Halomonas lutea sp. nov., a moderately halophilic bacterium isolated from a salt lake

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A Gram-negative, moderately halophilic bacterium, designated YIM 91125^T, was isolated from a salt lake in Xinjiang province, north-west China. The isolate grew at salinities in the range 1–20 % (w/v) and at 4–45 °C. Optimal growth occurred at 37 °C, pH 7.5 and 5–10 % (w/v) NaCl. Cells were short rods motile by means of single polar flagella. The major fatty acids were $C_{18:1}\omega7c$, $C_{16:0}$, $C_{19:0}$ cyclo $\omega8c$ and $C_{12:0}$ 3-OH. The DNA G+C content was 60.8 mol%. The predominant respiratory quinone was Q-9. A comparison of 16S rRNA gene sequences revealed its relationship to *Halomonas* species, its closest neighbours being *Halomonas pantelleriensis* (95.9 % similarity to the type strain) and *Halomonas muralis* (95.4 % similarity). On the basis of chemotaxonomic, phylogenetic and phenotypic evidence, strain YIM 91125^T represents a novel member of the genus *Halomonas*, for which the name *Halomonas lutea* sp. nov. is proposed. The type strain is YIM 91125^T (=KCTC 12847^T =CCTCC AB 206093^T).

The family *Halomonadaceae* of the class *Gammaproteobacteria* currently comprises seven genera, *Carnimonas*, *Chromohalobacter*, *Cobetia*, *Halomonas*, *Halotalea*, *Modicisalibacter* and *Zymobacter*, and more than half of the taxa in the family have been reclassified according to their heterogeneous features (Franzmann *et al.*, 1988; Mellado *et al.*, 1995; Dobson & Franzmann, 1996; Arahal *et al.*, 2002a, b). At the time of writing, *Halomonas* is the largest genus in the family *Halomonadaceae* and comprises 52 species with validly published names; most of these species were isolated from saline environments (Dobson & Franzmann, 1996; Mata *et al.*, 2002; Ventosa *et al.*, 1998; Cabrera *et al.*, 2007; Kim *et al.*, 2007; Soto-Ramírez *et al.*, 2007; Wang *et al.*, 2007a, b). *Halomonas* was described as comprising Gram-negative, aerobic, moderately halophilic bacteria. Some members of this genus have been recognized for their potential use in biotechnology, such as fermented food production, enzyme production (amylases, DNases, lipases, proteases and pullulanases) and the degradation of toxic compounds (Margesin & Schinner, 2001; Ventosa & Neito, 1995; Ventosa *et al.*, 1998; Sánchez-Porro *et al.*, 2003).

In the course of a programme of screening for halophilic bacteria, a moderately halophilic bacterium belonging to the genus *Halomonas* was isolated from Ebinur Lake (82° 35′–83° 16′ E 44° 05′–45° 08′ N), in Xinjiang, north-west China, a highly saline lake that has been a long-term target for the study of element cycling and microbial biota under extremely high-salinity conditions (Cui *et al.*, 2006). The results of a polyphasic characterization of this *Halomonas* strain, designated YIM 91125^T, are described here. On the basis of the data presented below, a novel species of the genus *Halomonas* is proposed to accommodate this isolate.

Strain YIM 91125^T was isolated using the dilution plating method on modified ISP 5 medium at 37 °C. This medium contained the following [(l distilled water)$^{-1}$]: 1 g L-asparagine, 10 g glycerol, 5 g yeast extract, 1 g K_2HPO_4, 5 g KNO_3, 100 g NaCl and 15 g agar. NaCl was sterilized separately before being added to the medium. The medium was adjusted to pH 7.5. Strain YIM 91125^T was maintained on modified ISP 5 agar slants (5 % NaCl, w/v; pH 7.5) at 4 °C and as 20 % (w/v) glycerol suspensions at −20 °C. Biomass for chemical and molecular studies was obtained

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from cultivation in shaken flasks (about 150 r.p.m.) in modified ISP 5 medium (5 % NaCl, w/v; pH 7.5) at 37 °C for about 1 week.

