Salinivibrio proteolyticus sp. nov., a moderately halophilic and proteolytic species from a hypersaline lake in Iran

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A novel moderately halophilic, Gram-negative, curved-rod-shaped bacterium, designated strain AF-2004^T, was isolated from Bakhtegan Lake, a hypersaline lake with 17 % (w/v) total salt located in the southern region of Iran. Strain AF-2004^T was a facultative anaerobe, motile by one polar flagellum, non-sporulating and oxidase- and catalase-positive. It grew at salinities of 1–17 % (w/v) NaCl, showing optimal growth at 5 % (w/v). Growth occurred at 10.0–45.0 °C and pH 5.0–9.5, with optimum growth at 32.0–35.0 °C and pH 8.0–8.5. Phylogenetic analyses based on 16S rRNA gene sequence comparisons indicated that strain AF-2004^T is a member of the genus *Salinivibrio*. Strain AF-2004^T had C_{18:1ω7c} (31.6 %), C_{16:1ω7c} (22.1 %) and C_{16:0} (20.7 %) as the predominant fatty acids and Q-8 as the major respiratory lipoquinone. The DNA G + C content was 49.5 mol%, which is in the range of values for members of the genus *Salinivibrio*. On the basis of differentiation from *Salinivibrio costicola* by phenotypic and chemotaxonomic characteristics, 16S rRNA sequence analysis and DNA–DNA relatedness, it is proposed that strain AF-2004^T (=DSM 19052^T =CIP 109598^T) should be placed in the genus *Salinivibrio* as the type strain of a novel species, *Salinivibrio proteolyticus* sp. nov.

The genus *Salinivibrio* includes moderately halophilic bacteria belonging to the family *Vibrionaceae* within the *Gammaproteobacteria* and, at the time of writing, is represented by a single species, *Salinivibrio costicola*, with three subspecies with validly published names: *S. costicola* subsp. *costicola* (Mellado *et al.*, 1996), *S. costicola* subsp. *vallismortis* (Huang *et al.*, 2000) and *S. costicola* subsp. *alcaliphilus* (Romano *et al.*, 2005). *S. costicola*, formerly classified as *Vibrio costicola*, was first described by Smith (1938) and then included in editions of *Bergey's Manual* and the *Approved Lists of Bacterial Names* (Skerman *et al.*, 1980). In 1987, Garcia and others amended the description of *V. costicola* (Garcia *et al.*, 1987) and, finally, Huang *et al.* (2000) described *S. costicola* subsp. *vallismortis* and further amended the description of *S. costicola*. *S. costicola* is considered to be a representative model for studies of moderately halophilic bacteria and it has been used to study osmoregulatory and other physiological mechanisms in moderate halophiles (Oren, 2003).

Bakhtegan Lake is a hypersaline lake located in the southern region of Iran and its area varies between 1210 and 2400 km², with a maximum depth of 1.1–1.7 m and a mean depth of 0.5 m. Temperatures range from 15 to 45 °C in the shallow parts. Bakhtegan is a seasonal lake with maximum depth in the winter and it dries up in the summer. This unique lake has salty water which contains mostly NaCl, MgCl₂ and Na₂SO₄. The lake is also rich in several organisms, including different kinds of extremophiles that have not been well studied. Our research aimed to screen, isolate and identify moderately halophilic bacteria in this region of Iran. In this paper, a moderately halophilic, aerobic, Gram-negative, curved rod, designated strain AF-2004^T, isolated from Bakhtegan Lake is described. The phenotypic and chemotaxonomic characteristics, DNA G + C content, DNA–DNA relatedness to other subspecies and 16S rRNA gene sequence of this strain have been determined. The data obtained strongly support the suggestion that this strain represents a novel species of the genus *Salinivibrio*.

The salinity of samples obtained from regions near the shore of the lake was determined to be about 17 % (w/v, as total salt) and the pH of the sample was 7.4. Aliquots of the

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AF-2004^T is DQ092443.

sample were added to nutrient broth (NB; Merck) supplemented with 5 % (w/v) NaCl. The pH was adjusted to 7.2–7.4 with 1 M KOH prior to autoclaving. Incubation was carried out at 35 °C under aerobic conditions on an orbital shaking incubator (orbital incubator SI 50; Stuart Scientific) at 150 r.p.m. After 2 days of incubation, agar plates were streaked, resulting in different types of colonies. Pure cultures were isolated after repeated restreaking.

