Halogeometricum borinquense gen. nov., sp. nov., a novel halophilic archaeon from Puerto Rico

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A novel extremely halophilic archaeon was isolated from the solar salterns of Cabo Rojo, Puerto Rico. The organism is very pleomorphic, motile and requires at least 8% (w/v) NaCl to grow. Polar lipid composition revealed the presence of a novel non-sulfate-containing glycolipid and the absence of the glycerol diether analogue of phosphatidylglycerosulfate. The G+C content of the DNA is 59 mol%. On the basis of 16s rRNA sequence data, the new isolate cannot be classified in one of the recognized genera, but occupies a position that is distantly related to the genus Haloferax. All these features justify the creation of a new genus and a new species for the family Halobacteriaceae, order Halobacterales. The name Halogeometricum borinquense gen. nov., sp. nov. is proposed. The type strain is ATCC 700274T.

Keywords: halophilic bacteria, Halogeometricum borinquense gen. nov., sp. nov., archaea

INTRODUCTION

The family Halobacteriaceae (order Halobacterales, domain Archaea) presently contains ten recognized genera: Halobacterium, Haloarcula, Haloferax, Halococcus, Natronobacterium, Natronococcus, (Grant & Larsen, 1990; Tindall et al., 1984; Tindall, 1992; Torreblanca et al., 1986), Halorubrum (McGenity & Grant, 1995), Halobaculum (Oren et al., 1995), Natriiblu (Kamekura & Dyall-Smith, 1995) and Natronomonas (Kamekura et al., 1997). A primary characteristic of all Halobacteriaceae is a requirement for high salt concentrations, with a minimum of 1·5 M sodium chloride needed for growth (Norton, 1992). Most halobacteria grow optimally at 210–270 g NaCl l−1 (Grant & Larsen, 1990; Norton, 1992).

In 1994, we isolated 20 halophilic archaeal strains from the solar salterns of Cabo Rojo, Puerto Rico. Three strains (designated PR3, PR7 and PR19) were similar in morphological and physiological properties and in lipid composition. They showed unique features that did not correspond to any previously described species (Grant & Larsen, 1990; Kamekura & Dyall-Smith, 1995; Tindall, 1992; Kamekura et al., 1997). Strain PR3T (T = type strain) was chosen for further characterization. Based on 16S rRNA sequences, polar lipid content and physiological analysis the creation of a new genus for the family Halobacteriaceae, order Halobacterales appears to be justified. In this paper we present the characteristics of strain PR3T.
METHODS

Collection of samples and isolation of halophilic archaea. Five water samples (400 ml each) were collected in sterile plastic bags (Whirl-Pak) from the solar salterns in Cabo Rojo, Puerto Rico, between May and August of 1994. Each sample was divided into 50 ml aliquots, which were then filtered through 0.45 μm and 0.22 μm nitrocellulose membranes (Millipore). The membranes were transferred onto agar plates containing Seghal–Gibbons medium (Seghal & Gibbons, 1960) (SG) or glycerol solar salt medium (GSS) (250 g solar salt l⁻¹, 10 g glycerol l⁻¹ and 1 g Casamino acids l⁻¹). Inoculated plates were inoculated in sealed bags at 40°C. After 7 d incubation, representative colonies were transferred to the quadrant streak plate method.

The strains Halobacterium salinarum ATCC 19700, Halofex ax mediterranei ATCC 33500T, Halorubrum saccharovorum ATCC 29252T, Haloarcula hispanica ATCC 33960T, Haloarcula marismortui ATCC43094T, Haloarcula Vallisaltrarum ATCC 29715T, Halomonas elongata ATCC 33173T, Excherichia coli ATCC 867T and Pseudomonas aeruginosa ATCC 142 were used as reference strains in biochemical tests and in total and polar lipid analysis.

