Marinobacter szutsaonensis sp. nov., isolated from a solar saltern

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A Gram-negative, aerobic, non-spore-forming, halophilic bacterial strain, NTU-104^T, was isolated from the Szutsao saltern in southern Taiwan, which was previously used as salt production field. The novel isolate grew optimally at 35–40 °C, at pH 7.5–8.0 and in the presence of 5% (w/v) NaCl. The major fatty acids were C_{16:0}, C_{18:1}ω₉C, C_{16:1}ω₉C, C_{12:0} 3-OH and C_{12:0}. The predominant quinone was Q-9. Phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine were the predominant polar lipids. The DNA G+C content was 56.5 mol%. Phylogenetic analyses based on 16S rRNA gene sequences revealed the affiliation of the novel isolate to the genus *Marinobacter*. DNA–DNA hybridization results between strain NTU-104^T and the type strains of the most closely related species, *Marinobacter pelagius* and *Marinobacter koreensis*, were 36.4% and 33.2%, respectively. On the basis of phenotypic, phylogenetic and genetic analyses, strain NTU-104^T is considered to represent a novel species of the genus *Marinobacter*. The name *Marinobacter szutsaonensis* sp. nov. is proposed, with strain NTU-104^T (=BCRC 17809^T=CGMCC 1.7011^T=JCM 15751^T) as the type strain.

The genus *Marinobacter*, a member of the class *Gammaproteobacteria*, was first proposed by Gauthier *et al.* (1992). The type species of the genus, *Marinobacter hydrocarbonoclasticus*, was isolated from sediments collected in the Gulf of Fos (French Mediterranean coast) at the mouth of a petroleum refinery outlet chronically polluted by hydrocarbons (Gauthier *et al.*, 1992). Recently, many further species, for example *Marinobacter gudaonensis* (Gu *et al.*, 2007), *Marinobacter salsuginis* (Antunes *et al.*, 2007), *Marinobacter segnicrescens* (Guo *et al.*, 2007), *Marinobacter salicampi* (Yoon *et al.*, 2007), *Marinobacter pelagius* (Xu *et al.*, 2008), *Marinobacter guineae* (Montes *et al.*, 2008), *Marinobacter psychrophilus* (Zhang *et al.*, 2008), *Marinobacter mobilis* and *Marinobacter zhejiangensis* (Huo *et al.*, 2008) have been described and, at the time of writing, the genus includes 23 recognized species. Species of this genus are Gram-negative, aerobic, motile, rod-shaped bacteria. All previously described members of this genus have been found to contain C_{16:0}ω₉C, C_{16:1}ω₉C and C_{18:1} as the predominant fatty acids and to have DNA G+C contents ranging from 53 to 59.6 mol% (Liebgott *et al.*, 2006). In this study, a morphological, biochemical,

chemotaxonomic, genetic and phylogenetic characterization of a halophilic *Marinobacter*-like strain, NTU-104^T, was conducted.

A single isolate of strain NTU-104^T was obtained from soil sediment that was collected from Szutsao saltern, southern Taiwan. The isolate was cultured by the dilution method (Wang *et al.*, 2008). Briefly, soil sediment was dissolved in distilled water, diluted 1:1 with solutions of 5, 10, 15 and 20% (w/v) NaCl and plated on basal medium agar plates. The pH of the agar plate was adjusted to 7.0 by the addition of 1 M NaOH. The plates were then incubated at 37 °C for five days. Single colonies with various morphologies were selected for the growth condition tests. The salt requirements of these isolates were determined using basal medium [l⁻¹: 5 g yeast extract (Difco), 5 g Casamino acid (Difco), 5 g MgSO₄·7H₂O] with 0–30% (w/v) NaCl content. The optimal conditions for growth were determined on basal medium with various temperatures (10–70 °C) and pH values (4–10). The growth rate was monitored using a spectrophotometer at OD₆₆₀. The Gram-reaction was monitored by Gram staining using Gram Stain kits (BD), according to the manufacturer's instructions. The ability of the novel strain to utilize various carbohydrates and amino acids was tested by using the GN2 MicroPlate Identification Test Panel (Biolog). The results were read with a MicroPlate reader, using MicroLog 3.59 software to perform automated reading and identification. Tests for the hydrolysis of aesculin, gelatin, starch, Tween 20 and Tween 80, for oxidase and catalase reactions and for H₂S production were performed as

Abbreviations: EPS, exopolysaccharides; PHA, poly-β-hydroxyalkanoate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NTU-104^T is EU164778.

