Halomonas gudaonensis sp. nov., isolated from a saline soil contaminated by crude oil

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Two moderately halophilic strains, SL014B-69^T and SL014B-62A2, were isolated from a saline soil contaminated with crude oil in Gudao in the coastal Shengli oilfield in China; the isolates were Gram-negative, rod-shaped and carried lateral flagella. Growth occurred at NaCl concentrations of 1–20 % (w/v), at temperatures of 10–42 °C and at pH 8.0–9.0. Strain SL014B-69^T had C_{18:1}ω7c (28.61 %), C_{19:1} cyclo ω7c (27.97 %), C_{16:0} (19.66 %) and C_{12:0} 3-OH (8.87 %) as the predominant fatty acids and Q9 as the major ubiquinone, with the G + C content of genomic DNA being 64.0 mol%. Phylogenetic analyses based on 16S rRNA gene sequences indicated that the two strains belonged to genus of *Halomonas* in the *Gammaproteobacteria*, with the highest 16S rRNA gene sequence similarities of 96.4 % with *Halomonas campisalis* ATCC 700597^T and 96.0 % with *Halomonas desiderata* FB2^T. DNA–DNA relatedness of strain SL014B-69^T with strain SL014B-62A2, *H. campisalis* ATCC 700597^T and *H. desiderata* DSM 9502^T was 97.4, 42.9 and 36.8 %, respectively. On the basis of these data, a novel species of the genus *Halomonas*, *Halomonas gudaonensis* sp. nov., is proposed for strain SL014B-69^T and SL014B-62A2. The type strain is SL014B-69^T (=LMG 23610^T =CGMCC 1.6133^T).

With the description of *Halomonas elongata* in 1980 (Vreeland *et al.*, 1980), the species of the genus *Halomonas* were described as rod-shaped, Gram-negative, halotolerant bacteria with flagella that grow aerobically on a wide range of salt concentrations of 0.1–32.5 % (w/v) NaCl (Franzmann *et al.*, 1987). By the end of 2005, the genus *Halomonas* contained almost 40 species, isolated from various environments, including saline and hypersaline lakes (Franzmann *et al.*, 1987; Mormile *et al.*, 1999), a saltern (Hebert & Vreeland, 1987; Lim *et al.*, 2004), saline wetland (Martínez-Cánovas *et al.*, 2004), saline soil (García *et al.*, 2004), a mineral pool (Romano *et al.*, 2005), seawater (Yoon *et al.*, 2001), seafood (Yoon *et al.*, 2002), a deep-sea hydrothermal vent (Kaye *et al.*, 2004) and gill tissue of an ascidian (Romanenko *et al.*, 2002), as well as alkaline waste (Berendes *et al.*, 1996) and a mural painting (Heyrman *et al.*, 2002). In this study, we report the characterization of two novel strains, SL014B-69^T and SL014B-62A2, that were isolated from an oil-polluted saline soil in a coastal oilfield in eastern China. The results indicate that the two isolates represent a novel species of the genus *Halomonas*.

Strains SL014B-69^T and SL014B-62A2 were isolated from a saline soil from Gudao, in the Shengli oilfield of eastern

China, that was contaminated with crude oil; they were isolated by 10-fold dilution plating on agar plates containing only oil-produced water, and incubated for 7 days at 30 °C. The isolates were purified with restreaking on plates of artificial seawater (ASW) agar (l⁻¹: peptone, 5 g; yeast extract, 1 g; Na₂SO₄, 4 g; KCl, 0.68 g; KBr, 0.1 g; H₃BO₃, 0.025 g; MgCl₂·H₂O, 5.4 g; CaCl₂·2H₂O, 1.5 g; SrCl₂·6H₂O, 0.024 g; NaHCO₃, 0.2 g; Na₂HPO₄, 0.04 g; NH₄Cl, 0.5 g; NaF, 0.002 g; pH 8.0) with 2.4 % NaCl (Eguchi *et al.*, 1996) for 2 days at 30 °C. Growth was tested at different temperatures (5–50 °C) and at pH 2.0–12.0 in ASW medium containing 5 % (w/v) NaCl. Requirement for and tolerance of different concentrations of NaCl were determined in ASW medium with NaCl concentrations of 0–20 % (w/v).

