Litoribacter ruber gen. nov., sp. nov., an alkaliphilic, halotolerant bacterium isolated from a soda lake sediment

Shi-Ping Tian,^{1†} Yong-Xia Wang,^{1†} Bin Hu,¹ Xiao-Xia Zhang,² Wei Xiao,¹ Yun Chen,¹ Yong-Hong Lai,¹ Meng-Liang Wen¹ and Xiao-Long Cui¹

Correspondence

Xiao-Long Cui
xlcuiynu@yahoo.com.cn or
xlcui@ynu.edu.cn

¹The Key Laboratory for Microbial Resources of the Ministry of Education, and Laboratory for Conservation and Utilization of Bio-Resources, Yunnan Institute of Microbiology, Yunnan University, Kunming, Yunnan 650091, PR China

²Agricultural Cultural Collection of China, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100080, PR China

A novel alkaliphilic, halotolerant, rod-shaped bacterium, designated strain YIM CH208^T, was isolated from a soda lake in Yunnan, south-west China. The taxonomy of strain YIM CH208^T was investigated by a polyphasic approach. Strain YIM CH208^T was Gram-negative, strictly aerobic and non-motile and formed red colonies. Optimal growth conditions were 28 °C, pH 8.5 and 0.5–2.5% NaCl. Phylogenetic analysis based on 16S rRNA gene sequence comparisons showed that the isolate formed a distinct line within a clade containing the genus *Echinicola* in the phylum *Bacteroidetes* and was related to the species *Echinicola pacifica* and *Rhodonellum psychrophilum*, with sequence similarity of 91.7 and 91.6% to the respective type strains. The DNA G+C content was 45.1 mol%. The major respiratory quinone was menaquinone-7 (MK-7). The predominant cellular fatty acids were iso-C_{17:1}ω9c (19.9%), C_{15:0} 3-OH (12.1%), iso-C_{17:0} 3-OH (11.3%), summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c; 10.7%) and C_{17:1}ω6c (8.7%). On the basis of the phenotypic, chemotaxonomic and phylogenetic data, strain YIM CH208^T represents a novel species of a new genus, for which the name *Litoribacter ruber* gen. nov., sp. nov. is proposed. The type strain of *Litoribacter ruber* is YIM CH208^T (=AACC 05414^T =KCTC 22899^T).

Bacteria belonging to the phylum *Bacteroidetes* (Garrity & Holt, 2001) are considered to be of special importance in aquatic environments. In marine and freshwater environments, bacteria of the phylum *Bacteroidetes* can be abundant. This phylum includes Gram-negative, heterotrophic bacteria with the capacity to degrade complex organic compounds such as chitin, cellulose (Cottrell & Kirchman, 2000) and agar (Nedashkovskaya *et al.*, 2003), and they play an important role in carbon cycling in the environment. In volume 4 of *Bergey's Taxonomic Outline* (Ludwig *et al.*, 2008), the phylum *Bacteroidetes* includes four classes, 'Bacteroidia', *Flavobacteria*, 'Sphingobacteria' and 'Cytophagia'. The class 'Cytophagia' includes three families, of which the *Cytophagaceae* (previously 'Flexibacteraceae') is

the largest. In this paper, the characterization and classification of a strain that represents a novel genus-level group of the family *Cytophagaceae* is reported.

During the investigation of cultured microbial diversity of alkaline environments in Yunnan, south-west China, strain YIM CH208^T was isolated from a sediment sample collected from the shore of a soda lake in Yongsheng. The strain was isolated by the standard dilution-plating technique on marine agar 2216 (MA; Difco) at 28 °C. Strain YIM CH208^T was subsequently maintained on MA slants and stored as 20% (v/v) glycerol suspensions at –80 °C.

Morphological, physiological and biochemical characteristics of strain YIM CH208^T were investigated using routine cultivation on MA. Gram staining was carried out using the standard Gram reaction combined with the KOH lysis test method (Gegersen, 1978). Cell morphology was examined by light microscopy (Olympus) after staining. Gliding motility was determined using phase-contrast light microscopy (Olympus) after growth on quarter-strength MA solidified with 1% agar according to

†These authors contributed equally to this work.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM CH208^T is GU254164.

A 16S rRNA gene sequence-based maximum-likelihood tree and the absorption spectrum of an acetone/methanol extract of strain YIM CH208^T are available as supplementary material with the online version of this paper.

