides substantive evidence that

short chain alkane degradation is occurring in enrichments and that the microbial community at Zodletone spring is capable of metabolizing some short, straight chain alkanes under sulfate-reducing conditions.

## Introduction

Due to a lack of functional groups, alkanes (a major constituent of fossil fuels) suffer from low-chemical reactivity. However, the formation and existence of these compounds over long geologic periods of times has allowed microorganisms to evolve mechanisms to functionalize these hydrocarbons and thereby exploit them as a carbon and energy source (36-38).

Since the early twentieth century the degradation of hydrocarbons under aerobic conditions has been known to occur via activation by mono and dioxygenases, in which oxygen is used in the initial enzymatic attack on the hydrocarbon (6, 8, 16, 24). Evidence of anaerobic hydrocarbon degradation was reported as early as the mid-twentieth century, but the results from these experiments were not authenticated and the cultures were not preserved (25, 30). It was not until 1991 that an alkane degrading culture was successfully isolated, and shown to grow on hexadecane as the sole carbon and energy source while reducing sulfate (1). Since this pioneering study a range of hydrocarbon substrates have been identified to be degraded under denitrifying (13, 29), sulfate reducing (2, 9, 11, 12, 22, 31, 35) and methanogenic (4, 41) conditions in both hydrocarbon degrading enrichments and pure cultures.

Two pathways for anaerobic alkane degradation have been described. The first pathway is the addition of fumarate to the parent alkane at the double bond, to produce an alkylsuccinate (10-12, 22, 28, 39). Strain Hxd3, which produces C-odd fatty acids when grown on C-even alkanes and C-even fatty acids when grown on C-odd alkanes, appeared to have a different method of carbon activation (34). The method used by strain Hxd3 is thought to be carboxylation (10, 34).

Early work on anaerobic hydrocarbon degradation found that n-hexane was the lowest molecular weight hydrocarbon degraded (18). However, $C_1$-$C_5$ hydrocarbons also exist in nature and less is known about the ability of these compounds to be degraded under anaerobic conditions. It was not until 2007, that pure cultures were identified that were able to use ethane, propane and butane as substrates under sulfate-reducing conditions. Fumarate addition was identified as the pathway used (21).

The source of Zodletone spring, an anaerobic sulfur and sulfide-rich spring located in southwestern Oklahoma, USA, is contaminated by the short chain gaseous alkanes methane, ethane and propane. Culture independent analysis of the source community revealed that many sequences were affiliated with uncultured organisms from hydrocarbon contaminated environments and hydrocarbon degrading enrichments (14). This suggested that the presence of these low molecular weight hydrocarbons were impacting the microbial community. This study presents evidence that the resident source microflora were capable of degrading low-molecular weight hydrocarbons under sulfate-reducing conditions. Phylogenetic

analysis of the community shows that these enrichments are dominated by organisms related to known hydrocarbon degraders, or similar to clones from other hydrocarbon degrading enrichments.

## Materials and Methods

**Site Description.** Zodletone Spring is located in southwestern Oklahoma, USA. The spring flows for 20 m at a rate of approximately 8 l/min, where it then empties into nearby Saddle Mountain Creek (40)**.** The spring is characterized by high concentrations of dissolved sulfide (8-10 mM at the source) and sulfur. The high sulfide concentrations maintain anaerobic conditions throughout the spring. The source of the spring is an area measuring 1 $m^2$ by 50 cm in depth and the bottom 15 cm is a layer of biomass and soft sediment. The source water is fed from underneath the Anadarko basin and remains approximately 22°C. Gas is bubbling up from the source and was identified to be a mixture of methane, ethane and propane. A detailed site description can be found (14, 17, 32, 33).

**Enrichment of anaerobic hydrocarbon degrading organisms.**

Enrichment cultures were developed under anaerobic conditions using fresh source sediment as the incoculum and groundwater from the spring. The groundwater was buffered with sodium bicarbonate (0.04 M) and amended with vitamins, minerals and sulfate (26). Bottles were sealed with teflon stoppers, the headspace was flushed with a 80:20 $N_2/CO_2$ mixture and then bottles were amended with either 1μl pentane

or 4 ml of propane gas. Enrichments were set-up in 40 ml serum bottles and the bottles were incubated in the dark at room temperature.

