Marinobacter daqiaonensis sp. nov., a moderate halophile isolated from a Yellow Sea salt pond

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A Gram-negative, oxidase- and catalase-positive, moderately halophilic strain, designated YCSA40^T, was isolated from sediment of Daqiao saltern in Qingdao, on the east coast of China. Growth occurred at 10–45 °C, at pH 5–9 and with 1–15 % NaCl. Strain YCSA40^T showed the highest 16S rRNA gene sequence similarity to *Marinobacter segnicrescens* SS011B1-4^T (97 %) and *M. gudaonensis* SL014B61A^T (96.9 %) and 16S rRNA gene sequence phylogenetic analysis assigned the isolate to the genus *Marinobacter*. Strain YCSA40^T contained C_{18:1}ω9c (34.8 %), C_{16:0} (11.6 %), C_{19:0} cyclo ω10c/C_{19:1}ω6c (10.5 %), C_{16:1}ω9c (8.4 %), C_{17:0} (6.3 %) and C_{12:0} 3-OH (5.8 %) as the predominant fatty acids. The DNA G + C content was 60.8 mol% and the major ubiquinone was Q-9. These chemotaxonomic characters were all consistent with membership of the genus *Marinobacter*. DNA–DNA relatedness between the isolate and *M. segnicrescens* CGMCC 1.6489^T, *M. gudaonensis* CGMCC 1.6294^T and other type strains of species of the genus *Marinobacter* was ≤30 %. On the basis of the aforementioned data, it was concluded that strain YCSA40^T represents a novel species of the genus *Marinobacter*, for which the name *Marinobacter daqiaonensis* sp. nov. is proposed. The type strain is YCSA40^T (=CGMCC 1.9167^T =NCCB 100308^T =LMG 25365^T).

Recent decades have seen a surge in the number of studies on micro-organisms that thrive in hypersaline environments because of their ability to adapt to highly saline conditions and produce enzymes that exhibit optimal activities at ranges of salt concentration and pH (Setati, 2010; Hedi *et al.*, 2009). Many halophilic and halotolerant bacteria within different genera have been isolated from various marine and hypersaline environments, e.g. *Marinobacter*, *Halomonas*, *Chromohalobacter*, *Salibacillus* and *Salinivibrio*. In the course of an investigation of bacteria in a saltern in China, a Gram-negative and *Marinobacter*-like bacterium, strain YCSA40^T, was isolated.

The genus *Marinobacter*, which is a member of the *Gammaproteobacteria*, was proposed by Gauthier *et al.* (1992) with one species, *Marinobacter hydrocarbonoclasticus*. At the time of writing, 27 species of the genus *Marinobacter* have been recognized, most of which are characterized chemotaxonomically as having ubiquinone-9 (Q-9) as the predominant ubiquinone, C_{16:0}, C_{18:1}ω9c and C_{16:1}ω9c as the predominant fatty acids and a DNA G + C content of 54–63.5 mol% (Yoon *et al.*, 2003, 2004, 2007; Gorshkova *et al.*, 2003; Márquez & Ventosa, 2005; Kim *et al.*, 2006; Green *et al.*, 2006; Xu *et al.*, 2008; Wang *et al.*, 2009; Aguilera *et al.*, 2009; Kharroub *et al.*, 2011). The

exception is *Marinobacter lutaoensis*, which contains Q-8 as the predominant ubiquinone and iso-C_{15:0} as the major fatty acid (Shieh *et al.*, 2003).

Strain YCSA40^T was isolated from sediment sampled at Daqiao saltern at Jimo, Qingdao, on the east coast of China (36.50° N 120.82° E). Approximately 1 g sample was suspended in 100 ml sterile seawater and vortexed for 10 min. Isolates were obtained by incubating 10-fold serial dilutions of the dispersed suspension on modified marine agar (2216E-S) (per litre seawater from the Yellow Sea: 5 g tryptone, 1 g yeast extract, 15 g agar; pH 7.5; Qu *et al.*, 2011) for 7 days at 30 °C. Strain YCSA40^T was purified by subcultivation on 2216E-S agar for 5 days at 30 °C. Culture purity was confirmed by homogeneity of the cell and colony morphologies. Strain YCSA40^T was maintained on 2216E-S agar at 30 °C and stored at –80 °C in 2216E-S broth supplemented with 30 % (v/v) glycerol.

