Marinobacter mobilis sp. nov. and *Marinobacter zhejiangensis* sp. nov., halophilic bacteria isolated from the East China Sea

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Three Gram-negative, aerobic, motile, halophilic, rod-shaped strains (CN46^T, CN71 and CN74^T) were isolated from sediment of the East China Sea and subjected to a polyphasic taxonomic study. Strains CN46^T and CN71 had identical 16S rRNA gene sequences and phenotypic characteristics. Strain CN46^T was moderately halophilic. Growth of strain CN46^T was observed between 0.5 and 10.0 % (w/v) NaCl (optimal growth at 3.0–5.0 %) and between pH 6.5 and 9.0. Strain CN74^T grew over a wider range of pH (pH 6.0–9.5); the optimum NaCl concentration for growth was 1.0–3.0 %. The major fatty acids of strain CN46^T were $C_{16:1}\omega9c$, $C_{16:0}$ and $C_{12:0}$, whereas strain CN74^T contained $C_{16:0}$, $C_{16:1}\omega9c$, $C_{18:1}\omega9c$ and $C_{12:0}$. The DNA G+C contents of the three isolates were between 58.0 and 58.9 mol%. Phylogenetic analyses based on 16S rRNA gene sequences showed that strains CN46^T, CN71 and CN74^T grouped together within the cluster of *Marinobacter* species. 16S rRNA gene sequence similarities of the three strains with the type strains of *Marinobacter* species ranged from 94.0 to 97.1 %. The DNA–DNA hybridization values of strain CN74^T with strains CN46^T and CN71 were 35.0 and 36.0 %, respectively. Levels of DNA–DNA relatedness between strains CN46^T and CN74^T and *Marinobacter pelagius* CGMCC 1.6775^T, *Marinobacter gudaonensis* CGMCC 1.6294^T and *Marinobacter koreensis* DSM 17924^T were 15.3–45.2 %. The results of DNA–DNA hybridizations, fatty acid analysis, and physiological and biochemical tests allowed genotypic and phenotypic differentiation of the isolates from closely related species. Two novel species are proposed, named *Marinobacter mobilis* sp. nov. (type strain CN46^T =CGMCC 1.7059^T =JCM 15154^T) and *Marinobacter zhejiangensis* sp. nov. (type strain CN74^T =CGMCC 1.7061^T =JCM 15156^T).

The genus *Marinobacter*, which belongs to the family *Alteromonadaceae*, class *Gammaproteobacteria*, was first proposed by Gauthier *et al.* (1992) to accommodate aerobic, halophilic, rod-shaped bacteria that are capable of degrading a variety of hydrocarbons. The type species, *Marinobacter hydrocarbonoclasticus*, was isolated from seawater near a petroleum refinery. Over the past few years, a further 19 *Marinobacter* species have been described (Euzéby, 1997). Most of them have been isolated from saline environments, including seawater (Yoon *et al.*, 2003, 2004; Shivaji *et al.*, 2005), marine sediment (Gorshkova *et al.*, 2003; Romanenko *et al.*, 2005; Guo *et al.*, 2007), saline soil (Martín *et al.*, 2003; Gu *et al.*, 2007), sea sand (Kim *et al.*, 2006), a brine–seawater interface (Antunes *et al.*, 2007), a coastal hot spring (Shieh *et al.*, 2003) and a wastewater pond (Liebgott *et al.*, 2006), although some have been isolated from animal tissue (Romanenko *et al.*, 2005) and algae (Green *et al.*, 2006). Three strains, CN46^T, CN71 and CN74^T, were isolated from sediment of the East China Sea. The aim of this study was to determine whether these isolates represent novel species within the genus *Marinobacter* by a polyphasic approach.

The sediment sample was collected by a multicorer from the East China Sea (27° 19′ 57″ N 120° 34′ 29″ E).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CN71, CN46^T and CN74^T are EU293411–EU293413, respectively.

Transmission electron micrographs and detailed fatty acid compositions are available as supplementary material with the online version of this paper.

