Halopelagius longus sp. nov., a member of the family *Halobacteriaceae* isolated from a salt mine, and emended description of the genus *Halopelagius*

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A thermotolerant, extremely halophilic archaeon, BC12-B1^T, was isolated from a salt mine in Baicheng county, Xinjiang province, China. Colonies were off-white–grey. The cells stained Gram-negative, were motile and irregularly long-rod-shaped (variation in both width and length) with abundant gas vesicles. The strain was able to grow at 20–55 °C (optimum, 48 °C), at pH 6.0–8.0 (optimum, 7.0–7.3), with 1.8–6.0 M NaCl (optimum, 3.0–3.5 M) and with 0.02–2.2 M Mg^{2+} (optimum, 0.1–0.2 M). Cells lysed in distilled water and the minimal NaCl concentration to prevent cell lysis was 8 % (w/v). Phylogenetic analysis based on the 16S rRNA gene sequences showed that strain BC12-B1^T was most closely related to *Halopelagius inordinatus* RO5-2^T (98.5 %) with less than 95 % sequence similarity to other described species. The genomic DNA G+C content of strain BC12-B1^T was 64.0 mol%. The DNA–DNA hybridization value between strain BC12-B1^T and *Hpl. inordinatus* RO5-2^T was 43.6 %. The major polar lipids of strain BC12-B1^T were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, four glycolipids and an unknown lipid. Based on phenotypic, chemotaxonomic and genotypic characteristics, strain BC12-B1^T represents a novel species of the genus *Halopelagius*, for which the name *Halopelagius longus* sp. nov. is proposed. The type strain is BC12-B1^T (=CGMCC 1.12397^T=JCM 18758^T). An emended description of the genus *Halopelagius* is also provided.

The genus *Halopelagius* (order *Halobacteriales*, family *Halobacteriaceae*) was proposed by Cui *et al.* (2010) and at the time of writing contains one species, *Halopelagius inordinatus*. Cells of the type strain *Hpl. inordinatus* RO5-2^T were described as staining Gram-negative, pleomorphic and motile. Colonies were red-pigmented. This paper reports a non-pigmented, extremely halophilic strain, BC12-B1^T, isolated from a salt mine in Baicheng County, Xinjiang province, China. 16S rRNA gene sequence analysis revealed that strain BC12-B1^T was closely related to *Hpl. inordinatus* RO5-2^T. The aim of this study was to clarify the taxonomic status of this isolate and it is proposed that strain BC12-B1^T represents a novel species of the genus *Halopelagius* based on the results of these studies.

Neutral oligotrophic haloarchaeal medium (NOM-3) containing 100 μg streptomycin ml^{-1} and 100 μg ampicillin ml^{-1} was used in the initial enrichment (Cui *et al.*, 2011). The soil samples were collected from a salt mine located in Baicheng County, Xinjiang province, China. Three grams of sample was incubated in 30 ml NOM-3 medium at 37 °C, 130 r.p.m. for 2 weeks. The turbid cultures were diluted onto NOM-3 plates (with 2.0 % agar added) which were incubated at 37 °C. After 2 weeks of

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BC12-B1^T is JX518988.

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

incubation, an off-white–grey colony was picked up and purified by repeated restreaking. The isolate was routinely cultured in modified NOM-1 medium containing (l^{-1}): 0.2 g yeast extract, 0.2 g fish peptone, 2.0 g sodium pyruvate, 2.0 g sodium lactate, 5.4 g KCl, 0.3 g K_2HPO_4, 0.5 g NH_4Cl, 20.0 g $MgSO_4.7H_2O$ and 200.0 g NaCl (pH 7.0–7.2). Cells were stored at –80 °C with 25 % (v/v) glycerol.

