Extremophiles (2002) 6:445–452
Digital Object Identifier (DOI) 10.1007/s00792-002-0278-3
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ORIGINAL PAPER

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Halosimplex carlsbadense gen. nov., sp. nov., a unique halophilic archaeon, with three 16S rRNA genes, that grows only in defined medium with glycerol and acetate or pyruvate

Received: February 8, 2002 / Accepted: May 10, 2002 / Published online: August 13, 2002

Abstract A halophilic archaeon has been isolated from unsterilized salt crystals taken from the 250-million-year-old Salado formation in southeastern New Mexico. This microorganism grows only on defined media supplemented with either a combination of acetate and glycerol, glycerol and pyruvate, or pyruvate alone. The archaeon is unable to grow on complex media or to use carbohydrates, amino acids, fats, proteins, or nucleic acids for growth. Unlike other halophilic microbes, this organism possesses four glycolipids, two of which may be novel. The microbe is unique in that it has three dissimilar 16S rRNA genes. Two of the three genes show only 97% similarity to one another, while the third gene possesses only 92%–93% similarity to the other two. Inferred phylogenies indicate that the organism belongs to a deep branch in the line of *Haloarcula* and *Halorhabdus*. All three lines of taxonomic evidence: phenotype, lipid patterns, and phylogeny, support creation of a new genus and species within the halophilic Archaea. The name suggested for this new genus and species is *Halosimplex carlsbadense*. The type strain is 2–9-1^T (= ATCC BAA-75 and JCM 11222) as written in the formal description.

Key words Halophile · Halophilic archaeon · *Halosimplex* · *Halosimplex carlsbadense* · Permian

Communicated by W.D. Grant

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Introduction

Since the first description of the genus *Halobacterium* appeared in the seventh edition of *Bergey's manual* (Elazari-Volcani 1957), extremely halophilic Archaea have been generally regarded as a microbial group that required complex media for growth and reproduction. There are currently 17 different genera in the order *Halobacteriales* and family *Halobacteriaceae*. These halophilic Archaea have been isolated from numerous saline sources including terminal lakes, saline soils, salted hides, salterns and underground salt formations (Grant and Larsen 1989; Montalvo-Rodriguez et al. 2000). In most of these instances, the organisms have been grown on media containing yeast extract, peptones, amino acids, and carbohydrates (Hochstein 1988).

In addition to expanding upon their original, rather restricted, nutritional needs, recent descriptions of halophilic genera have included a variety of differences beyond the normal physiological characters. These differences include numerous ether lipids (Kamekura 1993; Morita et al. 1998; Kamekura and Kates 1999), and even multiple copies of rRNA genes (Oren et al. 1999; Mylvaganan and Dennis 1992).

In general, each genus within the *Halobacteriales* can be distinguished from the others partly on the presence or absence of specific marker lipids. While most of the genera possess the same primary phospholipids, each genus typically has zero to two specific glycolipids (Kamekura and Kates 1999). Two of the genera, *Halobacterium* and *Haloarcula* are unique in that they contain the phosphosulfolipid, phosphatidyl glycerosulfate (PGS). *Halobacterium* is also the only genus known to possess two sulfated glycolipids: S-TGD-1 (3-HSO_3-Gal_p-β-(1 → 6) Man_p-α (1 → 2) Glc_p α (1 → 1) sn-glycerol diether, and S-TeGD (3-HSO_3-Gal_p-β-(1 → 6) [Gal_f α (1 → 3)] Man_p-α (1 → 2) Glc_p α (1 → 1) sn-glycerol diether.

Since the mid 1980s, 16S rRNA analyses have been important for the description of new bacterial taxa, including members of the *Halobacteriaceae*. The largest portion of the halophilic Archaea have been found to possess one 16S

rRNA gene. Four species [*Haloarcula* (*Har.*) *quadrata*, *Har. sinaiiensis*, *Har. vallismortis* and *Har. marismortui*] have been shown to possess two divergent 16S rRNA genes. However, the family as a whole appears to represent a single microbial evolutionary lineage, distantly related to the *Methanobacteriales*.

This paper describes a new genus of halophilic archaeon that is distinctly different from other *Halobacteriaceae*. The organism is completely unable to use complex nutrients, possesses two unknown glycolipids, and has three distinct 16S rRNA genes. Phylogenies inferred from these genes show that the organism represents a new genus of halophilic Archaea evolutionarily related to *Halorhabdus* and *Haloarcula*. However, the exact evolutionary relationship between this organism and other halophilic Archaea is difficult to determine.

