Limimonas halophila gen. nov., sp. nov., an extremely halophilic bacterium in the family *Rhodospirillaceae*

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A novel, Gram-staining-negative, non-pigmented, rod-shaped, strictly aerobic, extremely halophilic bacterium, designated strain IA16^T, was isolated from the mud of the hypersaline Lake Aran-Bidgol, in Iran. Cells of strain IA16^T were not motile. Growth occurred with 2.5–5.2 M NaCl (optimum 3.4 M), at pH 6.0–8.0 (optimum pH 7.0) and at 30–50 °C (optimum 40 °C). Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain IA16^T belonged in the family *Rhodospirillaceae* and that its closest relatives were *Rhodovibrio sodomensis* DSM 9895^T (91.6 % sequence similarity), *Rhodovibrio salinarum* NCIMB 2243^T (91.2 %), *Pelagibius litoralis* CL-UU02^T (88.9 %) and *Fodinicurvata sediminis* YIM D82^T (88.7 %). The novel strain's major cellular fatty acids were $C_{19:0}$ cyclo $\omega 7c$ and $C_{18:0}$ and its polar lipid profile comprised phosphatidylglycerol, diphosphatidylglycerol, four unidentified phospholipids, three unidentified aminolipids and two other unidentified lipids. The cells of strain IA16^T contained the ubiquinone Q-10. The G+C content of the novel strain's genomic DNA was 67.0 mol%. The physiological, biochemical and phylogenetic differences between strain IA16^T and other previously described taxa indicate that the strain represents a novel species in a new genus within the family *Rhodospirillaceae*, for which the name *Limimonas halophila* gen. nov., sp. nov. is proposed. The type strain of *Limimonas halophila* is IA16^T (=IBRC-M 10018^T =DSM 25584^T).

The family *Rhodospirillaceae*, belonging to the order *Rhodospirillales* (Pfennig & Trüper, 1971) of the class *Alphaproteobacteria*, is a morphologically, metabolically and ecologically diverse group. Members of this family include chemo-organotrophs, chemolithotrophs and facultative photoheterotrophs, and some of them are also able to grow photoautotrophically (Garrity *et al.*, 2005). At the time of writing, this family comprises 29 genera, species of which have been isolated from various habitats, such as freshwater, activated sludge biomass, air, soil, roots, cystic fibrosis patients, Antarctic white rock and desert sand (Skerman *et al.*, 1983; Coenye *et al.*, 2002; Garrity *et al.*, 2005; Weon *et al.*, 2007; Yamada *et al.*, 2011; Liu *et al.*, 2011). Each of the genera *Rhodovibrio* (Mack *et al.*, 1993), *Rhodospira* (Pfennig *et al.*, 1997), *Thalassospira* (Kodama *et al.*, 2008), *Thalassobaculum* (Zhang *et al.*, 2008), *Nisaea* (Urios *et al.*, 2008), *Marispirillum* (Lai *et al.*, 2009a), *Oceanibaculum* (Lai *et al.*, 2009b) and *Caenispirillum* (Ritika *et al.*, 2012) include species that were isolated from

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Three supplementary figures are available with the online version of this paper.

saline environments such as seawater and solar salterns and are halotolerant or moderate halophiles. In this paper we present the isolation and polyphasic characterization of an extremely halophilic bacterial strain that represents a novel species in a new genus of the family *Rhodospirillaceae*.

Strain IA16^T was isolated from a saline mud sample [pH 7.5, salinity 22 % (w/v)] collected from the hypersaline Lake Aran-Bidgol in Iran (35° 70′ N 51° 39′ E). The modified growth medium (MGM) with 23 % (w/v) total salt concentration described in the *Halohandbook* was used for the isolation procedure (Dyall-Smith, 2008). This medium was made by mixing 5 g peptone (Oxoid), 1 g yeast extract and 200 ml pure water with 767 ml of a stock salt solution that contained (l^{-1}) 240 g NaCl, 35 g $MgSO_4.7H_2O$, 30 g $MgCl_2.6H_2O$, 7 g KCl and 1 g $CaCl_2$. The pH of the medium was adjusted to pH 7.2–7.4 with Tris-base (Merck). When necessary, agar was added to the medium to give a final concentration of 1.5 % (w/v). The mud sample was diluted in sterile 20 % (w/v) salt solution and then the dilutions were spread on plates of MGM agar. Inoculated plates were incubated aerobically at 40 °C for up to 2 months. After successive cultivation, a pure isolate, designated strain IA16^T, was obtained and routinely grown on MGM agar at 40 °C. Characterization of this strain was achieved by following a polyphasic approach, including the investigation of phenotypic features, chemotaxonomy (polar lipid, fatty acid and quinone analyses) and 16S rRNA gene sequence analysis. For the phenotypic characterization, the standard methods of Smibert & Krieg (1994) were used, after supplementation with salt to provide suitable conditions for the growth of extremely halophilic bacteria.

