***Haloterrigena thermotolerans* sp. nov., a halophilic archaeon from Puerto Rico**

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An extremely halophilic Archaeon belonging to the order *Halobacteriales* was isolated from the solar salterns of Cabo Rojo, Puerto Rico. The organism, designated strain PR5^T, is rod-shaped, non-motile and requires at least 12% (w/v) NaCl to grow. The strain is highly thermotolerant: its temperature optimum is 50 °C and growth is possible up to 60 °C. Polar lipid analysis revealed the presence of the bis-sulfated glycolipid S₂-DGD-1 as sole glycolipid and the absence of the glycerol diether analogue of phosphatidylglycerosulfate. Both C₂₀:C₂₀ and C₂₀:C₂₅ core lipids are present. The G+C content of the DNA is 63.3 mol%. According to 16S rDNA sequence data, strain PR5^T is closely related to the representatives of the genera *Haloterrigena* and *Natrinema*, but on the basis of its phenotypic properties, 16S rDNA sequence and DNA–DNA hybridization studies, strain PR5^T cannot be assigned to any of the recognized species within these genera. On the basis of its polar lipid composition, the isolate has been assigned to the genus *Haloterrigena*. The creation of a new species, *Haloterrigena thermotolerans*, is therefore proposed to accommodate this isolate. The type strain is strain PR5^T (= DSM 11552^T = ATCC 700275^T).

Keywords: *Haloterrigena thermotolerans* sp. nov., *Natrinema*, halophilic, hypersaline, Puerto Rico

INTRODUCTION

Halophilic Archaea belonging to the order *Halobacteriales* are found in large numbers in the crystallizer ponds of solar salterns worldwide. Their massive populations often impart a red colour to the brines (Oren, 1993, 1994). A variety of species of red halophiles have been isolated from saltern ponds, including *Halobacterium salinarum*, *Halorubrum saccharovorum*, *Haloferax mediterranei*, *Haloferax*

gibbonsii, *Haloferax denitrificans*, *Haloarcula hispanica*, *Haloarcula japonica*, *Halococcus morrhuae* and *Halococcus saccharolyticus*. However, the dominant type of halophilic Archaea inhabiting such ponds still defies microbiologists' attempts toward its isolation (Benlloch *et al.*, 1995; Oren *et al.*, 1996).

In 1994, 20 halophilic archaeal strains were isolated from the solar salterns of Cabo Rojo, Puerto Rico. Some of these isolated strains could be classified in the genera *Halobacterium* and *Haloferax* on the basis of cell morphology and polar lipid composition (Montalvo-Rodríguez, 1996). Three isolates (designated PR3^T, PR7 and PR19), while being similar to each other, did not resemble any of the hitherto described taxa within the *Halobacteriales*. A new genus and species were created to accommodate these strains, *Halogeometricum borinquense*, with strain PR3^T (=

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Abbreviations: PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerophosphate methyl ester; PGS, phosphatidylglycerosulfate.

The GenBank accession number for the 16S rRNA gene sequence of *Haloterrigena thermotolerans* strain PR5^T is AF115478.

ATCC 700274^T) as type strain (Montalvo-Rodríguez *et al.*, 1998).

One isolate, designated PR5^T, consisted of long non-motile rods, and contained the bis-sulfated glycolipid S₂-DGD-1 as sole glycolipid. In this paper, the characteristics of strain PR5^T, which appeared to be sufficiently different from the other recognized species of halophilic Archaea to be described as a new species, are presented.

METHODS

Collection of samples and isolation of halophilic Archaea.

Water samples (400 ml portions) were collected in sterile plastic bags (Whirl-Pak) from the solar salterns in Cabo Rojo, Puerto Rico, in June 1994. Each sample was divided in 50 ml aliquots, which were then filtered through 0.45 µm sterile nitrocellulose membranes (Millipore). The membranes were transferred onto agar plates containing a modified Sehgal–Gibbons (SG) medium (Sehgal & Gibbons, 1960). This medium contained (g l⁻¹): NaCl, 250; MgSO₄·7H₂O, 20; KCl, 2; sodium citrate (trisodium salt), 3; Casamino acids, 7.5; yeast extract, 1.0; and FeSO₄·7H₂O, 0.0023. For solid media, 20 g agar l⁻¹ was added. Plates were incubated in sealed plastic bags at 40 °C. After 7 d incubation, representative colonies were transferred to SG broth medium and isolated in pure culture.