Bowman (2000). The pigment composition was examined using sonicated cell suspensions, as well as acetone/methanol (7:2, v/v) extracts. Absorption spectra were determined between 300 and 800 nm with a UV spectrophotometer. Growth was also tested on trypticase soy agar (TSA; BBL) and nutrient agar. Growth at 4–50 °C and pH 5.0–11.0 was determined in trypticase soy broth (TSB). To test NaCl tolerance, TSA containing 0–15 % (w/v) NaCl was used. Growth under anaerobic conditions was determined after incubation in an anaerobic jar (GasPak Anaerobic Systems; BBL) on TSA. Degradation of aesculin, cellulose, gelatin, starch and Tweens 20, 40 and 80 was determined according to the protocols described by Cowan & Steel (1965). Catalase activity was determined by assessing bubble production in 3 % (v/v) H₂O₂, and oxidase activity was determined using a 1 % (w/v) solution of tetramethyl-*p*-phenylenediamine (Kovács, 1956). Carbon utilization was tested using artificial seawater medium (Cho & Giovannoni, 2006) as the basal medium containing each carbon source at a final concentration of 0.5 % (w/v or v/v). Acid production from carbon sources was assessed by using API 50CH and enzyme activity tests were performed using the API ZYM system (bioMérieux) according to the manufacturer's instructions. Other biochemical tests were carried out with the API 20NE kit (bioMérieux). Antibiotic sensitivities were determined using the disc-diffusion method (Bauer *et al.*, 1966).

Isoprenoid quinones were extracted according to the method of Collins *et al.* (1977) and analysed by HPLC as described by Tamaoka *et al.* (1983). Biomass for

quantitative fatty acid analysis of the isolate was obtained by scraping cells from TSA plates that had been incubated for 5 days at pH 8.5 and at 28 °C. Analysis of the whole-cell fatty acid pattern followed the method of Sasser (1990) using the Microbial Identification System (MIDI).

Genomic DNA extraction, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out as described previously (Cui *et al.*, 2001). The sequence was compared to those available in GenBank/EMBL/DBJ using BLAST (Altschul *et al.*, 1990). Alignments and similarities were obtained by the CLUSTAL_X method. Phylogenetic analyses were carried out using MEGA3 (Kumar *et al.*, 2004). Distances (corrected by Kimura's two-parameter model; Kimura, 1980) were calculated and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). A maximum-likelihood (Felsenstein, 1981) tree was generated using the treeing algorithm contained in the PHYLIP package (Felsenstein, 1993). Bootstrap analysis was used to evaluate the tree topology by means of 1000 resamplings (Felsenstein, 1985).

Phylogenetic analysis of the almost-complete 16S rRNA gene sequence (1450 bp) revealed that the isolate occupied a distinct lineage with *Echinicola* species within the family *Cytophagaceae* (Fig. 1 and Supplementary Fig. S1, available in IJSEM Online). The topology of the phylogenetic tree constructed using the neighbour-joining algorithm was similar to that of the tree constructed using maximum-likelihood analysis. Sequence analysis revealed that strain YIM CH208^T was most closely related to *Echinicola pacifica* KMM 6172^T (91.7 % 16S rRNA gene sequence

Fig. 1. Neighbour-joining tree based on almost-complete 16S rRNA gene sequences showing the phylogenetic position of strain YIM CH208^T. Numbers at nodes indicate percentages of 1000 bootstrap resamplings; only values >50 % are given. Bar, 0.01 substitutions per nucleotide position. *Roseivirga echinicomitans* KMM 6058^T was used as an outgroup.

similarity), *Rhodonellum psychrophilum* GCM71^T (91.6 %), *Algoriphagus hitonicola* 7-UAH^T (91.6 %), *Echinicola vietnamensis* LMG 23754^T (91.3 %) and *Belliella baltica* BA134^T (91.2 %) and exhibited less than 91 % sequence similarity to other members of the phylum *Bacteroidetes*. Because of these low similarities, DNA–DNA hybridization experiments were not performed. The rather low levels of sequence similarity also suggest that the isolate belongs to a novel genus-level taxonomic group.