Approximately 100 mg sample was suspended in 3 ml sterile seawater and vortexed for 15 min. The dispersed sediment suspension was diluted and added to modified ZoBell medium (ZoBell, 1941). The modified ZoBell medium contained (per l distilled water): 19.45 g NaCl, 8.8 g $MgCl_2$, 3.24 g Na_2SO_4, 1.8 g $CaCl_2$, 0.55 g KCl, 0.16 g $NaHCO_3$, 0.1 g ferric citrate, 0.08 g KBr, 34 mg $CsCl_2$, 22 mg H_3BO_3, 4.0 mg Na_2SiO_3, 2.4 mg NaF, 1.6 mg NH_4NO_3, 8.0 mg Na_3PO_4, 0.5 g peptone (Difco) and 0.1 g yeast extract (Difco); pH 7.4. After 3 days of aerobic incubation at 25 °C, three colonies, named CN46^T, CN74 and CN74^T, were picked. All strains were purified by repeated restreaking and maintained on halophilic medium (HM) (Ventosa *et al.*, 1982) at 30 °C. The HM medium contained (per l distilled water): 40.0 g NaCl, 2.0 g KCl, 1.0 g $MgSO_4$, 0.36 g $CaCl_2.2H_2O$, 0.23 g NaBr, 0.06 g $NaHCO_3$, trace $FeCl_3$, 10.0 g yeast extract (Difco), 5.0 g peptone (Difco), 1.0 g glucose; pH 7.2.

The optimal conditions for growth were determined in HM with different salt concentrations (0, 0.5, 1, 3, 5, 7.5, 10, 15 and 20 %, w/v). The pH range for growth was determined by adding MES (pH 5.0–6.0), PIPES (pH 6.5–7.0), Tricine (pH 7.5–8.5), CAPSO (pH 9.0–9.5) or CAPS (pH 10.0–10.5) to HM at a concentration of 40 mM. The temperature range for growth was determined by incubating cultures at 4–48 °C. Cell morphology and motility were examined by optical microscopy (Olympus BX40) and transmission electron microscopy (JEM-1230). Strains CN46^T and CN74^T were motile by a polar flagellum (see Supplementary Fig. S1 available in IJSEM Online).

Physiological and biochemical characteristics were determined using previously described methods (Xu *et al.*, 2008; Mata *et al.*, 2002). Susceptibility to antibiotics was detected on HM plates by using antibiotic discs containing the following: amoxicillin (10 μg), ampicillin (10 μg), bacitracin (0.04 IU), carbenicillin (100 μg), cefoxitin (30 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), nitrofurantoin (300 μg), novobiocin (30 μg), nystatin (100 μg), penicillin (10 μg), polymyxin B (300 IU), streptomycin (10 μg), tobramycin (10 μg) and tetracycline (30 μg). Detailed results are given in the species descriptions.

Fatty acid methyl esters, prepared from lipids that had been extracted from cells grown in HM plates for 48 h at 30 °C, were analysed by using GC-MS (Kuykendall *et al.*, 1988). Genomic DNA G+C contents were determined by thermal denaturation (T_m) (Marmur & Doty, 1962) using *Escherichia coli* K-12 DNA as a calibration standard.

The 16S rRNA gene was amplified and analysed as described previously (Xu *et al.*, 2007). PCR products were cloned into a pMD18-T vector (TaKaRa) and then sequenced to determine the almost-complete 16S rRNA gene sequence. The sequences were compared with those of closely related reference organisms from the FASTA network service.

Sequence data were aligned with CLUSTAL W 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods with the MEGA3 program package (Kumar *et al.*, 2004) and by the maximum-likelihood method (Felsenstein, 1981) with the PHYLIP 3.6 program. Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980) for the neighbour-joining method.