Materials and methods

Isolation and purification

The archaeon was isolated from unsterilized rock salt taken from the Permian-aged Salado Formation located in southeastern New Mexico. The crystals were removed from the mine wall using a rock hammer and chisel. Ten grams of salt were immediately dissolved in 90 ml of sterile brine containing 15% (w/v) NaCl to give a final concentration of 25% (w/v) NaCl. These samples were then serially diluted and plated onto a defined glycerol–acetate medium (GA) containing (g l^{-1}): NaCl 200; $MgSO_4{\cdot}7H_2O$ 20; KCl 4.0; K_2HPO_4 0.5; $(NH_4)_2SO_4$ 1.0; acetate (sodium salt) 2.5; glycerol 2.5. This medium was brought to pH 7.4 with 10 M NaOH and sterilized at 121°C for 20 min. This medium was used in three other formulations in which the glycerol and acetate were replaced with 5.0 g l^{-1} pyruvate (P medium); 2.5 g l^{-1} pyruvate plus 2.5 g l^{-1} glycerol (PG medium) and 2.5 g l^{-1} pyruvate plus 2.5 g l^{-1} acetate (PA medium).

Due to the limited growth capacity and apparent uniqueness of the isolate, extensive measures were taken to ensure culture purity. Upon first isolation, the culture was purified using multiple single colony isolations. Following this first isolation, individual colonies were selected and spread onto solid complex media and onto the GA medium. All of the colony streaks grew as identical colonies on the GA medium while none grew on any of the complex media used. Once growth studies showed that the organism was able to use solid PG medium, additional single colony isolations were performed using this medium.

Culture purity has also been assessed via phase contrast examination of living cells, and through comparison of polar lipid profiles from cultures grown with glycerol and acetate, pyruvate alone, and pyruvate and glycerol as carbon sources.

Colonial and cell characteristics were determined following growth on solidified PG and GA medium at 40°C. Cell photographs were taken using a Nikon, Cool-pix 990 digital camera attached to an Olympus Labophot phase contrast microscope. Images were electronically processed and labeled using Adobe Photoshop 6.0 on a Dell Optiplex GX 300 computer.

Biochemical characterization

The organism's temperature, pH, and salt tolerance were tested in both GA and PG broth media. Growth was monitored by measurement of optical density at 600 nm. Optimal growth conditions were determined based upon maximum optical densities. Temperatures tested included 22°, 30°, 35°, 40°, 45°, 50°, 60°, and 70°C. Tolerance to pH was tested in PG broth medium set at pH 4, 5, 6, 7, 8, 9, and 10. Salt tolerance was tested in PG broth medium using 0%, 5%, 10%, 15%, 20%, 25%, and 30% (w/v) NaCl.

The biochemical capability of the isolate was also tested in broth containing the salts listed in the GA medium but replacing the acetate and glycerol with various organic compounds at 5.0 g l^{-1}. Growth was determined visually using the production of both a red pigment and increased medium turbidity as indicators. Medium in negative flasks remained clear for at least 14 days after inoculation. Due to the very limited metabolic capability of this archaeon, biochemical comparisons between it and other halophilic Archaea grown simultaneously in identical media were not performed. The biochemical characteristics of this organism are presented in this manuscript in order to complete the description of the taxon.

Catalase activity was tested using hydrogen peroxide contained inside a capillary tube. Oxidase tests were performed using the dry slide oxidase test (Difco Laboratories, Detroit, MI, USA).

Antibiotic sensitivity was determined using solidified PG medium. Sterile disks containing various antibiotics were placed onto the surface of an inoculated Petri plate. The antibiotics tested were: chloramphenicol (30 μg), erythromycin (15 μg), gentamycin (10 μg), penicillin G (10 units), tetracycline (30 μg), vancomycin (30 μg), bacitracin (10 μg), sulfasoxazole (300 μg), novobiocin (30 μg), rifampin (5 μg), monensin (100 μg ml^{-1}), valinomycin (100 μg ml^{-1}), aphidocolin (100 μg ml^{-1}), kanamycin (100 μg ml^{-1}), clindamycin (100 μg ml^{-1}), neomycin sulfate (100 μg ml^{-1}), and anisomycin (100 μg ml^{-1}). Prior to being added to the sterile disk, valinomycin was dissolved in pure methanol, anisomycin was dissolved in a solution of methanol:water (1:1 v/v), and monensin was dissolved in methanol:water (1:10 v/v). Aphidocolin, clindamycin, kanamycin, and neomycin sulfate were dissolved in distilled water. The other antibiotics were purchased on disks from Difco (Detroit MI). Cultures were scored as being sensitive to the antibiotic if the zone of inhibition extended beyond 8 mm. This distance was chosen since sterile disks containing distilled water lysed the cells of this archaeon in a ring 8 mm in diameter around the disk.