Substrate loss was monitored using a Shimadzu GC-14A with a Chemipak C-18 column using a flame ionization detector (125°C). The oven temperature was at 30°C and 100°C to measure propane and pentane respectively, the injector was kept at 100°C. Sulfate loss was monitored using Dionex DX500 ion chromatography system (Dionex Corp. Sunnyvale, CA) with an AS4A column.

After more than 80% of the substrate was lost, enrichments were transferred into a mineral medium containing. Transfers were made by taking 1.0 ml of liquid from the active enrichment and injecting it into a serum tube containing 9.0 ml of a mineral medium (26) prepared anaerobically (5, 7) containing the following (ml or g $l^{-1}$) $NH_4Cl$ (0.27), $KH_2PO_4$ (0.03), NaCl (11.7), $MgCl_2 \bullet 6H_2O$ (1.22), $CaCl_2 \bullet 2H_2O$ (0.44), vitamin solution (6.25), mineral solution (6.25) 0.1% resazurin (0.5). The bottles were then amended with ferrous sulfate (final concentration 0.015 M) and the same hydrocarbon substrate. All bottles and tubes were monitored for sulfate and substrate loss. Attempts to isolate the organism(s) responsible was attempted after at least one transfer if hydrocarbon degrading activity was maintained. This was done using a dilution to extinction method where the transferred enrichment was diluted from $10^{-1}$ to $10^{-5}$ in triplicate. These dilutions later served as the source of DNA for a community survey of active enrichments.

**Isotope Fractionation.** A method used to differentiate the biotic versus abiotic mineralization of a compound is stable isotope fractionation. Compounds that are

biodegraded become enriched in the heavy carbon isotope $C^{13}$, this is reflected in a change in the ratio of $C^{13}/C^{12}$ which can be quantified (3, 23). The $\delta C^{13}$ value were determined for propane and then compared to the $\delta C^{13}$ value of residual propane in propane degrading enrichments, as well as the propane from sterile controls and non-propane degrading enrichments. Isotope analysis was done at Sarkey's Energy Center (University of Oklahoma) in Dr. Paul Philp's laboratory. Isotope ratios were determined using gas chromatography and isotope ratio mass spectrometry (GC-IRMS) (Finnigan MAT 252 IRMS).

**Metabolite Analysis.** Several transferred cultures that had lost greater than 80% of the substrate added were used for metabolite analysis. Cultures were acidified to a pH less than 2 by adding HCl. Ethyl acetate (15 ml) was added to the acidified samples, the sample was shaken and the layers were allowed to separate in a separatory funnel. The bottom aqueous layer was collected in the original tube and the top layer, containing ethyl acetate, was passed through a filter paper lined funnel containing anhydrous sodium sulfate (changed after each sample), and this step was repeated two more times. The ethyl acetate layer was concentrated using rotary evaporation to a volume of approximately 1-2 ml. This concentrated layer was then transferred into a small glass vial. The sample was further concentrated by placing under a stream of nitrogen and then derivitized by adding 50 μl of *N, O*-bis(trimethylsilyl)trifluoroacetimide (BSTFA).

The derivatized sample was analyzed on a Agilent HP 6890 gas chromatograph and an Agilent 5973 mass spectrometer with a J&W DB5-MS

column. Peaks identified as propylsuccinic acid based on the mass spectral profile were confirmed by running a known standard of *n*-propylsuccinic acid.