Colony morphology and pigmentation of the isolates were observed following growth on HM agar and milk HM agar (HM agar supplemented with 1 % skimmed milk) for 48 h at 30 °C. Strain CN74^T was semitransparent and cream-coloured; strains CN46^T and CN71, however, were transparent and non-pigmented. When the incubation time was extended to 14 days, colonies of strain CN46^T became cream. Cells of strains CN74^T and CN46^T were cultivated under identical conditions for comparison of the cellular fatty acid content. The major fatty acids (>5 %) of strain CN46^T were $C_{16:1}\omega9c$, $C_{16:0}$ and $C_{12:0}$, whereas strain CN74^T contained $C_{16:0}$, $C_{16:1}\omega9c$, $C_{18:1}\omega9c$ and $C_{12:0}$. A more detailed fatty acid composition is given in Supplementary Table S1. In addition, strain CN46^T could be distinguished from strain CN74^T by pH range for growth, hydrolysis of lecithin and utilization of ethanol. The three isolates could also be differentiated from *Marinobacter* species with validly published names by some phenotypic and genotypic characteristics, such as salinity and temperature ranges for growth, hydrolysis of substrates, utilization of some hydrocarbons and DNA G+C content (Table 1).

16S rRNA gene sequence similarities between the isolates and the type strains of *Marinobacter* species ranged from 94.0 to 97.1 %. Strains CN46^T and CN71 had identical 16S rRNA gene sequences. Strain CN46^T showed the highest sequence similarities to CN74^T (97.8 %) and the type strains of *Marinobacter pelagius* (97.1 %) and *Marinobacter gudaonensis* (97.1 %); sequence similarities between strain CN46^T and the type strains of other *Marinobacter* species were less than 97.0 %. 16S rRNA gene sequence similarities between strain CN74^T and the type strains of *Marinobacter* species were below 96.5 %. The phylogenetic trees indicated that strains CN46^T, CN71 and CN74^T clustered together in a distinct branch within the genus *Marinobacter* (Fig. 1).

To verify the species status of the two novel *Marinobacter* species, DNA–DNA hybridizations were performed by the thermal denaturation and renaturation method of De Ley *et al.* (1970) as modified by Huß *et al.* (1983), using a Beckman DU 800 spectrophotometer. The tests were carried out in triplicate. The DNA–DNA hybridization values of strain CN74^T with CN46^T and CN71 were 35.0 and 36.0 %, respectively. DNA–DNA hybridization values between strain CN46^T and *M. pelagius* CGMCC 1.6775^T, *M. gudaonensis* CGMCC 1.6294^T and *M. koreensis* DSM 17924^T were 32.6, 45.2 and 37.0 %, respectively. The DNA–DNA hybridization values of strain CN74^T to *M. pelagius* CGMCC 1.6775^T, *M. gudaonensis* CGMCC 1.6294^T and *M. koreensis* DSM 17924^T were 15.3, 32.5 and 31.0 %,

Table 1. Differential phenotypic characteristics of strains CN46^T and CN74^T and the type strains of related *Marinobacter* species

Strains: 1, CN46^T; 2, CN74^T; 3, *M. pelagius* CGMCC 1.6775^T; 4, *M. koreensis* DSM 17924^T; 5, *M. gudaonensis* CGMCC 1.6294^T; 6, *M. lutaoensis* T5054^T; 7, *M. bryozoorum* KMM 3840^T; 8, *M. hydrocarbonoclasticus* ATCC 49840^T. Data are based on our comparative studies with *M. pelagius* CGMCC 1.6775^T, *M. koreensis* DSM 17924^T and *M. gudaonensis* CGMCC 1.6294^T, as well as on literature data derived from Gauthier *et al.* (1992), Shieh *et al.* (2003), Romanenko *et al.* (2005), Kim *et al.* (2006), Gu *et al.* (2007) and Xu *et al.* (2008). +, Positive; −, negative; (+) weakly positive; ND, no data. All taxa are positive for motility, oxidase and catalase.

