Marinobacter gudaonensis sp. nov., isolated from an oil-polluted saline soil in a Chinese oilfield

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Two novel strains, SL014B61A^T and SL014B11A, were isolated from an oil-polluted saline soil from Gudao in the coastal Shengli Oilfield, eastern China. Cells of strains SL014B61A^T and SL014B11A were motile, Gram-negative and rod-shaped. Growth occurred at NaCl concentrations of between 0 and 15% and at temperatures of between 10 and 45 °C. Strain SL014B61A^T had Q9 as the major respiratory quinone and C16 : 0 (21.2%), C18 : 1 ω 9c (20.3%), C16 : 1 ω 7c (7.3%) and C16 : 1 ω 9c (6.4%) as predominant fatty acids. The G + C content of the DNA was 57.9 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain SL014B61A^T belonged to the genus *Marinobacter* in the class *Gammaproteobacteria*. Strain SL014B61A^T showed the highest 16S rRNA gene sequence similarity with *Marinobacter bryozoorum* (97.9%) and showed 97.8% sequence similarity to *Marinobacter lipolyticus*. DNA–DNA relatedness to the reference strains *Marinobacter bryozoorum* and *Marinobacter lipolyticus* was 35.5% and 33.8%, respectively. On the basis of these data, it is proposed that strains SL014B61A^T and SL014B11A represent a novel species, *Marinobacter gudaonensis* sp. nov. The type strain is strain SL014B61A^T (=DSM 18066^T=LMG 23509^T=CGMCC 1.6294^T).

The genus *Marinobacter* was proposed by Gauthier *et al.* (1992) with a single species *Marinobacter hydrocarbonoclasticus*. The genus currently contains 14 species with validly published names (Gorshkova *et al.*, 2003; Martín *et al.*, 2003; Shieh *et al.*, 2003; Yoon *et al.*, 2003, 2004; Romanenko *et al.*, 2005; Shivaji *et al.* 2005; Green *et al.* 2006; Kim *et al.* 2006; Liebgott *et al.* 2006). The type species, *Marinobacter hydrocarbonoclasticus*, is able to utilize various hydrocarbons as the sole source of carbon and energy (Gauthier *et al.*, 1992). The second species of the genus to be recognized, *Marinobacter aquaeolei*, was isolated from an oil-producing well on an offshore platform in southern Vietnam (Nguyen *et al.*, 1999). However, research by Márquez and Ventosa has suggested that *M. aquaeolei* is a later heterotypic synonym of *M. hydrocarbonoclasticus* based on fatty acid composition, DNA G + C content and DNA–DNA hybridization studies (Márquez & Ventosa, 2005). In this study, we report the

characterization of two novel strains, SL014B61A^T and SL014B11A, that were isolated from an oil-polluted saline soil in a coastal oilfield in eastern China. The results indicate the two isolates represent a novel species of the genus *Marinobacter*.

An oil-polluted soil was sampled from a ditch containing discharged oil recovery wastewater in Gudao Oil-Product, a coastal Shengli Oilfield in Shandong Province, eastern China. The temperature of the soil was around 30 °C all year round and the salinity of the soil was around 1% NaCl (w/w). The main organic compounds in the soil were petroleum hydrocarbons. Strains SL014B61A^T and SL014B11A were isolated from the soil by a 10-fold dilution plating technique on inorganic salts agar containing (w/v) 0.5% NaCl, 0.1% NH₄H₂PO₄, 0.1% (NH₄)₂SO₄, 0.02% MgSO₄·7H₂O, 0.3% KNO₃, 0.1% K₂HPO₄ and distilled oil recovery wastewater instead of pure water. The isolates were purified by restreaking on plates of inorganic salts agar incubated for 3–5 days at 30 °C.

After the strains had grown to late exponential phase on marine agar 2216 (MA), cell morphology and flagellum type were examined using transmission and scanning electron

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SL014B61A^T and SL014B11A are DQ414419 and DQ629025, respectively.

