Halomonas shengliensis sp. nov., a moderately halophilic, denitrifying, crude-oil-utilizing bacterium

Ya-Nan Wang,¹ Hua Cai,¹ Chang-Qiao Chi,¹ An-Huai Lu,² Xian-Gui Lin,³ Zheng-Feng Jiang¹ and Xiao-Lei Wu¹

Correspondence

Xiao-Lei Wu

xiaolei_wu@tsinghua.edu.cn

¹Department of Environmental Science and Engineering, Tsinghua University, Beijing 100084, China

²Department of Geology, Peking University, Beijing 100871, China

³Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China

A moderately halophilic bacterium, designated strain SL014B-85^T, was isolated from a crude-oil-contaminated saline soil from Shengli oilfield, Shandong Province, China. Cells were Gram-negative, aerobic, short rods with lateral flagella. Growth occurred at NaCl concentrations of 0–15% (optimum 5–15%), at 10–42 °C (optimum 30 °C) and at pH 8.0–9.0 (optimum pH 8.5). The only respiratory quinone was Q9, and the main cellular fatty acids were C_{18:1}ω7c, C_{16:0} and C_{19:0} cyclo ω8c. The G + C content of the DNA was 66.6 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain SL014B-85^T belonged to the genus *Halomonas* in the *Gammaproteobacteria*, with highest sequence similarity of 98.1 and 97.8% to *Halomonas alimentaria* DSM 15356^T and *Halomonas ventosae* DSM 15911^T, respectively. DNA–DNA relatedness values were below 40% with members of closely related *Halomonas* species. Results of phenotypic, biochemical and phylogenetic analyses revealed that strain SL014B-85^T could be classified as representing a novel species of the genus *Halomonas*, for which the name *Halomonas shengliensis* sp. nov. is proposed. The type strain is SL014B-85^T (=CGMCC 1.6444^T=LMG 23897^T).

The bacterial genus *Halomonas* contains numerous moderately halophilic species with different biochemical functions including: denitrification [*Halomonas desiderata* (Berendes *et al.*, 1996), *H. campisalis* (Mormile *et al.*, 1999)], production of exopolysaccharides [*Halomonas eurihalina* (Mellado *et al.*, 1995), *H. maura* (Bouchotroch *et al.*, 2001), *H. ventosae* (Martínez-Cánovas *et al.*, 2004)] and degradation of aromatic compounds (*Halomonas organivorans*; García *et al.*, 2004). Because of the diverse functions of these moderately halophilic bacteria, in recent years we have focused on the characterization of the moderately halophilic bacterial community that is able to degrade petroleum hydrocarbons in several saline oilfields of China. Bacterial strains that could use crude oil as the sole carbon source were isolated (Gu *et al.*, 2007; Wang *et al.*, 2007), among which was strain SL014B-85^T. Based on its phenotypic, physiological, biochemical and phylogenetic characteristics, strain SL014B-85^T is considered to represent a novel species of the genus *Halomonas*.

Strain SL014B-85^T was isolated from an oil-polluted saline soil from the coastal Shengli oilfield, in Shandong Province in eastern China, by a 10-fold dilution plating technique on

oil-produced water (OPW) agar plates (Wang *et al.*, 2007) at 30 °C. As the organic and mineral contents of the oil-producing water were constantly changing, cultivation of strain SL014B-85^T for identification purposes was carried out in artificial seawater (ASW) medium, consisting of 5 g peptone, 1 g yeast extract, 4 g Na₂SO₄, 0.68 g KCl, 0.1 g KBr, 0.025 g H₃BO₃, 5.4 g MgCl₂·H₂O, 1.5 g CaCl₂·2H₂O, 0.024 g SrCl₂·6H₂O, 0.2 g NaHCO₃, 0.04 g Na₂HPO₄, 0.5 g NH₄Cl and 0.002 g NaF, per litre of water, with 2.4% (w/v) NaCl (pH 8.0) (Eguchi *et al.*, 1996).

After growth in ASW medium for 2 days at 30 °C, cell morphology was examined via transmission electron microscopy. Salt requirement for growth was tested by using ASW medium with NaCl concentrations ranging from 0 to 30% (w/v) (pH 8.0, at 30 °C) (Bouchotroch *et al.*, 2001). pH and temperature requirements for growth were determined in ASW medium by adjusting pH values between 2.0 and 12.0 (30 °C) and by incubation at 4–50 °C (pH 8.0).