The phenotypic characteristics of strain YCSA40^T were investigated using routine cultivation at 30 °C on marine agar 2216 (MA; Difco) with the NaCl concentration increased to 6.0 %. The reference strains *Marinobacter segnicrescens* CGMCC 1.6489^T, *M. gudaonensis* CGMCC 1.6294^T, *M. bryozorum* DSM 15401^T and *M. lacisalsi* LMG 24237^T were used as controls. Cellular morphology and the presence of flagella were observed using transmission electron microscopy (H-7000; Hitachi). Gram-staining, catalase and oxidase activities, indole production, nitrate reduction and H₂S production from L-cysteine were

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YCSA40^T is FJ984869.

Two supplementary figures are available with the online version of this paper.

examined according to standard methods (Dong & Cai, 2001). Oxidative or fermentative utilization of glucose was determined following the description of Dong & Cai (2001). Hydrolysis of starch, urea, aesculin, gelatin, Tweens 20 and 80 and DNA was assessed according to methods described elsewhere (Smibert & Krieg, 1994; Dong & Cai, 2001), using media supplemented with 6% (w/v) sea salts. Growth at 5–55 °C (at intervals of 5 °C) and 42 °C was determined in marine broth (MB; Difco) with the NaCl concentration increased to 6.0%. Growth with 0, 0.5, 1–8 (at intervals of 1%), 10, 12 and 15–30% (at intervals of 5%) (w/v) NaCl was tested using Luria–Bertani medium (Sambrook *et al.*, 1989). Growth at pH 3–10 (at intervals of 1 pH unit) was tested in MB adjusted to the appropriate pH with HCl or NaOH. Growth was determined by monitoring the OD₆₆₀ with a spectrophotometer. Acid production from carbohydrates and enzyme production were carried out using the API 50 CH and API ZYM systems (bioMérieux), according to the manufacturer's instructions, with the NaCl concentration increased to 6.0% for the preparation of inocula. Carbon source assimilation was tested using the mineral medium solution of Shivaji *et al.* (2005), with each carbon source added to a concentration of 0.2% (w/v). Growth was examined after incubation at 30 °C for 7 and 14 days (Gu *et al.*, 2007). In addition, antibiotic susceptibility tests were performed on MA using filter-paper discs (Hangzhou Microbial Reagent Company) containing various antibiotics.

Cultures for fatty acid analysis were grown on MA at 30 °C for 3 days. Fatty acid methyl esters were prepared and analysed using the standard Microbial Identification System (MIDI) for an automated gas chromatographic analysis (Sasser, 1990). Isoprenoid quinones were obtained from whole cells according to the standard method (Dong & Cai, 2001) and determined by HPLC according to Komagata & Suzuki (1987). Genomic DNA for the determination of the G+C content and 16S rRNA gene sequence analysis was prepared following the method of Rainey *et al.* (1996) and DNA purity was assessed by determining the A₂₈₀/A₂₆₀ and A₂₃₀/A₂₆₀ ratios. The G+C content of the chromosomal DNA was determined according to the methods described by Mesbah & Whitman (1989) using reversed-phase HPLC. DNA–DNA hybridization to determine genomic relatedness between strain YCSA40^T and the reference strains was performed according to the description of Liu & Shao (2005).

The 16S rRNA gene was amplified from extracted genomic DNA using PCR with universal primers 27F and 1492R, as described by Lane (1991). PCR products were purified and analysed as described previously (Qu *et al.*, 2011). 16S rRNA gene sequence similarities were investigated using the BLASTN search engine (Altschul *et al.*, 1990) and the EzTaxon server (Chun *et al.*, 2007), with which the sequences of related taxa were retrieved. After multiple sequence alignment using BioEdit version 7.0.9 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA), phylogenetic analysis

was performed using MEGA version 4 (Tamura *et al.*, 2007). A phylogenetic tree was reconstructed using the neighbour-joining method of Saitou & Nei (1987) with distance options according to Kimura's two-parameter model and the tree topology was evaluated by bootstrapping based on 1000 replications. As a robustness test, an alternative phylogenetic tree was constructed using the minimum-evolution method (Rzhetsky & Nei, 1992).

