Marinobacterium zhoushanense sp. nov., isolated from surface seawater

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A Gram-stain-negative, facultatively anaerobic bacterium, designated WM3^T, was isolated from surface seawater collected from the East China Sea. Cells were catalase- and oxidase-positive, short rods and motile by means of a single polar flagellum. Growth occurred at 15–43 °C (optimum 37–40 C), pH 5.5–9.5 (optimum pH 6.5–7.5) and with 0.25–9.0 % (w/v) NaCl (optimum 1.0–1.5 %). Chemotaxonomic analysis showed that the respiratory quinone was ubiquinone-8, the major fatty acids included $C_{16:0}$ (23.6 %), $C_{18:1}\omega7c$ (26.2 %) and summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, 22.1 %). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain WM3^T was most closely related to the genus *Marinobacterium*, sharing the highest 16S rRNA gene sequence similarity of 95.5 % with both *Marinobacterium litorale* KCTC 12756^T and *Marinobacterium mangrovicola* DSM 27697^T. The genomic DNA G+C content of the strain WM3^T was 55.8 mol%. On the basis of phenotypic, chemotaxonomic and genotypic characteristics presented in this study, strain WM3^T is suggested to represent a novel species of the genus *Marinobacterium*, for which the name *Marinobacterium zhoushanense* sp. nov. is proposed. The type strain is WM3^T (=KCTC 42782^T=CGMCC 1.15341^T).

The genus *Marinobacterium*, belonging to the family *Alteromonadaceae*, was originally proposed by González *et al.* (1997). At the time of writing, the genus *Marinobacterium* contained 15 species with validly published names, isolated from various environments: *Marinobacterium georgiense* from pulp mill effluent (González *et al.*, 1997); *M. halophilum* (Chang *et al.*, 2007), *M. rhizophilum* (Kim *et al.*, 2008), *M. lutimaris* (Kim *et al.*, 2010) and *M. aestuariivivens* (Park *et al.*, 2016) from tidal flats; *M. litorale* (Kim *et al.*, 2007) and *M. marisflavi* (Kim *et al.*, 2009a) from seawater; *M. nitratireducens*, *M. sediminicola* (Huo *et al.*, 2009), *M. maritimum* (Kim *et al.*, 2009b) and *M. profundum* (Hwang *et al.*, 2016) from marine sediment; *M. coralli* from mucus of coral (Chimetto *et al.*, 2011); *M. mangrovicola* from mangrove roots (Alfaro-Espinoza & Ullrich, 2014); and *M. jannaschii* and *M. stanieri* (Satomi *et al.*, 2002) which were transferred from *Oceanospirillum jannaschii* and *Pseudomonas stanieri*, respectively, were isolated from coastal seawater. Cells of members of the genus *Marinobacterium* are Gram-negative, oxidase-positive rods and most are motile by means of a single polar flagellum. Colonies of most species in this genus are circular, smooth, convex, opaque and creamy white. $C_{18:1}\omega7c$, $C_{16:1}\omega7c$ and $C_{16:0}$ are the major fatty acids, and the respiratory quinone is Q-8. The DNA G+C content of the genus *Marinobacterium* varies from 54.9 mol% to 62.5 mol%.

In this paper, we describe a novel facultatively anaerobic strain, designated WM3^T, isolated from surface seawater collected in April 2014 around the Zhoushan Islands (30° 07′ 59.56″ N 122° 47′ 41.55″ E) of the East China Sea. The pH of the seawater was 7.9 and the salinity was 1.5 % (w/v). Based on phenotypic and phylogenetic data presented in this study, the isolate represents a novel species of the genus *Marinobacterium*. *M. litorale.* KCTC 12756^T, *M. mangrovicola* DSM 27697^T, *M. lutimaris* DSM 22012^T and *M. georgiense* KCTC 12422^T were used as reference strains in this study.

We obtained the novel isolate by the following procedure. The seawater was diluted and spread onto marine agar 2216 (MA) plates using a tenfold dilution series method. Obvious colonies formed after incubation for 3 days at 30 °C. Distinctive colonies were picked and purified by repeated

Abbreviations: FAME, fatty acid methyl ester; PHB, polyhydoxybutyrate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WM3^T is KT248536.