Characteristic	1	2	3	4	5	6	7	8
Colony pigmentation	Cream	Cream	Cream	Cream	Cream	Off-white	White	White
Range for growth								
NaCl (%, w/v)	0.5–10.0	0.5–10.0	0.5–15.0	0.5–20.0	0–15	0.5–12.0	1–18	0.5–20.0
pH	6.5–9.0	6.0–9.5	6–9	5–9	6.0–9.5	5–9	ND	6.0–9.5
Temperature (°C)	15–42	15–42	4–48	10–45	10–45	25–50	7–42	10–45
Nitrate reduction	+	+	+	+	+	−	+	+
Amylase	−	−	−	−	+	−	−	−
Lecithinase	+	(+)	−	−	+	ND	ND	+
Hydrolysis of Tween 80	+	+	+	+	+	ND	−	ND
Utilization of:								
Acetate	+	+	+	+	+	+	−	+
L-Alanine	−	−	+	−	+	+	−	−
L-Arabinose	−	−	−	−	−	−	+	−
Cellobiose	−	−	−	−	(+)	−	+	−
Citrate	−	−	−	−	+	−	−	+
Ethanol	−	+	−	−	+	ND	ND	ND
D-Fructose	−	−	−	−	+	ND	ND	−
Glutamate	+	+	+	+	+	+	−	+
Glucose	−	−	−	−	+	+	−	−
Glycerol	−	−	−	−	+	ND	+	−
Mannitol	−	−	−	−	−	+	+	−
Succinate	+	+	+	−	+	ND	ND	+
Sucrose	−	−	−	−	+	−	+	−
DNA G+C content (mol%)*	58.0–58.9	58.4	59.0	54.1	57.9	63.5	59.6	52.7

*All values were determined using the T_m method except that of *M. koreensis* DSM 17924^T, which was determined by HPLC.

respectively. All the values are sufficiently low to classify strains CN46^T and CN74^T as representatives of two genotypically distinct species within the genus *Marinobacter*.

Based on differential phenotypic properties, as well as 16S rRNA gene sequence analysis and DNA–DNA hybridization data, it is concluded that strains CN46^T and CN74^T represent two novel species within the genus *Marinobacter*.

Description of *Marinobacter mobilis* sp. nov.

Marinobacter mobilis (mo′bi.lis. L. masc. adj. *mobilis* motile).

Cells are Gram-negative and motile by a polar flagellum. Young cultures show rod-like cells (1.5–3.0 × 0.5–0.8 μm). Colonies on HM agar are 1–2 mm in diameter, circular, smooth, elevated, transparent and non-pigmented after 48 h at 30 °C. Moderately halophilic. No growth occurs in the absence of salt. Growth occurs at NaCl concentrations of 0.5–10.0 % (w/v), with optimum growth at 3.0–5.0 %. Grows at pH 6.5–9.0 and 15–42 °C (optimum growth at pH 7.0–7.5 and 30–35 °C). Oxidase- and catalase-positive. Nitrate is reduced. Tweens 20 and 80 are hydrolysed. Aesculin, casein, DNA, gelatin, starch and tyrosine are not hydrolysed. Lecithinase-positive. Negative for gluconate oxidation, indole production, *o*-nitrophenyl-*β*-D-galactopyranosidase and urease. H_2S is produced from thiosulfate. The following substrates are utilized for growth: acetate, glutamate, L-isoleucine, lactate, malate, propionate, pyruvate, succinate and L-valine. The following compounds are not utilized as sole carbon sources: L-alanine, L-arabinose, L-arginine, cellobiose, citrate, L-cysteine, ethanol, formate, D-fructose, fumarate, D-galactose, gluconate, glucose, glycerol, glycine, L-histidine, *myo*-inositol, lactose, lysine, malonate, maltose, mannitol, D-mannose, L-methionine, raffinose, rhamnose, ribose, L-serine, sorbitol, L-sorbose, sucrose, trehalose, tyrosine and D-xylose. Acid is not produced from L-arabinose, D-fructose, D-galactose, glucose, *myo*-inositol, lactose, maltose, mannitol, D-mannose, rhamnose, sorbitol, L-sorbose, trehalose or sucrose. Susceptible to amoxicillin, ampicillin, carbenicillin, cefoxitin, ceftriaxone, chloramphenicol, erythromycin, nitrofurantoin, novobiocin, penicillin, polymyxin B, tobramycin