The genomic DNA of the novel strain was extracted with a High Pure PCR template preparation kit (Roche) according to the manufacturer's instructions. The 16S rRNA gene was then amplified using the bacterial universal primers 27F and 1492R (Lane *et al.*, 1985). Direct sequencing of the PCR-amplified DNA was performed commercially, on an ABI 3730XL DNA sequencer (Applied Biosystems), by Macrogen (Seoul, South Korea). Phylogenetic analysis was performed using version 5 of the MEGA software package (Tamura *et al.*, 2011) after multiple alignments of 16S rRNA gene sequences were made within CLUSTAL_X (Thompson *et al.*, 1997). Clustering was performed using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and minimum-evolution (Rzhetsky & Nei, 1992) methods. Bootstrap analysis with 1000 resamplings (Felsenstein, 1985) was used to evaluate the topology of the neighbour-joining tree.

An almost-complete 16S rRNA gene sequence of strain IA16^T (1407 nt) was obtained. The results of the 16S rRNA gene sequence analysis indicated that strain IA16^T belonged in the family *Rhodospirillaceae*. However, the highest sequence similarities observed in pairwise comparisons between the novel strain and type strains of members of this family (91.6 %, 91.2 %, 88.9 % and 88.7 % with *Rhodovibrio sodomensis* DSM 9895^T, *Rhodovibrio salinarum* NCIMB 2243^T, *Pelagibius litoralis* CL-UU02^T and *Fodinicurvata sediminis* YIM D82^T, respectively) were relatively low. In the neighbour-joining phylogenetic tree, the novel strain clustered with the halophilic members of the family *Rhodospirillaceae* although in a separate clade (Fig. 1). The minimum-evolution tree (Fig. S1, available in IJSEM Online) and maximum-parsimony tree (Fig. S2) each showed a similar relationship.

Cell morphology and motility were examined under an Olympus BX41 microscope equipped with phase-contrast optics. Colony morphology was observed on MGM agar after incubation at 40 °C for 10 days. The Gram reaction was determined by following the method of Dussault (1955). Physiological tests were conducted using MGM broth or agar, unless stated otherwise. Broth cultures were incubated at 40 °C in an orbital incubator at 200 r.p.m. Growth rates were determined by monitoring the increase in optical density at 600 nm. The temperature range for growth was examined in MGM broth at 20–55 °C (at intervals of 5 °C). The pH range for growth was assessed in MGM broth at pH 5.0–9.0 (at intervals of 0.5 pH unit). For this, the pH of the medium was adjusted with 50 mM MES (pH 5.0–6.5), 50 mM HEPES (pH 7.0–8.0) or 50 mM CHES (pH 8.5–9.0). The concentration of NaCl or $MgCl_2$ required for growth was assessed in MGM broth containing 0–5 M NaCl (at 0.5 M intervals) or 0–1 M $MgCl_2$ (at 0.05 M intervals), respectively.

Acid production from carbohydrates (0.1 %, w/v) was tested in unbuffered MGM broth and was determined by measuring the initial and final pH of the medium; the culture was considered positive for acid production if the pH decreased by at least 1 pH unit. In testing for carbon source utilization (1 %, w/v), peptone was omitted from the MGM broth and the yeast extract concentration was reduced to 0.1 g l^{-1} (Oren *et al.*, 1997). The ability of strain IA16^T to grow anaerobically in the presence of DMSO (5.0 g l^{-1}) and to ferment arginine (5.0 g l^{-1}) was tested in MGM broth prepared anaerobically in serum tubes according to the procedures described by Bryant (1972) and Balch & Wolfe (1976). Growth and gas formation with nitrate as an electron acceptor were tested in 10 ml stoppered tubes, completely filled with MGM broth to which $NaNO_3$ (5 g l^{-1}) had been added, and containing an inverted Durham tube (Oren *et al.*, 1997). Hydrolysis of Tweens 20, 40, 60 and 80 was tested as described by Gutiérrez & González (1972). Casein, gelatin and starch hydrolysis was investigated as described by Oren *et al.* (1997). The tests for catalase and oxidase activities were performed as described by González *et al.* (1978). Indole production, hydrolysis of DNA and arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activities were investigated as recommended by Smibert & Krieg (1994). Production of H_2S was tested by growing strain IA16^T in MGM broth supplemented with 0.5 % (w/v) $Na_2S_2O_3$. Susceptibility to antimicrobial compounds was determined by the disc diffusion method, using MGM agar and incubating for 10 days at 40 °C (Oren *et al.*, 1997).