A supplementary figure and a supplementary table are available with the online Supplementary Material.

restreaking. Purity was confirmed by the uniformity of cell morphology. The isolate was routinely cultured in marine broth 2216 (MB) medium and maintained at −80 °C with 20 % (v/v) glycerol.

Cell morphology and motility were examined by optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL) during the late-exponential or stationary growth phases. Growth at various NaCl concentrations (0.25, 0.5, 1.0, 1.5 and 2.0–13.0 %, at increments of 1 %, w/v) was investigated in modified MB medium without Na^+ ions. The temperature range for growth was tested by incubating cells in MB medium at various temperatures (4, 15, 20, 25, 28, 32, 37, 40, 43, 45 and 50 °C). The pH range for growth was determined in MB medium with the pH adjusted (from pH 5.5 to 10.0, at intervals of 0.5 pH units) by the addition of 30 mM buffering agents, including MES (for pH 5.5–6.5), PIPES (pH 6.5–7.5), Tricine buffer (pH 7.5–8.5) and CAPSO (pH 9.0–10.0). Anaerobic growth was determined at 30 °C for 15 days in modified MB medium supplemented with sodium thiosulfate (20 mM), sodium sulfite (5 mM), sodium sulfate (20 mM), sodium nitrite (5 mM) and sodium nitrate (20 mM) as electron acceptors under a gas phase of 100 % N_2.

Utilization of single carbon sources was tested using the GN2 MicroPlate (Biolog) according to the manufacturer's instructions and the description of Park *et al.* (2009), with modified BM medium (Farmer & Hickman-Brenner, 2006). The medium contained (per litre distilled water): 0.5 g NH_4Cl, 0.0375 g $K_2HPO_4 . 7H_2O$, 0.014 g $FeSO_4 . 7H_2O$, 5.85 g NaCl, 6.15 g $MgSO_4 . 7H_2O$, 0.375 g KCl, 0.725 g $CaCl_2$, 10 ml Tris-HCl (10 mM, pH 8.0), and 25 mM PIPES, pH 7.0. Catalase and oxidase activities, polyhydroxybutyrate (PHB) production, H_2S production from thiosulfate and L-cysteine, and hydrolysis of starch, casein, L-tyrosine and cellulose were tested according to Zhu *et al.* (2011). Acid production from carbohydrates was determined at 30 °C using MOF medium [per litre distilled water: NaCl optimal concentration, $MgCl_2 . 6H_2O$ 3.87 g, $MgSO_4$ 0.48 g, KCl 0.5 g, $CaCl_2$ 0.25 g, $(NH_4)_2SO_4$ 0.5 g, $FeSO_4$ trace, casitone (BD) 1.0 g, yeast extract (BD) 0.1 g, Tris 0.5 g, and phenol red 0.01 g; adjusted to pH 7.5] (Leifson, 1963). API ZYM and 20NE kits (bioMérieux) were also used according to the manufacturer's instructions. Antibiotic susceptibility tests were determined on MA plates at 30 °C using antibiotic discs containing the following (μg per disc, unless indicated): amikacin (30), ampicillin (10), bacitracin (0.04 IU), carbenicillin (100), cefazolin (30), cefoxitin (30), cefradine (30), ceftriaxone (30), cephalothin (30), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), furazolidone (100), gentamicin (10), kanamycin (30), lincomycin (2), macrodantin (300), nalidixan (30), neomycin (30), polymyxin B (300 IU), rifampicin (5), streptomycin (10), tetracycline (30) and vancomycin (30). The test on the plates was performed with 1.5×10^8 cells ml^{-1} (McFarland standard 0.5). The strains were considered susceptible, intermediate and resistant when the diameter of the inhibition zone was >5 mm, 2–5 mm and <2 mm, respectively, according to Nokhal & Schlegel (1983).