Gram staining was carried out using the standard Gram reaction and was confirmed using the KOH lysis test (Cerny, 1978). Cell motility was confirmed by the presence of turbidity throughout a tube containing semisolid medium (Leifson, 1960). Morphology was examined using transmission electron microscopy (H-800 microscope; Hitachi) with cells from exponentially growing cultures. Poly-β-hydroxybutyrate inclusions in cells were stained with Sudan black B (Drews, 1983) and viewed with phase-contrast microscopy. Colony morphology was observed on modified ISP 5 medium containing 5 % NaCl (pH 7.5) after incubation at 37 °C for 3 days. Growth was tested at various temperatures (0, 4, 10, 20, 28, 37, 45 and 55 °C) on modified ISP 5 medium containing 5 % NaCl. The pH range for growth was investigated between pH 4.0 and 10.0 (in increments of 1 pH unit) with the buffer system described by Xu *et al.* (2005). Liquid cultures were grown in tubes at 37 °C for 2–3 weeks, using modified ISP 5 as the basal medium. Tolerance of chlorides of sodium, potassium, magnesium and calcium at concentrations between 0 and 30 % (in increments of 1 %) were tested using the same basal medium. Catalase activity was determined by assessing bubble production after the addition of a drop of 3 % H_2O_2. Oxidase activity was determined by assessing the oxidation of tetramethyl-*p*-phenylenediamine. The methyl red and Voges–Proskauer tests were performed as described by Lányí (1987). Production of DNase and hydrolysis of casein, starch and Tweens 40 and 80 were tested as described by Smibert & Krieg (1994). The API 20E system (bioMérieux) was used to determine the following: hydrolysis of gelatin and ONPG, activity of lysine and ornithine decarboxylases, urease and arginine dihydrolase and production of acetoin, H_2S and indole. Nitrate reduction and aesculin hydrolysis were tested using the API 20NE system (bioMérieux). The utilization of different compounds as sole carbon, nitrogen and energy sources was determined using GN2 microplates with the Microlog system (Biolog; 95 substrates). Acid production from carbohydrates was determined by using the API 50 CH system (bioMérieux) and enzyme activities were determined by means of the API ZYM system (bioMérieux). Antibiotic susceptibility was determined using the disc diffusion method on agar (Reva *et al.*, 1995).

Strain YIM 91125^T comprised Gram-negative, short rods, approximately 0.4–0.7 μm wide and 0.6–1.0 μm long after cultivation for 3 days at 37 °C on modified ISP 5 agar containing 5 % NaCl (w/v). Cells were motile by means of single polar flagella. On modified ISP 5 agar containing 5 % NaCl (w/v), colonies were orange, flat, opaque and mucoid with slightly irregular edges. Cells were oxidase- and catalase-positive. The other physiological and biochemical properties determined for strain YIM 91125^T are given in Table 1 and in the species description.

For the analysis of fatty acids, strain YIM 91125^T was cultivated at 37 °C for 48 h on tryptic soy agar (Difco) containing 5 % NaCl and investigated as described by Sasser (1990), using the Microbial Identification System (MIDI). Isoprenoid quinones were extracted and purified as described by Komagata & Suzuki (1987). The purified ubiquinones were dissolved in acetone and separated by reversed-phase HPLC. The predominant respiratory quinone found in strain YIM 91125^T was Q-9, similar to members of the genus *Halomonas*. The fatty acids (>1 %)

Table 1. Differential characteristics of strain YIM 91125^T and its closest neighbours in the genus *Halomonas*

Strains: 1, YIM 91125^T; 2, *H. muralis* LMG 20969^T; 3, *H. pantelleriensis* DSM 9661^T; 4, *H. desiderata* DSM 9502^T; 5, *H. kribbensis* KCTC 12584^T. Data for reference strains are from Heyrman *et al.* (2002), Romano *et al.* (1997), Berendes *et al.* (1996) and Jeon *et al.* (2007), respectively. +, Positive; −, negative; NA, data not available.

Characteristic	1	2	3	4	5
Cell morphology	Short rods	Rods	Rods	Rods	Cocci or short rods
Pigmentation	Orange	Cream	Cream	NA	Yellow-cream
Flagellation	Single flagellum	Peritrichous	Peritrichous	Peritrichous	Single flagellum
NaCl range (%, w/v)	1–20	0–15	1–15	0–18	1–14
pH range	5.0–9.0	5.5–10.0	6.0–11.0	7.0–11.0	5.5–9.5
Temperature range (°C)	4–45	10–35	10–45	10–48	10–40
Oxidase	+	+	+	+	−
Hydrolysis of:					
Aesculin	−	+	+	NA	+
Gelatin	−	−	−	−	+
Urea	−	−	+	−	−
Major fatty acids	$C_{18:1}\omega7c$, $C_{16:0}$, $C_{19:0}$ cyclo $\omega8c$, $C_{12:0}$ 3-OH	$C_{18:1}\omega7c$, $C_{16:0}$, $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH	NA	$C_{16:0}$, $C_{16:1}\omega7c$, $C_{18:1}\omega7c$	$C_{16:0}$, $C_{19:0}$ cyclo $\omega8c$, $C_{17:0}$ cyclo
DNA G+C content (mol%)	60.8	62.4	65.0	66.0	66.0