**Community Analysis**. DNA was extracted from active diluted transferred enrichments, propane ($10^{-2}$) and pentane ($10^{-4}$) using the MoBio Power Soil DNA Isolation Kit. 1.5 ml of the liquid culture was used for the analysis and the provided directions were followed. Extracted DNA was kept frozen at -20°C until use. Bacterial specific primers (Invitrogen Corp., Carlesbad, CA) 8F 5'-AGAGTTTGATCCTGGCTCAG-3' and 805R 5'-GACTACCAGGGTATCTAATCC-3' were used for PCR amplification. Amplified products were then cloned into *Escherichia coli* using a TOPO-TA cloning kit (Invitrogen). Clones were picked and digested using endonuclease MspI (available from New England Biolabs). Digested products were run on a 2% agarose gel. Representative clones for each pattern were selected and sent to the Oklahoma Medical Research Foundation (Oklahoma City, Oklahoma) for sequencing. Neighbor joining trees were constructed by aligning sequences using Clustal X and submitting aligned sequences to PAUP for neighbor joining tree construction (Jukes Cantor corrected). Phylogenentic affiliations were obtained using the available online Ribosomal Database (available online http://rdp.cme.msu.edu/).

## Results and Discussion

To determine if the native microflora at Zodletone were indeed capable of metabolizing low molecular weight hydrocarbons, enrichments were started using source sediment and groundwater that was amended with one of five low molecular weight hydrocarbons ($C_2$-$C_6$). This was repeated under several different electron accepting conditions (sulfate-reducing, sulfur-reducing, phototrophic and methanogenic).

Under sulfate reducing conditions a loss of 95% (0.167 ± .007 mmoles) of propane in the headspace was measured after 600 days, compared to a loss of 0.043 (± .020) mmoles of propane in the sterile-control (Fig 1). Loss in sterile control can likely be attributed to leaking during sampling. If propane loss is corrected for loss in the sterile controls (0.124 mmols propane) theoretical sulfate loss is 0.31 mmoles sulfate. Sulfate loss corrected for background sulfate reduction measured 0.230 (±0.081) mmoles sulfate, slightly less than the expected amount.

To further enrich for organisms capable of degrading pentane and propane and to better correlate propane loss to sulfate reduction. Active propane degrading cultures were transferred (1:10) to a mineral medium containing sulfate and amended with propane after 670 days of incubation. After 280 days of incubation the transferred cultures degraded 80% (0.031 ±0.05 mmoles per 10 ml sample) of the propane. Sulfate loss was in excess (120%) of the theoretical amount expected to account for the propane lost. Because activity was maintained in the transferred

enrichment at 270 days the enrichments were transferred again (1:10) and diluted out to $10^{-5}$ to attempt to isolate the hydrocarbon degrading organisms.

Enrichments amended with pentane showed a complete loss of pentane (6.49 ±0.90 μmoles) under sulfate reducing conditions after 450 days, compared to the sterile control that only lost 1.15 ±1.31 μmoles of the substrate (Fig. 1)**.** Pentane degrading cultures were subcultured after 440 days of incubation. The subcultured enrichments lost approximately 80% (3.89 ±1.25 μmoles) of the substrate after 390 days of incubation with 133.57 (±28.02) μmoles of sulfate lost, which is in stoichiometric excess. These cultures were then transferred a second time (1:10) into the same mineral medium used for the propane degrading enrichments. Isolation of the hydrocarbon degrading organism(s) was attempted using a dilution to extinction method.

In order to show that propane was being biodegraded, and loss was not due to some abiotic factor, the change in the $\delta C^{13}$ value for propane before and after incubation was determined. It has been shown that microorganisms will preferentially use the light carbon isotope, because less energy is required to break bonds with the lighter carbon isotope (23). Ultimately this results the parent substrate becoming heavier as it is being biodegraded. Active propane degrading enrichments showed that residual propane was indeed enriched in the heavy carbon isotope with a $\delta C^{13}$= -22.75‰ (± 0.92) at the start of the incubation and a $\delta C^{13}$= -16.03‰ (± 2.00) after the incubation. (Table 1). Furthermore the change in the isotopic ratio of the $CO_2$ of the active propane degrading enrichment showed that the carbon dioxide was

enriched in $C^{12}$ by approximately the same amount that propane was depleted in $C^{12}$ (Table 1) providing evidence that the propane was being mineralized to carbon dioxide. This is in accordance with results from Kniemeyer *et al.* which also showed that propane and butane became heavier in active hydrocarbon degrading enrichments (21).