The strains *Haloterrigena turkmenica* VKM B-1734^T, *Halo-bacterium salinarum* NRC 817, *Haloferax mediterranei* ATCC 33500^T, *Halorubrum saccharovororum* ATCC 29252^T, *Haloarcula hispanica* ATCC 33960^T, *Haloarcula marismortui* ATCC 43049^T, *Haloarcula vallismortis* ATCC 29715^T and *Natrialba asiatica* JCM 9576^T were used as reference strains. Cultures of *Natrinema pellirubrum* NCIMB 786^T and *Natrinema pallidum* NCIMB 777^T were obtained as a gift from W. D. Grant (Leicester, UK).

Morphological, cultural and physiological characteristics. In most experiments described below, SG medium was used for growth of strain PR5^T, modified by lowering the NaCl concentration to 200 g l⁻¹ and decreasing the yeast extract concentration to 0.5 g l⁻¹. Cultures were examined for motility and morphological features in wet mounts. Optimal conditions for growth were determined by growing cultures on a rotary shaker (120 r.p.m.) in 40 ml portions of medium in 100 ml Erlenmeyer flasks, and changing concentrations of medium components and temperature as indicated. Unless otherwise indicated, growth temperature was 35 °C. The extent of growth was assessed by measuring the turbidity of the cultures at 600 nm. The pH tolerance was tested in modified SG medium buffered with 20 mM MES (pH 5.0, 5.5), PIPES (pH 6.0, 6.5), Tricine (pH 7.0, 7.5, 8.0) or TAPS (pH 8.0, 8.5). For growth experiments in buffered medium above pH 7.5, the Mg²⁺ concentration of the medium was reduced to 20 mM to avoid the formation of massive precipitates. Gram stains were prepared as described by Dussault (1955).

Antibiotic sensitivity was tested by spreading bacterial suspensions on plates of SG medium and applying antibiotic discs (penicillin, 10 U; bacitracin, 10 µg; novobiocin, 30 µg; kanamycin, 30 µg; ampicillin, 30 µg; vancomycin, 30 µg; sulfamethazone and trimethoprim, 25 µg each; and chloramphenicol, 30 µg) (Bonelo *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 2 mm beyond the antibiotic disc. Sensitivity to neomycin and anisomycin was tested in liquid cultures, to which the antibiotics were added to a concentration of 25 µg ml⁻¹.

Lipid analyses. Polar lipid extraction and characterization by TLC on silica gel plates (20 × 20 cm; Sigma) was performed as described by Torreblanca *et al.* (1986), using single development in chloroform/methanol/acetic acid/water (85:22:5:10:4, v/v). Plates were stained for glycolipids and other lipids using the α-naphthol stain, the CeSO₄/H₂SO₄ stain, the orcinol/FeCl₃ stain and the molybdate stain for detection of phospholipids (Kates, 1972; Oren *et al.*, 1996; Torreblanca *et al.*, 1986). The presence of C₂₀, C₂₀ and C₂₀, C₂₅ core lipids was assessed by hydrolysing lyophilized cell material overnight at 50 °C in methanol/toluene/sulfuric acid (5:5:0.2, v/v), extracting the core lipids with hexane, followed by chromatography on silica gel plates, using hexane/diethylether (4:1, v/v) as eluent (Tindall, 1985). Spots were visualized with iodine vapours. Carotenoid pigments were extracted with methanol/acetone (1:1, v/v) and absorption spectra were recorded in a Hewlett Packard model 8452A diode array spectrophotometer.