were $C_{18:1}\omega7c$ (25.1 %), $C_{16:0}$ (17.0 %), $C_{19:0}$ cyclo $\omega8c$ (13.6 %), $C_{12:0}$ 3-OH (10.7 %), $C_{12:0}$ (7.9 %), $C_{10:0}$ (6.0 %) and $C_{17:0}$ cyclo (4.6 %). The profile of major fatty acids in strain YIM 91125^T was also similar to those of members of the genus *Halomonas* (Heyrman *et al.*, 2002; Romano *et al.*, 1997; Berendes *et al.*, 1996; Jeon *et al.*, 2007).

To determine the G+C content of strain YIM 91125^T, genomic DNA was prepared according to the method of Marmur (1961). The G+C content of the DNA was determined by reversed-phase HPLC (Mesbah *et al.*, 1989) as 60.8 mol%.

Extraction of genomic DNA and PCR amplification of 16S rRNA gene were done as described by Li *et al.* (2007). The sequence obtained was compared with reference 16S rRNA gene sequences retrieved from GenBank/EMBL by means of a BLAST search. Multiple alignments and calculations of evolutionary distances were carried out using CLUSTAL_X software (Thompson *et al.*, 1997). Gaps at the 5′ and 3′ ends of the alignment were omitted for further analysis. Phylogenetic analyses were performed using three tree-making algorithms: neighbour joining (Saitou & Nei, 1987), maximum likelihood (Felsenstein, 1981) and maximum parsimony (Fitch, 1971). A neighbour-joining phylogenetic tree was constructed from K_{nuc} values (Kimura, 1980) using MEGA, version 3.0 (Kumar *et al.*, 2004). The topology of the phylogenetic tree was evaluated using the bootstrap resampling method, with 1000 replicates (Felsenstein, 1985).

An almost-complete 16S rRNA gene sequence was determined for strain YIM 91125^T. Alignment data showed that strain YIM 91125^T had the highest levels of 16S rRNA gene sequence similarity with respect to members of the *Gammaproteobacteria*, in particular with respect to the genus *Halomonas*. In the phylogenetic tree based on the neighbour-joining algorithm, strain YIM 91125^T clustered together with the type strains of *Halomonas kribbensis*, *Halomonas desiderata*, *Halomonas muralis* and *Halomonas pantelleriensis* (Fig. 1); this relationship was supported by all of the tree-making methods used in this study. The results of the 16S rRNA gene sequence comparisons clearly demonstrated that strain YIM 91125^T was a member of the genus *Halomonas*. The similarities between the 16S rRNA gene sequence of strain YIM 91125^T and those of the type strains of 52 *Halomonas* species with validly published names ranged from 92.0 to 95.9 %. The closest relatives of strain YIM 91125^T were the type strains of *H. pantelleriensis* (95.9 % sequence similarity) and *H. muralis* (95.4 %).

On the basis of chemotaxonomic and phylogenetic data and some phenotypic features that distinguish the novel isolate from its closest neighbours in the genus *Halomonas* (Table 1), strain YIM 91125^T represents a novel species of the genus *Halomonas*, for which the name *Halomonas lutea* sp. nov. is proposed.