Lipid profiles

The lipid characteristics of the isolate were determined using the Bligh and Dyer extraction technique (Bligh and Dyer 1959) and thin layer chromatography (Torreblanca et

al. 1986). Lipids were separated and analyzed using both single and double developments as well as 1- and 2-dimensional separations. Separated phospholipids were stained with molybdenum blue, glycolipids were stained with orcinol reagent, and sulfolipids were visualized with azure A stain according to the method of Tadano-Aritomi and Ishizuka (1983). The lipids were identified by comparison with extracts from previously identified halophilic cultures obtained from the American Type Culture Collection (ATCC).

DNA characterization and sequencing of 16S rRNA genes

The guanine plus cytosine content of the DNA was determined using the reversed phase high performance liquid chromatography method of Tamaoka and Komagata (1984).

Total DNA was extracted as described by Ng et al. (1995). The 16S rRNA gene(s) were amplified by PCR with the following forward and reverse primers: 5′-ATT CCG GTT GAT CCT GCC GG (positions 6–25 according to *Escherichia coli* numbering) and 5′-AGG AGG TGA TCC AGC CGC AG (positions 1,540–1,521).

The 16S rRNA genes were amplified with LA Taq polymerase (Takara Shuzo, Japan) using both the forward and reverse primers described above. LA Taq was chosen for this work over Pyrobest polymerase because of its higher fidelity when amplifying longer DNA sequences. The DNA sequences were amplified with a thermal cycle of 1 min incubation at 94°C, followed by 30 cycles at 98°C for 10 s, and extension at 68°C for 10 min. The amplified DNA were cloned into the *sma*I site of pUC119 and introduced into *E. coli* JM 109. Following growth, 12 transformants were randomly selected, the plasmids were isolated and sequences determined from both the 5′ and 3′ termini. The inserts were sequenced with a combination of dye-primer and dye-terminator methods using a Perkin Elmer Biosystems DNA sequencer. The sequences obtained were aligned with those deposited in databases using the Clustal W v1.7 program (Thompson et al. 1994), and a similarity tree was constructed by the neighbor joining method.

In order to confirm that sequence heterogeneity was not caused by PCR artifacts from the use of the LA Taq, a 740-bp region of the 5′ terminus was amplified using Ex Taq (Takara, Japan). The amplification conditions in this experiment were 25 cycles of 30 s at 94°C, 30 s at 55°C and 2 min at 72°C. In this case, the same 6–25 position sense primer was used while the reverse primer used was 5′-ACC CGG GTA TCT AAT CCG GT (*E. coli* 801–782). This product was inserted and transformed as described above. Eleven clones were randomly selected for sequencing.

Ribosomal DNA sequences were also compared to known GenBank sequences via a BLAST search. The 16S rRNA sequences of all type species of members of the family *Halobacteriaceae* were downloaded to a personal computer, combined into a single data file with the sequences from the isolate, and aligned using Clustal W v1.7. Phylogenetic inferences on the aligned sequences were drawn using the maximum likelihood method in PHYLIP (Felsenstein 1993). The phylogenetic inferences used the global rearrangement option, with jumbled data entry. The data file used to produce the phylogenetic tree includes the type strains of each of the currently accepted halophilic archaeal genera, as well as another halophile isolated from underground salts (*Halococcus salifodinae*). The comparison also included all 16S rRNA sequences from the four species known to possess multiple divergent rRNA genes (*Har. vallismortis*, *Har. quadrata*, *Har. sinaiiensis* and *Har. marismortui*). *Methanobacterium thermoautotrophicum* served as the outgroup.

Results and discussion

According to Murray et al. (1990) and Oren et al. (1997), the establishment of new taxa should be based upon the use of polyphasic methods that incorporate at least some phenotypic, chemotaxonomic, and phylogenetic data. In the present study, all three forms of taxonomic data clearly show that strain 2–9-1^T represents a distinct halophilic group.

The halophilic strain was isolated from an unsterilized salt crystal taken from the north wall of the US Waste Isolation Pilot Plant (WIPP) at the S90 W550 location. The sample was taken from a halite bed that contained <5 wt% impurities. Ten grams of salt were dissolved in 90 ml of sterile 15% (w/v) salt brine. Plates from this sample produced no growth on complex casamino acids medium (Vreeland et al. 1984, 1998) and only a single red colony type on the GA medium. The strain was isolated from this GA medium and given the laboratory tracking number 2–9-1^T. The organism grew in 7 days at 40°C in GA or PG broth containing 20–30% (w/v) NaCl. Optimum growth occurs at 25% (w/v) NaCl. Strain 2–9-1^T grew best on solid medium containing pyruvate or pyruvate plus glycerol. This archaeal strain grew at temperatures between 22° and 50°C, with optimal growth occurring at 37°–40°C. Growth did not occur at 60° or 70°C. Growth tests were not conducted at temperatures below 22°C. The archaeon produced a pink to red pigment. Colonies are small, circular and raised, with entire margins. The colony surface is smooth and shiny, becoming wrinkled with age. Growth occurs only between pH 7.0 and 8.0. The archaeon is an obligate aerobe and requires vigorous aeration for growth. It cannot use nitrate, nitrite, or any other alternate electron acceptors. The cells are rod shaped (Fig. 1). Log phase cells are approximately 0.95 μm wide by 5.0 μm in length. Actual cell sizes changed from short rods in log phase cultures to somewhat elongated rods in stationary phase cultures. The cells were motile and lysed in distilled water. Cells stained Gram-negative using the three-step Gram stain procedure (Difco, Detroit, MI, USA).