Cells were Gram-negative, catalase- and oxidase-positive rods, 0.3–0.5 µm wide and 1.2–1.5 µm long. Gliding motility could not be observed by phase-contrast microscopy. Growth occurred on TSA and nutrient agar. Colonies were smooth, circular, convex, opaque and bright red on MA and TSA. Growth did not occur under anaerobic conditions. Growth occurred in media containing 0–9 % (w/v) NaCl and optimal growth was observed in media containing 0.5–2.5 % (w/v) NaCl. Strain YIM CH208^T grew at pH 7.5–10.5 and showed optimal growth at pH 8.5. Strain YIM CH208^T was alkaliphilic and halotolerant, but the isolate differs from two recently described alkaliphilic members of the family ‘*Cyclobacteriaceae*’, *Nitritalea halalkaliphila* and *Indibacter alkaliphilus* (Anil Kumar *et al.*, 2010a, b), in pH and salinity ranges for growth, nitrate reduction, hydrolysis of gelatin and starch and DNA G+C content (Table 1). The temperature range for growth of strain YIM CH208^T was 20–37 °C and the optimal temperature was 28 °C, whereas the temperature ranges for growth of *Echinicola* and *Rhodonellum* species are 6–44 °C and

0–22 °C, respectively (Table 1). In addition, strain YIM CH208^T differs from *Echinicola* and *Rhodonellum* species in the capacity for nitrate reduction and salinity ranges (Table 1). Strain YIM CH208^T shows differences from *Belliella* species with respect to salinity and temperature ranges and DNA G+C content (Table 1). Other features that distinguish the isolate from its phylogenetic relatives are shown in Table 1.

The genomic DNA G+C content was determined by HPLC according to Mesbah *et al.* (1989) after DNA extraction according to the method of Cui *et al.* (2001). Genomic DNA from *Escherichia coli* DH5α was used as a standard. The DNA G+C content of the isolate was 45.1 mol%.

The major cellular fatty acids of strain YIM CH208^T were iso-C_{17:1}ω9c (19.9 %), C_{15:0} 3-OH (12.1 %), iso-C_{17:0} 3-OH (11.3 %), summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c, 10.7 %) and C_{17:1}ω6c (8.7 %). Strain YIM CH208^T could be differentiated chemotaxonomically from closely related species of the family ‘*Cyclobacteriaceae*’. For instance, strain YIM CH208^T differs from related species on the basis of the relatively large amount of C_{15:0} 3-OH and small amount of iso-C_{15:0} (Table 2). *Nitritalea halalkaliphila* (Anil Kumar *et al.*, 2010b) differs clearly from strain YIM CH208^T in its fatty acid composition and respiratory quinone (Table 2; Anil Kumar *et al.*, 2010a). The isoprenoid quinones of the strain were MK-7 (97 %) and MK-5 (3 %). The absorbance spectrum of strain YIM CH208^T showed a broad peak with maximum absorbance

Table 1. Differential phenotypic properties of *Litoribacter ruber* gen. nov., sp. nov. YIM CH208^T and its closest phylogenetic neighbours

Taxa: 1, strain YIM CH208^T (*Litoribacter ruber* gen. nov., sp. nov.); 2, *Echinicola* (data from Nedashkovskaya *et al.*, 2006, 2007); 3, *Rhodonellum* (Schmidt *et al.*, 2006); 4, *Belliella* (Brettar *et al.*, 2004a; Arun *et al.*, 2009); 5, *Algoriphagus* (Bowman *et al.*, 2003; Liu *et al.*, 2009); 6, *Nitritalea* (Anil Kumar *et al.*, 2010b); 7, *Indibacter* (Anil Kumar *et al.*, 2010a); 8, *Aquiflexum* (Brettar *et al.*, 2004b). +, Positive; –, negative; v, variable; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8
Source	Soda lake	Marine	Tufa column	Marine, mud volcano	Marine	Haloalkaline lake water	Haloalkaline lake water	Marine
Cell morphology	Rods	Regular rods	Rods	Regular rods	Coccobacillus to short filaments with rounded ends	Regular rods	Regular rods	Regular rods
Motility	–	+	–	–	–	–	–	–
Salinity range for growth (%)	0–9	0–15	0–3	0–6	0–10	1–22	0–8	0–6
pH range for growth	7.5–10.5	ND	7.5–10.7	6–9/6–10	v	7.5–12	7.5–12	7–9
Temperature for growth (°C)								
Range	20–37	6–44	0–22	4–40	4–42	25–40	15–40	4–40
Optimum	28	25–28/30–32	5	25/37	v	37	30–37	30
Nitrate reduction to nitrite	+	–	–	v	v	–	+	+
Hydrolysis of:								
Gelatin	–	v	ND	v	v	+	–	+
Starch	+	+	ND	+/ND	v	–	+	+
DNA G+C content (mol%)	45.1	44–45.9	44.2	35.4–40	37–49	49	43	38.3–38.7

Table 2. Cellular fatty acid compositions of *Litoribacter ruber* gen. nov., sp. nov. YIM CH208^T and its closest phylogenetic neighbours