Biochemical tests. Unless specified otherwise, tests for phenotypic properties were carried out as indicated in the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Nitrate reduction was tested in liquid SG medium supplemented with 0.5% NaNO₃. Formation of gaseous products from nitrate was detected by the presence of gas bubbles in Durham tubes. Anaerobic growth in the presence of either nitrate or arginine was tested as described previously (Hartmann *et al.*, 1980; Mancinelli & Hochstein, 1986). Production of acids from sugars was examined in SG medium supplemented with 0.5% of the sugars tested. After 2 d, the pH was measured with a pH electrode. To determine the ability to use sugars as energy sources, the yeast extract and Casamino acids concentration in the SG medium were reduced to 0.025% each, and the medium was amended with 0.05% NH₄Cl and 0.5% of the sugar to be tested. After 3 d incubation at 35 °C, the OD of the cultures was compared with that of the control culture without added sugar.

Indole production was detected with Kovacs' reagent after having grown the cells in modified SG medium amended with 0.25% tryptone. To determine starch hydrolysis, the strains were streaked onto modified SG plates with 0.2% soluble starch solidified with 2% (w/v) agar. Starch hydrolysis was detected by flooding the plates with iodine solution after the colonies were fully grown. Presence of catalase was determined by adding a 1% (v/v) H₂O₂ solution to colonies on modified SG agar medium. Gelatin hydrolysis was determined by growing colonies on agar plates amended with 0.4% gelatin and flooding the plates with a solution of 15% (w/v) HgCl₂ in 20% (w/v) HCl after growth was obtained (Gutiérrez & González, 1972). Tween 80 hydrolysis was tested as outlined by Gutiérrez & González (1972). The presence of oxidase was determined with tetramethyl-*p*-phenylenediamine·2HCl (Holding & Collee, 1971). Appropriate positive and negative controls were run for all the above tests.

Phase-contrast and electron microscopy. Cells were examined for motility using a Zeiss standard microscope equipped with phase-contrast optics. For photography, drops of culture were mixed on a microscope slide with an equal volume of melted 2% (w/v) agar containing 20% (w/v) NaCl and covered with a cover-slip. For EM examination, 4 µl drops of culture were applied to carbon-coated

collodion-stabilized copper grid for 30 s. The drops were washed away with 4–5 drops of 1% (w/v) aqueous uranyl acetate and the grids were allowed to air-dry. Specimens were examined in a JEOL-JEM model 100CX TEM operating at 80 kEv.

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

16S rDNA characterization. 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 at the DSMZ, 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 were identified. Phylogenetic tree reconstructions based on the sequence obtained and the sequences available in databases were performed as described by Kamekura & Dyall-Smith (1995).

Preparation of labelled DNA and DNA–DNA hybridization experiments. DNA was labelled by the multiprime system with a commercial kit (RPN 1601 Y; Amersham) with deoxy[1',2',5-³H]cytidine 5'-triphosphate (Amersham). The mean specific activity obtained with this procedure was 8.4×10^6 c.p.m. μg^{-1} DNA. The labelled DNA was denatured prior to hybridization by heating at 100 °C for 5 min and then placed on ice.

DNA–DNA hybridization studies were performed by the competition procedure of the membrane method described by Johnson (1981). Competitor DNAs were sonicated in a Braun sonifier at 50 W for two 15 s time intervals. Membrane filters (HAHY; Millipore) containing reference DNA ($25 \mu\text{g cm}^{-2}$) were placed in 5 ml screw-cap vials that contained the labelled, sheared, denatured DNA and the denatured, sheared competitor DNA. The ratio of the concentrations of competitor to labelled DNA was at least 150:1. The final volume and concentrations were adjusted to 140 μl , $2 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate) and 30% formamide. Hybridization was performed for 18 h in a water-bath with slow shaking at 56 °C, which is within the limits of validity for the filter method (De Ley & Tijtgat, 1970). After hybridization, the filters were washed in $2 \times \text{SSC}$ at the optimal renaturation temperature (56 °C). The radioactivity bound to the filters was measured in a liquid scintillation counter (Beckman) and the percentage of homology was calculated according to Johnson (1981). Two independent determinations were carried out for each experiment.