Electron micrographs of cells of strain SL014B61A^T are available as a supplementary figure in IJSEM Online.

microscopy. Carbon source assimilation was tested using the mineral medium solution of Shivaji *et al.* (2005). Each carbon source was added at a concentration of 0.2 % (w/v) after the mineral base solution had been autoclaved. Growth was examined after incubation at 30 °C for 1, 7, 10 and 14 days. Hydrolysis of starch, gelatin and Tween 80 was assessed as described by Smibert & Krieg (1994). Nitrate and nitrite reduction were assessed as described by Lanyi (1987). Optimum pH and temperature for growth were determined using marine broth 2216. The requirement for and tolerance of various NaCl concentrations were determined in a medium containing (l^{-1}): 1.0 g $MgCl_2 \cdot 6H_2O$, 5.0 g $MgSO_4 \cdot 7H_2O$, 0.7 g KCl, 0.15 g $CaCl_2 \cdot 2H_2O$, 0.5 g NH_4Cl , 0.1 g KBr, 0.27 g KH_2PO_4 , 0.04 g $SrCl_2 \cdot 6H_2O$, 0.025 g H_3BO_3 , 5.0 g peptone and 1.0 g yeast extract (pH 8.0) with various NaCl concentrations (0, 0.5, 1, 3, 5, 10, 15, 18, 20 and 25 %). Sensitivity to various antibiotics (kanamycin, tetracycline, chloramphenicol, ampicillin, streptomycin, erythromycin and gentamicin) was tested by using the method described by Cho & Giovannoni (2003).

Cells of strain SL014B61A^T, *Marinobacter bryozorum* DSM 15401^T and *Marinobacter lipolyticus* SM19^T were grown on MA at 28 °C for 3 days for cellular fatty acid analyses. Cellular fatty acid methyl esters were prepared and analysed using GC according to the instructions of the Microbial Identification System (MIDI). Fatty acid profiles were analysed by the Sherlock system (Microbial ID). Lipopigments were extracted from lyophilized cells with chloroform/methanol (2:1, v/v) as described by Tindall (1990). Respiratory lipopigments were analysed using reversed-phase HPLC (Shim-pack, VP-ODS, Shimadzu). Genomic DNA was extracted and purified by the method of Marmur (1961) and DNA purity was assessed by the A_{280}/A_{260} and A_{230}/A_{260} ratios (Johnson, 1994). The DNA G+C content 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 previously (Rainey *et al.*, 1996), except that the following pair of bacterial universal primers was used: 8f, 5'-AGAGTTTGA-TTCCTGGCTCAG-3' and 1492r, 5'-GGTTACCTTGTTAC-GACTT-3'. 16S rRNA gene sequence alignments were performed with the CLUSTAL_X program (version 1.64b; Thompson *et al.*, 1997). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) and evaluated by bootstrap analysis based on 1000 resampling replicates with the SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs of the PHYLIP software package version 3.6 (Felsenstein, 2004). DNA-DNA hybridization was performed in triplicate by the thermal denaturation and renaturation method of Huß *et al.* (1983), modified from that of De Ley *et al.* (1970). The temperature of renaturation was 76.5 °C in $2 \times$ SSC buffer (0.15 M NaCl buffered with 0.015 M trisodium citrate, pH 7.0).

The two novel isolates were Gram-negative, rod-shaped and motile with a polar flagellum (see Supplementary Fig. S1a, b in IJSEM Online). The small creamy colonies (about

1–2 mm) were produced on MA after incubation at 30 °C for 3–5 days. Colonies were smooth, uniformly circular, flat and a little transparent. The pH range and NaCl concentrations for growth were pH 6.0–9.5 (optimum pH, 7.5–8.0) and 0%–15 % NaCl (w/v) (optimum NaCl 2.0–3.0 %). Growth was observed at temperatures of 10–45 °C, but not at 4 °C or 50 °C. The novel isolates gave a positive reaction in tests for catalase and oxidase and reduced nitrate to nitrite. Nitrite was not reduced to N_2 . Starch and Tween 80 were hydrolysed, but no hydrolysis of urea or gelatin was detected. Both strains were susceptible to kanamycin, tetracycline, ampicillin, chloramphenicol, streptomycin, erythromycin and gentamicin. The other main characteristics that differentiate the novel strains from the type strains of species of the genus *Marinobacter* are listed in Table 1.