Morphological, cultural and physiological characteristics. Isolated strains were examined for motility and morphological features in wet mounts (Vreeland, 1993). Optimal conditions for growth were determined by growing each isolate in SG broth supplemented with 5, 8, 10, 15, 25 and 30% (w/v) NaCl and temperatures of 4, 22, 28, 37, 40 and 50°C. The pH tolerance of each isolate was also tested in SG medium with pH values 4, 6, 7, 8 and 10. The cultures were inoculated with 100 μl of a late-exponential-phase culture grown at 37°C without shaking. All of the optimal conditions tests were incubated for 7 d. The extent of growth was determined visually (Gonzalez et al., 1978; Vreeland, 1993). Gram stains were prepared as described by Dussault et al. (1955). Spore and capsule stains were performed according to the methods described by Murray et al. (1994). Minimal magnesium requirement for growth was determined using the optimal growth conditions determined above with variable amounts of MgSO₄ (Ihara et al., 1997; Takashina et al., 1990).

Antibiotic sensitivity was tested by spreading bacterial suspensions on plates of SG medium and applying antibiotic discs (penicillin 10 IU, bacitracin 10 μg, novobiocin 30 μg, kanamycin 30 μg, ampicillin 30 μg, vancomycin 30 μg, sulfamethazine and trimethoprim 25 μg, tetracycline 30 μg, erythromycin 15 μg and chloramphenicol 30 μg) (Bonello et al., 1984; Colwell et al., 1979; Torreblanca et al., 1986). The results were recorded as sensitive or resistant after 14 d incubation at 40°C, with sensitive being defined as the appearance of a zone of inhibition extending at least to 2 mm beyond the antibiotic disc.

Lipid chromatography. Total lipid analysis was performed as described by Ross et al. (1981), without freeze-drying the cells. Three hundred milligrams of wet cells were used for lipid extraction. Polar lipid extraction and TLC was performed as described by Torreblanca et al. (1986) using single or double development. Plates were stained for glycolipids and other lipids using the α-naphthol stain (Torreblanca et al., 1986) or the CeSO₄ stain (Kates, 1972). The presence of sulfate groups on the separated lipids was determined using Azure A stain as described by Matsubara et al. (1994).

Biochemical tests. Nitrate reduction was tested in liquid SG medium supplemented with 0.1% (w/v) KNO₃ (Tindall et al., 1984). Formation of gaseous products from nitrate was detected by the presence of bubbles in Durham tubes (Vreeland, 1993). Anaerobic growth in the presence of either nitrate or arginine was tested as described previously (Hartmann et al., 1980; Hochstein & Tomlinson, 1985; Mancinelli & Hochstein, 1986). Production of acids from sugars was examined in phenol red broth base (BB) supplemented with 25% (w/v) NaCl, 0.2% (w/v) KCl and 2% (w/v) MgSO₄, 7H₂O (Thongthai et al., 1992). To determine the ability to use sugars as energy sources, the minimal medium of Rodriguez-Valera et al. (1980) was supplemented with 1% of the test sugar and 0.01% yeast extract. Control plates with 1% (w/v) or 0.01% yeast extract were also inoculated. The result was considered positive when growth appeared on the control medium with 1% yeast extract and on the medium with the sugar being tested, but not on the 0.01% yeast extract control (Rodriguez-Valera et al., 1983).

Indole production was detected by adding the Kovacs' reagent to the SG broth supplemented with 1% (w/v) tryptone (Holding & Collee, 1971; Takashina et al., 1990). To determine starch hydrolysis, the strains were streaked onto SG with 1% (w/v) soluble starch and 2% (w/v) Bactoagar, and the plates were flooded with iodine solution after growth was obtained (Takashina et al., 1990). Catalase was determined by adding 1% (w/v) H₂O₂ solution to colonies on SG agar medium. Gelatin hydrolysis was detected in SG supplemented with 1% (w/v) gelatin. After 14 days, the tubes were examined for the presence of liquefaction (Holding & Collee, 1971; Tomlinson & Hochstein, 1976). The presence of oxidase was determined using the DRY SLIDE (Difco) biochemical test.

