Nitritalea halalkaliphila gen. nov., sp. nov., an alkaliphilic bacterium of the family 'Cyclobacteriaceae', phylum *Bacteroidetes*

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A novel Gram-negative, rod-shaped, non-motile bacterium, designated strain LW7^T, was isolated from a water sample collected at a depth of 4.5 m from Lonar Lake in Buldhana district, Maharashtra, India. The cell suspension was dark-reddish orange due to the presence of carotenoids. The fatty acids were dominated by large amounts of iso-C_{15:0} (59.6 %) and iso-C_{17:0} 3-OH (8.9 %). Strain LW7^T contained MK-4 and MK-5 as the major respiratory quinones and phosphatidylglycerol and phosphatidylethanolamine as the major phospholipids. 16S rRNA gene sequence analysis indicated that *Belliella baltica*, a member of family 'Cyclobacteriaceae' (phylum *Bacteroidetes*), is the closest related species, with a sequence similarity of 94.0 % to the type strain. Other members of the family 'Cyclobacteriaceae' had sequence similarities of <93.3 %. Based on the above-mentioned phenotypic and phylogenetic characteristics, strain LW7^T is proposed as a representative of a new genus and species, *Nitritalea halalkaliphila* gen. nov., sp. nov. The type strain of *Nitritalea halalkaliphila* is LW7^T (=CCUG 57665^T =JCM 15946^T =NCCB 100279^T). The genomic DNA G + C of strain LW7^T is 49 mol%.

Members of the 'Cyclobacteriaceae' have rod- or horseshoe-shaped cells and have been isolated from a wide variety of habitats such as marine surface water (Brettar *et al.*, 2004a), oilfield sediment (Ying *et al.*, 2006), a sea urchin (Nedashkovskaya *et al.*, 2006), alkaline groundwater, coral, marine sediments, soil enriched with boron (Nedashkovskaya *et al.*, 2007), microbial mats from Antarctic lakes (Van Trappen *et al.*, 2004), freshwater lakes (Liu *et al.*, 2009), brown and green algae, tidal flat sediment (Nedashkovskaya *et al.*, 2004), salt-water lagoons (Copa-Patiño *et al.*, 2008), marine solar salterns (Yoon *et al.*, 2005), sea ice (Bowman *et al.*, 2003) and soil (Yoon *et al.*, 2006). Lonar Lake, located in the town of Lonar, Buldhana district, Maharashtra, India (19° 58' N 76° 30' E), is a halo-alkaline water body; several strains belonging to the *Firmicutes*, *Proteobacteria* and *Actinobacteria* have been isolated from the lake (Joshi *et al.*, 2008). *Indibacter alkaliphilus*, a member of the family 'Cyclobacteriaceae', was recently described from Lonar Lake (Anil Kumar *et al.*, 2010). In the present study, we describe a novel bacterium isolated from Lonar Lake that is assigned to a new genus, *Nitritalea* gen. nov., that also belongs to the family 'Cyclobacteriaceae'.

Strain LW7^T was isolated from a water sample collected from Lonar Lake on 25 August 2008. The sample that yielded strain LW7^T had a pH of 10. For isolation of bacteria, 100 µl water sample was plated on ZoBell marine

agar medium (ZoBell, 1941) adjusted to pH 10.0 with sodium carbonate solution (20 %, w/v) and incubated at room temperature for 15 days. Based on the colony morphology, a dark reddish-orange-coloured colony was selected and characterized in the present study.

Cell morphology and motility were studied using a light microscope. Motility was assessed on TSA medium containing (l⁻¹) 17 g pancreatic casein digest, 3 g papaic soyabean meat digest, 5 g sodium chloride, 2.5 g dipotassium hydrogen phosphate, 2.5 g glucose and 0.4 g agar. Growth at different temperatures, salt tolerance, biochemical characteristics, carbon assimilation, H₂S production and the sensitivity of the culture to different antibiotics were determined by previously described methods (Lányi, 1987; Smibert & Krieg, 1994). Biochemical characteristics were also double checked with the Hi25 *Enterobacteriaceae* identification kit (catalogue number KB003) and HiCarbohydrate kit parts A, B and C (catalogue number KB009) (both from HiMedia) according to the manufacturer's protocol. Growth of LW7^T at different pH was checked on TSA medium buffered with citric acid/NaOH (pH 5 and 6), phosphate (pH 7 and 8), glycine/NaOH (pH 9 and 10) or Tris/HCl (pH 11 and 12).