RESULTS

Strain PR5^T was isolated from a saltern crystallizer pond in Cabo Rojo, Puerto Rico, using the same set of samples from which *Halogeometricum borinquense* was obtained (Montalvo-Rodríguez *et al.*, 1998). The bacterium is a non-motile, slender rod, measuring $4\text{--}13 \times 0.7\text{--}1.0 \mu\text{m}$ (Fig. 1). No flagella were observed by EM after negative staining with uranyl acetate. After fixation with acetic acid (Dussault, 1955), the

cells stained Gram-negative. Colonies on agar are pale red, 0.5–1.0 mm in diameter, opaque, circular and convex with an entire margin. Absorption spectra of cell extracts in methanol/acetone (1:1, v/v) showed maxima at 496 nm and 528 nm, with a shoulder at 474 nm, typical of α -bacterioruberin and derivatives characteristically found in most members of the *Halobacteriales*.

Strain PR5^T required at least 2 M NaCl for growth. Optimum growth was obtained between 3.0 and 3.5 M NaCl. At 4.3 M NaCl, growth was significantly slower than at 3.5 M. Cells lysed when suspended in media with NaCl concentrations below 1 M. In contrast to most neutrophilic members of the *Halobacteriales*, strain PR5^T showed only a low requirement for Mg^{2+} ions. Magnesium concentrations as low as 5 mM were sufficient for optimal growth and the rod-shaped morphology was maintained at suboptimal Mg^{2+} concentrations. The pH range for growth was 6.5–8.2, with an optimum between 7.0 and 7.5. In the standard medium used (modified SG broth), the isolate grew optimally at 50 °C with a doubling time of 6 h. Growth was obtained at temperatures as high as 60 °C, but not at 62 °C. At 60 °C, most cells were highly elongated, with lengths of 20–25 μm and higher, suggesting that cell division was inhibited at this high temperature.

Isolate PR5^T is chemo-organotrophic and strictly aerobic. Oxidase and catalase reactions were positive. Casamino acids were an excellent source of carbon, nitrogen and energy. Tests with different sugars and related compounds (glucose, sucrose, fructose, mannose, maltose, galactose, lactose, raffinose, cellobiose, trehalose, arabinose, ribose, xylose, mannitol, dulcitol, glycerol) showed that none of these stimulated growth, either when added to the standard growth medium, or when used at a concentration of 5 g l⁻¹ in medium in which the concentrations of Casamino acids and yeast extract were reduced to 0.25 g l⁻¹ each. Production of acids from sugars was never observed. Amylase was not produced. No anaerobic growth was obtained in the presence of L-arginine or nitrate. Some nitrite was produced from nitrate in aerobic cultures. Gas formation on nitrate was never observed. Tween 80 was hydrolysed. Weak gelatinase activity was detected only after prolonged (2–3 weeks) incubation; casein was not hydrolysed. Indole was not produced.

The isolate proved sensitive to anisomycin, bacitracin, novobiocin, vibriostatic agent (O/129) and sulfamethazone plus trimethoprim. No inhibition was observed by ampicillin, penicillin, chloramphenicol, erythromycin, neomycin, streptomycin, tetracycline, kanamycin or vancomycin.

Strain PR5^T possessed both C₂₀,C₂₀ and C₂₀,C₂₅ diether core lipids. Polar lipids detected were phosphatidylglycerol (PG), phosphatidylglycerophosphate methyl ester (PGP-Me) and a single glycolipid, chromatographically identical to S₂-DGD-1 of *Natrialba asiatica* (Kamekura & Dyall-Smith, 1995; Matsubara *et al.*, 1994). Phosphatidylglycerosulfate (PGS) was absent

Fig. 1. Strain PR5^T, as viewed by phase-contrast microscopy. Bar, 10 μm.

Fig. 2. Phylogenetic tree showing the relationship between isolate PR5^T, species allocated to the genera *Haloterrigena* and *Natrinema*, and close relatives, based on 16S rRNA sequences. The tree was constructed using the neighbour-joining method. Bootstrap values (1000 replicates) are shown at the nodes. Bar, 0.014 expected changes per site. The outgroup is *Natronobacterium gregoryi*.

or below the detection limit. A minor, as yet unidentified lipid, running somewhat slower than PGS, was sometimes found.