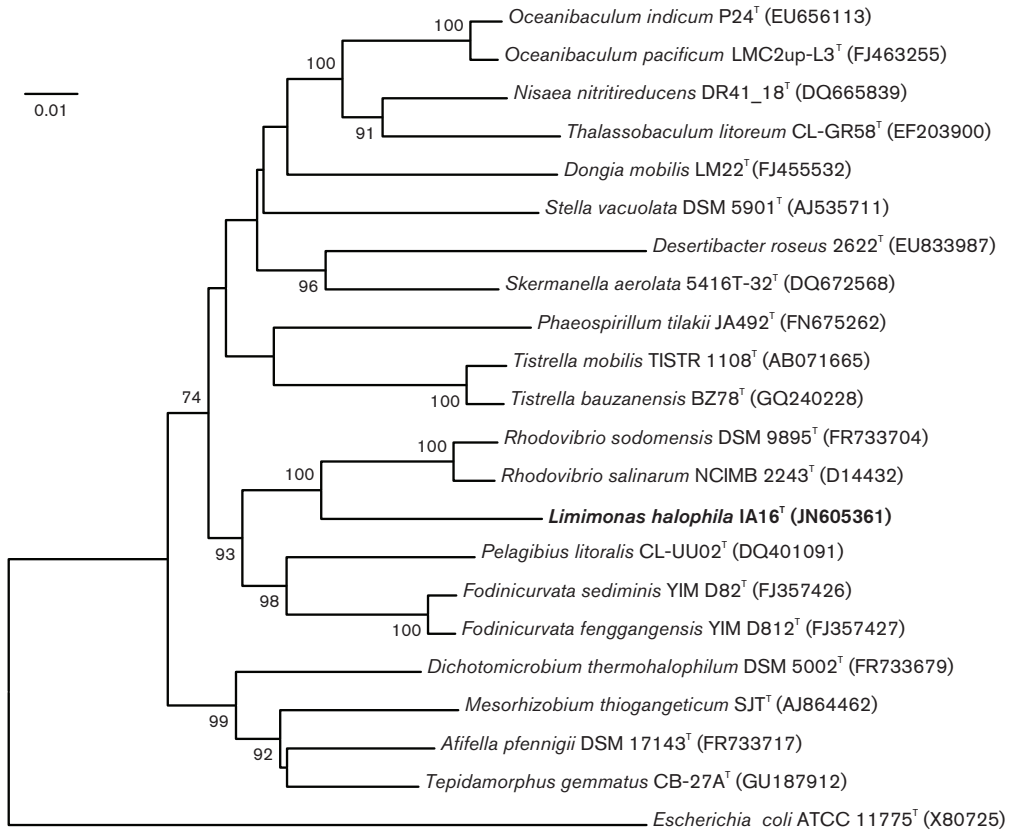

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between strain IA16^T and its close relatives within the family *Rhodospirillaceae*. *Escherichia coli* ATCC 11775^T was used as an outgroup. Bootstrap values (%), based on 1000 replicates, are shown at branching points. Bar, 0.01 substitutions per nucleotide position.

Strain IA16^T was Gram-staining-negative, non-motile, catalase- and oxidase- positive and strictly aerobic. Cells were rods with widths of 0.1–0.2 μm and lengths of 1.5–2.0 μm. After 10 days at 40 °C on MGM, colonies were circular, entire, smooth, non-pigmented and with a diameter of 1 mm. The novel strain was able to grow with 2.5–5.2 M NaCl (optimum 3.4 M), indicating that it was extremely halophilic. Growth did not occur in the absence of magnesium and optimum growth was achieved with 0.2 M $MgCl_2$. The detailed physiological and biochemical characteristics of strain IA16^T are listed in Table 1 as well as in the genus and species descriptions.