Fatty acid methyl esters were prepared and analysed by the Sherlock Microbial Identification System (MIDI) according to the protocol described by Agilent Technologies. For this purpose, strain LW7^T and reference strains *Belliella baltica* DSM 15883^T and *I. alkaliphilus* LW1^T were grown

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LW7^T is FM991866.

on TSA medium at 30 °C for 2 days. Polar lipids were extracted and analysed according to the method described by Komagata & Suzuki (1987). Menaquinones and polar lipids were determined from freeze-dried cells. Menaquinones were extracted as described by Collins *et al.* (1977) and analysed by HPLC (Groth *et al.*, 1997). DNA was isolated according to the procedure of Marmur (1961) and the G+C content was determined from melting point (T_m) curves (Sly *et al.*, 1986) obtained by using a Lambda 2 UV-Vis spectrophotometer (Perkin Elmer) equipped with the Templab 2.0 software package (Perkin Elmer). DNA from *Escherichia coli* DH5 α was used as a standard in determining the G+C content of the DNA. For 16S rRNA gene sequencing, DNA was prepared using a MoBio microbial DNA isolation kit (MoBio Laboratories Inc.) and sequenced as described previously (Lane, 1991). The resultant almost-complete sequence of

the 16S rRNA gene contained 1502 nt. The 16S rRNA gene sequence of the isolate was subjected to BLAST sequence similarity search (Altschul *et al.*, 1990) and EzTaxon (Chun *et al.*, 2007) to identify the nearest taxa. All 16S rRNA gene sequences belonging to members of the family 'Cyclobacteriaceae' were downloaded from the NCBI database (<http://www.ncbi.nlm.nih.gov>) and aligned using the CLUSTAL_X program (Thompson *et al.*, 1997) and the alignment was corrected manually. Phylogenetic trees were constructed using the maximum-likelihood algorithm using the PhyML program (Guindon & Gascuel, 2003) and the neighbour-joining method (Saitou & Nei, 1987) using the PHYLIP package, version 3.5 (Felsenstein, 1993), and the resultant tree topologies were evaluated by bootstrap analysis based on 1000 resamplings using the SEQBOOT and CONSENSE programs in the PHYLIP package. Pairwise evolutionary distances were computed using the

Table 1. Fatty acid compositions of strain LW7^T and closely related members of the family 'Cyclobacteriaceae'

Strains: 1, LW7^T; 2, *B. baltica* DSM 15883^T; 3, *I. alkaliphilus* LW1^T. Results are percentages of total fatty acids; fatty acids amounting to 5% or more of the total are in bold. Data for all strains are from the present study, and were obtained from cultures grown on TSA at 30 °C for 48 h. —, Not detected; tr, trace (<1 %).

Fatty acid	1	2	3
C _{14:0}	tr	—	—
iso-C _{14:0}	tr	—	—
iso-C _{15:1} G	—	8.7	—
iso-C _{15:0}	59.6	21.1	47.0
anteiso-C _{15:0}	3.1	3.8	7.6
C _{15:0} 3-OH	—	2.1	3.7
iso-C _{15:0} 3-OH	2.1	2.9	2.1
C _{15:1} ω 6c	1.6	—	—
C _{16:0}	1.2	6.0	3.5
iso-C _{16:0}	1.8	2.9	2.3
C _{16:0} 3-OH	—	1.6	—
iso-C _{16:0} 3-OH	tr	—	—
iso-C _{16:1} H	tr	—	—
C _{16:1} ω 5c	2.4	8.9	—
C _{17:0} 2-OH	—	—	—
iso-C _{17:0}	1.4	1.8	3.5
anteiso-C _{17:0}	tr	—	—
iso-C _{17:0} 3-OH	8.9	6.5	8.2
C _{17:1} ω 6c	1.4	4.0	—
iso-C _{17:1} ω 9c	—	9.5	17.6
anteiso-C _{17:1} ω 9c	—	—	—
C _{18:0}	—	—	—
iso-C _{18:0}	1.5	—	—
iso-C _{19:0}	tr	—	—
Summed feature 3*	3.7	20.2	3.3
Summed feature 4*	1.4	—	—
Summed feature 9*	2.7	—	—

*Summed features are groups of two or three fatty acids that could not be separated by the MIDI system. Summed feature 3 contained iso-C_{15:0} 2-OH and/or C_{16:1} ω 7c; summed feature 4 contained iso-C_{17:1} I and/or anteiso-C_{17:1} B; summed feature 9 contained iso-C_{17:1} ω 9c and/or 10-methyl C_{16:0}.

