Pseudidiomarina donghaiensis sp. nov. and *Pseudidiomarina maritima* sp. nov., isolated from the East China Sea

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Two Gram-negative, aerobic, motile, rod-shaped bacteria, designated strains 908033^T and 908087^T, were isolated from a seawater sample collected from the East China Sea. Chemotaxonomic characteristics of the two isolates included the presence of iso-$C_{15:0}$, iso-$C_{17:0}$ and iso-$C_{17:1}\omega9c$ as the major cellular fatty acids and Q-8 as the predominant ubiquinone. The genomic DNA G+C contents of strains 908033^T and 908087^T were 45.5 and 45.2 mol%, respectively. Phylogenetic analyses based on 16S rRNA gene sequences revealed that the new isolates were related to members of the genus *Pseudidiomarina*, showing levels of similarity of 95.8–96.6 % with the type strains of recognized species of the genus. The results of DNA–DNA hybridization experiments among these two isolates and *Pseudidiomarina sediminum* CICC 10319^T, in combination with chemotaxonomic and phenotypic data, demonstrated that the new isolates represent two novel species of the genus *Pseudidiomarina*, for which the names *Pseudidiomarina donghaiensis* sp. nov. (type strain 908033^T=CGMCC 1.7284^T=JCM 15533^T) and *Pseudidiomarina maritima* sp. nov. (type strain 908087^T=CGMCC 1.7285^T=JCM 15534^T) are proposed.

The family *Idiomarinaceae*, belonging to the class *Gammaproteobacteria*, was proposed by Ivanova *et al.* (2004) based on a comprehensive phylogenetic analysis, and the family comprises two recognized genera: *Idiomarina* (Ivanova *et al.*, 2000) and *Pseudidiomarina* (Jean *et al.*, 2006). At the time of writing, the genus *Pseudidiomarina* comprised two recognized species, namely *Pseudidiomarina taiwanensis* (Jean *et al.*, 2006) and *Pseudidiomarina sediminum* (Hu & Li, 2007). Members of the genus contain iso-branched fatty acids with 15 and 17 carbons as the predominant components. In this study, we present a polyphasic study describing two motile bacteria isolated from a seawater sample. The resultant phylogenetic and phenotypic data showed that the new isolates represent two novel species of the genus *Pseudidiomarina*.

A seawater sample was collected from the East China Sea (125° 59′ 24″ E 30° 58′ 16″ N) at a depth of 70 m (temperature 16.7 °C; salinity 33.95 ‰). Approximately 150 µl seawater was plated on marine agar 2216 (MA; Difco). After 3 days aerobic incubation at 25 °C, two non-pigmented colonies, designated strains 908033^T and 908087^T, were picked. The isolates were purified by repeated restreaking; purity was confirmed based on uniformity of cell morphology. Unless stated otherwise, strains 908033^T and 908087^T were maintained on halophilic medium (HM) containing 3 % NaCl (w/v) at 37 °C. HM contained (per litre distilled water): NaCl as indicated, 2.0 g KCl, 1.0 g $MgSO_4 . 7H_2O$, 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) and 1.0 g glucose (pH 7.5) (Ventosa *et al.*, 1982).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 908033^T and 908087^T are EU600204 and EU600203, respectively.

Transmission electron micrographs of cells of strains 908033^T and 908087^T, maximum-parsimony and maximum-likelihood phylogenetic trees based on 16S rRNA gene sequences, and a table giving the cellular fatty acid contents of strains 908033^T and 908087^T and related *Pseudidiomarina* species are available as supplementary material with the online version of this paper.

Optimal conditions for growth were determined in MYP medium with various NaCl concentrations (0, 0.5, 1, 3, 5, 7.5, 10, 15, 20 and 25, w/v). MYP medium contained (per litre distilled water): 1.0 g $MgSO_4 . 7H_2O$, 10.0 g yeast extract (Difco), 5.0 g peptone (Difco) and 1.0 g glucose (pH 7.5). The pH range for growth (from pH 5.0 to 10.0, at intervals of 0.5 pH units) was determined in HM with the addition of MES (50 mM; pH 5.0–6.5), PIPES (50 mM; pH 6.5–7.5), Tris (50 mM; pH 7.5–9.0) or $Na_2CO_3/NaHCO_3$ (pH 9.0–10.0). The temperature range for growth was determined by incubating at 4, 10, 15, 20, 25, 30, 35, 37, 40, 42, 45 and 50 °C. Cell morphology and motility were examined by using optical microscopy (BX40; Olympus) and electron microscopy (JEM-1200EX; JEOL).

Single carbon-source assimilation tests were performed by using a basal medium (Kämpfer *et al.*, 1991) containing (per litre distilled water): 30.0 g NaCl, 0.5 g $MgSO_4 . 7H_2O$, 0.1 g $CaCl_2 . 2H_2O$, 1.74 g K_2HPO_4, 1.36 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 0.02 g yeast extract (Difco), 0.02 g peptone (Difco), 1 ml vitamin mixture solution (Wolin *et al.*, 1963), 5 ml mineral mixture solution (Balch *et al.*, 1979) and 25 mM PIPES (pH 7.2). The corresponding filter-sterilized sugar (0.2 %), alcohol (0.1 %), organic acid (0.1 %) or amino acid (0.1 %) was added to the liquid medium. Acid production was tested by using modified MOF medium supplemented with 1 % sugars (Leifson, 1963; Xu *et al.*, 2008). Biochemical and nutritional tests were performed in HM according to Xu *et al.* (2007) as described by Mata *et al.* (2002). Additional enzyme activities and biochemical characteristics were determined by using API 20E, API 20NE and API ZYM kits at 37 °C as recommended by the manufacturer (bioMérieux). *P. sediminum* CICC 10319^T was used as a control in these tests.