Phase-contrast microscopy. One millilitre of stationary phase cells was centrifuged into a pellet and resuspended in 0.5 ml filter-sterilized brine containing 20% (w/v) NaCl. The cells were then mixed with an equal volume of melted 2% (w/v) agarose containing 20% (w/v) NaCl. A single drop of this cell suspension was placed onto a clean microscope slide and allowed to harden. A cover slip was placed onto the drop of agar and the slide was placed into a 45°C oven and allowed to melt to form a thin (10 μm) layer. The slide was then cooled and viewed using an Olympus BH phase-contrast microscope. Photographs of individual cells were obtained using an MTK-1 television camera fitted with a 20 x ocular lens. Images were digitized and electronically enhanced using the Paint Shop Pro and Power Point editing programs. To enhance the view of the gas vesicles, selected images were digitally treated to a special effect of hot waxing using the Paint Shop Pro editing program. These digital images were printed using an Hewlett Packard 870Cse Deskjet printer system or a Techtronix Phaser II sublimation printer.

Electron microscopy. A 4 μl drop of the culture was applied to a carbon-coated collodion-stabilized copper grid for 30 s. The drop was washed away with 4–5 drops of 1% (w/v) aqueous uranyl acetate and the grid allowed to air dry. Specimens were examined in a Philips CM12 transmission electron microscope operating at 100 kV. Micrographs were recorded at a nominal magnification of ×60000 on Kodak emulsion SO-163 (Oren, 1983).

DNA base composition and 16S rRNA analysis. The G+C content of the DNA and the 16S rRNA phylogenetic analyses were performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSMZ). The DNA for the G+C content was
isolated and purified by chromatography on hydroxyapatite. The G+C content was determined by HPLC.

Genomic DNA extraction, PCR mediated amplification of the 16S rDNA and purification of the PCR products was carried out as described previously (Barns et al., 1994; Rainey et al., 1996). Purified PCR products were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) as directed in the manufacturer’s protocol. Sequence reactions were electrophoresed using the Applied Biosystems 373A DNA Sequencer. A total of 1451 bases corresponding to positions 19–1469 of the Halofexerus mediterranei sequence (accession no. D11107) were identified during these analyses. Distance analysis of the resulting sequence of PR3 was performed using the PHYLIP program (version 3.5.1) (Felsenstein, 1993). A multiple-sequence alignment was made by using the CLUSTAL W program with 16S rRNA gene sequences of organisms belonging to the extremely halophilic archaea (McGenity & Grant, 1995; Kamekura & Dyall-Smith, 1995; Oren et al., 1995; Maidak et al., 1996). The halophiles used and their corresponding accession numbers in parentheses were: Halobacterium salinarum Y12 (D14127), Halofexerus mediterranei (D11107), Halobacterium saccharovorum (X82167), Halococcus salinarum (D50851), Halofexerus denitrificans (D14128), Halofexerus gibbonsii (D13378), Halofexerus volcanii (K00421), Halobaculum gomorrense (L37444), Halobacterium sodomense (D13379) and Natronobacter asiatica BIT (D14124). Methanospirillum hungatei (M60880) served as the outgroup. The 16S rRNA gene similarity values were calculated by pairwise comparison of the sequences within the alignment. SEQBOOT was used to generate 100 bootstrapped data sets. Distance matrices were calculated with DNADIST. One hundred trees were inferred by using NEIGHBOR. Any bias introduced by the order of sequence addition was minimized by randomizing the input order. CONSENSE was used to determine the most frequent branching order. The final tree was drawn using TREEVIEW (Page, 1996).

RESULTS

Cell and colony morphology

Three strains (designated PR3, PR7 and PR19) all isolated from isolated from Cabo Rojo proved to have very similar morphology, physiology and lipid compositions. Strain PR3 was studied in further detail and was designated the type strain of the new genus and species described below. Cells of isolate PR3T were extremely pleomorphic showing different shapes (short and long rods, squares, triangles, ovals) when grown on SG broth (Fig. 1). Mean cell dimensions of the rod forms were 1–3 x 1–2 μm. Motility in SG broth was due to peritrichous flagella (Fig. 2). Cells stained Gram-negative. Spores or capsules were not observed.