A pure culture of strain AF-2004^T was grown and maintained on nutrient agar (NA; Merck) supplemented with 5 % NaCl at pH 8.0 and 35 °C. The morphological, physiological and biochemical characteristics of the isolate were studied in NB supplemented with 5 % NaCl at pH 8.0 and 35 °C, unless stated otherwise. Reference strains *S. costicola* subsp. *costicola* DSM 11403^T, *S. costicola* subsp. *vallismortis* DSM 8285^T and *S. costicola* subsp. *alcaliphilus* DSM 16359^T were obtained from the DSMZ.

Cell morphology was examined by light microscopy and scanning electron microscopy using cells from exponentially growing cultures. Strain AF-2004^T was prepared for electron microscopy according to the method of Bozzola & Russell (1999) and specimens were observed on a Zeiss DSM 960 EM. Gram staining (Burke method) (Murray *et al.*, 1994) was performed and the result was confirmed by the KOH test (Baron & Finegold, 1990). Motility was analysed by the wet-mount method (Murray *et al.*, 1994). The presence of flagella was examined as described by Kodaka *et al.* (1982). Growth under anaerobic conditions was determined by incubating the isolate in an anaerobic chamber in NB supplemented with 5 % (w/v) NaCl. Cells of the isolate were Gram-negative, non-spore-forming, short, curved rods (Fig. 1) that were highly motile by one polar flagellum. The isolate was a facultative anaerobe. On NA supplemented with 5 % NaCl after 2 days at 35 °C,

Fig. 1. Scanning electron micrograph of cells of *Salinivibrio proteolyticus* sp. nov. AF-2004^T from an exponentially growing culture. Bar, 2 μm.

colonies were creamy white, round with entire edges, smooth, opaque and approximately 2 mm in diameter.

To determine the optimum temperature and pH for growth of the strain, cultures were incubated at 5–55 °C at intervals of 5 °C and pH 4–11 at intervals of 0.5 pH units. The medium was adjusted to below pH 6, pH 6–9 and above pH 9 with sodium acetate/acetic acid, Tris/HCl and glycine/sodium hydroxide buffers, respectively. Growth at different salt contents (0, 2.5, 5, 7.5, 10, 15, 17, 18 and 20 %, w/v) was tested on NB at pH 8.0. Growth was monitored by turbidity at OD₆₀₀ using a photometric method (Shimadzu model UV-160 A). Strain AF-2004^T grew at NaCl concentrations in the range 1–17 % (w/v) in NB. Growth did not occur at concentrations of NaCl greater than 17 % (w/v). The temperature range for growth was 10–45 °C and the pH range was 5.0–9.5. Optimal growth occurred in NB supplemented with 5 % (w/v) NaCl at 32–35 °C and pH 8.0–8.5.

Catalase, oxidase, lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase and urease activities, nitrate reduction, hydrolysis of aesculin and indole production were checked as recommended by Smibert & Krieg (1994). Hydrolysis of Tween 80 was examined as described by Harrigan & McCance (1976). Methyl red and Voges–Proskauer tests were done as recommended by Smibert & Krieg (1994). Acid production from carbohydrates and sugar, carbon and nitrogen source utilization were assessed as recommended by Ventosa *et al.* (1982). Other physiological and biochemical tests, including H₂S production, gelatin hydrolysis, casein hydrolysis, starch hydrolysis and DNase activity, were performed as described previously (Mata *et al.*, 2002; Quesada *et al.*, 1984). Antibiotic susceptibility was determined according to the conventional Kirby–Bauer method (Bauer *et al.*, 1966). Plates were incubated at 35 °C for 48 h and the inhibition zone was interpreted according to the manufacturer's manual. The phenotypic, biochemical and physiological characteristics of strain AF-2004^T are summarized and compared with those of the type strains of related *Salinivibrio* subspecies in Table 1 and in the species description. Although the novel strain shared numerous morphological, biochemical and physiological properties with related *Salinivibrio* subspecies, it could be readily distinguished from these organisms on the basis of the characteristics listed in Table 1.

The 16S rRNA gene of the isolate was amplified using the universal primers 8F (5'-AGAGTTTGGATCCTGGCTCAG-3') and 1525R (5'-AAGGAGGTGATCCAGCC-3'). The amplification was done by initial denaturation at 95 °C for 5 min followed by 10 cycles of 93 °C for 1 min, 63 °C for 1 min and 71 °C for 1.5 min, 20 cycles of 93 °C for 1 min, 67 °C for 1 min and 71 °C for 2 min and a final extension at 71 °C for 5 min. The purified PCR product was sequenced in both directions using an automated sequencer by SeqLab (Göttingen, Germany). Phylogenetic analysis was performed using the neighbour-joining, maximum-parsimony and