Oxidase activity was tested as described by Smibert & Krieg (1994) and catalase activity was determined by use of a 3% (v/v) hydrogen peroxide solution. Nitrite and nitrate reduction were tested in ASW medium by growing the cells separately in the presence of nitrite and nitrate (Berendes *et al.*, 1996). Denitrification was tested by growing the cells

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SL014B-85^T is EF121853.

anaerobically in the presence of nitrate (Zumft, 1992). Hydrolysis of starch, gelatin and Tween 80, urease activity, and growth on sole carbon sources were examined according to the procedures of Williams *et al.* (1983) on ASW medium without organic compounds at 30 °C for 5–7 days. H₂S production was tested in ASW medium supplemented with 0.01 % L-cysteine, the indicator being a strip of paper impregnated with lead acetate placed in the neck of the tube (Clarke, 1953; Mata *et al.*, 2002).

Strain SL014B-85^T, *Halomonas alimentaria* DSM 15356^T and *H. ventosae* DSM 15911^T were grown in ASW medium for 3 days at 30 °C; their cellular fatty acids were analysed as described by Komagata & Suzuki (1987), and then tested by using GC/MS following the instructions of the Microbial Identification System (MIDI; Microbial ID Inc.). Polar lipid analyses were performed following a standard extraction procedure, and polar lipids were then 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). Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987), by using an HPLC fitted with a reversed-phase column (Shim-pack, VP-ODS; Shimadzu).

Fig. 1. Transmission electron micrograph of negatively stained cells of strain SL014B-85^T. Bar, 0.5 μm.

Genomic DNA was extracted from cells grown in ASW medium for 2 days at 30 °C according to the method of Marmur (1961). Purity was assessed based on A_{280}/A_{260} and A_{230}/A_{260} ratios (Johnson, 1994). The G + C content of the

Table 1. Differential physiological and biochemical characteristics between strain SL014B-85^T and closely related *Halomonas* type strains

Data for *H. alimentaria* and *H. ventosae* are from Yoon *et al.* (2002) and Martínez-Cánovas *et al.* (2004). All are Gram-negative rods, positive for oxidase, catalase, aerobic and anaerobic nitrate reduction and negative for hydrolysis of gelatin, starch and Tween 80 and production of H₂S. All can utilize glucose, sucrose, trehalose, DL-malate and succinate, but not ribose, arabinose, fructose, cellobiose, maltose, sorbose or xylose as sole carbon sources in ASW medium. +, Positive; –, negative; w, weak growth; ND, no data available.

Characteristic	SL014B-85 ^T	<i>H. alimentaria</i> YKJ-16 ^T	<i>H. ventosae</i> Al12 ^T
Cell size (μm)	0.6–0.8 × 1.0–1.6	0.8–1.2 × 1.3–1.9	0.7–0.8 × 1.2–1.4
Flagella	Several lateral flagella	Absent	ND
Optimal temperature (°C)	30	30	32
Temperature range (°C)	10–42	4–45	15–50
Optimum pH	8.5	6.5–7.5	8.0
pH range	8.0–9.0	Above 5.0	6.0–10.0
Optimum NaCl (%)	5.0–15.0	1.0–13.0	8.0
NaCl range (%)	0–15.0	0.5–23.0	3.0–15.0
Growth on:			
Galactose	+	–	+
Mannose	+	–	+
Lactose	+	w	+
Dextrin	+	w	+
Mannitol	–	–	+
Sorbitol	+	–	+
Inositol	–	–	+
Malonate	+	–	+
Lactate	–	+	–
Gluconate	+	–	+
Hydrolysis of urea	+	+	–
Aerobic nitrite reduction	w	+	+
DNA G + C content (mol%)	66.6	63.0	74.3

genomic DNA was determined by thermal denaturation (Marmur & Doty, 1962) with DNA from *Escherichia coli* K-12 as a control. DNA–DNA hybridization experiments were performed in triplicate following the methods of De Ley *et al.* (1970) and Huß *et al.* (1983). The 16S rRNA gene was amplified (Embley, 1991) with universal bacterial primers corresponding to *E. coli* positions 8F (5'-AGAGT-TTGATCCTGGCTCAG) and 1492R (5'-GGTTACCTTGT-TACGACTT). The 16S rRNA gene sequence of strain SL014B-85^T was aligned with those of related *Halomonas* species by using MEGA software (Kumar *et al.*, 2004). Phylogenetic trees were constructed via the neighbour-joining method (Saitou & Nei, 1987) and maximum-parsimony algorithm of MEGA, version 5.0 (Kumar *et al.*, 2004), and re-evaluated with the interior branch test of phylogeny.