Phenotypic tests were performed according to the proposed minimal standards for the description of new taxa in the order *Halobacteriales* (Oren *et al.*, 1997). Cell morphology and motility were examined by phase-contrast microscopy (DM5000B; Leica) and transmission electron microscopy (JEM-1230; JEOL) (Huo *et al.*, 2010). For pigment analysis, pigment was extracted with methanol and acetone (7 : 2; v/v), and scanned by a Beckman Coulter DU800 spectrophotometer. Gram staining was performed according to Dussault (1955). To determine the salt dependence of strain BC12-B1^T, the strain was cultured at various NaCl concentrations (1.0–6.0 M, at intervals of 0.5 M) and the effects of different Mg^{2+} concentrations (0.01–2.2 M) were assessed at the optimal NaCl concentration. The pH range for growth was determined at pH 5.0–10.0 (with intervals of 0.5 pH unit) using the following buffers: ammonium acetate (pH 4.0–5.0), MES (pH 5.5–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5) and CAPSO (pH 9.0–10.0) at a concentration of 30 mM. The temperature range for growth was determined by incubating the strain at 10, 15, 20, 30, 40, 45, 48, 50, 55 and 58 °C. Minimal salt concentration to prevent cell lysis was tested by suspending washed cells in a set of sterile saline solutions containing 0–15 % NaCl (w/v) and the stability of the cells was detected by light microscopic examination.

All biochemical and nutritional tests were performed in NOM-1 medium with *Hpl. inordinatus* RO5-2^T as the reference strain. Briefly, oxidase and catalase activities were tested according to Dong & Cai (2001). Growth with nitrate as the electron acceptor was tested as described by Mancinelli & Hochstein (1986). Esterase activity and hydrolysis of gelatin were detected as outlined by Gutiérrez & González (1972). Tests for indole, starch and casein hydrolysis were performed as described by Shen & Chen (2008). Anaerobic growth in the presence of L-arginine and DMSO was examined as described by Oren *et al.* (1997). Phosphatase activity, β-galactosidase activity and urease activity were determined using the API system (bioMérieux), with the modification that the cells were suspended in 12 % NaCl (w/v) solution. The strips were read after incubation at 37 °C for 48 h (API 20 NE) and 8 h (API ZYM). Reduction of nitrate to nitrite, production of gas from nitrate, production of H_2S, utilization of single carbon sources and production of acids from carbohydrates were performed according to Cui *et al.* (2010). Susceptibility to antibiotics was determined on NOM-1 agar plates using antibiotic discs (μg per disc, unless otherwise indicated): ampicillin (10), bacitracin (0.04 IU), carbenicillin (100), cefotaxime (30), cephalothin (30), chloramphenicol (30), ciprofloxacin (5), ceftriaxone (30), erythromycin (15), gentamicin (10), kanamycin (30), nalidixic acid (30), neomycin (30), nitrofurantoin (300), norfloxacin (10), novobiocin (30), nystatin (100), penicillin G (10 IU), rifampicin (5), streptomycin (10), tetracycline (30) and vancomycin (30).

More than 2 weeks were needed for strain BC12-B1^T to form colonies on NOM-1 solid medium. Colonies were less than 1 mm in diameter, off-white–grey, rounded, elevated and opaque. Pigment extract analysis showed that no pigments were detected. Cells of strain BC12-B1^T stained Gram-negative and were motile and irregular long-rod-shaped (Fig. 1). Gas vesicles were easily observed and they appeared as phase-bright inclusions inside the cells. The strain grew at 20–55 °C (optimum 48 °C), pH 6.0–8.0 (optimum 7.0–7.3), 1.8–6.0 M NaCl (optimum 3.0 – 3.5 M) and 0.02–2.2 M Mg^{2+} (optimum 0.1–0.2 M). Cells lysed in distilled water and the minimal NaCl concentration to prevent cell lysis was 8 % (w/v). The strain was strictly aerobic. Catalase, oxidase and β-galactosidase activities and H_2S production from sodium thiosulfate were positive. Phosphatase activity, urease activity, nitrate reduction, gas production from nitrate, indole production from tryptophan, and hydrolysis of starch, casein, gelatin and Tweens 20, 40, 60 and 80 were negative. More detailed characteristics of strain BC12-B1^T are given in the species description.