Table 1. Comparison of characteristics of strain AF-2004^T and the type strains of the subspecies of *S. costicola*

Strains: 1, AF-2004^T; 2, *S. costicola* subsp. *costicola* DSM 11403^T; 3, *S. costicola* subsp. *alcaliphilus* DSM 16359^T; 4, *S. costicola* subsp. *vallismortis* DSM 8285^T. Data from Garcia *et al.* (1987), Huang *et al.* (2000), Romano *et al.* (2005) and this study. +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4
Sampling site	Hypersaline lake	Salted food	Saltish spring	Death Valley, USA
Cell size (µm)	0.5–0.6 × 1.0–2.3	0.5–0.6 × 1.0–3.2	0.5–0.6 × 3.2–3.5	0.5–0.6 × 1.0–1.8
Oxygen requirement	Facultative anaerobe	Facultative anaerobe	Aerobe	Facultative anaerobe
Growth without NaCl	–	–	–	+
Growth in 17 % NaCl	+	+	+	–
Growth in 20 % NaCl	–	+	+	–
NaCl optimum (%)	5	10	10	2.5
Nitrate reduction	–	–	+	–
Temperature for growth (°C)				
Range	10–45	5–45	10–40	20–50
Optimum	32–35	37	30	37
pH for growth				
Range	5.0–9.5	5–10	7.0–10.5	5.5–8.2
Optimum	8.0–8.5	7.5	9	7.3
Hydrolysis of:				
Starch	+	–	–	+
Tween 80	–	ND	ND	+
Acid production from				
D-Galactose	+	+	ND	–
D-Xylose	+	–	ND	–
D-Lactose	+	–	ND	–
Inositol	+	–	ND	–
Sucrose	–	+	ND	–
Carbon source utilization				
D-Galactose	–	–	+	–
D-Fructose	+	–	+	ND
D-Mannose	–	–	+	+
D-Xylose	–	+	–	+
Sucrose	–	+	+	+
Maltose	+	–	+	–
Lactose	–	–	+	–

maximum-likelihood methods with the software packages PHYLIP (Felsenstein, 1993) and MEGA version 3 (Kumar *et al.*, 2004) after multiple alignment of data available from public databases by CLUSTAL_X (Thompson *et al.*, 1997).

Pairwise evolutionary distances were computed using the correction of Jukes & Cantor (1969). Bootstrap analysis was used to evaluate tree topology by performing 1000 resamplings (Felsenstein, 1985). An almost-complete 16S rRNA gene sequence (1489 bp) of strain AF-2004^T was determined. Alignments of this sequence with sequences from GenBank belonging to the subspecies of *S. costicola* showed that the closest relative of strain AF-2004^T was *S. costicola* subsp. *vallismortis* DSM 8285^T, with a sequence similarity of 99 %. The sequence similarities of strain AF-2004^T to *S. costicola* subsp. *alcaliphilus* DSM 16359^T and *S. costicola* subsp. *costicola* NCIMB 701^T were 97 % and 96.8 %, respectively. The level of 16S rRNA gene sequence similarity between strain AF-2004^T and other Gram-negative rods was less than 93 %. The phylogenetic tree

constructed by the neighbour-joining method is shown in Fig. 2. This phylogenetic tree is also supported by maximum-parsimony and maximum-likelihood methods (not shown). The different algorithms gave consistent results, placing strain AF-2004^T in a cluster together with *S. costicola* subsp. *vallismortis*.

Analysis of isoprenoid quinones was carried out as described by Groth *et al.* (1996). Fatty acid methyl esters were prepared by using cells cultivated on marine agar 2216 (Difco) and analysed following Klatte *et al.* (1994) using the standard Microbial Identification system (MIDI system; <http://www.midi-inc.com/>). The major lipokinone of strain AF-2004^T was Q-8. The fatty acid profile of strain AF-2004^T was dominated by C_{18:1}ω7c (31.6 %), C_{16:1}ω7c (22.1 %) and C_{16:0} (20.7 %). Minor components were C_{12:0} (4.0 %), C_{14:0} 3-OH (3.6 %), C_{14:0} (3.5 %), C_{12:0} 3-OH (2.8 %), C_{18:1}ω9c (2.1 %), C_{16:1}ω9c (1.9 %), C_{18:0} (1.7 %) and C_{19:1}ω6c (1.3 %) (components of less than 1 % were not considered). These data are compatible

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences showing the position of strain AF-2004^T among members of the genus *Salinivibrio*. Bootstrap values greater than 50% are indicated. Bar, 2% estimated sequence divergence.

with the assignment of strain AF-2004^T to the genus *Salinivibrio* (Mellado *et al.*, 1996; Romano *et al.*, 2005).