Cells of strain SL014B-85^T were Gram-negative, short-rods (0.6–0.8 × 1.0–1.6 µm) with several lateral flagella (Fig. 1). Colonies on ASW agar plates were creamy and circular. Growth occurred at 0–15 % (w/v) NaCl (optimum 5–15 %), pH 8.0–9.0 (optimum pH 8.5) and 10–42 °C (optimum 30 °C). The strain was positive for oxidase, catalase and urease activities, aerobic nitrate and nitrite reduction, as well as for anaerobic nitrate reduction, but negative for hydrolysis of starch, gelatin and Tween 80 and H₂S production (Table 1). Strain SL014B-85^T was able to use glucose, sucrose, lactose, mannose, trehalose, galactose, dextrin, gluconate, malate, malonate, succinate and sorbitol, but not ribose, arabinose, fructose, cellobiose, maltose, sorbose, xylose, mannitol, inositol or lactate as sole carbon sources in ASW medium (Table 1). In addition, it was able to grow with the crude oil from Shengli oilfield as the sole carbon source.

The predominant cellular fatty acids of strain SL014B-85^T were C_{18:1}ω7c (43.05 % of total fatty acids), C_{16:0} (24.81 %), C_{19:0} cyclo ω8c (10.14 %), C_{12:0} 3-OH (8.41 %), C_{16:1}ω7c and/or iso-C_{15:0} 2-OH (6.98 %), C_{12:0} (2.70 %) and C_{10:0} (1.73 %) (Table 2), and ubiquinone with nine isoprene units (Q9) was the only isoprenoid quinone. The predominant polar lipids of strain SL014B-85^T were phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine, which are characteristic of members of the genus *Halomonas*. The G + C content of the DNA of strain SL014B-85^T was 66.6 mol%, within the range of 52–68 mol% reported for the genus *Halomonas* by Franzmann & Tindall (1990), but distinct from the most closely related species *H. alimentaria* (63.0 mol%; Yoon *et al.*, 2002) and *H. ventosae* (74.3 mol%; Martínez-Cánovas *et al.*, 2004).

The nearly complete 16S rRNA gene sequence (1460 bp) was determined for strain SL014B-85^T. Phylogeny of the 16S rRNA gene sequences revealed that strain SL014B-85^T was a member of the *Gammaproteobacteria* and had a close phylogenetic relationship with species of the genus *Halomonas*, forming a cluster with *H. alimentaria* DSM 15356^T, *H. ventosae* DSM 15911^T, *Halomonas cupida* DSM 4740^T (Baumann *et al.*, 1983; Dobson & Franzmann, 1996) and *Halomonas halodenitrificans* DSM 735^T (Dobson &

Table 2. Fatty acid profiles of strain SL014B-85^T and closely related type strains

Cells of all three strains were grown in ASW medium at 30 °C for 2 days before harvest. Values are percentages of total fatty acids. Data for *H. alimentaria* YKJ-16^T were taken from Yoon *et al.* (2002).

Fatty acid	SL014B-85 ^T	<i>H. alimentaria</i> YKJ-16 ^T	<i>H. ventosae</i> DSM 15911 ^T
C _{10:0}	1.73	1.9	2.20
Unknown ECL 11.799	0.57	—	—
C _{12:0}	2.70	—	6.79
C _{12:0} 3-OH	8.41	5.2	—
C _{14:0}	—	1.2	—
Summed feature 3*	6.98	24.2	16.44
C _{16:0}	24.81	27.0	27.26
C _{17:0} cyclo	0.48	5.1	1.35
C _{17:0}	0.54	0.2	—
C _{18:0}	0.57	0.2	0.69
C _{18:1} ω7c	43.05	19.4	30.23
11-Methyl C _{18:1} ω7c	—	0.4	0.71
Unknown ECL 18.846	—	0.1	—
C _{19:0} cyclo ω8c	10.14	12.9	14.32
C _{20:2} ω6,9c	—	0.2	—