After the strains were grown to late exponential phase on ASW agar, cell morphology and flagellum type were examined using transmission and scanning electron microscopy. Oxidase activity was tested as described by Smibert & Krieg (1994). Catalase activity was determined with 3 % (v/v) hydrogen peroxide solution. Denitrification was assessed as described by Zumft (1991). Hydrolysis of starch, gelatin and Tween 80, urease activity and growth on sole carbon sources and sole nitrogen sources were examined according to Williams *et al.* (1983) on ASW medium after 5–7 days incubation at 30 °C. After cells of

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SL014B-69^T is DQ421808.

SL014B-69^T, *Halomonas campisalis* ATCC 700597^T and *Halomonas desiderata* DSM 9502^T were grown on ASW agar at 30 °C for 2 days, cellular fatty acid methyl esters were prepared and analysed using gas chromatography according to the instructions of the Microbial Identification System (MIDI). Fatty acid profiles were analysed with the Sherlock system (Microbial ID Inc.). Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987), using HPLC fitted with a reversed-phase column (Shim-pack, VP-ODS; Shimadzu). Polar lipids were extracted and tested by one- and two-dimensional TLC on Merck silica gel 60 F₂₅₄ aluminium-backed thin-layer plates according to the methods of Kates (1986) and Collins *et al.* (1980).

Genomic DNA was extracted and purified by the method of Marmur (1961) from cells grown in ASW medium for 2 days at 30 °C. The purity was assessed from the A_{280}/A_{260} and A_{230}/A_{260} ratios (Johnson, 1994). The G + C content of genomic DNA was determined by thermal denaturation (Marmur & Doty, 1962) using DNA from *Escherichia coli* K-12 as a control. The 16S rRNA gene was amplified as described by Embley (1991) with universal bacterial primers 8F (5'-AGAGTTTGATCCTGGCTCAG) and 1492R (5'-GGTTACCTTGTTACGACTT). DNA-DNA hybridization was performed in triplicate following the methods of De Ley *et al.* (1970) and Huß *et al.* (1983). The 16S rRNA gene sequence of strain SL014B-69^T was aligned with those of related *Halomonas* species by using the MEGA software (Kumar *et al.*, 2004). The phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) available in the MEGA software package, version 5.0 (Kumar *et al.*, 2004), and re-evaluated with the interior branch test of phylogeny.

Cells of strains SL014B-69^T and SL014B-62A2 were Gram-negative rods, 0.3–0.4 × 0.75–1.65 µm, with a lateral flagellum. Spores were not present (Fig. 1). The two isolates formed creamy, smooth, irregular circular colonies on ASW agar. The ranges of NaCl concentration, pH and temperature for growth were 1–20 % NaCl (w/v) (optimum 10–15 % NaCl), pH 8.0–9.0 (optimum pH 8.0) and 10–42 °C

(optimum 30 °C). The isolates were positive for oxidase, catalase, urease and denitrifying activities, but negative for hydrolysis of gelatin, starch and Tween 80. The other main characteristics that differentiate the strains from other type strains of *Halomonas* are listed in Table 1 and in the species description.