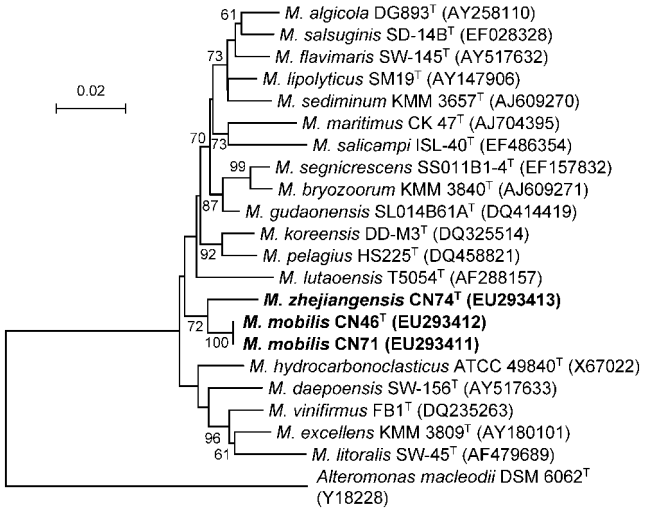

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship between the isolates and related taxa. Bootstrap percentages are based on 1000 replicates; only values >60% are shown. Bar, 0.02 substitutions per nucleotide position.

and tetracycline, but not to bacitracin, nystatin or streptomycin. Major fatty acids are $C_{16:1}\omega9c$, $C_{16:0}$ and $C_{12:0}$. The DNA G+C content is 58.0–58.9 mol% (T_m).

The type strain is CN46^T (=CGMCC 1.7059^T =JCM 15154^T), isolated from a marine sediment sample, Zhejiang, China. Strain CN71, a reference strain, was isolated from the same source.

Description of *Marinobacter zhejiangensis* sp. nov.

Marinobacter zhejiangensis (zhe.ji.ang.en′sis. N.L. masc. adj. *zhejiangensis* pertaining to Zhejiang province in China, where the type strain was isolated).

Cells are Gram-negative and motile. Young cultures show rod-like cells (1.0–2.5 × 0.4–0.8 μm), occurring singly or in pairs. Colonies on HM agar are 2–3 mm in diameter, circular and slightly irregular, elevated, semitransparent and cream-coloured after 48 h at 30 °C. No growth occurs in the absence of salt. Growth occurs at NaCl concentrations of 0.5–10.0 % (w/v), with optimum growth at 1.0–3.0 %. Grows at pH 6.0–9.5 and 15–42 °C (optimum growth at pH 7.0–7.5 and 30–35 °C). Oxidase- and catalase-positive. Nitrate is reduced. Tweens 20 and 80 are hydrolysed. Aesculin, casein, DNA, gelatin, starch and tyrosine are not hydrolysed. Weakly positive for lecithinase. Negative for gluconate oxidation, indole production, *o*-nitrophenyl-β-D-galactopyranosidase and urease. H_2S is produced from thiosulfate. The following substrates are utilized for growth: acetate, ethanol, glutamate, L-isoleucine, lactate, malate, propionate, pyruvate, succinate and L-valine. The following compounds are not utilized as sole carbon sources: L-alanine, L-arabinose, L-arginine, cellobiose, citrate, L-cysteine, formate, D-fructose, fumarate, D-galactose, gluconate, glucose, glycerol, glycine, L-histidine, *myo*-inositol, lactose, lysine, malonate, maltose, mannitol, D-mannose, L-methionine, raffinose, rhamnose, ribose, L-serine, sorbitol, L-sorbose, sucrose, trehalose, tyrosine and D-xylose. Acid is not produced from L-arabinose, D-fructose, D-galactose, glucose, *myo*-inositol, lactose, maltose, mannitol, D-mannose, rhamnose, sorbitol, L-sorbose, trehalose or sucrose. Susceptible to amoxicillin, ampicillin, carbenicillin, cefoxitin, ceftriaxone, chloramphenicol, erythromycin, nitrofurantoin, novobiocin, penicillin, polymyxin B, tobramycin and tetracycline, but not to bacitracin, nystatin or streptomycin. Major fatty acids are $C_{16:0}$, $C_{16:1}\omega9c$, $C_{18:1}\omega9c$ and $C_{12:0}$.

The type strain is CN74^T (=CGMCC 1.7061^T =JCM 15156^T), isolated from a marine sediment sample of Zhejiang, China. The DNA G+C content of the type strain is 58.4 ± 0.1 mol% (T_m).

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