The G + C content of the DNA of strain PR5^T was 63.3 mol % (HPLC). Comparison of the 16S rDNA sequence (1451 bases determined, with 16 bases missing at the 5' side and 6 bases at the 3' end; GenBank accession number AF115478) with published sequences of other members of the *Halobacteriales* shows strain PR5^T to be related to the genera *Haloterrigena* (Ventosa *et al.*, 1999) and *Natrinema* (McGenity *et al.*, 1998) (Fig. 2).

The phylogenetic distance between strain PR5^T and

the type strains of its closest relatives (Fig. 2) and the differences in phenotypic properties (Table 1) all suggest that PR5^T is sufficiently different from the recognized species within the *Haloterrigena*–*Natrinema* group to warrant its classification as a new species. On the basis of its polar lipid composition it is proposed that strain PR5^T be assigned to the genus *Haloterrigena*. Its DNA relatedness with *Haloterrigena turkmenica*, the only species of the genus described thus far, is sufficiently low (48% hybridization of *Haloterrigena turkmenica* DNA with ³H-labelled DNA from strain PR5^T) and phenotypic properties are sufficiently different to justify the creation of a new species.

Table 1. Some properties differentiating *Haloterrigena thermotolerans* from the validly described species within the genera *Haloterrigena* and *Natrinema*

Strains: 1, *Haloterrigena thermotolerans*; 2, *Haloterrigena turkmenica* VKM B-1734^T; 3, *Natrinema pellirubrum*; 4, *Natrinema pallidum*. +, Positive; –, negative; NR, not reported. Data on *Haloterrigena turkmenica*, *Natrinema pellirubrum* and *Natrinema pallidum* were derived from McGenity *et al.* (1998), Ventosa *et al.* (1999), Zvyagintseva & Tarasov (1987) and from our own observations.

Property	1	2	3	4
Morphology	Rods	Coccoid	Rods*	Rods
Cell size (µm)	4–13	1.5–2.0	1–4	1.5–6.0
Lysis in distilled water	+	–	+	+
Temperature range (°C)	25–60	NR	20–45	NR
Temperature optimum (°C)	50	45	NR	37–40
Use of sugars as carbon source	–	+	+	+
Acid formation from sugars	–	+	–	–
Gelatin liquefaction	Weak	–	+	+
Glycolipid content	S ₂ -DGD-1	S ₂ -DGD-1	One major glycolipid, distinct from S ₂ -DGD-1, running very slowly on TLC plates	Several, unidentified, distinct from S ₂ -DGD-1
Presence of PGS	–	–	–	+
DNA G + C content (mol %)	63.3	59.2–60.2	69.9 (major) [†] , 60.0 (minor)	NR

* *Natrinema pellirubrum* was reported to be rod-shaped (McGenity *et al.*, 1998), but in our cultures cells are extremely pleomorphic.

[†] These data were presented by McGenity *et al.* (1998) as representative for the genus. However, they relate to *Natrinema pellirubrum* only.

DISCUSSION

The 16S rDNA sequence of strain PR5^T clearly demonstrates its affiliation with representatives of the genera *Haloterrigena* (Ventosa *et al.*, 1999) and *Natrinema* (McGenity *et al.*, 1998). These genera were created independently to accommodate a number of strains formerly classified in other genera but phylogenetically unrelated to them and a few new isolates. Some strains have been assigned to both genera and they may thus overlap at least in part. Additional studies will be required to reassess the taxonomic status of the two genera. Similarity percentages between the PR5^T sequence and the type strains of *Haloterrigena turkmenica*, *Natrinema pellirubrum* and *Natrinema pallidum* were 96.2, 96.6 and 96.2%, respectively, sufficiently high to enable classification within either of these genera, but sufficiently low to warrant the creation of a separate taxon at the species level (Devereux *et al.*, 1990).