Nearly complete 16S rRNA gene sequences were determined for strains SL014B-69^T and SL014B-62A2. Phylogeny of the 16S rRNA gene sequences revealed that strains SL014B-69^T and SL014B-62A2 were members of the *Gammaproteobacteria* and had a close phylogenetic relationship with the species of the genus *Halomonas* (Fig. 2). The 16S rRNA gene sequence of strain SL014B-69^T showed 100, 96.4 and 96.0 % sequence similarity with those of strain SL014B-62A2, *H. campisalis* ATCC 700597^T and *H. desiderata* FB2^T, respectively. Whole-genome DNA–DNA hybridization was carried out between strains SL014B-69^T and SL014B-62A2, *H. campisalis* ATCC 700597^T and *H. desiderata* DSM 9502^T. The DNA–DNA relatedness values for strain SL014B-69^T with SL014B-62A2, *H. campisalis* ATCC 700597^T and *H. desiderata* DSM 9502^T were respectively 96.4 % (SD 4.4 %), 42.9 % (SD 2.7 %) and 36.8 % (SD 5.2 %).

Results of the cellular fatty acid composition analysis are given in Table 2. The predominant cellular fatty acids of strain SL014B-69^T were C_{18:1}ω7c (28.61 %), C_{19:1} cyclo ω7c (27.97 %), C_{16:0} (19.66 %), C_{12:0} 3-OH (8.87 %), which are characteristic of *Halomonas* species. In contrast with other closely related *Halomonas* species, strain SL014B-69^T contained a distinct unknown fatty acid ECL 11.799 (2.98 %) and did not have C_{14:0} (Table 2). The major polar lipids of strain SL014B-69^T included phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and, distinctively, an unknown glycolipid that was not detected in *H. campisalis* ATCC 700597^T or *H. desiderata* DSM 9502^T (Fig. 3). The predominant isoprenoid quinone in strain SL014B-69^T was ubiquinone with nine isoprene units (Q9). The DNA G + C content of strain SL014B-69^T was 64.0 mol%, which is in the range reported previously for *Halomonas* (52–68 mol%; Franzmann *et al.*, 1987).

Fig. 1. Scanning electron micrograph of cells (a) and electron micrograph of a negatively stained cell (b) of strain SL014B-69^T. Bars, 2.0 µm.

Table 1. Characteristics that differentiate strain SL014B-69^T from closely related *Halomonas* type strains

Data for carbon utilization are from the present study and from Berendes *et al.* (1996) and Mormile *et al.* (1999). All strains are Gram-negative rods, positive for catalase, oxidase and denitrification activities and utilization of sucrose, trehalose, ethanol, glycerol, acetate and gluconate. All strains are negative for hydrolysis of gelatin, starch and Tween 80 and utilization of cellobiose and D-xylose. –, Negative; +, positive; +/-, weakly positive.

Characteristic	SL014B-69 ^T	<i>H. desiderata</i> DSM 9502 ^T	<i>H. campisalis</i> ATCC 700597 ^T
Cell size (µm)	0.30–4.0 × 0.75–1.65	0.4–0.6 × 0–2.6	1.0 × 3–5
Flagellum	Lateral	Peritrichous	Absent
Growth temperature (°C)			
Optimum	30	37	30
Range	10–42	10–45	4–50
pH for growth			
Optimum	8.0	9.7	9.5
Range	8.0–9.0	7.0–11.0	6.0–11.0
NaCl concentration for growth (%)			
Optimum	10.0–15.0	9.0	8.0
Range	1.0–20.0	0–18.0	1.0–20.0
Growth on:			
D-Glucose	+	+	+/-
Galactose	+	+/-	–
Mannose	+	+	–
Fructose	–	–	+
Maltose	+	+	–
L-Arabinose	–	+/-	–
Ribose	–	+/-	–
Lactose	+	+/-	–
Dextrin	+/-	+	+/-
Mannitol	+	+	+/-
Lactate	+	+/-	+/-
Hydrolysis of urea	+	–	+
DNA G + C content (mol%)	64.0	66.0	66.0

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between members of the genus *Halomonas* and related genera within the family *Halomonadaceae*. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more than 50 % bootstrap support. Bar, 0.02 expected changes per site.