Almost complete 16S rRNA gene sequences were determined for strains SL014B61A^T and SL014B11A. Analysis of the 16S rRNA gene sequences revealed that strain SL014B61A^T was a member of the class *Gammaproteobacteria* and had a close phylogenetic relationship with species of the genus *Marinobacter*; 16S rRNA gene sequence similarity ranged from 94.2 to 97.9 % (Fig. 1). The 16S rRNA gene sequence of strain SL014B61A^T had 100 % similarity to that of strain SL014B11A. The novel strains were most closely related to *M. bryozorum* DSM 15401^T (97.9 %) and *M. lipolyticus* SM19^T (97.8 %). Lower similarity values were observed with other *Marinobacter* species, such as *M. hydrocarbonoclasticus* ATCC 49840^T (94.9 %) and *Marinobacter litoralis* SW-45^T (94.2 %).

Fig. 1. Phylogenetic position of *Marinobacter gudaonensis* sp. nov. and other *Marinobacter* species according to 16S rRNA gene sequence analysis. The topology shown was obtained by using neighbour-joining methods. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at branch points. Bar, 1 % sequence divergence.

Table 1. Characteristics that differentiate *Marinobacter gudaonensis* sp. nov. from related species of the genus *Marinobacter*

Strains: 1, *M. gudaonensis* SL014B61A^T; 2, *M. bryozoorum* DSM 15401^T; 3, *M. lipolyticus* SM19^T. Data for carbon utilization are from the present study and other data were obtained from Martín *et al.* (2003) and Yoon *et al.* (2004). All strains are positive for motility, catalase and oxidase activities and utilization of acetate, pyruvate and glycerol. All strains are negative for hydrolysis of gelatin and urea and do not utilize L-arginine, D-melezitose, D-erythritose, formate, L-lysine, malonic acid, *myo*-inositol, D-melibiose, α -lactose, D-ribose, glycine, lactic acid, L-glutamic acid or –, Negative; +, positive; (+), weakly positive.

Characteristic	1	2	3
Cell size (μm)	0.3–0.5 $\times$ 1.2–1.8	0.4–0.5 $\times$ 1.0–1.3	0.3–0.5 $\times$ 2.5–3.5
Min. growth temperature ($^{\circ}\text{C}$)	10	7	15
Max. growth temperature ($^{\circ}\text{C}$)	45	42	40
NaCl range for growth (%)	0–15	1–18	1–15
Utilization of:			
L-Alanine	+	–	–
D-Arabinose	–	–	+
D-Cellobiose	(+)	–	+
Citrate	+	–	+
Dextrin	+	–	+
Ethanol	+	(+)	(+)
D-Fructose	+	–	+
D-Galactose	(+)	(+)	–
Gluconate	–	–	+
D-Glucose	+	–	+
L-Isoleucine	+	–	+
D-Maltose	+	–	+
D-Mannitol	–	–	+
D-Mannose	–	+	(+)
L-Proline	+	–	+
Propionate	+	–	+
D-Raffinose	–	–	–
L-Rhamnose	–	+	+
D-Sorbitol	+	–	+
L-Sorbose	–	–	–
Succinate	+	–	+
Sucrose	+	+	–
D-Trehalose	+	(+)	(+)
D-Xylose	+	+	(+)
DNA G + C content (mol%)	57.9	59.6	57.0

Whole-genome DNA–DNA hybridization studies were performed with strains SL014B61A^T and SL014B11A, *M. bryozoorum* DSM 15401^T and *M. lipolyticus* SM19^T. DNA–DNA relatedness values (based on three independent determinations) for the strain SL014B61A^T with strain SL014B11A, *M. bryozoorum* and *M. lipolyticus* were 95.5 % (SD = 4.7 %), 35.5 % (SD = 5.8 %) and 33.8 % (SD = 5.5 %), respectively.