Taxa: 1, YIM CH208^T (*Litoribacter ruber* gen. nov., sp. nov.); 2, *Echinicola* (data from Nedashkovskaya *et al.*, 2006, 2007); 3, *Rhodonellum* (Schmidt *et al.*, 2006); 4, *Belliella* (Arun *et al.*, 2009); 5, *Algoriphagus* (Bowman *et al.*, 2003; Liu *et al.*, 2009); 6, *Nitritalea* (Anil Kumar *et al.*, 2010b); 7, *Indibacter* (Anil Kumar *et al.*, 2010a); 8, *Aquiflexum* (Kämpfer *et al.*, 2010). Values are percentages of total fatty acids; fatty acids present at less than 1 % in all strains are not shown. Values for predominant fatty acids (≥ 5 %) are in bold; NA, not applicable (reported as a component of a summed feature); —, not detected/not reported.

Fatty acid	1	2	3	4	5	6	7	8
iso-C _{14:0}	—	0.1–0.2	—	0.7–1.3	0.9	—	—	2.0
anteiso-C _{14:0}	—	—	—	—	—	5.0	—	—
C _{15:0}	—	0.8–1.5	0.4	0.5–0.7	0.2	56.8	—	—
iso-C _{15:0}	—	17.3–20.0	7.6	23.9–31.2	13.4	—	47.9	11.2
anteiso-C _{15:0}	4.7	1.4–2.8	1.4	2.9–5.4	3.8	—	6.7	16.5
iso-C _{15:1} F	—	—	—	—	—	—	1.6	—
iso-C _{15:1} G	3.0	0–0.6	6.3	1.9–9.9	—	—	—	5.6
C _{16:0}	1.8	0.8–0.9	0.6	1.2–1.5	1.5	0.9	1.6	—
iso-C _{16:0}	1.7	1.1–1.2	2.2	1.9–2.2	2.7	—	2.1	4.7
iso-C _{16:1} H	1.7	0.8–1.0	7.3	1.7–2.9	—	—	0.8	11.1
iso-C _{17:0}	2.5	0–1.0	0.2	0.6–0.7	0.2	—	2.4	—
iso C _{19:1} I	—	—	—	—	—	—	—	1.5
C _{15:1} ω6c	3.1	1.1–1.2	2.7	1.2–6.4	0.1	—	1.5	1.0
iso-C _{15:1} ω10c	—	—	—	—	8.1	—	—	—
C _{16:1} ω5c	6.5	4.9–7.8	1.8	4.2–4.9	11.2	—	0.9	2.0
iso-C _{16:1} ω6c	—	—	—	—	6.9	—	—	—
iso-C _{16:1} ω11c	—	—	—	—	—	21.7	—	—
C _{16:1} ω7c	NA	NA	NA	NA	34.1	—	—	NA
C _{16:1} ω9c	—	—	—	—	—	5.1	—	—
iso-C _{17:1} ω5c	—	—	—	—	1.2	—	—	—
iso-C _{17:1} ω7c	—	—	—	—	3.9	—	—	—
iso-C _{17:1} ω9c	19.9	4.4–6.9	17.5	4.4–7.3	—	—	1.0	5.3
anteiso-C _{17:1} ω9c	—	—	—	—	—	—	—	1.1
C _{17:1} ω6c	8.7	4.3–4.8	6.8	5.6–7.9	0.5	—	1.2	5.3
C _{17:1} ω8c	1.6	—	0.2	1.0–1.1	—	—	0.4	—
C _{18:1} ω9c	—	—	—	—	—	8.0	—	—
iso-C _{15:0} 2-OH	NA	NA	NA	0.9–1.2*	4.2	—	—	NA
iso-C _{15:0} 3-OH	4.1	3.4–5.0	3.7	3.9–4.2	0.3	—	1.2	4.3
anteiso-C _{15:0} 2-OH	—	—	—	—	3.6	—	—	—
C _{15:0} 2-OH	—	—	—	—	—	—	—	2.3
C _{15:0} 3-OH	12.1	2.5–2.6	—	—	—	—	—	—
C _{16:0} 3-OH	0.8	0.9–2.3	0.3	1.9–2.5	—	—	0.8	1.5
iso-C _{16:0} 3-OH	0.5	0.6–0.7	2.8	0.7–1.1	—	—	0.6	4.1
C _{17:0} 2-OH	0.4	0.4	1.1	—	—	—	1.8	3.3
iso-C _{17:0} 3-OH	11.3	9.4–10.0	17.5	5.2–6.7	—	—	10.7	5.3
C _{18:0} 3-OH	—	—	—	—	—	1.9	—	—
anteiso-C _{17:1} B/iso-C _{17:1} I†	—	0–5.0	—	0–4.4	—	—	—	5.9‡
C _{16:1} ω7c/iso-C _{15:0} 2-OH†	10.7	34.5	12.6	12.3–12.7	—	—	—	8.7
C _{14:0} 2-OH/iso-C _{15:0} 2-OH†	—	—	6.5	—	—	—	—	—
Not identified	—	—	—	—	—	—	12.5	1.1