Genomic DNA was obtained using the method described by Ng *et al.* (1995). The 16S rRNA gene was amplified via PCR by using the universal archaea primers: forward 5′-ATTCCGGTTGATCCTGCCGGA-3′; reverse 5′-AGGAGGTGATCCAGCCGCAG-3′ (Cui *et al.*, 2009). The amplified products were cloned into vector pMD18-T (TaKaRa) and transformed into *Escherichia coli* TOP10 cells. Twenty-two transformants of strain BC12-B1^T were

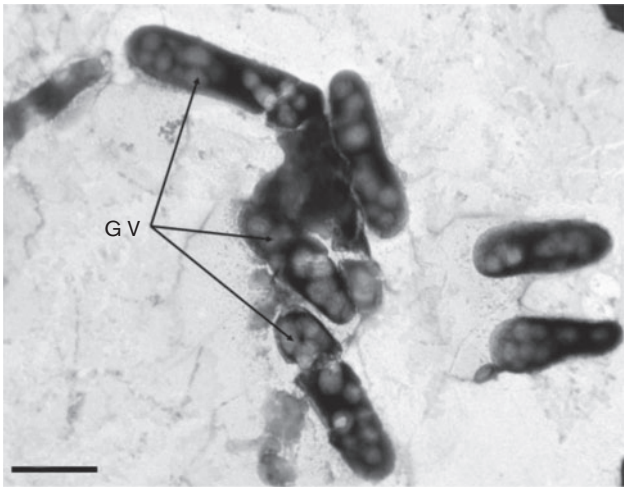

Fig. 1. Transmission electron photomicrograph of strain BC12-B1^T. GV, gas vesicles. Bar, 1 μm.

picked randomly and sequenced to determine whether the strain possessed multiple distinct 16S rRNA genes. Multiple sequence alignments were performed as described by Cui *et al.* (2011). The sequence was aligned with closely related 16S rRNA gene sequences by the EzTaxon-e server (Kim *et al.*, 2012) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods with the MEGA 5 software package (Tamura *et al.*, 2011). Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980) for the neighbour-joining method. The DNA G+C content was determined by reversed-phase HPLC as described by Mesbah & Whitman (1989). DNA–DNA hybridizations were performed using the thermal denaturation and renaturation method (De Ley *et al.*, 1970) as modified by Huss *et al.* (1983), using a Beckman DU 800 Spectrophotometer. The experiments were carried out in triplicate.

Seventeen complete 16S rRNA gene sequences of strain BC12-B1^T were obtained. Sequence comparisons indicated that the strain contained a single 16S rRNA gene and their sequences were 99.9 % similar. Strain BC12-B1^T was affiliated with the family *Halobacteriaceae* and showed high sequence similarity to *Hpl. inordinatus* RO5-2^T (98.5 %), but shared lower levels of 16S rRNA gene sequence similarity with other species (<95 %). Phylogenetic analysis using the neighbour-joining algorithm revealed that strain BC12-B1^T and *Hpl. inordinatus* RO5-2^T clustered together and formed a single clade distant from all other genera (Fig. 2). The phylogenetic position was also confirmed by trees generated using the maximum-parsimony and maximum-likelihood algorithms (Fig. S1, available in IJSEM Online). The genomic DNA G+C content of strain BC12-B1^T was 64.0 mol%. The DNA–DNA relatedness value of strain BC12-B1^T with *Hpl. inordinatus* RO5-2^T was 43.6 %.

Polar lipids were extracted by using a chloroform/methanol system and separated by two-dimensional TLC as described by Kates (1986). The optimized solvent system chloroform/methanol/water (65 : 25 : 4, by vol.) was used in the first dimension, followed by the second solvent of chloroform/glacial acetic acid/methanol/water (80 : 12 : 15 : 4, by vol.). The resulting TLC plates were sprayed with sulfuric acid/ethanol (1 : 1, v/v) and then heated at 130 °C for 5 min to detect phospholipids and glycolipids. Strain BC12-B1^T contained phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), four glycolipids including sulfated mannosyl glucosyl diether (S-DGD-1) and mannosyl glucosyl diether (DGD-1), and an unknown lipid (Fig. S2).