DNADIST program with Kimura's two-parameter model (Kimura, 1980).

Cells of strain LW7^T were rods, 0.7–0.8 µm wide and 2–3 µm long, and multiplied by binary fission. Cells stained Gram-negative and were non-motile. Colonies were circular, 2–3 mm in diameter, smooth, dark reddish orange, opaque, convex and entire on half-strength marine agar. The strain grew aerobically and chemoheterotrophically. Growth was observed at 25–40 °C, with optimum growth at 37 °C. Growth was observed at 1–22 % NaCl (w/v), with optimum growth between 2 and 5 % NaCl (w/v), and at pH 7.5–12, with optimum growth at pH 10. The spectrum of the ethanol extract of strain LW7^T showed a broad peak with a maximum around 480 nm that is typical of carotenoids. Alkalinization did not show a bathochromic shift of the peaks. Therefore, it was concluded that strain LW7^T contains carotenoids but no flexirubins, as often observed for marine bacteria belonging to the *Bacteroidetes* (Brettar *et al.*, 2004a). The cellular fatty acid composition of strain LW7^T showed a spectrum of 20 fatty acids, with a pronounced dominance of the saturated fatty acid iso-C_{15:0} (Table 1). The saturated fatty acids constituted 85.6 % of the total, with iso-C_{15:0} forming the major fraction (59.6 %). When compared with *B. baltica* DSM 15883^T, the number of fatty acids detected in strain LW7^T was higher and the composition differed considerably (Table 1). The menaquinones present in LW7^T were MK-4 (36.3 %) and MK-5 (63.7 %), and the phospholipids were phosphatidylglycerol and phosphatidylethanolamine. The other characteristics of LW7^T are listed in the species description. The DNA G + C content of strain LW7^T was 49 mol%.

The phylogenetic relationship of strain LW7^T was ascertained based on 16S rRNA gene sequence similarity to

other reported sequences using BLAST sequence similarity search (NCBI BLAST/EzTaxon). The results indicated that, at the 16S rRNA gene sequence level, strain LW7^T was close to *B. baltica* BA134^T, with a pairwise similarity of 94 %. The BLAST results indicated that strain LW7^T probably belongs to a new genus. Phylogenetic analyses based on maximum-likelihood and neighbour-joining trees further indicated that strain LW7^T clustered with *B. baltica*, at a phylogenetic distance of 92.5 %, and distinct from the clades represented by the genera *Algoriphagus*, *Aquiflexum*, *Cyclobacterium*, *Echinicola* and *Indibacter* (Fig. 1).

Strain LW7^T could also be differentiated phenotypically from the closely related species *B. baltica* (Brettar *et al.*, 2004a) (Table 2). For instance, LW7^T differs from *B. baltica* BA134^T with respect to colony colour, cell size, optimal salt concentration for growth, salt tolerance, growth at pH 12 and DNA G + C content (Table 2). In addition, LW7^T shows specific differences from *B. baltica* BA134^T with respect to acid production and utilization of various carbon sources. *I. alkaliphilus*, a member of the family 'Cyclobacteriaceae', was described from the same source as strain LW7^T (Anil Kumar *et al.*, 2010), although at a different depth. Strain LW7^T differed from *I. alkaliphilus* phylogenetically (strain LW7^T clustered with *B. baltica* but *I. alkaliphilus* forms a separate clade from this cluster; Fig. 1), phenotypically (nitrate reduction, salinity range, hydrolysis of gelatin and starch, acid production and substrate utilization; Table 2) and chemotaxonomically (DNA G + C content and fatty acid composition; Tables 1 and 2). Thus, the cumulative differences that strain LW7^T exhibits from these closely related species unambiguously support the creation of a new genus and species, for which the name *Nitritalea halalkaliphila* gen. nov., sp. nov. is proposed.