The results of the cellular fatty acid content analysis are given in Table 2. Fatty acids C12:0 3-OH, C16:0, C16:1 ω 9 c and C18:1 ω 9 c have been reported to be predominant in other known *Marinobacter* species (Spröer

et al., 1998; Nguyen *et al.*, 1999; Martín *et al.*, 2003; Yoon *et al.*, 2003, 2004). The predominant cellular fatty acids of strain SL014B61A^T were C16:0 (21.2 %), C18:1 ω 9 c (20.3 %), C18:3 ω 6 c (6, 9, 12) (8.5 %), C16:1 ω 7 c (7.3 %) and C16:1 ω 9 c (6.4 %). This differed from those of the reference species *M. bryozoorum* DSM 15401^T, but was similar to those of *M. lipolyticus* SM19^T. The G + C content of strain SL014B61A^T was 57.9 mol% (T_m).

On the basis of physiological and molecular properties, it is proposed that strains SL014B61A^T and SL014B11A represent a novel species of the genus *Marinobacter*, for which we propose the name *Marinobacter gudaonensis* sp. nov.

Table 2. Cellular fatty acid content of strain SL014B61A^T and related species of the genus *Marinobacter* grown on MA

Species: 1, *M. gudaonensis* SL014B61A^T; 2, *M. bryozoorum* DSM 15401^T; 3, *M. lipolyticus* SM19^T. Values are percentages of total fatty acids; values <1 % are not shown.

Fatty acid	1	2	3
C12:0	2.8	4.3	3.7
C12:0 3-OH	3.4	8.3	5.8
C14:0	1.3	0.8	1.0
C16:0 N alcohol	7.1	0	5.6
C16:0	21.2	20.0	23.0
C16:1 ω 7c	7.3	0.5	6.3
C16:1 ω 9c	6.4	2.6	8.5
10-Methyl C16:0	2.5	0	1.1
C17:0	0.9	2.9	0.7
C17:1 ω 8c	1.1	1.1	1.2
10-Methyl C17:0	4.5	0	3.0
C18:0	5.6	4.9	2.9
C18:1 ω 7c	5.4	0.7	4.9
C18:1 ω 9c	20.3	45.8	27.4
C18:3 ω 6c (6,9,12)	8.5	0	4.1
C19:0 ω 10c cyclo	0	7.0	0

Description of *Marinobacter gudaonensis* sp. nov.

Marinobacter gudaonensis (gu.dao.nen'sis. N.L. masc. adj. *gudaonensis* pertaining to Gudao of the Shengli Oilfield, P. R. China, from where the type strain was first isolated.)

Cells are Gram-negative, rod-shaped (0.3–0.5 × 1.2–1.8 µm) and motile with a polar flagellum on semi-solid medium. Growth occurs in 0–15 % NaCl at temperatures of between 10 and 45 °C. Colonies on MA are smooth, uniformly circular, flat and a little transparent after 3–5 days. Positive results in tests for catalase, oxidase and nitrate-reducing activities and for the hydrolysis of starch and Tween 80. Negative results in tests for gelatin hydrolysis and urease and nitrite-reducing activities. The following substrates are utilized as a sole carbon source for growth: D-maltose, L-proline, dextrin, D-glucose, citrate, D-xylose, L-alanine, propionate, ethanol, D-fructose, pyruvate, D-trehalose, sucrose, D-sorbitol, acetate, succinate and D-galactose. Sensitive to kanamycin (10 µg), tetracycline (5 µg), chloramphenicol (10 µg), ampicillin (10 µg), streptomycin (10 µg), erythromycin (10 µg) and gentamicin (10 µg). Q9 is a main respiratory quinone and C16:0 (21.2 %), C18:1 ω 9c (20.3 %), C18:3 ω 6c (6, 9, 12) (8.5 %), C16:1 ω 7c (7.3 %) and C16:1 ω 9c (6.4 %) are the predominant fatty acids. The G + C content of the DNA is 57.9 mol% (T_m).

The type strain, SL014B61A^T (=DSM 18066^T=LMG 23509^T=CGMCC 1.6294^T), was isolated from an oil-polluted saline soil in Gudao in the coastal Shengli Oilfield, eastern China. Strain SL014B11A is a reference strain.

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