Fumarate addition is a known mechanism of anaerobic hydrocarbon degradation (10-12, 22, 28, 39). Propane and pentane degrading enrichments that had lost a significant amount of substrate (>80%) were sacrificed to look for key metabolites in the fumarate addition pathway. The characteristic (M-15) ion for the propane derived alkylsuccinate would be ion 289 (15). Two peaks were present, in propane degrading enrichments, at ion 289 they were identified as *n*-propylsuccinic acid and isopropylsuccinic acid (Fig. 2) These peaks were not detected in unamended enrichments (data not shown). The *n*-propylsuccinate peak was confirmed by running an *n*-propylsuccinate standard (15) and comparing the m/z fragmentation pattern. A standard was not available for isopropylsuccinic acid, but the mass spectra was similar to that of the *n*-propylsuccinic acid. Although neither peak was quantified the relative abundance suggests that the sub-terminal addition was more prevalent. These results are in complete agreement with those found by Kniemeyer *et al.* (21) who suggested that there were two routes for propane activation, with activation at the secondary carbon requiring more energy, therefore is less abundant. The analysis of metabolites from propane degrading enrichments support the findings of Kniemeyer *et al.* (21) that the mechanism for pentane degradation

involves fumarate addition to both the primary and secondary carbons of the propane molecule. In addition to the presence of the metabolite the detection of an alkyl succinate synthase-like gene in propane degrading enrichment cultures (Amy Callaghan, personal communication) provides additional evidence of the biodegradation of propane and that the mechanism likely involves a fumarate addition product as an intermediate. Metabolites reflective of known pathways of anaerobic hydrocarbon degradation were not identified in pentane degrading enrichments (data not shown) this could be due to the metabolites being present at undetectable levels.

The community composition of the most highly enriched propane and pentane degrading enrichments was analyzed. The majority of clones (95.8 and 82.1%) for the propane and pentane degrading cultures respectively fell within three major phyla *Delta-Proteobacteria*, *Firmicutes* and *Chloroflexi*. *Delta-Proteobacteria* dominated both clone libraries comprising 50.1 and 69.2% of the propane and pentane degrading enrichments respectively.

The most abundant group of clones in the propane degrading enrichment were closely related to unaffiliated bacterial clones from an oil-polluted retention basin (27) they fall within the family *Desulfobacteraceae* (Table 2). Other numerically abundant groups included OTUs C3-C12 and C3-E1 which are related to uncultured bacterial clones from a 4-Methylbenzoate-degrading consortium and *Chloroflexi* clones from benzene contaminated groundwater respectively (Fig. 4). Almost a quarter of the propane enrichment clone library clustered with the gram

positive *Peptococcaceae* (Fig. 4) this family contains organisms capable of syntrophic growth (19, 20). The presence of potential syntrophic organisms indicates the possibility that degradation of these low molecular weight alkanes could possibly be a cooperative effort.

The pentane degrading enrichment was dominated by clones within the family *Desulfobacteraceae* as well, however, they were closely related (95% similarity) to the isolate Hxd3, *Desulfococcus oleovorans* (Fig. 3). Strain Hxd3 was one of the first isolates shown to grow on alkanes under anaerobic conditions, and can grow on alkanes $C_{12}$-$C_{20}$ (1).

It is not surprising that under sulfate reducing conditions that both libraries were dominated by the *Delta-Proteobacteria* however, the most abundant OTU's in the propane and pentane enrichments clustered within different genera (Table 2) this indicates that the same organism is most likely not responsible for degrading both propane and pentane. The diversity within the *Delta-Proteobacteria* varied between each enrichment suggesting that the alkane substrates selected for two different sulfate-reducing communities (Table 2 and Fig. 3,4). Propane degrading enrichments had an abundance of clones that were related to the phyla *Firmicutes* and *Chloroflexi.* The pentane degrading enrichments had a large population that did not cluster closely with any known bacterial divisions. The only difference in these two enrichments was the alkane substrate either propane or pentane, only a two carbon chain length difference. This could be a downstream affect of the dominant organisms in each enrichment and the community structure could depend solely on

the organism responsible for the hydrocarbon degradation. It is also interesting to note that 25% of the clones from the propane degrading enrichment were in the same clade as strain BuS5, a propane degrading isolate (21), but shared only a 90% similarity to this isolate. If this OTU is responsible for the biodegradation of propane the *Desulfobacteraceae* may contain a diverse group capable of degrading low-molecular weight hydrocarbons in various ecosystems. Likewise, the majority of clones from the pentane degrading enrichment were related to the hydrocarbon degrading *Desulfococcus* (1) suggesting that this genus might use a broader range of hydrocarbons to support growth than previously believed.