Table 2. Major cellular fatty acids (%) of strain SL014B-69^T and related type strains

Fatty acid	SL014B-69 ^T	<i>H. desiderata</i> DSM 9502 ^T	<i>H. campisalis</i> ATCC 700597 ^T
C _{10:0}	0.98	1.83	2.76
Unknown 11.799	2.98		
C _{12:0}	1.25		0.86
C _{12:0} 3-OH	8.87	6.27	5.87
C _{14:0}		3.03	3.35
Sum in feature 3	4.44	1.77	8.07
C _{16:0}	19.66	18.03	16.44
C _{17:0} cyclo	2.70	1.12	2.96
C _{17:0}	0.56	0.42	0.28
C _{18:0}	0.65	0.84	
C _{18:1} ω7c	28.61	35.58	49.12
11-Methyl C _{18:1} ω7c	0.82	0.78	
C _{19:1} cyclo ω7c	27.97	29.68	10.28
C _{20:2} ω6,9c	0.51	0.65	

Based on phenotypic and genotypic data, strains SL014B-69^T and SL014B-62A2 are clearly distinguishable from their closest phylogenetic relatives and other species of the genus *Halomonas*. It is therefore proposed that strains SL014B-69^T and SL014B-62A2 represent a novel species of the genus *Halomonas*, for which we propose the name *Halomonas gudaonensis* sp. nov.

Fig. 3. (a) One-dimensional TLC of glycolipids of *H. campisalis* ATCC 700597^T (lane 1), strain SL014B-69^T (lane 2) and *H. desiderata* DSM 9502^T (lane 3). (b) Two-dimensional TLC of phospholipids from strain SL014B-69^T. DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol. Phospholipids were detected by spraying the plate with Zinzadze reagent (Kates, 1986). TLC was carried out on Merck silica gel 60 F₂₅₄ aluminium-backed thin-layer plates. Plates were subjected to development in chloroform/methanol/water (65:25:4, by vol.) (first dimension) followed by chloroform/methanol/acetic acid/water (85:12:15:4, by vol.) [second dimension in (b)].

Description of *Halomonas gudaonensis* sp. nov.

Halomonas gudaonensis (gu.dao.nen'sis. N.L. fem. adj. *gudaonensis* pertaining to Gudao, in the Shengli oilfield, PR China, where the type strain was isolated).

Cells are Gram-negative, aerobic rods (0.3–0.4 × 0.75–1.65 µm) with lateral flagella. Colonies on ASW medium are creamy, circular, smooth, opaque and approximately 1.5–2 mm in diameter after incubation at 30 °C for 2 days. Growth occurs at temperatures of 10–42 °C (optimum 30 °C), at NaCl concentrations of 1.0–20.0 % (optimum 10.0–15.0 %) and at pH 8.0–9.0. Positive for catalase, oxidase, urease and denitrifying activities. Negative for hydrolysis of Tween 80, starch and gelatin. The following substrates are utilized as sole carbon sources for growth: D-glucose, galactose, mannose, sucrose, maltose, trehalose, lactose, dextrin, ethanol, glycerol, mannitol, lactate, acetate and gluconate. Fructose, cellobiose, L-arabinose, D-xylose and ribose are not utilized. The following substrates are utilized as sole nitrogen sources for growth: L-alanine, glutamine, isoleucine, L-proline and arginine. Lysine and glycine are not utilized. The predominant isoprenoid quinone is Q9 and the cellular polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and an unknown glycolipid. The major cellular fatty acids are C_{18:1}ω7c (28.61 %), C_{19:1} cyclo ω7c (27.97 %), C_{16:0} (19.66 %) and C_{12:0} 3-OH (8.87 %). The DNA G + C content is 64.0 mol%.

The type strain, SL014B-69^T (=LMG 23610^T =CGMCC 1.6133^T), was isolated from a saline soil contaminated by crude oil, collected from Gudao oil-product from the coastal Shengli oilfield in China.

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