For determination of DNA base composition, DNA was isolated using a French pressure cell (Thermo Spectronic) before being purified by chromatography on hydroxyapatite, as described by Cashion *et al.* (1977). The novel strain's genomic DNA G+C content, which was determined by reversed-phase HPLC of nucleosides (Mesbah *et al.*, 1989), is 67.0 mol%.

Cell biomass for the fatty acid, isoprenoid quinone and polar lipid analyses was obtained by cultivation in MGM broth at 150 r.p.m. and 40 °C. Cells were harvested in the mid-exponential growth phase. The whole-cell fatty acid composition of strain IA16^T was determined according to the standard protocol of version 6.1 of the Sherlock Microbial Identification System (MIDI). Extracts were analysed on an HP6890A gas chromatograph (Hewlett Packard) equipped with a flame-ionization detector, by the method of Kämpfer & Kroppenstedt (1996). Fatty acid peaks were identified using the TSBA40 database. The major fatty acids of strain IA16^T were identified as $C_{19:0}$ cyclo $\omega 7c$ (30.1 %) and $C_{18:0}$ (22.7 %) but $C_{18:1}\omega 7c$ (13.8 %), $C_{16:0}$ (12.7 %), $C_{18:1}\omega 9c$ (7.0 %), $C_{14:0}$ 2-OH (3.0 %), $C_{18:1}$ 2-OH (2.4 %), 11 methyl $C_{18:1}\omega 7c$ (2.1 %), $C_{20:2}\omega 6,9c$ (1.9 %), $C_{18:0}$ 3-OH (1.8 %), $C_{16:1}\omega 5c$ (1.8 %) and $C_{14:0}$ (1.1 %) were also detected. This fatty acid profile is distinct from those of the novel strain's closest phylogenetic neighbours, which, like most members of the class *Alphaproteobacteria*, have $C_{18:1}\omega 7c$ as their predominant fatty acid (Labrenz *et al.*, 2000).

Polar lipids were extracted according to the method described by Minnikin *et al.* (1979), separated by two-dimensional TLC and identified by comparison with authentic standards (Sigma) and by spraying with ninhydrin, molybdenum blue and α-naphthol (Embley & Wait,

Table 1. Characteristics that distinguish strain IA16^T from phylogenetically related genera within the family *Rhodospirillaceae*

Taxa: 1, strain IA16^T (data from this study); 2, *Rhodovibrio* (Mack *et al.*, 1993; Imhoff *et al.*, 1998; Garrity *et al.*, 2005; this study), 3, *Pelagibius* (Choi *et al.*, 2009), 4, *Fodinicurvata* (Wang *et al.*, 2009), 5, *Tistrella* (Shi *et al.*, 2002); 6, *Phaeospirillum* (Anil Kumar *et al.*, 2009). +, Positive; −, negative; NA, data not available; MP, monopolar; BP, bipolar.

Characteristic	1	2	3	4	5	6
Habitat	Saline mud	Seawater	Coastal seawater	Salt mine	Wastewater	Freshwater
Colony colour	Non-pigmented	Pink	Cream	Cream–white	NA	Brown
Cell shape	Rod	Vibrioid, spiral	Slightly curved rod	Rod and vibrioid	Rod	Spiral
Cell size (μm)	0.1–0.2 × 1.5–2.0	0.6–0.9 × 1.0–3.5	0.5–1.0 × 1.2–2.5	0.3–0.5 × 0.7–1.5	0.7–1.0	0.8–1.0 × 4–8
Flagella	−	+ (MP, BP)	+ (MP)	−	+ (MP)	+ (MP)
Temperature range (optimum) (°C)	30–50 (40)	25–47 (35–40)	15–33 (28–30)	15–42 (28)	20–40 (30)	25–35 (30)
pH range (optimum)	6–8 (7)	7–8 (7)	6–11 (7–8)	6.5–8.5 (7.5)	5–9 (7.4)	6.5–8.0 (7.0)
Salt requirement (%, w/v)	15–30	3–24	2–6	1.5–20	<1	0
Mg^{2+} requirement	+	−	−	−	−	−
Bacteriochlorophyll *a*	−	+	−	−	−	+
Utilization of carbon sources:						
L-Arabinose	+	−	+	+	+	NA
D-Glucose	+	−	+	+	+	−
D-Mannitol	−	−	+	+	+	−
D-Ribose	+	+	−	−	NA	NA
Sucrose	+	−	−	+	NA	NA
Major quinone(s)	Q-10	Q-10, MK-10	Q-10	Q-10	Q-10	Q-9, MK-9
$C_{18:1}\omega7c$ as predominant fatty acid	−	+	+	+	+	+
DNA G+C content (mol%)	67.0	66.2–68.1	66.3	61.5	67.5	60.54

1994). The polar lipids detected in strain IA16^T were phosphatidylglycerol, diphosphatidylglycerol, four unidentified phospholipids, three unidentified aminolipids and two other unidentified lipids (Fig. S3).