Strain 2–9-1^T proved to have only limited nutritional ability. Growth occurred in defined medium containing inorganic salts and small-molecular-weight carbon sources. Pyruvate was the only carbon compound able to act as a sole carbon source to support the growth of this halophile. The organism grew in clumps in the pyruvate medium. The archaeon was able to grow in media containing both glyc-

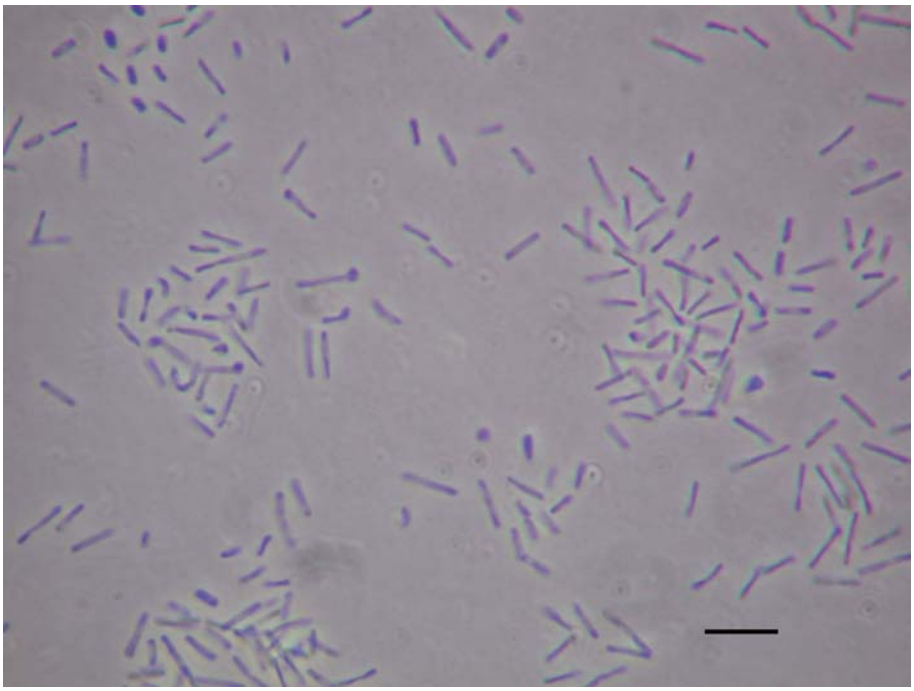

Fig. 1. Light microscope photograph of log phase cells of *Halosimplex carlsbadense*. *Bar* equals 2 μm

erol and acetate, as well as both glycerol and pyruvate, but neither glycerol nor acetate supported growth as sole carbon sources. When the organism was supplied with glycerol (either in the presence of pyruvate or acetate) growth was evenly distributed throughout the medium. Interestingly, the organism did not grow in media containing acetate plus pyruvate. No other carbon compounds or complex nutrients supported growth of this organism. Table 1 presents the complete list of amino acids, alcohols, aldehydes, vitamins, nucleotide bases, fatty acids, and Krebs cycle intermediates that did not support growth when used as sole sources of carbon. The archaeon was catalase and oxidase positive.

These phenotypic data show this organism to be the most fastidious halophile described to date. The fact that this archaeon is only able to grow on simple carbon compounds is both surprising and exciting. The exclusive use of defined medium without any ability to use complex materials such as yeast extract, peptones, or vitamins is unusual among halophilic Archaea. Prior to the isolation of this strain, the defined media used by halophilic Archaea have generally been complicated and contained a significant number of compounds (Rodriguez-Valera et al. 1980; Dundas et al. 1963; Ducharme et al. 1972). Waino et al. (2000) recently described another halophilic organism, *Halorhabdus utahensis*, which utilized a relatively limited number of carbon sources. The species description of *Hrd. utahensis* shows that it utilizes glucose, xylose, and fructose in media containing yeast extract (Waino et al. 2000). In comparison, strain 2–9-1^T is unable to use even these 5- and 6-carbon compounds. Furthermore, the presence of materials such as yeast extract, amino acids, and peptones actually appears to inhibit growth of strain 2–9-1^T. Having such a restricted metabolic ability represents a significant difference between strain 2–9-1^T and all other previously described halophilic Archaea.