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 comprised C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

Franzmann, 1996) (Fig. 2). Strain SL014B-85^T showed 16S rRNA gene sequence similarity of 98.1 and 97.8 %, respectively, to the type strains of the most closely related species *H. alimentaria* and *H. ventosae*. DNA–DNA hybridization experiments were carried out in triplicate and results showed mean levels of relatedness of 30.5 ± 0.06 % to *H. alimentaria* DSM 15356^T and 15.5 ± 0.07 % to *H. ventosae* DSM 15911^T, indicating that strain SL014B-85^T was genotypically distinct from these type strains (Wayne *et al.*, 1987).

Based on the above data and the full description provided below, we propose that strain SL014B-85^T represents a novel species of the genus *Halomonas*, for which the name *Halomonas shengliensis* sp. nov. is proposed.

Description of *Halomonas shengliensis* sp. nov.

Halomonas shengliensis (sheng.li.en' sis. N.L. fem. adj. *shengliensis* pertaining to Shengli oilfield, China, where the type strain was isolated).

Cells are moderately halophilic, Gram-negative, crude-oil-utilizing short rods (0.6–0.8 × 1.0–1.6 µm) with several lateral flagella. Colonies on ASW agar are creamy and circular. Grows at 0–15 % (w/v) NaCl, pH 8.0–9.0 and 10–42 °C; optimum growth occurs at 5–15 % (w/v) NaCl, pH 8.5 and 30 °C. Utilizes glucose, sucrose, lactose,

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain SL014B-85^T and 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% support. Bar, 0.02 expected changes per site.

mannose, galactose, dextrin, gluconate, malate, malonate, succinate and sorbitol as sole carbon sources. Oxidase-, urease- and catalase-positive, but negative for hydrolysis of starch, Tween 80 and gelatin and production of H₂S. It is able to reduce nitrate anaerobically as well as nitrate and nitrite aerobically. Q9 is the only isoprenoid quinone and phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine are the predominant polar lipids. The main cellular fatty acids are C_{18:1}ω7c (43.05%), C_{16:0} (24.81%) and C_{19:0} cyclo ω8c (10.14%). The DNA G + C content of the type strain is 66.6 mol%.

The type strain, SL014B-85^T (=CGMCC 1.6444^T=LMG 23897^T), was isolated from a saline soil from the coastal Shengli oilfield in eastern China.

Acknowledgements

We would like to thank G.-F. Zhao, S.-L. Yu and Y.-F. Guo for their valuable help. This study was supported by the National Natural Science Foundation of China (30570033, 30300008) and the National Basic Research Program of China (2005CB221308).