To determine the DNA base composition and DNA–DNA hybridization, DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). The G+C content was determined by reversed-phase HPLC of nucleosides according to Mesbah *et al.* (1989). DNA–DNA hybridization was carried out in 2 × SSC at 68 °C by the Identification Service of the DSMZ using the method described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) with a model Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). Genomic DNA of the isolate was extracted with a Genelute DNA extraction kit (Sigma) by following the manufacturer's recommended procedure. The G+C content of strain AF-2004^T was 49.5 mol%. DNA–DNA hybridization experiments revealed low levels of relatedness between strain AF-2004^T and *S. costicola* subsp. *costicola* DSM 11403^T (10.1%). According to Wayne *et al.* (1987), less than 70% DNA–DNA relatedness is considered to be the threshold value for the delineation of genospecies, so the value is low enough to separate strain AF-2004^T from the type species of the genus *Salinivibrio*.

Considering the phylogenetic, phenotypic and chemotaxonomic characteristics of the isolate, it is concluded that strain AF-2004^T belongs to the genus *Salinivibrio*. The phylogenetic data presented in this paper indicate that strain AF-2004^T is related to *S. costicola* subsp. *vallismortis*. However, our strain exhibited differences from this and other known subspecies of this genus. Phenotypically, strain AF-2004^T and *S. costicola* subsp. *vallismortis* DSM 8285^T differed from each other in their growth temperature and pH range, salt tolerance, esterase activity, their ability to produce acid from galactose, inositol, lactose and xylose and their utilization of maltose, mannose, sucrose and xylose.

On the basis of evidence obtained from phenotypic data, phylogenetic characteristics and DNA–DNA hybridization, it is proposed that strain AF-2004^T be placed as the type strain of a novel species of the genus *Salinivibrio*, *Salinivibrio proteolyticus* sp. nov.

Description of *Salinivibrio proteolyticus* sp. nov.

Salinivibrio proteolyticus (pro.te.o.ly'ti.cus. N.L. masc. adj. *proteolyticus* proteolytic).

Cells are Gram-negative, short, curved rods, 0.5–0.6 × 1.0–2.3 µm. Highly motile by one polar flagellum. Facultative anaerobe. Endospores are not formed. Colonies are round with entire edges, smooth, slightly convex and creamy white in colour. Growth occurs in media containing 1.0–17.0% (w/v) NaCl; 5% (w/v) is optimal for growth. No growth occurs in the absence of NaCl. The pH range for growth is 5.0–9.5 (optimum at pH 8.0–8.5). Growth occurs at 10.0–45.0 °C (optimum at 32–35 °C). Catalase, oxidase, H₂S production, arginine dihydrolase and Voges–Proskauer tests are positive, but indole, lysine and ornithine decarboxylase, β-galactosidase, urease and methyl red tests are negative. Casein, DNA, gelatin and starch are hydrolysed, whereas aesculin and Tween 80 are not. Acid is produced from D-fructose, D-galactose, D-glucose, inositol, D-lactose, maltose, D-mannose, trehalose and D-xylose, but not from L-arabinose or sucrose. Nitrate is not reduced. Utilizes acetate, dextrin, D-fructose, glycerol, D-glucose, DL-lactate, D-mannitol, maltose, trehalose and starch as sole carbon and energy sources, but not aesculin, citrate, D-galactose, inositol, D-lactose, D-mannose, salicin, sucrose or D-xylose. L-Asparagine, L-glycine, L-ornithine, L-proline and yeast extract are utilized as sole nitrogen and energy sources, but L-arginine, L-phenylalanine and urea are not used. Susceptible to carbenicillin (100 µg), chloramphenicol (30 µg), cotrimoxazole (25 µg), nitrofurantoin (300 µg), polymyxin B (100 U), rifampicin (5 µg) and tetracycline (30 µg); resistant to amoxicillin (30 µg), bacitracin (10 U), cephoxitin (30 µg), kanamycin (30 µg), penicillin G (10 U), streptomycin (10 µg) and tobramycin (10 µg). The major respiratory lipoquinone is

Q-8. The major cellular fatty acids are C_{18:1}ω7c, C_{16:1}ω7c and C_{16:0}.

The type strain is AF-2004^T (=DSM 19052^T =CIP 109598^T), isolated from Bakhtegan Lake, a hypersaline lake with 17 % (w/v) total salt located in southern Iran. The DNA G+C content of strain AF-2004^T is 49.5 mol%.

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