Cells of strain YCSA40^T were Gram-negative, oxidase- and catalase-positive rods (approximately 0.4–0.7 × 2.5–4.0 µm) and were motile with a single polar flagellum (Supplementary Fig. S1, available in IJSEM Online). Small cream, flat and circular colonies (1.0–2.0 mm in diameter) were produced on MA after 2–3 days at 30 °C. Growth occurred at 10–45 °C, at pH 5–9 and with 1–15% (w/v) NaCl. Detailed results of the morphological and phenotypic characterization are given in the species description. Phenotypic features that differed between strain YCSA40^T and the reference strains are summarized in Table 1. Strain YCSA40^T contained C_{18:1ω9c} (34.8%), C_{16:0} (11.6%), C_{19:0 cyclo ω10c}/C_{19:1ω6c} (10.5%), C_{16:1ω9c} (8.4%), C_{17:0} (6.3%) and C_{12:0 3-OH} (5.8%) as the predominant fatty acids (Table 2). The fatty acid composition of strain YCSA40^T was similar to those of the reference strains, although there were some differences in the proportions. Specifically, strain YCSA40^T had relatively large differences in the content of C_{16:0} compared with *M. segnicrescens* CGMCC 1.6489^T and *M. gudaonensis* CGMCC 1.6294^T (11.6% compared with 22.2–24.4%) and C_{18:1ω9c} compared with *M. bryozoorum* DSM 15401^T and *M. lacsalsi* LMG 24237^T (34.8% compared with 41.2–42.2%) (Table 2). As the fatty acid profiles of these five strains were determined under the same conditions, these differences could be used to distinguish strain YCSA40^T. Q-9 was the major quinone of strain YCSA40^T, which is as given for the majority of members of the genus *Marinobacter*. The G+C content of the chromosomal DNA of strain YCSA40^T was 60.8 mol%, which is within the range (54–63.5 mol%) for the genus *Marinobacter* (Shieh *et al.*, 2003; Kim *et al.*, 2006). DNA–DNA relatedness between strain YCSA40^T and *M. segnicrescens* CGMCC 1.6489^T, *M. gudaonensis* CGMCC 1.6294^T, *M. bryozoorum* DSM 15401^T and *M. lacsalsi* LMG 24237^T was 30, 20, 10 and 20%, respectively. According to the cut-off value of 70% recommended by Wayne *et al.* (1987) for discriminating bacterial species, strain YCSA40^T represented a novel species.

Sequence analysis of the 16S rRNA gene (1410 bp) revealed that strain YCSA40^T was affiliated with the genus *Marinobacter*. 16S rRNA gene sequence similarity ranged from a minimum of 92.8% with *Marinobacter litoralis* SW-45^T to a maximum of 97% with *M. segnicrescens* SS011B1-4^T. According to the cut-off value of 98–99% 16S rRNA gene sequence similarity for the delineation of bacterial species (Stackebrandt & Ebers, 2006), the divergence of the 16S rRNA gene sequences of strain YCSA40^T and other members of the genus *Marinobacter* supported the view that strain YCSA40^T represented a novel species. In addition, the

Table 1. Differential characteristics of strain YCSA40^T and its closest phylogenetic neighbours