A Quick bacteria genomic DNA extraction kit (DongSheng Biotech) was used to obtain high quality DNA template. An almost-complete 16S rRNA gene sequence of strain WM3^T was obtained by PCR using the primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), and the PCR products were cloned into the pMD19-T vector (Takara) for sequencing (Xu *et al.*, 2007). The primer pair 27F/1492R was also used for sequencing. The complete 16S rRNA gene sequence of strain WM3^T was identified on the EzTaxon-e service (Kim *et al.*, 2012) by using EzTaxon-e tool. Multiple sequences were aligned with CLUSTAL W1.8 (Thompson *et al.*, 1994). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods within the MEGA 5 program package. Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980) for the neighbour-joining method. In addition, the 16S rRNA gene sequence of strain WM3^T was also compared with other related sequences in the SILVA rRNA Databases Project libraries (http://www.arb-silva.de/) and subjected to phylogenetic analyses using ARB (Ludwig *et al.*, 2004). The DNA G+C content was determined by reversed-phase HPLC as described by Mesbah & Whitman (1989).

Isoprenoid quinones were extracted from freeze-dried cells (grown on MB medium for 24 h at 30 °C) with chloroform/methanol (2 : 1, v/v) and analysed by reversed-phase HPLC. For the preparation of cellular fatty acid methyl esters (FAMEs), strains were harvested and freeze-dried at the exponential stage of growth according to Kuykendall *et al.* (1988). Identification and quantification of the FAMEs were performed using the Sherlock Microbial Identification System (MIDI) with the standard MIS Library Generation software (Microbial ID).

After incubation for 24 h, strain WM3^T formed white, smooth, circular, elevated and cream colonies with diameters of approximately 0.5~1 mm. Cells of strain WM3^T were Gram-negative, rod-shaped and motile by means of a single flagellum (Fig. 1). Strain WM3^T grew at 15–43 °C (optimum 37–40 °C), at pH 5.5–9.5 (optimum pH 6.5–7.5) and with 0.25–9.0 % NaCl (optimum 1.0–1.5 %, w/v). Detailed results of physiological and biochemical tests are given in Table 1 and species description.

The almost-complete 16S rRNA gene sequence (1486 nt) of strain WM3^T was obtained. Analysis of 16S rRNA gene sequence similarity between strain WM3^T and other representative species revealed that the novel isolate was closely related to the genus *Marinobacterium* and shared the highest 16S rRNA gene sequence similarity of 95.5 % with both *M. litorale* KCTC 12756^T and *M. mangrovicola* DSM 27697^T. Strain WM3^T was also closely related to *M. lutimaris* DSM 22012^T (95.4 % similarity), but shared low 16S rRNA gene sequence similarity (<94 %) with all other

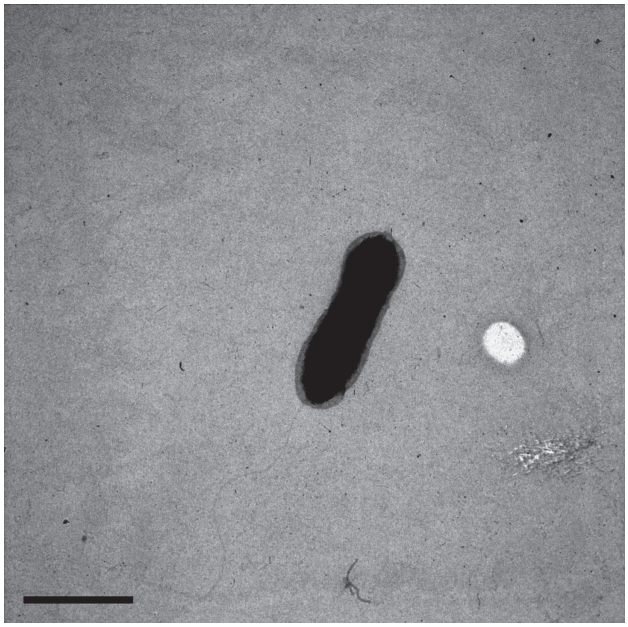

Fig. 1. Transmission electron micrograph of a cell of strain WM3^T growing on MA plates at 37 °C for 24 h; Bar, 1 μm.

species. Conformably, phylogenetic analysis based on the multiple sequences alignment indicated that strain WM3^T belonged to the genus *Marinobacterium* by clustering with *M. litorale* KCTC 12756^T, *M. mangrovicola* DSM 27697^T and *M. lutimaris* DSM 22012^T in neighbour-joining, maximum-likelihood and maximum-parsimony trees (Fig. 2). The result was consistent with the ARB tree (Fig. S1, available in the online Supplementary Material). The DNA G+C content of strain WM3^T was 55.8 mol% (HPLC).