Colonies of PR3T on SG medium were 0.5–1 mm in size, pink, very mucoid, circular in size, convex, with an entire margin after 1 week incubation at 40 °C, and had a milky appearance due to the presence of gas vesicles (Fig. 1k–m, and Fig. 2). On plates of GSS medium, growth was slower, and colonies were less pigmented.

Cultural and biochemical characteristics

Strain PR3T required at least 8% (w/v) NaCl to grow in SG medium. Optimum growth occurred at 20–25% (w/v) NaCl at 40 °C and a pH of 7. No growth was
observed below 22 °C or above 50 °C and at pH values below 6 and above 8. At optimal conditions of salinity, temperature and pH, strain PR3T could grow at very low magnesium concentrations. The best growth was obtained with 40–80 mM Mg²⁺.

The isolate showed positive catalase and oxidase reactions. Nitrite was produced from nitrate with gas production. PR3T could grow anaerobically in the presence of nitrate. Strain PR3T strongly hydrolysed gelatin. Starch was not hydrolysed. Indole was formed from tryptophan. Glucose, mannose, fructose, xylose, maltose, trehalose, cellobiose, raffinose and glycero were used as carbon sources. Acid was produced from fructose, arabinose, ribose, xylose, lactose and sucrose. The organism was unable to grow anaerobically on arginine.

The strain was resistant to penicillin, kanamycin, ampicillin, vancomycin, tetracycline, erythromycin and chloramphenicol. Isolate PR3T showed susceptibility to novobiocin, bacitracin and sulfamethazine plus trimethoprim.

**Lipid analyses**

Total lipid analysis of PR3T revealed spots attributable to glycerol diether moieties, characteristic of the Archaea (Ross et al., 1981).

TLC of polar lipids showed that isolate PR3T contains the glycerol diether analogues of phosphatidylglycerol (PG), phosphatidylglycerolphosphate (PGP) and a single glycolipid (Fig. 3). This glycolipid runs somewhat faster on the TLC plate than TGD-2, the glycolipid marker of Haloarcula. Phosphatidylglycerosulfate (PGS), which is present in halophilic archaeal species of Haloarcula, Halobacterium, Halorubrum and Halococcus, was not detected in isolate PR3T.

**DNA base composition**

The G+C content of the DNA of strain PR3T was 59 mol%. The G+C content of the two similar strains designated PR7 and PR19, was 59.6 and 60.5 mol% respectively.

**Phylogeny**

The data obtained from the 16S rRNA 1451 bases and the phylogenetic analysis reveal that strain PR3T occupies a position that is distantly related to the members of the genus Haloferax (Fig. 4). The highest similarity value (93.7%) exists with Haloferax volcanii ATCC 29605T (Table 1).

**DISCUSSION**

The presence of glycerol diether moieties indicates that isolate PR3T is an extremely halophilic archaeon (Ross et al., 1981; Torreblanca et al., 1986). High salt requirement, resistance to kanamycin, chloramphenicol and penicillin (which inhibit growth of halophilic eubacteria) and susceptibility to bacitracin and novobiocin also place this strain into the Halobacteriales (Tindall, 1992; Torreblanca et al., 1986).

The polar lipid chromatogram of strain PR3T showed a novel pattern (Fig. 3). The sole glycolipid of PR3T behaved similar to the glycolipid of Haloarcula species (1-O-[β-D-glucose-(1'→ 6')-α-D-mannose-(1'→ 2')-α-D-glucose]-2,3-di-O-phytanyll-sn-glycerol [TGD-2])
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Fig. 3. Left-hand parts: thin-layer chromatogram of polar lipids extracted from strain PR3 (lanes 2 and 4), *Haloarcula marismortui* (lane 1) and *Haloarcula vallismortis* (lane 3). The silica gel plate was developed once with chloroform/methanol/acetic acid/water (80:12:15:4, by vol.) and was stained with CeSO₄ (a) or with α-naphthol (b). Dark spots indicate the position of glycolipids and clear spots indicate phospholipids. Right-hand parts: single development chromatography of polar lipids extracted from strain PR3 (lane 1), *Haloarcula vallismortis* (lane 2), and a mixture of strain PR3 and *Haloarcula vallismortis* (lane 3). The solvents used were chloroform/methanol/acetic acid/water [80:12:15:4, by vol. for (a); 85:22.5:10:4 for (b)], and plates were stained with CeSO₄ to reveal all lipids. In all parts, dark spots indicate the position of glycolipids and clear spots indicate phospholipids. PGP, phosphatidylglycerophosphate; PG, phosphatidylglycerol; and PGS, phosphatidylglycerosulfate.