Strains: 1, *Marinobacter daqiaonensis* sp. nov. YCSA40^T; 2, *M. segnicrescens* CGMCC 1.6489^T; 3, *M. gudaonensis* CGMCC 1.6294^T; 4, *M. bryozorum* DSM 15401^T; 5, *M. lacisalsi* LMG 24237^T. All data were obtained in this study unless otherwise stated. All strains are Gram-negative, oxidase- and catalase-positive, motile with a single flagellum and positive for hydrolysis of Tween 20. All strains are negative for methyl red, Voges–Proskauer and nitrite reduction tests, gelatin and DNA hydrolysis and activities of α -chymotrypsin, α - and β -galactosidases, trypsin, β -glucuronidase, α - and β -glucosidases, α -mannosidase and α -fucosidase. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5
Cell dimensions (μm)					
Width	0.4–0.7	0.6–0.7 ^{a*}	0.3–0.5 ^b	0.4–0.5 ^c	0.6–0.9 ^d
Length	2.5–4.0	0.9–1.2 ^a	1.2–1.8 ^b	1.0–1.3 ^c	1.9–2.4 ^d
pH range for growth	5–9	6–10	6–9	6–9	5–9
Temperature for growth ($^{\circ}\text{C}$)					
Range	10–45	10–45	5–50	5–42	20–40
Optimum	30	35	35	30	35
Nitrate reduction	–	+	+	+	– (+ ^d)
Indole production	–	+	–	–	–
H ₂ S production	+	+	+	–	–
Hydrolysis of:					
Tween 80	–	+	+	–	+
Starch	+	–	+	–	+
Urea	+	–	–	–	–
Growth on:					
Citrate	–	+	+	–	+
D-Mannose	+	–	–	+	–
L-Arabinose	–	+ (– ^a)	–	–	–
Cellobiose	+	–	+	–	–
Ethanol	+	–	+	w	–
D-Sorbitol	–	– (w ^a)	+	–	–
Gluconate	+	–	+	–	+
Glycerol	–	+	+	+	+
myo-Inositol	–	w	w	–	–
Enzyme activities (API ZYM)					
Alkaline phosphatase	w	w	+	w	w
Esterase (C4)	w	w	+	w (– ^c)	w
Lipase (C14)	–	–	+	–	–
Leucine aminopeptidase	+	+	+	+	w
Valine aminopeptidase	w	w	w	+	–
Acid phosphatase	w	w	w	–	–
N-Acetyl- β -glucosaminidase	w	+	+	+	+
Sensitivity to (μg):					
Novobiocin (30)	+	–	+	+	–
Streptomycin (10)	–	+	–	– (+ ^c)	–
Tetracycline (30)	–	+	+	+	–
Carbenicillin (100)	–	+	+	+	–
Erythromycin (15)	–	+	+	+	–
Ampicillin (10)	–	+	+	+	+
DNA G + C content (mol%)	60.8	62.2 ^a	57.9 ^b	59.6 ^c	58.6 ^d

*Data taken from: a, Guo *et al.* (2007); b, Gu *et al.* (2007); c, Romanenko *et al.* (2005); d, Aguilera *et al.* (2009).

phylogenetic analysis showed that strain YCSA40^T formed a distinct clade within the genus *Marinobacter*, which was supported by high bootstrap resampling values ($\geq 90\%$) in the neighbour-joining and minimum-evolution trees (Fig. 1 and Supplementary Fig. S2).

Thus, the combined phylogenetic, chemotaxonomic and phenotypic evidence clearly indicated that strain YCSA40^T should be classified in a novel species within the genus *Marinobacter*, for which the name *Marinobacter daqiaonensis* sp. nov. is proposed.

Table 2. Cellular fatty acid compositions of strain YCSA40^T and its closest phylogenetic neighbours

Strains: 1, *Marinobacter daqiaonensis* sp. nov. YCSA40^T; 2, *M. segnicrescens* CGMCC 1.6489^T; 3, *M. gudaonensis* CGMCC 1.6294^T; 4, *M. bryozorum* DSM 15401^T; 5, *M. lacisalsi* LMG 24237^T. Values are percentages of total fatty acids and were obtained in this study under identical growth conditions.