A unique feature of strain PR5^T is its ability to grow at elevated temperatures: optimum growth was recorded

at 50 °C and growth was possible up to 60 °C. The appearance of greatly elongated cells at 60 °C suggests that the first function to be damaged by the supra-optimal temperatures may be related to cell division. None of the other *Haloterrigena* or *Natrinema* strains described shows such thermophilic or thermotolerant behaviour. Within some of the other genera of the *Halobacteriaceae*, growth was recorded at quite high temperatures, and optima of up to 50 °C for growth have been reported for *Halobacterium salinarum*, *Halorubrum saccharovorum*, *Haloarcula marismortui* and *Haloferax denitrificans*. The most thermotolerant species described thus far seems to be *Haloarcula quadrata* (Oren *et al.*, 1999), which grows optimally between 50 and 53 °C but does not grow above 55 °C, and *Natrialba asiatica* strain B1T, which has its optimum between 35 and 40 °C but can grow up to 52 °C (Kamekura & Dyll-Smith, 1995). However, individual enzymes from halophilic Archaea may display a highly thermophilic character (Keradjopoulos & Holldorf, 1977).

Strain PR5^T shares the presence of S₂-DGD-1 with

Haloterrigena turkmenica VKM B-1734^T. This glycolipid is clearly distinct from the (yet unidentified) glycolipids of *Natrinema pellirubrum* and *Natrinema pallidum*. The genus *Natrinema* is heterogeneous with respect to the glycolipids encountered in the different species. Such a phenomenon may occur in other genera within the *Halobacteriaceae* as well (*Natrialba*, *Halo-rubrum*) (Kamekura, 1998, 1999).

Because of the affiliation of strain PR5^T with the genera *Haloterrigena* and *Natrinema* and the profound differences with the validly described species within these genera, strain PR5^T should be classified as a new species. On the basis of its polar lipid composition, its assignment to the genus *Haloterrigena* appears to be warranted and the name *Haloterrigena thermotolerans* is proposed in view of its ability to grow at high temperatures. For the time being, the description of the species is necessarily based on a single isolate. It is desirable to obtain additional isolates that belong to the same taxon. The property of growth at high temperatures may probably be used in the designation of specific enrichment procedures toward the isolation of other representatives of the species.

Description of *Haloterrigena thermotolerans* sp. nov.

Haloterrigena thermotolerans (ther.mo.to'le.rans. Gr. n. *therme* heat; *tolerans* L. pres. part. of *tolero* tolerate; M.L. adj. *thermotolerans* heat-tolerant).

Cells are rod-shaped, 4–13 µm by 0.7–1.0 µm. Non-motile. Require at least 2 M NaCl for growth. Cells lyse in less than 1 M NaCl. Optimum NaCl concentration for growth is 3.0–3.5 M. Magnesium requirement low, with optimal growth and normal morphology being obtained in 5 mM Mg²⁺. Colonies are circular, entire, convex, opaque, pale red, 0.5–1.0 mm in diameter. Chemo-organotrophic, strictly aerobic. Nitrogen and carbon source: Casamino acids. Sugars (glucose, fructose, sucrose, ribose and others) and glycerol are not used as carbon sources and do not give rise to acid production. Does not produce amylase. Reduces nitrate to nitrite. Does not grow anaerobically in the presence of nitrate or L-arginine. Liquefaction of gelatin weak. Tween 80 hydrolysed. Does not produce indole. Oxidase- and catalase-positive. Possesses C₂₀:C₂₀ and C₂₀:C₂₅ diether core lipids. Polar lipids are PG, PGP-Me and S₂-DGD-1 as sole or main glycolipid. PGS absent or below detection limit. Growth in the pH range 6.5–8.2; optimum 7.0–7.5. Temperature optimum 50 °C; weak growth is possible up to 60 °C. Sensitive to anisomycin, bacitracin, novobiocin, vibriostatic agent (O/129) and sulfamethazone plus trimethoprim. Insensitive to ampicillin, penicillin, chloramphenicol, erythromycin, neomycin, streptomycin, tetracycline, kanamycin and vancomycin. The G+C content of the DNA is 63.3 mol% (HPLC). Type strain: PR5^T, isolated from a saltern crystallizer pond in Puerto Rico, was deposited as DSM 11552^T (= ATCC 700275^T).