Table 1. Carbon compounds that do not support reproduction of strain 2–9-1^T

Amino acids	L-Alanine, L-arginine (HCl), L-aspartic acid, L-citrulline, Na-glutamate, glycine, DL-homocysteine, L-lysine (HCl), L-phenyla lanine, proline, L-serine, DL-tryptophan, L-tyrosine, L-valine
Carbohydrates	L-Arabinose, fructose, D-glucose, lactose, D-mannose, methyl cellulose, D-raffinose, rhamnose, sucrose, D-trehalose, D-xylose, starch, DL-glyceraldehyde
Krebs cycle intermediates	Citric acid, α-ketoglutarate, malic acid, oxaloacetate, succinic acid
Fatty acids	Formic acid, Na-formate, lactic acid, malonic acid
Alcohols	1-Butanol, ethanol, isobutanol, isopropanol, methanol, 1-propanol
Sugar alcohols	D-Sorbitol
Vitamins	L-Ascorbic acid, thiamine (HCl)
DNA bases	Guanine, thymine
Miscellaneous compounds	*p*-Aminobenzoic acid, benzoic acid, caffeine, formaldehyde, gluconolactone, tartaric acid, Tween 80, Na-glycerophosphate
Complex materials	Proteose–pepetone #3, casamino acids, yeast extract, gelatin, casein

The archaeon required at least 0.5% (w/v) Mg^{2+}, which could be supplied as either $MgSO_4$ or $MgCl_2$. Optimum growth occurred in the presence of 1–2% (w/v) magnesium.

The organism did not grow in any medium containing complex nutrients such as peptones, casamino acids, yeast

extract, proteins (gelatin, casein), or starch. Gelatin and starch hydrolysis tests were negative. Addition of a vitamin mix (1% w/v) (Franzmann et al. 1987), to media containing either pyruvate and glycerol or the other carbon compounds listed in Table 1 neither inhibited nor stimulated growth. The archaeon could not ferment arginine.

The growth pattern demonstrated by strain 2–9-1^T is difficult to understand. There are only two possible explanations for such restricted metabolic activity. Either this archaeon does not possess normal catabolic pathways, or it completely lacks the membrane transport enzymes needed to utilize other substrates.

Strain 2–9-1^T was sensitive to bacitracin [37-mm zone of inhibition (zoi)], sulfasoxazole (67-mm zoi), novobiocin (29-mm zoi), rifampin (12-mm zoi), and monensin (22-mm zoi). The organism was not sensitive to chloramphenicol, erythromycin, gentamycin, penicillin G, tetracycline, vancomycin, clindamycin, kanamycin, anisomycin, aphidocolin, neomycin, and valinomycin, making this organism the only halophilic archaeon not sensitive to either anisomycin or aphidicolin.

Thin-layer chromatograms of acid methanolysate extractions from whole cells (Ross et al. 1981) (data not shown) showed that the organism possessed ether lipids typical of the Archaea. Two-dimensional thin-layer chromatography of acetone-fractionated lipids showed that 2–9-1^T produced four glycolipids (Fig. 2). All four of the glycolipids stained positive for sulfate. One sulfated glycolipid (Fig. 2) chromatographs along with an unknown lipid that is also present in the type strain of *Haloterrigena thermotolerans*. The second sulfated glycolipid chromatographs along with disulfated diglycosyl diether (S_2-DGD) in all solvents and dimensions (Fig. 2). A third sulfated glycolipid is most likely sulfated tetraglycosil diether (S-TeGD). The fourth sulfated glycolipid does not correspond to other known lipids, although it does chromatograph close to sulfated diglycosyl diether (S-DGD) in chloroform:methanol:acetic acid:water (85:22.5:10:4 v/v). The organism also possesses a small amount of phosphatidyl glycerol (PG) (see Fig. 2, square inserted to show location) and methylated phosphatidyl glycerol phosphate (PGP-me) (Fig. 2). This lipid pattern provides a relatively easy distinction between this organism and other extreme halophiles. Four sulfated glycolipids are more than any other halophilic genus contains.

The DNA of strain 2–9-1^T contained 64.4 mol% guanine plus cytosine, which is only slightly higher than both *Htr. thermotolerans* and *Hrd. utahensis* and is similar to most other halophilic Archaea.