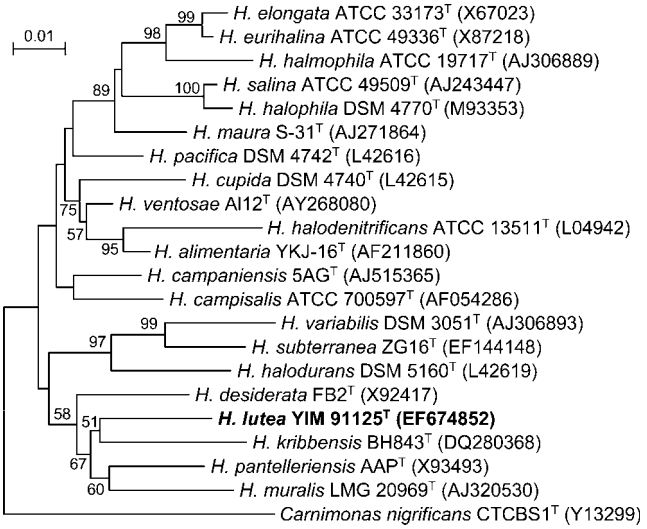

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain YIM 91125^T among species of genera within the family *Halomonadaceae*. The sequence of *Carnimonas nigrificans* CTCBS1^T was used as an outgroup. Bootstrap percentages (based on 1000 resamplings) >50 % are shown at branching points. Bar, 1 substitution per 100 nucleotide positions.

Description of *Halomonas lutea* sp. nov.

Halomonas lutea (lu′te.a. L. fem. adj. *lutea* orange-coloured).

Cells are aerobic, Gram-negative, short rods and are motile by means of single polar flagella. The type strain is unable to grow on modified ISP 5 medium supplemented with salts containing only K^+, Mg^{2+} and Ca^{2+} cations. Growth ranges for temperature, pH and NaCl are 4–45 °C, pH 5–9 and 1–20 % (w/v) NaCl, with optimal growth at 37 °C, pH 7.5 and 5–10 % (w/v) NaCl. Nitrate is reduced. DNase, aesculin, casein, starch and Tweens 40 and 80 are not hydrolysed. Negative for poly-β-hydroxybutyrate production and in the methyl red and Voges–Proskauer tests. In the API 20E system, the following enzyme activities are recorded as negative: β-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, urease, gelatinase, indole production from tryptophan, H_2S production from sodium thiosulfate and acetoin production from sodium pyruvate. The following substrates are utilized as sole carbon, nitrogen and energy sources (Biolog GN2 system): dextrin, *N*-acetyl-D-glucosamine, DL-arabinose, cellobiose, D-fructose, D-galactose, α-D-glucose, maltose, D-mannitol, methyl β-D-glucoside, D-psicose, sucrose, trehalose, methyl pyruvate, succinic acid monomethyl ester, acetic acid, formic acid, D-galacturonic acid, D-gluconic acid, α-ketobutyric acid, α-ketoglutaric acid, DL-lactic acid, succinic acid, bromo-succinic acid, succinamic acid, L-alaninamide, DL-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline,

L-serine, inosine, uridine, 2,3-butanediol, glycerol and glucose 6-phosphate. The remaining substrates of the Biolog GN2 system are not utilized. In the API 50 CH system, acid is produced from glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-galactose, D-glucose, D-fructose, L-sorbose, L-rhamnose, D-mannose, D-mannitol, D-sorbitol, aesculin, cellobiose, maltose, D-lactose, melibiose, starch, trehalose, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, potassium 2-ketogluconate and potassium 5-ketogluconate, but not from erythritol, inositol, D-adonitol, methyl β-D-xylopyranoside, arbutin, dulcitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, salicin, sucrose, inulin, melezitose, raffinose, glycogen, xylitol, turanose, potassium gluconate or L-arabitol. In the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), cystine arylamidase and naphthol-AS-BI-phosphohydrolase give positive results. Acid phosphatase, lipase (C14), leucine arylamidase, valine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, β-glucosidase, α-mannosidase and α-fucosidase are negative. The type strain is sensitive to the following antibiotics (μg per disc, unless indicated otherwise): amoxicillin (10), ampicillin (10), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), gentamicin (10), neomycin (30), netilmicin sulfate (30), norfloxacin (10), novobiocin (30), penicillin G (10), rifampicin (5), streptomycin sulfate (10), tetracycline (30), tobramycin (10), trimethoprim/sulfonamides (23.7/1.25) and vancomycin (30). Resistance is exhibited only to amikacin (30 μg) and lincomycin (2 μg). Q-9 is the predominant ubiquinone. The major cellular fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$, $C_{19:0}$ cyclo $\omega8c$ and $C_{12:0}$ 3-OH. The DNA G+C content of the type strain is 60.8 mol%.

The type strain, YIM 91125^T (=KCTC 12847^T =CCTCC AB 206093^T), was isolated from a salt lake in Xinjiang Province, north-west China.

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