ACKNOWLEDGEMENTS

We thank W. D. Grant (University of Leicester) for the gift of *Natrinema pellirubrum* and *Natrinema pallidum* strains, and H. G. Trüper (University of Bonn) for invaluable discussions. The work of R.M.-R. was supported by the Minority Research Career of Excellence (MRCE-NSF) program (HRD-9543554), UPR-NASA EPSCOR (NCC5-215) and the Department of Biology, University of Puerto Rico, Mayaguez Campus. R.H.V. received support from grant number EAR 9714203 from the National Science Foundation. This study was further supported by grant no. 95-00027 from the United States–Israel Binational Science Foundation (BSF, Jerusalem, Israel) (to A.O.), and grant BIO4-CT96-0488 (Extremophiles as Cell Factories) from the European Commission, grant BIO97-1876-CE from the Spanish Ministerio de Educación y Ciencia, and from the Junta de Andalucía (to A.V.).

REFERENCES

- Barns, S. M., Fundyga, R. E., Jeffries, M. W. & Pace, N. R. (1994). Remarkable archaeal diversity in Yellowstone National Park hot spring environment. *Proc Natl Acad Sci USA* **91**, 1609–1613.
- Benlloch, S., Martínez-Murcia, A. J. & Rodríguez-Valera, F. (1995). Sequencing of bacterial and archaeal 16S rRNA genes directly amplified from a hypersaline environment. *Syst Appl Microbiol* **18**, 574–581.
- Bonelo, G., Ventosa, A., Megias, M. & Ruiz-Berraquero, F. (1984). The sensitivity of halobacteria to antibiotics. *FEMS Microbiol Lett* **21**, 341–345.
- Colwell, R. R., Litchfield, C. D., Vreeland, R. H., Kiefer, L. A. & Gibbons, N. E. (1979). Taxonomic studies of red halophilic bacteria. *Int J Syst Bacteriol* **29**, 379–399.
- De Ley, J. & Tijtgat, R. (1970). Evaluation of membrane filter methods for DNA-DNA hybridization. *Antonie Leeuwenhoek* **36**, 461–474.
- Devereux, R., He, S.-H., Doyle, C. L., Orkland, S., Stahl, D. A., LeGall, J. & Whitman, W. B. (1990). Diversity and origin of *Desulfovibrio* species: phylogenetic definition of a family. *J Bacteriol* **172**, 3609–3619.
- Dussault, H. P. (1955). An improved technique for staining red halophilic bacteria. *J Bacteriol* **70**, 484–485.
- Gutiérrez, C. & González, C. (1972). Method for simultaneous detection of proteinase and esterase activities in extremely halophilic bacteria. *Appl Microbiol* **24**, 516–517.
- Hartmann, R., Sickinger, H.-D. & Oesterhelt, D. (1980). Anaerobic growth of halobacteria. *Proc Natl Acad Sci USA* **77**, 3821–3825.
- Holding, A. J. & Collee, J. G. (1971). Routine biochemical tests. *Methods Microbiol* **6A**, 1–32.
- Johnson, J. L. (1981). Genetic characterization. In *Manual of Methods for General Bacteriology*, pp. 450–472. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.
- Kamekura, M. (1998). Diversity of extremely halophilic bacteria. *Extremophiles* **2**, 289–296.
- Kamekura, M. (1999). Diversity of members of the family *Halobacteriaceae*. In *Microbiology and Biogeochemistry of Hypersaline Environments*, pp. 13–25. Edited by A. Oren. Boca Raton: CRC Press.
- Kamekura, M. & Dyll-Smith, M. L. (1995). Taxonomy of the

family *Halobacteriaceae* and the description of two new genera *Halorubrobacterium* and *Natrialba*. *J Gen Appl Microbiol* **41**, 333–350.

Kates, M. (1972). *Techniques of Lipidology*. New York: North Holland/American Elsevier.