The fatty acid compositions of strain WM3^T and type strains of related species of the genus *Marinobacterium* are shown in Table S1. The major cellular fatty acids of strain WM3^T were $C_{16:0}$ (23.6 %), $C_{18:1}\omega7c$ (26.2 %) and summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, 22.1 %), which was similar to those the reference strains, but there were also little differences in the proportions of some fatty acids. Ubiquinone-8 was detected as the sole respiratory quinone of strain WM3^T, which was in accordance with the members of the genus *Marinobacterium*.

Based on the phylogenetic, genomic and chemotaxonomic characteristics described above, strain WM3^T should belong to the genus *Marinobacterium*. However, in addition to the low 16S rRNA gene sequence similarity value, differential characteristics between strain WM3^T and the reference strains were evident. First and foremost, many carbon substrates such as L-arabinose, D-arabitol, D-galactose, D-mannitol, turanose, D-gluconic acid and D-glucuronic acid could be used by strain WM3^T but were not assimilated by the other four strains. Secondly, the maximal and optimal temperatures for growth of strain WM3^T were higher than those four reference strains of the genus *Marinobacterium*. Thirdly, acid production was detected from D-glucose and D-mannose, but not from the reference strains. Fourthly, strain WM3^T was resistant to amikacin, cefazolin and nalidixan, but the reference strains were all sensitive to those antibiotics. In addition, though the major fatty acids of strain WM3^T and the reference strains was the same, there was still some little differences between those strains. The content of $C_{12:0}$ in strain WM3^T (9.6 %) was higher than that in other strains and $C_{17:0}$ was only detected in strain WM3^T (6.6 %).

According to the physiological, biochemical and phylogenetic characteristics, strain WM3^{T}represents a novel species of the genus *Marinobacterium*, for which the name *Marinobacterium zhoushanense* sp. nov. is proposed.

Description of *Marinobacterium zhoushanense* sp. nov.

Marinobacterium zhoushanense (zhou.shan.en′se. N.L. neut. adj. *zhoushanense* referring to the Zhoushan Islands in China, from which the type strain was isolated).

Cells are Gram-stain-negative, PHB-accumulating, rod-shaped, approximately 0.4–0.6 μm wide and 1.0–2.0 μm long, and motile by means of a single polar flagellum. After incubation for 24 h on MA plates, colonies are 0.5–1.0 mm in diameter, cream, slightly convex, smooth and circular. Growth occurs at pH 5.5–9.5 (optimum pH 6.5–7.5). The temperature range for growth is 15–43 °C and no growth is detected at 4 or 45 °C. The NaCl concentration range for growth is 0.25–9.0 % (w/v), and optimal growth occurs at 1.0–1.5 % (w/v). Weak growth was observed in anaerobic conditions. Positive results in tests for catalase, oxidase, PHB accumulation, methyl red reaction and hydrolysis of tyrosine. Negative results in tests for Voges-Proskauer reaction, indole production, H_2S production (from thiosulfate or L- cysteine), hydrolysis of aesculin, casein, gelatin, starch or CM-cellulose. Acid is produced from arabinose, fructose, D-glucose and D-mannose. The following carbon substrates are utilized: L-arabinose, D-arabitol, D-fructose, D-galactose, α-D-glucose, maltose, D-mannitol, sucrose, trehalose, turanose, formic acid, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, β-hydroxybutyric acid, *p*-hydroxyphenlyacetic acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, succinic acid, L-alaninamide, D-alanine, L-alanine, L-glutamic acid, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, γ-aminobutyric acid, phenylethylamine, putrescine, glycerol. Those following carbon sources are utilized weakly: glycogen, Tween 40, Tween 80, pyruvic acid methyl ester, acetic acid, *cis*-aconitic acid, citric acid, γ-hydroxybutyric acid, D-saccharic acid, bromosuccinic acid, L-alanyl glycine, L-asparagine and L-aspartic acid. Can reduce nitrate to nitrite but not to nitrogen. The following enzymic activities are present: alkaline and acid phosphatase, esterase (C4), leucine arylamidase and α-glucosidase. Weakly positive