For the phylogenetic analysis of the organism, 12 separate *E. coli* clones were randomly selected for sequencing. Initial analysis of a 400-bp region of the 5′ end and a 400-bp region of the 3′ end of the 16S rRNA gene showed that the clones formed two groups. Group I consisted of five clones and group II contained seven. Two clones from each group were selected for complete sequencing of the inserted genes. The four clones produced three distinct 16S rRNA sequences. The sequences for genes A and B were present in group I, while that for gene C was found in group II. Genes A and B were found to differ primarily at the 3′ end of the gene. A total of 1,471 bases were determined for genes A and C and a total of 1,470 bases were determined for gene B. There were no ambiguous bases for gene A, and one ambiguous base each in the sequences for genes B and C. The similarity between genes A and B proved to be 97.7%, while gene C possesses only 93.8% and 92.2% similarity to genes A and B, respectively. The phylogenetic relationship of all available genetic sequences is shown in Fig. 3.

Strain 2–9-1^T is therefore unique among halophilic Archaea in that it possesses three divergent 16S rRNA genes. Phylogenetic inferences showed that all three genes consistently form a distinct separate cluster starting from a common node (Fig. 3). Further bifurcation then occurs between two of the three genes. In addition, examination of the sequences shows that the changes are not localized within one region but are scattered throughout the variable regions of the genes. None of the differences break into the conserved regions, making the three *Halosimplex* genes consistent with all other Archaea in this regard. Of even greater interest is the degree of difference between the three individual genes. Based purely on similarity values from a BLAST search, *Natronomonas pharaonis*, of all available halophiles, had the highest sequence similarity (89% to the A gene). The phylogenetic tree prepared from these sequences indicates that *Halosimplex carlsbadense* (2–9-1^T) represents a branch deep within the lineage of the genus *Haloarcula* and the *Halorhabdus* (Fig. 3). This result is also of interest, given the fact that *Haloarcula* is the only other halophilic group containing species that possess multiple copies of divergent genes (Gemmell et al. 1998). We should note, however, that a bootstrap analysis (R.H. Vreeland, J. Jones, D. Dunbar, and W. Rosenzweig, unpublished results) has shown that this branch only forms 29% of the time. Consequently, the phylogenetic position of this organism remains somewhat uncertain.

The fact that the strain has three highly divergent genes makes it unique amongst the halophilic Archaea (Table 2). The rather large divergence between the three genes (especially C relative to A and B), indicates that these genes have been separate and distinct entities for a very long period of time. In fact, the similarity existing between gene C and genes A and B is greater than that found in the same gene from individual species of different genera. If these three genes had actually been cloned and sequenced directly from the environment, rather than from a growing pure culture, the genes might be considered to represent two different uncultured genera (C vs. A and B) and perhaps two species (A and B) of one of those genera.

At this point there is extensive evidence to support the fact that all three 16S rRNA genes reside inside a single pure culture. First, the strain was extensively purified via several sequential single colony isolations. Second, the strain has consistently shown a unique highly restricted metabolic capability. One would expect that if *Halosimplex carlsbadense* (2–9-1^T) were an impure culture, then at least one of the strains would possess a less restricted metabolism. Third, the strain consistently yields a single lipid pattern regardless of the carbon source provided. Finally, the results from two different PCR experiments using different PCR conditions and polymerases yielded nearly identical