Fatty acid	1	2	3	4	5
C _{10:0}	0.6	1.1	0.4	1.2	3.5
C _{12:0}	3.8	6.2	5.6	4.9	—
C _{12:0} 3-OH	5.8	12.4	7.7	10.6	10.3
C _{14:0}	0.4	2.0	0.9	1.0	1.0
C _{16:0} N alcohol	—	—	1.9	—	1.0
C _{16:1} ω9c	8.4	8.9	5.8	3.2	6.5
C _{16:1} ω7c/C _{16:1} ω6c	2.6	—	8.3	0.8	—
C _{16:0}	11.6	22.2	24.4	17.3	16.1
iso-C _{17:1} ω9c	—	—	6.3	—	—
C _{17:1} ω8c	4.7	—	2.0	1.3	0.7
C _{17:0}	6.3	0.7	1.5	3.8	1.2
10-Methyl C _{17:0}	0.9	—	1.9	—	1.2
C _{18:3} ω6c(6,9,12)	—	—	3.4	—	—
C _{18:2} ω6,9c/anteiso-C _{18:0}	—	—	3.1	—	—
C _{18:1} ω9c	34.8	33.4	15.5	42.2	41.2
C _{18:1} ω7c	1.5	—	4.5	1.2	1.0
C _{18:0}	3.3	4.0	4.1	6.0	5.5
C _{19:0} cyclo ω10d/C _{19:1} ω6c	10.5	9.2	—	6.5	11.5

Description of *Marinobacter daqiaonensis* sp. nov.

Marinobacter daqiaonensis (da.qia.o.nen'sis. N.L. masc. adj. *daqiaonensis* of or belonging to the Daqiao Saltern, where the type strain was isolated).

Cells are Gram-negative, oxidase- and catalase-positive rods (0.4–0.7 µm wide and 2.5–4.0 µm long) and motile with a single polar flagellum. Colonies on MA are small, cream, flat and circular (1.0–2.0 mm in diameter) after 2–3 days at 30 °C. Grows with 1–15 % (w/v) NaCl (optimum 5–10 % NaCl), at 10–45 °C (optimum 30 °C) and at pH 5–9 (optimum pH 7.5). Positive for H₂S production, but negative for nitrate reduction, nitrite reduction, indole production, oxidation and fermentation of glucose and Voges–Proskauer and methyl red tests. Hydrolyses Tween 20, aesculin, starch and urease, but not Tween 80, gelatin or DNA. With API 50 CH, positive only for 5-ketogluconate. With API ZYM, positive for esterase lipase (C8) and leucine aminopeptidase, weakly positive for alkaline phosphatase, esterase (C4), valine aminopeptidase, cystine aminopeptidase, acid phosphatase, naphthol-AS-BI-phosphoamidase and *N*-acetyl-β-glucosaminidase, but negative for lipase (C14), trypsin, α-chymotrypsin, α- and β-galactosidases, β-glucuronidase, α- and β-glucosidases, α-mannosidase and α-fucosidase. Susceptible to (µg per disc unless otherwise stated) nalidixic acid (30), novobiocin (30), polymyxin B (300 U), chloramphenicol (30) and rifampicin (5), but resistant to doxycycline (30), streptomycin (10), tetracycline (30), penicillin G (10 U), carbenicillin (100), vancomycin (30), neomycin (30), kanamycin (30), ampicillin (10), lincomycin (2), clindamycin (2), oxacillin (1) and erythromycin (15). As sole carbon and energy sources, utilizes D-mannose, cellobiose, adonitol, starch, ethanol, D-mannitol, D-salicin, trehalose, D-fructose, lactose, D-galactose, gluconate, D-glucose, maltose, propionate and ribose, but not sorbitol, aesculin, formate, *myo*-inositol, malonate, L-arabinose, citrate, fumarate or glycerol. The main cellular fatty acids (>10 %) are C_{18:1}ω9c, C_{16:0} and C_{19:0} cyclo

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain YCSA40^T with respect to other members of the genus *Marinobacter*. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. *Marinimicrobium koreense* M9^T was used as the outgroup. Bar, 0.01 substitutions per nucleotide position.

$\omega 10d/C_{19:1}\omega 6c$. Q-9 is the major quinone. The DNA G + C content of the type strain is 60.8 mol%.

The type strain is YCSA40^T (=CGMCC 1.9167^T =NCCB 100308^T =LMG 25365^T), isolated from sediment of Daqiao saltern, Jimo, Qingdao, on the east coast of China.

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