This study provides strong evidence that the resident community at Zodletone not only shaped by the presence of hydrocarbons at the source of the spring, but these organisms are capable of metabolizing these low-molecular hydrocarbons. Unlike this study, however, a different group of organisms is responsible for the degradation of both propane and pentane. Further work needs to be done to confirm the mechanism of pentane degradation, and to positively identify the organism(s) responsible for the degradation of both propane and pentane.

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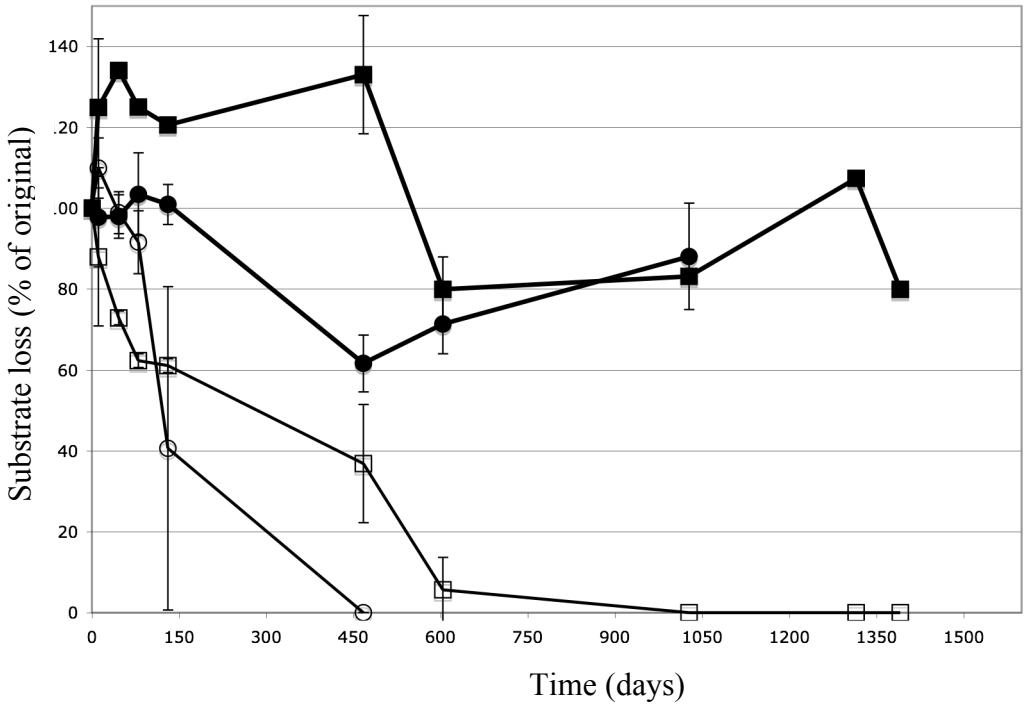


**Figure 1.** Propane loss in sterile (filled squares) compared to live (open squares) enrichments and pentane loss in sterile (filled circles) compared to live (open circles). Values are presented as percentage lost of original substrate. Original concentration of propane= 0.167 (± .007) mmoles, pentane= 6.49 ((± 0.91) μmoles.