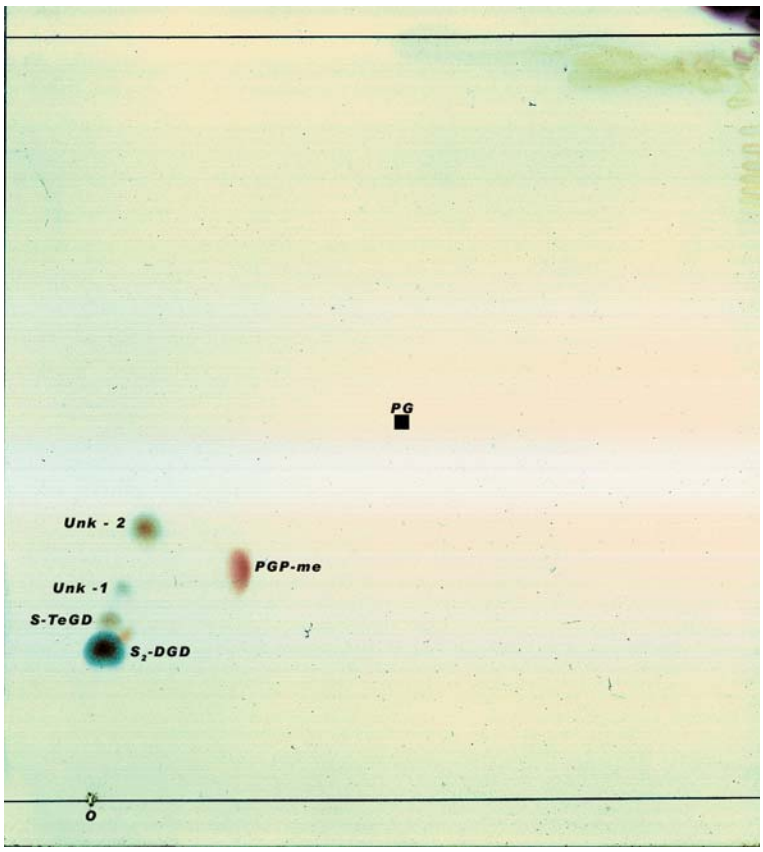

Fig. 2. Two-dimensional polar lipid pattern of *Halosimplex carlsbadense*. The origin of the plate is at the *bottom left*. The solvent system for the first dimension was chloroform:methanol:water (71.5:27.5:4.4 v/v). The first dimension was developed twice in this solvent. The second dimension was developed using chloroform:methanol:90% acetic acid (65:4:35 v/v). Lipids were visualized using 4.2 M sulfuric acid charring

Table 2. Main differential characteristics between *Halosimplex* (strain 2–9-1^T) and other Haloarchaea

	Cell shape	Growth on complex media	Growth on amino acids	Growth on sugars	pH range of growth	Glycolipids present	Number of divergent 16S rRNA genes
Halosimplex	Short and long rods	No	No	No	7–8	S-TeGD, S_2-DGD, two unknown[a]	3
Haloarcula	Irregular rods, triangles, rectangles	Yes	Yes	Yes	5–8	DGD, TGD	2
Halobacterium	Rods	Yes	Yes	No	5–8	TGD, S-TGD, S-TeGD	1
Halorhabdus	Rods and pleomorphic cells	Yes	Yes	Yes	5.5–8.5	TGD, S-TGD	1
Halogeometricum	Pleomorphic	Yes	Yes	Yes	5–8	NG[b]	1
Haloferax	Pleomorphic	Yes	Yes	Yes	5–8	S-DGD	1
Halorubrum	Rods and pleomorphic cells	Yes	Yes	Yes	5–8	S-DGD	1
Halococcus	Cocci	Yes	Yes	Yes	5–8	S-DGD, TGD	1
Natrialba	Rods	Yes	Yes	Yes	5–8	S_2-DGD	1
Natronobacterium	Rods	Yes	Yes	Yes	8–10	None	1
Natronococcus	Cocci	Yes	Yes	Yes	8–10	None	1
Natronomonas	Rods	Yes	Yes	Yes	8–10	None	1

[a] The organism possesses two other novel sulfated glycolipids
[b] *Halogeometricum* possesses an unidentified non-sulfated glycolipid

results within the first 740 bases (5′ end) of all three sequences.

In view of all the data we believe that establishing strain 2–9-1^T as the type strain and species of a new genus within the halophilic Archaea is warranted and defensible. We propose the specific epithet *Halosimplex carlsbadense* for this new genus and species. The primary differential characteristics for this genus are listed in Table 2.

Description of *Halosimplex* gen. nov.

Halosimplex Vreeland, Rosenzweig, Straight, Krammes, Dougherty, and Kamekura (Ha.lo.sim′.plex Gr. n. hals, halos, the sea, salts, L. adj. simplex, simple, uncomplicated. L. neut. n. *Halosimplex* simple salts, the simple halophile).

Gram-negative, primarily rod shaped cells, some pleomorphic forms can be found. Colonies are pink to red,