References

- Baumann, L., Bowditch, R. D. & Baumann, P. (1983). Description of *Deleya* gen. nov. created to accommodate the marine species *Alcaligenes aestus*, *A. pacificus*, *A. cupidus*, *A. venustus*, and *Pseudomonas marina*. *Int J Syst Bacteriol* **33**, 793–802.
- Berendes, F., Gottschalk, G., Heine-Dobbernack, E., Moore, E. R. B. & Tindall, B. J. (1996). *Halomonas desiderata* sp. nov., a new alkaliphilic, halotolerant and denitrifying bacterium isolated from a municipal sewage works. *Syst Appl Microbiol* **19**, 158–167.
- Bouchotroch, S., Quesada, E., Del Moral, A., Llamas, I. & Béjar, V. (2001). *Halomonas maura* sp. nov., a novel moderately halophilic, exopolysaccharide-producing bacterium. *Int J Syst Evol Microbiol* **51**, 1625–1632.
- Clarke, P. H. (1953). Hydrogen sulphide production by bacteria. *J Gen Microbiol* **8**, 397–407.
- Collins, M. D., Goodfellow, M. & Minnikin, D. E. (1980). Fatty acid isoprenoid quinine and polar lipid composition in the classification of *Curtobacterium* and related taxa. *J Gen Microbiol* **118**, 29–37.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Dobson, S. J. & Franzmann, P. D. (1996). Unification of the genera *Deleya* (Baumann *et al.* 1983), *Halomonas* (Vreeland *et al.* 1980) and *Halovibrio* (Fendrich 1988) and the species *Paracoccus halodenitrificans* (Robinson and Gibbons 1952) into a single genus, *Halomonas*, and placement of the genus *Zymobacter* in the family *Halomonadaceae*. *Int J Syst Bacteriol* **46**, 550–558.
- Eguchi, M., Nishikawa, T. & Macdonald, K. (1996). Responses to stress and nutrient availability by the marine ultramicrobacterium *Sphingomonas* sp. strain RB2256. *Appl Environ Microbiol* **62**, 1287–1294.
- Embley, T. M. (1991). The linear PCR reaction: a simple and robust method for sequencing amplified rRNA genes. *Lett Appl Microbiol* **13**, 171–174.
- Franzmann, P. D. & Tindall, B. J. (1990). A chemotaxonomic study of members of the family *Halomonadaceae*. *Syst Appl Microbiol* **13**, 142–147.
- García, M. T., Mellado, E., Ostos, J. C. & Ventosa, A. (2004). *Halomonas organivorans* sp. nov., a moderate halophile able to degrade aromatic compounds. *Int J Syst Evol Microbiol* **54**, 1723–1728.
- Gu, J., Cai, H., Yu, S.-L., Qu, R., Yin, B., Guo, Y.-F., Zhao, J.-Y. & Wu, X.-L. (2007). *Marinobacter gudaonensis* sp. nov., isolated from an oil-polluted saline soil in a Chinese oilfield. *Int J Syst Evol Microbiol* **57**, 250–254.

- HuB, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* 4, 184–192.
- Johnson, J. L. (1994). Similarity analysis of DNAs. In *Methods for General and Molecular Bacteriology*, pp. 655–681. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Kates, M. (1986). *Techniques of Lipidology*, 2nd edn. Amsterdam: Elsevier.
- Komagata, K. & Suzuki, K. (1987). Lipid and cell wall analysis in bacterial systematics. *Methods Microbiol* 19, 161–206.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 5, 150–163.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* 3, 208–218.
- Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* 5, 109–118.
- Martínez-Cánovas, M. J., Quesada, E., Llamas, I. & Béjar, V. (2004). *Halomonas ventosae* sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium. *Int J Syst Evol Microbiol* 54, 733–737.
- Mata, J. A., Martínez-Cánovas, J., Quesada, E. & Béjar, V. (2002). A detailed phenotypic characterisation of the type strains of *Halomonas* species. *Syst Appl Microbiol* 25, 360–375.
- Mellado, E., Moore, E. R. B., Nieto, J. J. & Ventosa, A. (1995). Phylogenetic inferences and taxonomic consequences of 16S ribosomal DNA sequence comparison of *Chromohalobacter marismortui*, *Volcaniella eurihalina*, and *Deleya salina* and reclassification of *V. eurihalina* as *Halomonas eurihalina* comb. nov. *Int J Syst Bacteriol* 45, 712–716.
- Mormile, M. R., Romine, M. F., Garcia, M. T., Ventosa, A., Bailey, T. J. & Peyton, B. M. (1999). *Halomonas campisalis* sp. nov., a denitrifying, moderately haloalkaliphilic bacterium. *Syst Appl Microbiol* 22, 551–558.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4, 406–425.
- Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Wang, Y.-N., Cai, H., Yu, S.-L., Wang, Z.-Y., Liu, J. & Wu, X.-L. (2007). *Halomonas gudaonensis* sp. nov., isolated from a saline soil contaminated by crude oil. *Int J Syst Evol Microbiol* 57, 911–915.
- Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* 37, 463–464.
- Williams, S. T., Goodfellow, M., Alderson, G., Wellington, E. M. H., Sneath, P. H. A. & Sackin, M. J. (1983). Numerical classification of *Streptomyces* and related genera. *J Gen Microbiol* 129, 1743–1813.
- Yoon, J.-H., Lee, K.-C., Kho, Y. H., Kang, K. H., Kim, C.-J. & Park, Y.-H. (2002). *Halomonas alimentaria* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. *Int J Syst Evol Microbiol* 52, 123–130.
- Zumft, W. G. (1992). The denitrifying bacteria. In *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, 2nd edn, pp. 554–582. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.