**Table 1.** $\delta C^{13}$ values for propane compared to the original propane used to amend the enrichments. Conditions include incubation in a sterile control, a live enrichment in which no propane loss was detected and an active enrichment in which propane loss was detected. Values for $\delta C^{13}$ of $CO_2$ are compared to the sterile control where $CO_2$ is from exchanging the headspace.

| | $\delta C^{13}$ (‰) | | | |
|---|---|---|---|---|
| **Enrichment Conditions** | **$C_3H_8$** | **$\delta C^{13}$ (‰)** | **$CO_2$** | **$\delta C^{13}$ (‰)** |
| **$C_3H_8$** | -22.75 (±0.92) | - | - | - |
| **Sterile Control** | -23.28 (±0.54) | -0.53 | -10.58 (±0.22) | - |
| **Live non-propane degrading enrichment** | -22.35 (±0.35) | +0.40 | -13.65 (±0.21) | -3.07 |
| **Propane degrading enrichment** | -16.03 (±2.00) | +6.72 | -17.23 (±0.15) | -6.65 |

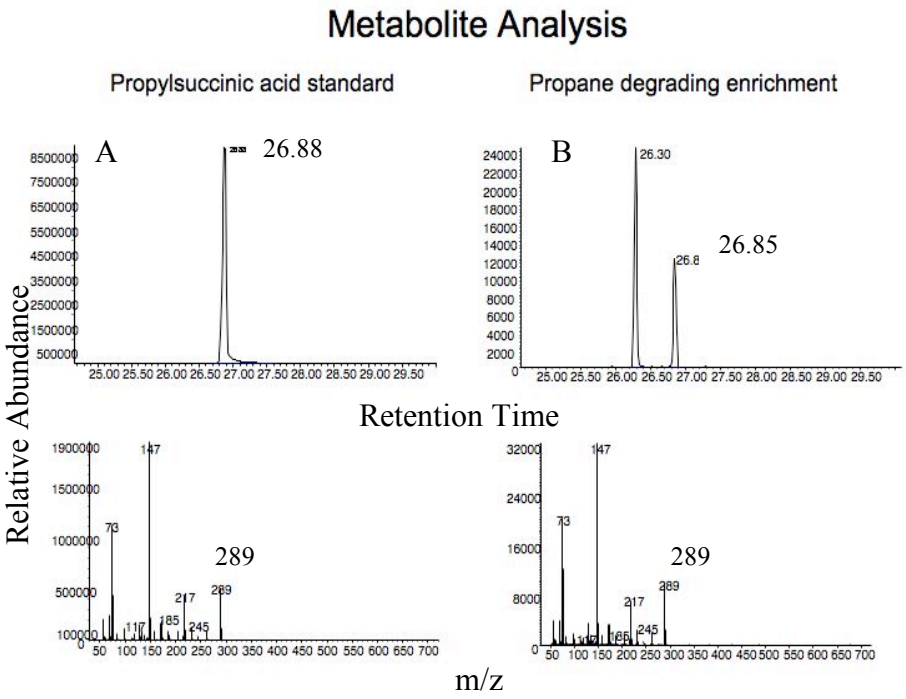


**Figure 2.** Mass spectral profiles from analysis of propane metabolites and a n-propylsuccinic acid standard, results for ion 289 and m/z signals. (A) Injection of an n-propylsuccinic acid standard. (B) Injection of metabolites from active propane degrading enrichment. The less abundant peak matches the retention time and ion chromatogram of the *n*-propylsuccinic acid standard. The second peak is believed to be isopropylsuccinic acid.

**Table 2.** Comparison of the communtiiy composition of propane and pentane amended enrichments. DNA was extracted from active transferred enrichments propane ($10^{-2}$) and pentane ($10^{-4}$).

| | **Hydrocarbon degrading enrichment community (% of total clones)** | |
|---|---|---|
| | **Propane** | **Pentane** |
| ***δ- Proteobacteria*** | **50.1** | **69.2** |
| *Desulfobacteraceae* | 27.1 | 43.7 |
| *Geobacteraceae* | 4.2 | |
| *Desulfarculaceae* | 18.8 | |
| *Syntrophaceae* | | 5.1 |
| *Desulfovibrionaceae* | | 15.3 |
| *Desulfuromonaceae* | | 5.1 |
| ***Firmicutes*** | **26.9** | **7.7** |
| *Peptococcaceae* | 26.9 | 7.7 |
| ***Chloroflexi*** | **18.8** | **5.2** |
| *Caldilineaceae* | 18.8 | 5.2 |
| **Other** | **4.2** | **17.9** |
| C5_A12 | | 10.2 |
| C5_C4 | | 5.1 |
| C5_B9, C3_C1 | 2.1 | 2.6 |
| C3_B1 | 2.1 | |