The only respiratory quinone detected in strain IA16^T, using the method described by Groth *et al.* (1996), was the ubiquinone Q-10.

When production of bacteriochlorophyll was investigated, spectrophotometrically (UV-160A, Shimadzu) according to the procedure of Cohen-Bazire *et al.* (1957) and following the recommendations of Allgaier *et al.* (2003), strain IA16^T was found not to produce this photosynthetic pigment.

In conclusion, the results obtained from the polyphasic study indicate that strain IA16^T represents a novel species of a new genus, for which the name *Limimonas halophila* gen. nov., sp. nov. is proposed.

Description of *Limimonas* gen. nov.

Limimonas [Li.mi.mo′nas. L. n. *limus* mud; L. fem. n. *monas* a unit, monad; N.L. fem. n. *Limimonas* a unit (bacterium) isolated from mud].

Cells are Gram-staining-negative, strictly aerobic, non-motile and rod-shaped. Catalase- and oxidase-positive. Extremely halophilic. The polar lipid pattern consists of phosphatidylglycerol, diphosphatidylglycerol, four unidentified phospholipids, three unidentified aminolipids and two other unidentified lipids. Ubiquinone Q-10 is the major isoprenoid quinone. The predominant fatty acids are $C_{19:0}$ cyclo $\omega7c$ and $C_{18:0}$. Phylogenetically affiliated to the *Rhodospirillaceae*. The type species is *Limimonas halophila*.

Description of *Limimonas halophila* sp. nov.

Limimonas halophila [ha.lo′phi.la. Gr. n. *hals*, *halos* salt; N.L. adj. *philus -a -um* (from Gr. adj. *philos -ê -on*) friend, loving; N.L. fem. adj. *halophila* salt-loving].

Exhibits the following properties in addition to those given in the genus description. Cells are rods measuring 0.1–0.2 × 1.5–2.0 μm. After 10 days at 40 °C, colonies on MGM agar are non-pigmented, about 1 mm in diameter, circular and convex, with entire margins. Growth occurs in the presence of 2.5–5.2 M NaCl (optimum 3.4 M) and 0.05–0.7 M $MgCl_2$ (optimum 0.2 M), at pH 6.0–8.0 (optimum pH 7.0), and at 30–50 °C (optimum 40 °C). Does not grow under anaerobic conditions in the presence of nitrate, arginine or DMSO. Nitrate is not reduced. Utilizes D-glucose, D-galactose, sucrose, lactose, D-mannose, trehalose, D-glycerol, D-sorbitol, D-ribose, sodium acetate, L-aspartic acid, L-alanine and L-glycine as sole sources of carbon and energy, but not D-melibiose, D-mannitol, D-xylose, sodium succinate or L-threonine. Acid is not produced from various carbohydrates, including L-arabinose, D-fructose, D-galactose, D-glucose, maltose, D-mannitol, D-ribose, sucrose, trehalose and D-xylose. Tweens 20 and 40 are hydrolysed but DNA, casein, gelatin, starch, Tweens 60 and 80 are not. Indole is not produced from tryptophan. Negative for arginine dihydrolase, lysine

decarboxylase, ornithine decarboxylase and urease activities and for H_2S production. Susceptible to (μg per disc unless indicated otherwise) nitrofurantoin (300), novobiocin (30) and rifampicin (5), but resistant to amikacin (30), amoxicillin (25), bacitracin (10 U), carbenicillin (100), chloramphenicol (30), erythromycin (5), gentamicin (10), kanamycin (5), polymyxin B (100 U), streptomycin (10), tetracycline (30), cephalothin (30), nalidixic acid (30), tobramycin (10) and penicillin G (10 U).

The type strain, IA16^T (IBRC-M 10018^T =DSM 25584^T), was isolated from saline mud collected from Lake Aran-Bidgol in Iran. The genomic DNA G+C content of the type strain is 67.0 mol%.

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