A table detailing the fatty acid contents of strain NTU-104^T and some related species of the genus *Marinobacter* and figures showing the polar lipid analysis and additional phylogenetic trees are available with the online version of this paper.

described by Mata *et al.* (2002). Enzyme activity was determined using the API ZYM system (bioMérieux). Sensitivity to antimicrobial agents was determined in basal medium that contained 50 mg l⁻¹ of each antimicrobial agent for at least three days. The antimicrobial agents used were ampicillin, bacitracin, carbenicillin, cefotaxime, chloramphenicol, erythromycin, kanamycin, nalidixic acid, neomycin, nitrofurantoin, novobiocin, nystatin, penicillin, polymyxin B, rifampicin, streptomycin and tetracycline. Cell morphology was observed by scanning electron microscopy. The sample was fixed and processed following the recommended procedure for preparing specimens (Antón *et al.*, 2002). Gold was used to coat the samples to an approximate thickness of 5 nm. The samples were subsequently observed under a scanning electron microscope (Topcon Co.). Exopolysaccharides (EPS) were observed by the method of Azeredo & Oliveira (1996). Poly- β -hydroxyalkanoate (PHA) detection was performed using GC as described by Mas-Castellà & Guerrero (1995).

The G+C content of DNA was determined using the method of Mesbah *et al.* (1989). The nucleotide mixtures were separated by HPLC (JASCO) using a Phenomenex C18 column. The set conditions were a flow rate of 1.0 ml min⁻¹ at a temperature of 37 °C, quantified by measurement of OD₂₆₀. Non-methylated bacteriophage λ DNA (Sigma) was used as the calibration reference. The composition of the fatty acid methyl esters (FAME) was analysed using the standard procedure of the Microbial Identification software (MIDI). The extraction of the fatty acids was performed as described by Heyrman *et al.* (1999). Grown cultures were transferred on to trypticase soy agar (TSA) plates, which contained 3 % (w/v) trypticase soy broth, 1.5 % (w/v) Bacto-Agar (Difco) and 7 % (w/v) NaCl for 24 h at 37 °C. Single colonies were removed using a platinum inoculating loop and transferred to 10 ml Teflon centrifuge tubes with Teflon screw caps (Nalge Nunc International). FAME profiles were obtained by GLC using a model 6890N GC (HP) as described by Descheemaeker & Swings (1995). The identity of the quinones was determined by HPLC (Shin *et al.*, 1996). Polar lipids were analysed by two-dimensional TLC, as described by Komagata & Suzuki (1987).

The nucleic acids of the novel isolates were extracted using a FastDNA Spin kit (Bio 101), following the manufacturer's instructions. Two universal primers (9F and 1492R) were used to amplify the 16S rRNA gene (Stackebrandt & Liesack, 1993). The amplification was performed using a GeneAmp PCR system 9700 (Applied Biosystems). Amplicons were later sequenced (Mission Biotech) and aligned with representatives from the genus *Marinobacter* and related taxa using multiple sequence alignment software (CLUSTAL W 1.82; Thompson *et al.*, 1994). A phylogenetic tree was constructed with the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms with a bootstrap robustness of 1000 using PHYLIP package 3.6b. DNA-DNA hybridization was per-

formed fluorometrically in triplicate using the method of Ezaki *et al.* (1989).

Strain NTU-104^T was found to be a Gram-negative, motile rod that grew optimally in medium that contained approximately 5 % (w/v) salt. This bacterium exhibited extreme halotolerance since it was able to grow in a medium containing 0–20 % (w/v) NaCl. *M. hydrocarbonoclasticus* is also an extremely halotolerant species, whereas *M. pelagius* is a moderately halophilic bacterium with optimal growth at 5 % NaCl. Strain NTU-104^T grew at 10–50 °C and at pH 6–8.5; optimal growth occurred at 35–40 °C and at pH 7.5–8.0. The new isolate was aerobic and catalase- and oxidase-positive. EPS and PHA were not produced. Detailed results are given in the species description.