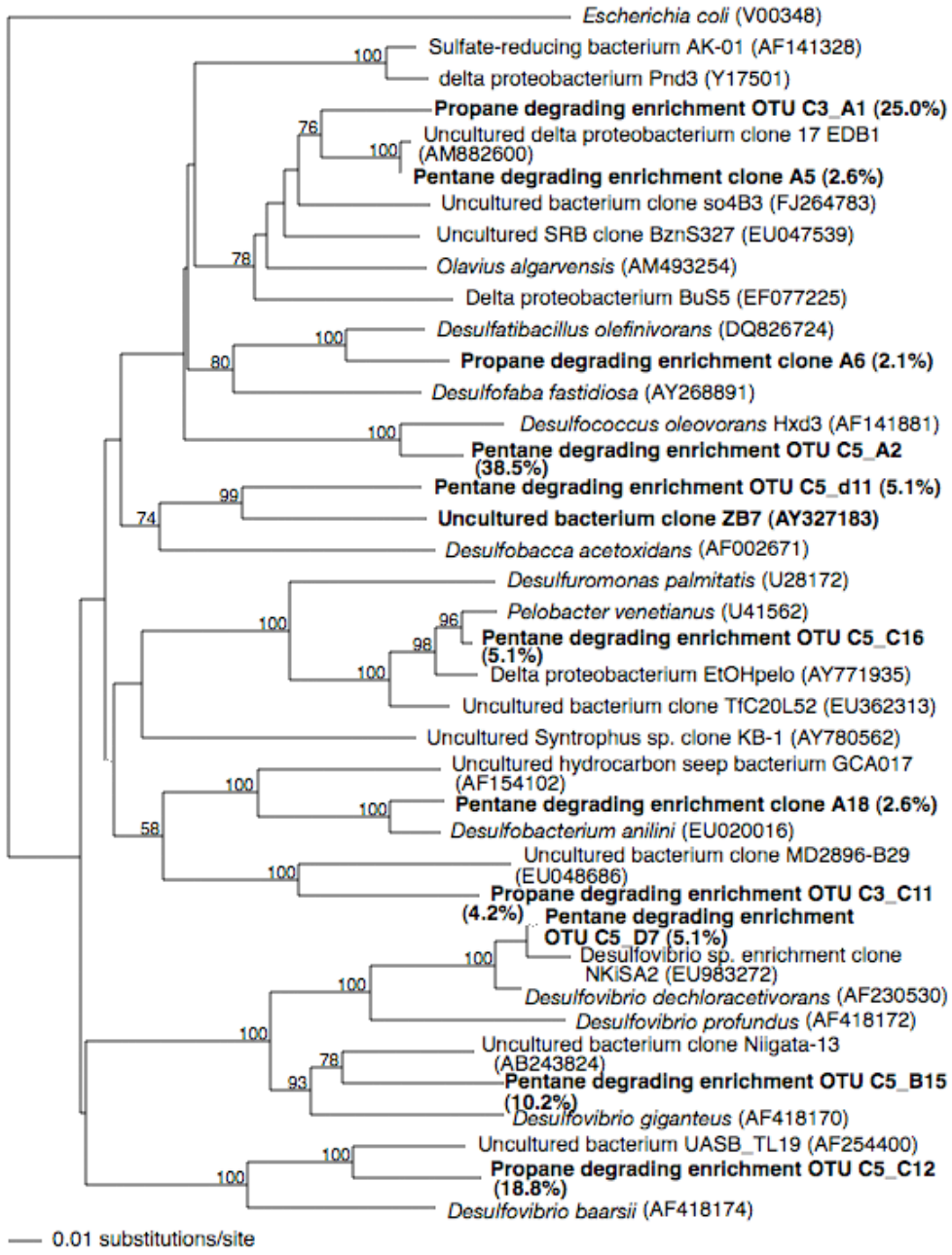


**Figure 3.** Neighbor-joining tree of enrichment clones (bold) from propane and pentane degrading enrichments belonging to the *Delta-Proteobacteria*