Keradjopoulos, D. & Holldorf, A. W. (1977). Thermophilic character of enzymes from extreme halophilic bacteria. *FEMS Microbiol Lett* **1**, 179–182.

McGenity, T. J., Gemmell, R. T. & Grant, W. D. (1998). Proposal of a new halobacterial genus *Natrinema* gen. nov., with two species *Natrinema pellirubrum* nom. nov. and *Natrinema pallidum* nom. nov. *Int J Syst Bacteriol* **48**, 1187–1196.

Mancinelli, R. L. & Hochstein, L. I. (1986). The occurrence of denitrification in extremely halophilic bacteria. *FEMS Microbiol Lett* **35**, 55–58.

Matsubara, T., Iida-Tanaka, N., Kamekura, M., Moldoveanu, N., Ishizuka, H., Hayashi, A. & Kates, M. (1994). Polar lipids of a non-alkaliphilic extremely halophilic archaeobacterium strain 172: a novel bis-sulfated glycolipid. *Biochim Biophys Acta* **1214**, 97–108.

Montalvo-Rodríguez, R. (1996). *Taxonomic studies on extremely halophilic archaeobacteria isolated from the solar salterns of Cabo Rojo, Puerto Rico*. PhD thesis, University of Puerto Rico.

Montalvo-Rodríguez, R., Vreeland, R. H., Oren, A., Kessel, M., Betancourt, C. & López-Garriga, J. (1998). *Halogeometricum borinquense* gen. nov., sp. nov., a novel halophilic archaeon from Puerto Rico. *Int J Syst Bacteriol* **48**, 1305–1312.

Oren, A. (1993). Ecology of extremely halophilic microorganisms. In *The Biology of Halophilic Bacteria*. Edited by R. H. Vreeland & L. I. Hochstein. Boca Raton: CRC Press.

Oren, A. (1994). The ecology of the extremely halophilic archaea. *FEMS Microbiol Rev* **13**, 415–440.

Oren, A., Duker, S. & Ritter, S. (1996). The polar lipid composition of Walsby's square bacterium. *FEMS Microbiol Lett* **138**, 135–140.

Oren, A., Ventosa, A. & Grant, W. D. (1997). Proposed minimal standards for description of new taxa in the order *Halobacteriales*. *Int J Syst Bacteriol* **47**, 233–238.

Oren, A., Ventosa, A., Gutiérrez, M. C. & Kamekura, M. (1999). *Haloarcula quadrata* sp. nov., a square, motile archaeon isolated from a brine pool in Sinai (Egypt). *Int J Syst Bacteriol* **49**, 1149–1155.

Rainey, F. A., Ward-Rainey, N., Kroppenstedt, R. M. & Stackebrandt, E. (1996). The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam. nov. *Int J Syst Bacteriol* **46**, 1088–1092.

Sehgal, S. N. & Gibbons, N. E. (1960). Effect of metal ions on the growth of *Halobacterium cutirubrum*. *Can J Microbiol* **6**, 165–169.

Tindall, B. J. (1985). Qualitative and quantitative distribution of diether lipids in haloalkaliphilic archaeobacteria. *Syst Appl Microbiol* **6**, 243–246.

Torreblanca, M., Rodríguez-Valera, F., Juez, G., Ventosa, A., Kamekura, M. & Kates, M. (1986). Classification of non-alkaliphilic halobacteria based on numerical taxonomy and polar lipid composition, and description of *Haloarcula* gen. nov. and *Haloferax* gen. nov. *Syst Appl Microbiol* **8**, 89–99.

Ventosa, A., Gutiérrez, M. C., Kamekura, M. & Dyal-Smith, M. L. (1999). Proposal to transfer *Halococcus turkmenicus*, *Halobacterium trapanicum* JCM 9743 and strain GSL-11 to *Haloterrigena turkmenica* gen. nov., comb. nov. *Int J Syst Bacteriol* **49**, 131–136.

Zvyagintseva, I. S. & Tarasov, A. L. (1987). Extreme halophilic bacteria from alkaline soils. *Mikrobiologiya* **56**, 839–844.