Table 1. Phenotypic and genotypic characteristics of strain WM3^T compared with type strains of other related species of the genus *Marinobacterium*

Strains: 1, WM3^T; 2, *M. litorale* KCTC 12756^T; 3, *M. mangrovicola* DSM 27697^T; 4, *M. lutimaris* DSM 22012^T; 5, *M. georgiense* KCTC 12422^T. All strains were positive for catalase and oxidase activities. All strains were negative in indole production, glucose fermentation, arginine dihydrolase, Voges-Proskauer reaction, H_2S production from thiosulfate, and hydrolysis of β-galactosidase, aesculin, gelatin, casein, starch and CM-cellulose. The following substrates could be used by all strains as sole carbon sources: β-hydroxybutyric acid, α-ketoglutaric acid, DL-lactic acid, propionic acid, quinic acid, succinic acid, L-alaninamide, D-alanine, L-alanine, L-glutamic acid, L-proline, putrescine, pyruvic acid methyl ester, acetic acid, *cis*-aconitic acid and citric acid. All strains were sensitive to chloramphenicol, kanamycin, erythromycin, streptomycin, tetracycline, neomycin, cefradine, ceftriaxone, macrodantin and rifampicin, but resistant to bacitracin and vancomycin. +, Positive; −, negative; W, weakly positive.

Characteristic	1	2	3	4	5
Temperature for growth (°C)					
Range	15–43	8–42[a]†	4–42[b]	15–40[c]	4–41[d]
optimum	37–40	30[a]	28–37[b]	25–30[c]	37[d]
Relationship with O_2	Facultatively anaerobic	Facultatively anaerobic	Strictly aerobic	Strictly aerobic	Strictly aerobic
PHB accumulation	+	−	+	+	+
Nitrate reduction	+	−	W	−	−
Methyl red test	+	−	−	−	−
Hydrolysis of:					
Tyrosine	+	−	−	−	−
Urea	−	+	+	+	−
Utilization of:*					
L-Arabinose	+	−	−	−	−
D-Arabitol	+	−	−	−	−
D-Galactose	+	−	−	−	−
D-Mannitol	+	−	−	−	−
Turanose	+	−	−	−	−
Trehalose	+	−	−	−	−
D-Gluconic acid	+	−	−	−	−
D-Glucuronic acid	+	−	−	−	−
Succinic acid monomethyl ester	−	+	+	+	+
Acid production from:					
Arabinose	+	−	−	+	+
Fructose	+	−	−	−	+
D-Glucose	+	−	−	−	−
D-Mannose	+	−	−	−	−
Antibiotic susceptibility					
Amikacin	−	+	+	+	+
Ampicillin	+	+	−	+	+
Cefazolin	−	+	+	+	+
Gentamicin	+	+	+	−	+
Nalidixan	−	+	+	+	+
Polymyxin B	−	+	+	−	−
API ZYM tests					
Alkaline phosphatase	+	W	+	+	+
Esterase lipase (C8)	W	+	+	+	+
Valine arylamidase	−	W	−	−	−
Acid phosphatase	+	+	+	+	W
Naphthol-AS-BI-phosphohydrolase	W	+	+	+	+
α-Glucosidase	+	−	−	−	−
β-Gglucosidase	−	−	−	W	−
DNA G+C content (mol%)	55.8	60.7[a]	57.0[b]	58.0[c]	54.6[d]

*Utilization of L-arabinose, D-galactose, D-mannitol, D-gluconic acid and trehalose was also tested in tubes with modified BM medium.
†Data obtained from: *a,* Kim *et al.* (2007); *b,* Alfaro-Espinoza & Ullrich (2014); *c,* Kim *et al.* (2010); *d,* González *et al.* (1997).