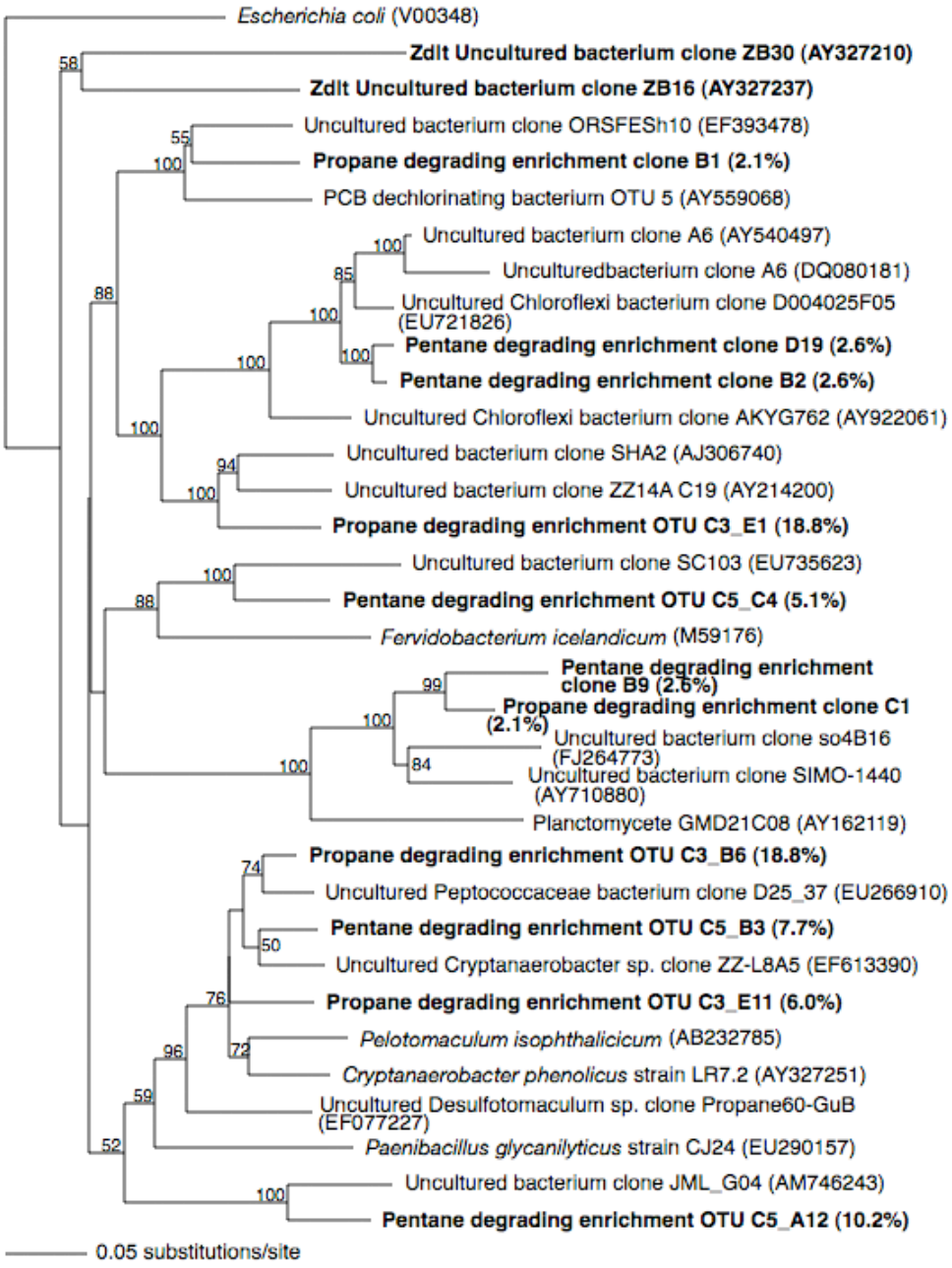


**Figure 4.** Neighbor-joining tree of enrichment clones (bold) from propane and pentane degrading enrichments non-*Delta-Proteobacteria.*