*Arun *et al.* (2009) reported iso-C_{15:0} 2-OH both as a component of summed feature 3 and as a distinct fatty acid.

†Summed features: groups of two or three fatty acids that cannot be separated by GC with the MIDI system.

‡Reported as anteiso-C_{17:1} B by Kämpfer *et al.* (2010).

at 473 nm with two shoulders at 450 and 504 nm (Supplementary Fig. S2), which suggests the presence of carotenoids. Bacteriochlorophyll *a* was not synthesized.

The close relative *Rhodonellum psychrophilum* showed a similar carotenoid absorption spectrum, with absorption peaks at 460, 489 and 517 nm (Schmidt *et al.*, 2006).

The data from our phenotypic, chemotaxonomic, genotypic and phylogenetic analyses suggest that the isolate cannot be assigned to any known taxon. Differences in some phenotypic features, as well as fatty acid compositions and 16S rRNA gene sequences, differentiate the isolate clearly from phylogenetically related taxa (Table 1) and indicate that the isolate represents a novel genus and species, for which the name *Litoribacter ruber* gen. nov., sp. nov. is proposed.

Description of *Litoribacter* gen. nov.

Litoribacter (Li.to.ri.bac'ter. L. n. *litus* -oris shore, beach, strand; N.L. masc. n. *bacter* rod; N.L. masc. n. *Litoribacter* rod from the shore).

Cells are Gram-negative, rod-shaped and oxidase- and catalase-positive. Growth is strictly aerobic. Major fatty acids are iso-C_{17:1}ω9c, C_{15:0} 3-OH, iso-C_{17:0} 3-OH, summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c) and C_{17:1}ω6c. Predominant isoprenoid quinone is MK-7. Cells contain carotenoids but no bacteriochlorophyll *a*. The genus *Litoribacter* is a member of the phylum *Bacteroidetes*. The type species is *Litoribacter ruber*.

Description of *Litoribacter ruber* sp. nov.

Litoribacter ruber (ru'ber. L. masc. adj. *ruber* rose coloured, red).

Main characteristics are as given for the genus. In addition, cells are 0.3–0.5 × 1.2–1.5 µm. Colonies are circular, 2–3 mm in diameter, convex, smooth and bright red. Grows at 20–37 °C (optimum 28 °C) and pH 7.5–10.5 (optimum pH 8.5). Growth occurs at 0–9% (w/v) NaCl (optimum 0.5–2.5%). Degrades aesculin, starch and Tween 40. Does not hydrolyse gelatin, cellulose (CM-cellulose) or Tween 20 or 80. In API 50CH test strips, acid is produced from amygdalin, aesculin, cellobiose, L-fucose, gentiobiose, D-glucose, lactose, maltose, D-mannose, methyl α-D-glucopyranoside, raffinose, salicin, starch and sucrose. In API 20NE, tests positive for nitrate reduction, aesculin hydrolysis, arginine dihydrolase, urease and β-galactosidase. Can utilize fructose, galactose, glycerol, glucose, lactose, D-mannitol, D-mannose, raffinose, ribose, starch, sucrose, D-sorbitol, trehalose, acetate, benzoate, citrate and malate as sole carbon sources. In API ZYM, tests positive for α-galactosidase, alkaline and acid phosphatases, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α- and β-glucosidases and N-acetyl-β-glucosaminidase, but negative for β-galactosidase, α-mannosidase, α-fucosidase and β-glucuronidase. Cells are sensitive to (µg per disc unless indicated) vancomycin (30), norfloxacin (10), erythromycin (15), novobiocin (5), tetracycline (30), ampicillin (10), amikacin (30), ciprofloxacin (5), penicillin (10 IU) and clindamycin (2), but resistant to tobramycin (10). The DNA G + C content of the type strain is 45.1 mol%.

The type strain, YIM CH208^T (=ACCC 05414^T =KCTC 22899^T), was isolated from a sediment sample collected from the shore of a soda lake in Yunnan, south-west China.

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