Sequence comparisons with representative bacteria with validly published names indicated that strain BC12-B1^T is phylogenetically closest to *Hpl. inordinatus* RO5-2^T (98.5 % sequence similarity). Phylogenetic analysis based on 16S rRNA gene sequences showed that the isolate formed a coherent cluster with strain *Hpl. inordinatus* RO5-2^T with a high bootstrap-resampling value (100 % by the neighbour-joining method) (Fig. 2). The topologies of the phylogenetic

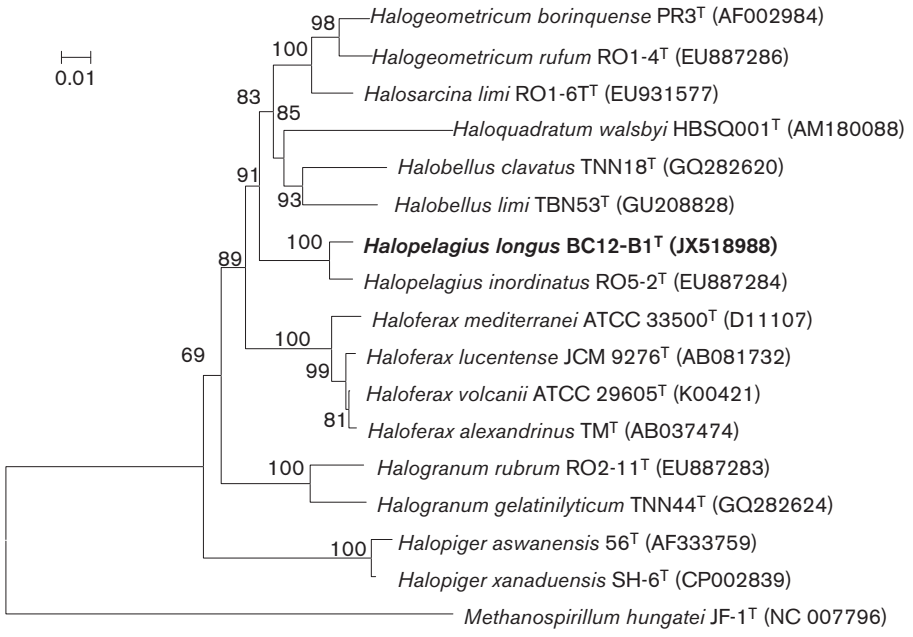

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic relationships of the novel isolate and related taxa. GenBank accession numbers are given in parentheses. Bootstrap values are based on 1000 replicates. Bar, 1 nt change per 100 nt.

Table 1. Differential phenotypic, physiological and genotypic characteristics that distinguish strain BC12-B1^T from *Hpl. inordinatus* RO5-2^T

Taxa: 1, strain BC12-B1^T; 2, *Hpl. inordinatus* RO5-2^T. Data were taken from this study. Both strains were motile, Gram-stain-negative, chemoorganotrophic and aerobic, positive for catalase and oxidase activities and production of H_2S, and negative for urease activity. Neither strain hydrolysed gelatin, casein, starch or Tweens 20, 40, 60 or 80. The following substrates could be used by both strains as the sole carbon source: acetate, L-arabinose, cellobiose, citrate, D-fructose, D-galactose, glycerol, DL-lactate, lactose, L-malate, maltose, D-mannose, pyruvate, D-sorbitol, succinate and sucrose. Other phenotypic characteristics of strain BC12-B1^T are summarized in the species description. +, Positive; −, negative; W, slightly utilized; R, resistant; S, susceptible.

Characteristic	1	2
Cell morphology	Irregular long rods	Pleomorphic
Pigments	No	Red
Growth temperature (°C)		
Optimum	48	37
Range	20–55	20–50
pH for growth		
Optimum	7.0–7.3	6.5–7.0
Range	6.0–8.0	5.5–8.0
NaCl concentration for growth (M)		
Optimum	3–3.5	3.4–3.9
Range	1.8–6.0	2.6–4.8
Mg^{2+} concentration for growth (M)		
Optimum	0.1–0.2	0.5
Range	0.02–2.2	0.03–0.7
Indole formation	−	+
Production of N_2	−	+
Carbon source used for growth		
L-Alanine	−	+
D-Xylose	+	−
D-Mannitol	+	−
Fumarate	−	+
D-Ribose	+	−
L-Glutamate	−	+
L-Lysine	W	−
Susceptible to (μg/disc):		
Rifampicin (5)	R	S
Bacitracin (0.04 IU)	R	S
Phosphatase activity	−	+
β-Galactosidase activity	+	−
Types of glycolipid present	S-DGD-1, DGD-1, GL-1, GL-2	S-DGD-1, DGD-1
DNA G+C content (mol%)*	64 %	62.3 %

*Determined by HPLC.