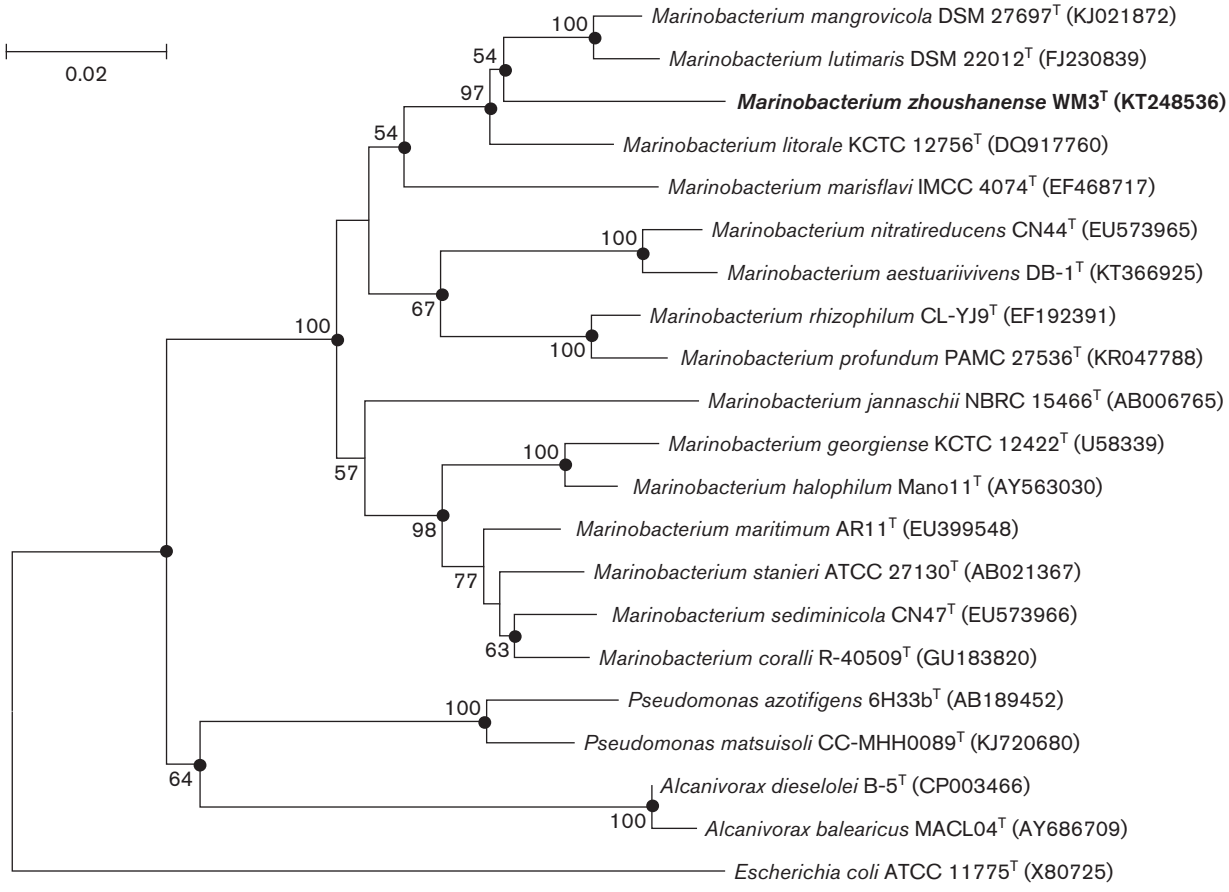

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships of strain WM3^T and related species. Bootstrap values based on 1000 replicates are listed as percentages at branching points; only values >50 % are shown. Filled circles indicate that the corresponding nodes were also recovered in both maximum-likelihood and maximum-parsimony trees. *Escherichia coli* ATCC 11775^T (GenBank accession no. X80725) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

enzyme activity for esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase. Susceptible to ampicillin, carbenicillin, cefradine, ceftriaxone, chloramphenicol, erythromycin, gentamicin, kanamycin, macrodantin, neomycin, rifampicin, streptomycin and tetracycline, but resistant to amikacin, bacitracin, cefazolin, lincomycin, nalidixan, polymyxin B and vancomycin. The respiratory quinone is Q-8. Major fatty acids are $C_{16:0}$ and $C_{18:1}\omega7c$ and summed feature 3 (comprising $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH).

The type strain, WM3^T (=KCTC 42782^T=CGMCC 1.15341^T), was isolated from surface seawater around Zhoushan Islands (30° 07′ 59.56″ N 122° 47′ 41.55″ E) of the East China Sea. The DNA G+C content of the type strain is 55.8 mol% (determined by HPLC).

Acknowledgements

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