The major components of the fatty acids detected in strain NTU-104^T were C_{16:0} (37.8 %), C_{18:1 ω 9c} (23.7 %),

Table 1. Differential phenotypic characteristics of strain NTU-104^T and other species of the genus *Marinobacter*

Species: 1, NTU-104^T (data from the present study); 2, *M. pelagius* HS225^T (data from the present study and Xu *et al.*, 2008); 3, *M. koreensis* DD-M3^T (data from the present study and Kim *et al.*, 2006); 4, *M. gudaonensis* SL014B61A^T (data from the present study and Gu *et al.*, 2007). All strains are positive in tests for motility, catalase- and oxidase-activities and for the utilization of acetate and pyruvate. All strains exhibit cream coloured colonies. All strains are negative for the hydrolysis of gelatin and urea.

Characteristic	1	2	3	4
Range for growth				
NaCl (% w/v)	0–20	0.5–15	0.5–20	0–15
pH	6–8.5	6–9	5–9	6–9.5
Temperature range (°C)	10–50	4–50	10–45	10–45
Nitrate reduction	+	+	+	+
Amylase	–	–	–	+
Hydrolysis of Tween 80	–	+	+	+
Utilization of:				
Acetate	+	+	+	+
L-Alanine	+	+	–	+
L-Arabinose	+	–	–	–
Cellobiose	–	–	–	–
Citrate	–	–	–	+
D-Fructose	–	–	–	+
Gluconate	–	–	–	–
D-Glucose	+	–	–	+
Glutamate	–	+	+	+
Glycerol	–	–	–	+
Maltose	+	–	–	+
D-Mannitol	+	–	–	–
Succinate	–	+	–	+
Sucrose	+	–	–	–
DNA G+C content (mol%)*	56.5	59.0 ^a	54.1 ^b	57.9 ^c

*Data from: a, Xu *et al.* (2008); b, Kim *et al.* (2006); c, Gu *et al.* (2007).

C_{16:1}ω9c (10.2 %), C_{12:0} 3-OH (9.3 %) and C_{12:0} (7.8 %). The fatty acid profile was similar to those of other species of the genus *Marinobacter*, in particular to that of *M. pelagius* (Gu *et al.*, 2007; Guo *et al.*, 2007; Xu *et al.*, 2008) (see Supplementary Table S1, available in IJSEM Online). The major isoprenoid quinone of strain NTU-104^T was Q9. Phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine were the major polar lipids (see Supplementary Fig. S2 in IJSEM Online). The DNA G + C content of strain NTU-104^T was 56.5 mol%, which was within the range found previously for recognized species of the genus *Marinobacter* (Table 1).

The 16S rRNA gene sequence of strain NTU-104^T as determined in this study comprised 1435 nt. Comparative 16S rRNA gene sequence analyses showed that strain NTU-104^T was most closely related to species of the genus *Marinobacter* (Fig. 1). Phylogenetic analysis based on 16S rRNA gene sequence comparisons showed that strain NTU-104^T formed a coherent cluster with *M. pelagius* with high bootstrap resampling value (98.7 % by the neighbour-joining method). Other phylogenetic trees were con-

structed by means of the maximum-likelihood and maximum-parsimony algorithms. Although the tree topologies were slightly different, the relationships between the species of the genus *Marinobacter* were similar (see Supplementary Figs S3 and S4 in IJSEM Online). Strain NTU-104^T exhibited high 16S rRNA gene sequence similarity to *M. pelagius* JCM 14804^T (98.7 %), *M. koreensis* DD-M3^T (97.2 %) and *M. gudaonensis* SL014B61A^T (97.0 %). The DNA–DNA relatedness of strain NTU-104^T to *M. pelagius* HS225^T, *M. koreensis* DD-M3^T and *M. gudaonensis* SL014B61A^T was 36.4 % (SD=3.8 %), 33.2 % (SD=4.1 %) and 30.7 % (SD=4.6 %), respectively. All of these values were clearly below the 70 % threshold that is generally used to delineate species. Thus, the 16S rRNA gene sequence data comparisons and DNA–DNA hybridization results demonstrated that strain NTU-104^T was most closely related to the genus *Marinobacter* but that the novel strain was not a member of any recognized species of this genus. In conclusion, these data, when combined with the differences revealed by biochemical, physiological and phylogenetic analyses, are sufficient to suggest that strain NTU-104^T represents a novel species of the genus