trees built by using the maximum-likelihood and maximum-parsimony algorithms also supported that strain BC12-B1^T was most closely related to strain *Hpl. inordinatus* RO5-2^T. To verify the species status of the strain, DNA–DNA hybridization results showed that the DNA–DNA relatedness value of strain BC12-B1^T with RO5-2^T was 43.6 %, which was significantly below the threshold value (70 %) to separate two species (Stackebrandt & Goebel, 1994). Polar lipid analysis showed that strain BC12-B1^T contained GL-1 and GL-2, which were absent in strain *Hpl. inordinatus* RO5-2^T. In addition, other characteristics that differentiated strain BC12-B1^T from *Hpl. inordinatus* RO5-2^T could also be found. For example, the two strains differed in genomic DNA G+C content and utilization of carbon sources. The detailed differences in physiological and biochemical characteristics between the two strains are summarized in Table 1.

On the basis of the phenotypic, chemotaxonomic and genotypic characteristics described above, strain BC12-B1^T represents a novel species within the genus *Halopelagius* for which the name *Halopelagius longus* sp. nov. is proposed. An emended description of the genus *Halopelagius* is also provided.

Emended description of the genus *Halopelagius*

The description is as given by Cui *et al.* (2010) with the following amendments. Cells are pleomorphic or irregular

long rods with variation in both width and length under optimal growth conditions. Gas vesicles are found in some species. Temperatures between 20 and 55 °C support growth. The genomic DNA G+C content is 59.9–64.0 mol%.

Description of *Halopelagius longus* sp. nov.

Halopelagius longus (lon′gus. L. masc. adj. *longus* long, extended, referring to the production of long rods in liquid medium).

Cells stain Gram-negative, are irregular long-rod-shaped, motile and contain gas vesicles. Colonies on agar plates are off-white–grey, rounded, elevated and opaque with diameter 0.5–1 mm. Chemo organotrophic. Growth occurs at 20–55 °C (optimum, 48 °C), pH 6.0–8.0 (optimum, pH 7.0–7.3), 1.8–6.0 M NaCl (optimum, 3.0–3.5 M NaCl) and 0.02–2.2 M Mg^{2+} (optimum, 0.1–0.2 M Mg^{2+}). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis is 8 % (w/v). Strictly aerobic, catalase- and oxidase-positive, shows β-galactosidase activity and produces H_2S from sodium thiosulfate. Phosphatase activity, urease activity, nitrate reduction, production of gas from nitrate, production of indole from tryptophan, and hydrolysis of starch, Tweens, casein and gelatin are negative. The following substrates are utilized as sole carbon sources: acetate, L-arabinose, cellobiose, citrate, D-fructose, D-galactose, D-glucose, glycerol, DL-lactate, lactose, L-lysine, L-malate, maltose, D-mannitol, D-mannose, L-ornithine, pyruvate, D-ribose, D-sorbitol, succinate, sucrose, trehalose and D-xylose. The substrates that yield the best growth are acetate, L-arabinose, D-fructose, D-galactose, glycerol, lactose, maltose, D-mannitol, D-mannose and D-xylose. Unable to utilize L-alanine, L-arginine, fumarate, L-glutamate, glycine, L-sorbose and starch. Acid is produced from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, lactose, maltose, D-mannitol, D-mannose, D-ribose, D-sorbitol, sucrose, trehalose and D-xylose. Resistant to the following antibiotics (μg per disc, unless otherwise indicated): ampicillin (10), bacitracin (0.04 IU), carbenicillin (100), cefotaxime (30), ceftriaxone (30), cephalothin (30), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), gentamicin (10), kanamycin (30), nalidixic acid (30), neomycin (30), norfloxacin (10), nystatin (100), penicillin G (10 IU), rifampicin (5), streptomycin (10), tetracycline (30) and vancomycin (30). Sensitive to nitrofurantoin (300) and novobiocin (30). The major polar lipids are phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), sulfated mannosyl glucosyl diether (S-DGD-1), mannosyl glucosyl diether (DGD-1), two unknown glycolipids and one unknown lipid.

The type strain, BC12-B1^T (=CGMCC 1.12397^T=JCM 18758^T), was isolated from a salt mine in Baicheng County, Xinjiang province, China. The DNA G+C content of the type strain is 64.0 mol% (as determined by HPLC).

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