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain LW7^T with members of the 'Cyclobacteriaceae'. The tree was constructed using the maximum-likelihood method. Numbers at nodes are bootstrap percentages. Bar, 0.02 substitutions per alignment position.

Table 2. Characteristics that differentiate strain LW7^T (*Nitritalea* gen. nov.) from closely related genera in the family 'Cyclobacteriaceae'

Data for reference genera were taken from this study (major fatty acids in *Belliella* and *Indibacter*), Brettar *et al.* (2004a) and Arun *et al.* (2009) (*Belliella*), Anil Kumar *et al.* (2010) (*Indibacter*), Bowman *et al.* (2003) and Liu *et al.* (2009) (*Algoriphagus*), Brettar *et al.* (2004b) (*Aquiflexum*), Raj & Maloy (1990), Nedashkovskaya *et al.* (2005) and Ying *et al.* (2006) (*Cyclobacterium*) and Nedashkovskaya *et al.* (2006) (*Echinicola*). +, Positive; –, negative; v, variable; ND, no data available.

Characteristic	Strain LW7 ^T	<i>Belliella</i>	<i>Indibacter</i>	<i>Algoriphagus</i>	<i>Aquiflexum</i>	<i>Cyclobacterium</i>	<i>Echinicola</i>
Cell morphology	Regular rods	Regular rods	Regular rods	Coccobacilli to short filaments with rounded ends	Regular rods	Ring-like/horseshoe-shaped	Regular rods
Gliding motility	–	–	–	–	–	–	+
Nitrate reduction	–	+	+	v	+	–	–
H ₂ S production	–	ND	–	–	ND	–	+
Salinity range (%)	1–22	0–6	0–8	0–10	0–6	0–10	0–12
Growth at 42 °C	–	–	–	v	–	v	–
Hydrolysis of:							
Agar	–	–	–	v	–	–	+
Gelatin	+	–	–	v	+	–	+
Starch	–	+	+	v	+	–	+
Major fatty acids*	i-C _{15:0} , i-C _{17:0} , 3-OH	i-C _{15:1} G, i-C _{15:0} , C _{16:0} , C _{16:1} ω5c, i-C _{17:0} 3-OH, i-C _{17:1} ω9c, C _{16:1} ω6c	i-C _{15:0} , ai-C _{15:0} , i-C _{17:0} 3-OH, i-C _{17:1} ω9c	i-C _{15:1} ω10c, i-C _{15:0} , i-C _{16:1} ω6c, C _{16:1} ω7c, C _{16:1} ω5c, i-C _{15:0} 2-OH, ai-C _{15:0} 2-OH	i-C _{15:0} , ai-C _{15:0} , i-C _{15:1} G, i-C _{16:1} H	i-C _{15:0} , ai-C _{15:0} , i-C _{15:1} , i-C _{17:1} ω9c, i-C _{17:0} 3-OH, SF3	i-C _{15:0} , C _{16:1} ω5c, i-C _{17:1} ω9c, C _{17:1} ω6c, i-C _{15:0} 3-OH, i-C _{17:0} 3-OH, i-C _{15:0} 2-OH, C _{16:1} ω7c
DNA G + C content (mol%)	49	35.4–40	43	37–49	38.3–38.7	41–45	44–45.9
Isolation source(s)	Haloalkaline water	Marine, estuarine, mud volcano	Haloalkaline water	Marine	Marine, estuarine	Marine	Marine

*ai, Anteiso-branched; i, iso-branched; SF3, summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c).

Description of *Nitritalea* gen. nov.

Nitritalea (Ni.tri.ta'le.a. L. n. *nitrum* natural soda; L. fem. n. *talea* a rod, a stick; N.L. fem. n. *Nitritalea* a rod or stick from natural soda, referring to the isolation of the first strain from a soda lake).