Fig. 1. Neighbour-joining tree showing the position of strain NTU-104^T with other species of the genus *Marinobacter* and related taxa based on 16S rRNA gene sequences. The bootstrap values from 1000 resamplings are indicated at nodes. Bar, 0.01 substitutions per site.

Marinobacter, for which the name *Marinobacter szutsaonensis* sp. nov. is proposed.

Description of *Marinobacter szutsaonensis* sp. nov.

Marinobacter szutsaonensis (szu.tsao.nen'sis. N.L. masc. adj. *szutsaonensis* of szutsao, a former salt field located in southern Taiwan).

Cells are Gram-negative, rod-shaped ($0.3\text{--}0.6 \times 1.3\text{--}2.2\ \mu\text{m}$) and motile with a single flagellum. Colonies are irregular to regular, flat and light translucent and milky cream colour after 48 h at $37\ ^\circ\text{C}$. Growth occurs at NaCl concentrations of 0–20% (w/v), with optimum growth at 5% NaCl. The optimum growth temperature is $35\text{--}40\ ^\circ\text{C}$; growth is observed at $10\text{--}50\ ^\circ\text{C}$. The pH range for growth is 6–8.5, with an optimum at pH 7.5–8.0. Growth occurs under aerobic conditions. Nitrate reduction is positive, H_2S production is negative. Aesculin, acetate, casein, pyruvate, urea and gelatin are hydrolysed, but starch, L-tyrosine, Tween 20, Tween 80, DNA and xanthine are not hydrolysed. Oxidase- and catalase-positive. Acid is produced from D-glucose, D-mannose, maltose, D-mannitol, sucrose and D-galactose. Acid is not produced from cellobiose, D-fructose, melibiose, D-ribose, L-arabinose, melezitose, D-sorbitol, raffinose, trehalose or D-xylose. The following substrates are used as sole carbon sources: L-alanine, fumarate, D-glucose, malate, succinate, D-mannose, maltose and sucrose. Melezitose, trehalose, D-salicin, D-sorbitol, D-xylose, benzoate, sorbose, lactose, D-fructose, L-tryptophan, formate, L-rhamnose, L-glutamate, glycerol, gluconate, glutamate, succinate and citrate are not used. When assayed with the API ZYM system, alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, trypsin, *N*-acetyl- β -glucosaminidase and α -mannosidase are present, but esterase (C4), esterase lipase (C8), lipase (C14), α -chymotrypsin, naphthol-AS-B1-phosphohydrolase, β -galactosidase, α -glucosidase, cystine arylamidase and α -fucosidase are absent. The type strain is susceptible to ampicillin, bacitracin, carbenicillin, cefotaxime, chloramphenicol, erythromycin, kanamycin, nalidixic acid, nitrofurantoin, nystatin, penicillin, polymyxin B and tetracycline, but is resistant to neomycin, novobiocin, rifampicin and streptomycin. The predominant isoprenoid quinone is Q-9. Phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine are the predominant polar lipids. The major fatty acids are $\text{C}_{16:0}$, $\text{C}_{18:1}\omega 9\text{c}$, $\text{C}_{16:1}\omega 9\text{c}$, $\text{C}_{12:0}$ 3-OH and $\text{C}_{12:0}$.

The type strain, NTU-104^T (=BCRC 17809^T=CGMCC 1.7011^T=JCM 15751^T), was isolated from Szutsao, a discarded salt field located in southern Taiwan. The DNA G+C content of the type strain is 56.5 mol%.

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