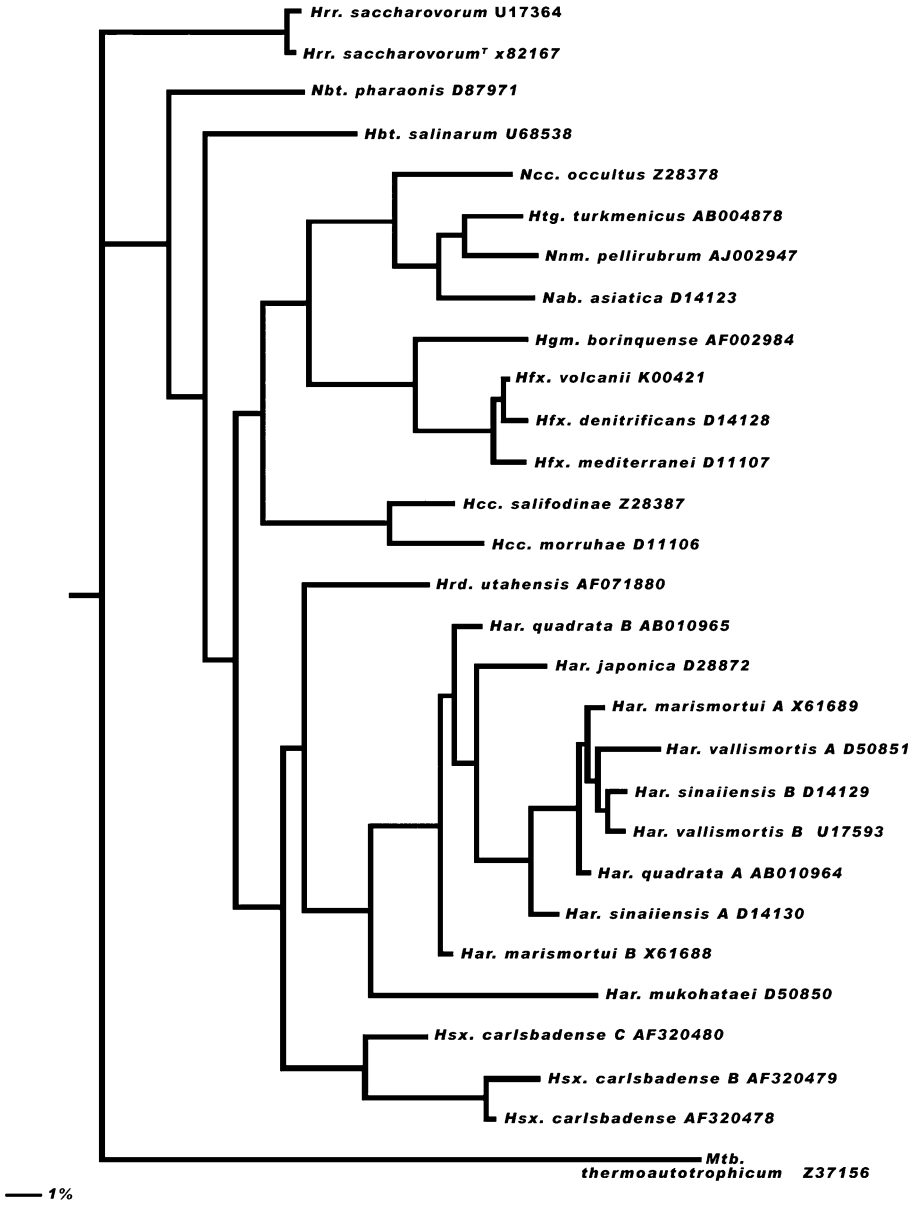

Fig. 3. Relationship between the three *Halosimplex* rRNA genes and other *Halobacteriaceae*. The accession numbers used to produce this tree follow the name of the archaeon. The tree is based upon maximum likelihood using global rearrangement

small, circular, opaque, smooth, shiny and raised, with entire margins. Colonies become wrinkled with age. Aerobic, oxygen is used as the final electron acceptor. Cannot use nitrate, nitrite, or any other alternate electron acceptors. Growth occurs only between pH 7.0 and 8.0, temperature 22°–50°C. Optimal growth occurs between 37° and 40°C. Extremely fastidious. Grows only on pyruvate, pyruvate plus glycerol, or glycerol plus acetate as carbon sources in defined medium. Pyruvate plus acetate will not support growth. Unable to grow on any other organic compounds tested, including amino acids, methanol, formaldehyde, vitamins, nucleotides, fatty acids, proteins, or Krebs cycle intermediates. Complex nutrients such as yeast extract, casamino acids, starch, or casein do not stimulate growth and may be inhibitory. Unable to ferment arginine or to grow autotrophically in the light. Catalase and oxidase positive.

Phosphatidyl glycerol and methylated phosphatidyl glycerol are produced. Possesses a total of four sulfated glycolipids. Two of the glycolipids have been identified as S-TeGD and S_2-DGD. The other two lipids are unidentified.

Guanine plus cytosine content 64.4 mol% by HPLC. Possesses three divergent 16S rRNA genes (designated A, B, and C).

The type species is *Halosimplex carlsbadense*.

Description of *Halosimplex carlsbadense* sp. nov.

carlsbadense (carls.bad′.ense M.L. n carlsbad isolated near Carlsbad, New Mexico, USA).

Requires at least 0.5% (w/v) Mg^{2+}, which can be supplied as either $MgSO_4$ or $MgCl_2$. Optimum growth occurs in the presence of 1–2% (w/v) magnesium.

Sensitive to bacitracin (10 μg), sulfasoxazole (300 μg), novobiocin (30 μg), rifampin (5 μg), and monensin (100 μg ml^{-1}). Resistant to chloramphenicol, erythromycin, gentamycin, penicillin G, tetracycline, vancomycin, clindamycin, kanamycin, anisomycin, aphidocolin, neomycin, and valinomycin.

The type strain is 2–9-1 (ATCC BAA 75; JCM 11222).

Genes A and B are related to each other at 97.7% similarity. Gene C is related to genes A and B at only 93.8% and 92.2%, respectively.

Genbank accession numbers Gene A AF320478; Gene B AF320479; Gene C AF320480.

Acknowledgments This research was supported by grants EAR 9714203 and EAR 0085371 from the Geosciences Directorate Life in Extreme Environments Program of the US National Science Foundation.

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