Fig. 4. Neighbour-joining distance tree of 16S rRNA sequences. Bar represents 10 substitutions per 100 nucleotides. Bootstrap values higher than 70% are shown. *Methanospirillum hungatei* DSM 864ᵀ was used as the outgroup.

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(Torreblanca et al., 1986), but it ran somewhat faster, indicating that they are not identical. The chromatogram and Azure A staining revealed that strain PR3ᵀ lacks PGS. Other genera that lack PGS are *Natrialba* (Kamekura & Dyall-Smith, 1995), *Haloferax* (Torreblanca et al., 1986), *Halobaculum* (Oren et al., 1995), *Natronobacterium*, *Natronococcus* (Tindall et al., 1984) and *Natronomonas* (Kamekura et al., 1997). Strain PR3ᵀ does not possess any of the common marker lipids associated with the more closely related halophilic genera such as *Haloferax* (Torreblanca et al., 1986), *Halobaculum* (Oren et al., 1995) or *Natrialba* (Kamekura & Dyall-Smith, 1995). Based upon its TLC migration behaviour and staining characteristics, the glycolipid present in isolate PR3ᵀ would appear to be a novel glycolipid which may be useful as a marker for the new genus represented by this strain (Table 1).

Analysis of the 16S rRNA phylogenetic tree (Fig. 4) revealed that strain PR3ᵀ belongs to a separate phylogenetic branch, distantly related to *Haloferax*. The highest similarity existed between strain PR3ᵀ and *Haloferax volcanii* ATCC 29605 (93.7%). Similarity values between PR3ᵀ and *Haloarcula* species are very low (86.2–87.4%), showing that this organism is not phylogenetically close to this genus. Some physio-
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Table 1. Main differences between strain PR3T (Halogeometricum) and known genera of neutrophilic haloarchaea

Data were obtained from Kamekura & Dyall-Smith (1995), Tindall (1992) and Torreblanca et al. (1986).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Haloarcula</th>
<th>Halobacterium</th>
<th>Halobaculum</th>
<th>Halococcus</th>
<th>Halofex</th>
<th>Halogenicum</th>
<th>Halorhabdium</th>
<th>Natridala</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell shape</td>
<td>Irregular rods, triangles, rectangles</td>
<td>Rods</td>
<td>Rods</td>
<td>Coccig</td>
<td>Irregular rods, discs</td>
<td>Short and long rods, squares, triangles, ovals</td>
<td>Rods</td>
<td>Rods</td>
</tr>
<tr>
<td>Mobility</td>
<td>D</td>
<td>+</td>
<td>+</td>
<td></td>
<td>D</td>
<td>+</td>
<td>NG†</td>
<td></td>
</tr>
<tr>
<td>Glycolipid present</td>
<td>TDG-2</td>
<td>S-TDG-1, S-TGD</td>
<td>S-DGD-1</td>
<td>S-DGD-1</td>
<td>S-DGD-1</td>
<td>S-DGD-1</td>
<td>NG†</td>
<td>S-DGD-3</td>
</tr>
<tr>
<td>PGSL present</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>S-DGD-1</td>
</tr>
<tr>
<td>G+C content</td>
<td>62.7-68</td>
<td>66-70.9</td>
<td>70</td>
<td>57-66.6</td>
<td>59.5</td>
<td>59-1</td>
<td>65.3-71.2</td>
<td>60.3-63.1</td>
</tr>
</tbody>
</table>

* D, different reactions in different species.
† NG, novel glycolipid not identified.

logical properties of PR3T also indicated that this organism did not belong to the genus Haloarcula. Characteristics such as the presence of gas vesicles, growth at 8% (w/v) NaCl, and the mucoid aspect of the colony do not fit into the description of the genus Haloarcula. These features are more typical of Haloferax species (Grant & Larsen, 1990; Torreblanca et al., 1986).