Cells stain Gram-negative and are aerobic rods. Positive for catalase, oxidase and gelatinase and negative for amylase, lipase and urease. The major fatty acids are iso-C_{15:0} and iso-C_{17:0} 3-OH and MK-4 and MK-5 are the predominant respiratory quinones. The phospholipids include phosphatidylglycerol and phosphatidylethanolamine. The genus is affiliated to the family 'Cyclobacteriaceae' of the order 'Sphingobacteriales' of class 'Sphingobacteria'. The type species is *Nitritalea halalkaliphila*.

Description of *Nitritalea halalkaliphila* sp. nov.

Nitritalea halalkaliphila (hal.al'ka.li.phi'la. Gr. n. *hals*, *halos* salt; N.L. n. *alkali* alkali; N.L. fem. adj. *phila* from Gr. fem. adj. *philê* friendly to, loving; N.L. fem. adj. *halalkaliphila* loving salt and alkaline conditions).

Displays the properties described for the genus as well as the following properties. Cells are 0.7–0.8 µm wide and 2–3 µm long, occur singly and multiply by binary fission. Colonies on half-strength marine agar are circular, 2–3 mm in diameter, smooth, dark reddish orange, opaque, convex and entire. Grows at 25–40 °C with optimum growth at 37 °C and tolerates up to 22.0% NaCl (w/v). Growth occurs at pH 7.5–12.0. Tests for ornithine decarboxylase and lysine decarboxylase are positive; tests for β-galactosidase, arginine dihydrolase and phenylalanine deaminase are negative. The methyl red and Voges–Proskauer reactions are negative. Gelatin is hydrolysed but starch, Tween 60 and aesculin are not. Nitrate is not reduced to nitrite and H₂S gas is not produced. Produces acid from sucrose, glucose, sorbitol, cellobiose, inositol, D-fructose, mannose, dulcitol, inulin and melibiose but not from xylose, erythritol, raffinose, methyl α-D-glucoside, arabinose, maltose, amygdalin, galactose, mannitol, glycogen, ribose, inositol, sorbose, mannoside or aesculin, even after 1 week of incubation at optimum temperature and pH. Assimilates pyruvic acid, succinic acid, citrate, sucrose, lactose, fructose, glucose, raffinose, trehalose, melibiose, sucrose, mannose, inulin, dulcitol, inositol, cellobiose, *o*-nitrophenyl β-galactoside, malonate, isoleucine, DL-aspartic acid, L-valine, L-histidine, glutamine and glutamic acid but not acetate, aesculin, xylose, maltose, galactose, L-arabinose, sodium gluconate, glycerol, salicin, glucosamine, sorbitol, mannitol, adonitol, methyl α-D-glucoside, ribose, rhamnose, melezitose, methyl α-D-mannoside, xylitol, D-arabinose, sorbose, L-proline, phenylalanine, L-arginine, threonine, L-tyrosine, L-serine, leucine, L-methionine or D-gluconic acid. Susceptible to (µg per disc unless indicated) amikacin (30), ampicillin (10), colistin (10), cefuroxime (30), norfloxacin (10), erythromycin (15), novobiocin (30), cefoperazone (75),

streptomycin (10), ciprofloxacin (5), gentamicin-G (30), cefazolin (30), nitrofurantoin (300), carbenicillin (100), penicillin G (10), lomefloxacin (30), co-trimoxazole (25), tetracycline (30), rifampicin (30), chloramphenicol (30), bacitracin (10), roxithromycin (30), polymixin B (50 U), lincomycin (2) and vancomycin (30) and resistant to kanamycin (30), nalidixic acid (30) and tobramycin (10). The cellular fatty acid composition of the type strain is detailed in Table 1. The genomic DNA G + C content of the type strain is 49 mol%.

The type strain, LW7^T (=CCUG 57665^T =JCM 15946^T =NCCB 100279^T), was isolated from a water sample collected at a depth of 4.5 m from Lonar Lake, Lonar, Buldhana district, Maharashtra, India.

Acknowledgements

We would like to thank Dr S. W. A. Naqvi, Scientist, National Institute of Oceanography, Goa, India, for providing the samples. S. S. is thankful to the National Centre for Antarctic and Ocean Research, Goa, Department of Biotechnology, New Delhi, and the CSIR Network Project on Biodiversity for funding. T. N. R. S. acknowledges the CSIR, Government of India, for the award of a Research Associateship.

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