Devereux et al. (1990) and Fry et al. (1991) have proposed that a similarity of less than 98% in a 16S rRNA sequence should be considered evidence for separate species, and that with a similarity of less than 93-95% strains should be classified in different genera. A similarity matrix constructed from a comparison of the 16S rRNA sequences showed that the closest relative to strain PR3 was the genus Haloferax at only 92.7-93.3%. The similarity of this strain to the other halophilic genera ranged between 86 and 89%. The similarity of the PR3 16S rRNA to that from both Halorubrum and Halobacterium was only 88%. Therefore, our proposal that isolate PR3T represents a new genus that is remotely related to Haloferax appears justified on this basis.

Recently, minimal standards for the description of the Halobacteriales have been established (Oren et al., 1997). One of the analyses proposed is the characterization of polar lipid patterns, since this property has been shown to be particularly important in classification at the generic level. Based on polar lipid analysis, PR3T could not be considered a species of Haloferax, which has S-DGD-1 as its major glycolipid. Similarity values of 16S rRNA between PR3T and members of the Haloferax are too low to consider PR3T a member of this genus. In addition, the GC content (59.1 mol%) is not in the range reported for Haloferax species (59.5-64 mol%; Table 1).

These data strongly suggest that PR3T represents a new genus and species. We propose that this strain be named Halogonomicum borinquense. Strain PR3T has been designated the type strain of this group and has been deposited in the American Type Culture Collection as strain ATCC 700274T.

Description of Halogonomicum gen. nov.

Halogonomicum Montalvo-Rodriguez, Vreeland, Oren, Kadell, Betancourt and Lopez-Garriga (Hal.o.ge.o.me'tri.cum. Gr. n. halos, halos the sea, salt. L. adj. geometricum geometrical; M. L. neut. n. Halogeometricum salty geometric shape).

Gram-negative pleomorphic cells (short and long rods, squares, triangles, and ovals). Colonies are pigmented (pink), small, circular, opaque, convex with an entire margin. Oxidase and catalase-positive. Chemorganotrophic and aerobic. Grows anaerobically in the presence of nitrate. Arginine not fermented. Halophilic, requiring at least 8% (w/v) NaCl for growth. The polar lipids are glycerol diether analogues of phosphatidylglycerol, phosphatidylglycerophosphatase, and a single, as-yet-unidentified glycolipid. Phosphatidylglycerosulfate is absent. Certain carbohydrates can stimulate growth with acid production. The G+C content of the type species is 59.1 mol%. The type species is Halogonomicum borinquense.

Description of Halogonomicum borinquense sp. nov.

Halogonomicum borinquense (bo .rin.quen’se. M. L. fem. adj. borinquense of Borinquen, the native Indian name for Puerto Rico).

Cells are extremely pleomorphic (rods 1-3 x 1–2 μm), showing different shapes (short and long rods, squares, triangles and ovals). Motile by peritrichous flagella. Gas vesicles are present creating a milky appearance. The colonies are pink, small (0.5-1 mm) and very mucoid. Chemoorganotrophic and aerobic. Anaerobic growth occurs with nitrate. Arginine not fermented. Requires at least 8% (w/v) NaCl for growth. The optimal NaCl concentration range is 20-25% (w/v) at 40 °C; the optimal Mg²⁺ concentration range is 40-80 mM. The optimal temperature is 40 °C (in medium containing 20-25% (w/v) NaCl and 40-80 mM Mg²⁺). Nitrate is reduced to nitrite with the production of gas. Indole is produced from tryptophan. Gelatin is strongly hydrolysed.

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REFERENCES


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