Genomic DNA was obtained by using the method described by Marmur (1961). The 16S rRNA gene was amplified and analysed as described by Xu *et al.* (2007). PCR products were cloned into pMD19-T vector (TaKaRa) and were then sequenced to determine the almost-complete sequence of the 16S rRNA gene. The sequence was compared with those of closely related reference organisms from the FASTA and EzTaxon services (Chun *et al.*, 2007). Sequence data were aligned with CLUSTAL W 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed according to the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) methods with the MEGA 3.1 program package (Kumar *et al.*, 2004), and according to the maximum-likelihood method (Felsenstein, 1981) with the TreePuzzle 5.2 program. Evolutionary distances were calculated based on the algorithm of the Kimura two-parameter model (Kimura, 1980) for the neighbour-joining method.

Cellular fatty acid methyl esters were prepared from cells grown on MA for 2 days at 30 °C and were analysed by using GC/MS (Kuykendall *et al.*, 1988), according to the instructions of the Microbial Identification System (MIDI Inc.). Isoprenoid quinones were extracted from freeze-dried cells (200 mg) with chloroform/methanol (2 : 1, by vol.) and were analysed by using reversed-phase HPLC. The purified DNA was hydrolysed with P1 nuclease and the nucleotides were dephosphorylated with calf intestine alkaline phosphatase (Mesbah & Whitman, 1989). The G+C contents of the resulting deoxyribonucleosides were determined by reversed-phase HPLC and were calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) (Mesbah & Whitman, 1989). DNA–DNA hybridizations were performed by using the thermal denaturation and renaturation method of De Ley *et al.* (1970) as modified by Huß *et al.* (1983), with a Beckman DU 800 spectrophotometer.

Cells of strains 908033^T and 908087^T were Gram-negative, slightly curved rods that were motile by means of peritrichous flagella (see Supplementary Fig. S1, in IJSEM Online). The NaCl concentration and temperature ranges for growth of strain 908033^T were 0.5–10.0 % (w/v) and 15–45 °C, whereas those of strain 908087^T were 0.5–15.0 % (w/v) and 10–45 °C. Strain 908033^T could be distinguished from strain 908087^T based on H_2S formation, selenite reduction, lecithinase production and tyrosine hydrolysis (Table 1). Physiological and chemotaxonomic characteristics of the two strains are summarized in the species descriptions below.

Almost-complete 16S rRNA gene sequences of strains 908033^T (1507 nt) and 908087^T (1507 nt) were obtained. The two strains were found to be phylogenetically closely related, showing 97.5 % 16S rRNA gene sequence similarity (Fig. 1). 16S rRNA gene sequence comparisons with representative bacteria with validly published names indicated that strains 908033^T and 908087^T were related most closely to members of the genera *Pseudidiomarina* (95.8–96.6 % similarity) and *Idiomarina* (93.7–97.5 %). Phylogenetic analysis based on 16S rRNA gene sequences showed that the novel isolates formed a coherent cluster with the type strains of *P. taiwanensis* and *P. sediminum* with a moderately high bootstrap resampling value (71 % based on the neighbour-joining method) (Fig. 1). The topologies of the phylogenetic trees constructed by using the maximum-likelihood and maximum-parsimony algorithms also supported the suggestion that strains 908033^T and 908087^T represent novel species of the genus *Pseudidiomarina* (Supplementary Fig. S2, in IJSEM Online). The major cellular fatty acids of strains 908033^T and 908087^T were iso-$C_{15:0}$ (22.9 and 26.8 % of the total, respectively), iso-$C_{17:0}$ (23.1 and 15.4 %) and iso-$C_{17:1}\omega 9c$ (12.5 and 11.6 %) (Supplementary Table S1, available in IJSEM Online). This profile was similar to that of recognized species of the genus *Pseudidiomarina* (Jean *et al.*, 2006; Hu & Li., 2007).

Strains 908033^T and 908087^T showed less than 97.0 % 16S rRNA gene sequence similarity to the type strains of the two recognized species of the genus *Pseudidiomarina*. Levels of DNA–DNA relatedness between strains 908033^T

Table 1. Differential taxonomic characteristics between strains 908033^T (*P. donghaiensis* sp. nov.) and 908087^T (*P. maritima* sp. nov.) and other related *Pseudidiomarina* and *Idiomarina* species

Strains: 1, 908033^T (data from the present study); 2, 908087^T (present study); 3, *P. sediminum* CICC 10319^T (present study); 4, *P. taiwanensis* PIT1^T (Jean *et al.*, 2006); 5, *I. salinarum* ISL-52^T (Yoon *et al.*, 2007). +, Positive; −, negative; ND, not determined. Phenotypic tests for *P. sediminum* CICC 10319^T were performed in parallel with those for strains 908033^T and 908087^T.

Characteristic	1	2	3	4	5
Motility	+	+	−	−	+
Growth in 12 % NaCl	−	+	+	−	+
Growth at 10 °C	−	+	−	−	+
Growth at 45 °C	+	+	−	−	−
Catalase	+	+	−*	+	+
Nitrate reduction	−	−	−	+	+
Production of H_2S	+	−	−	−	ND
Selenite reduction	−	+	+	ND	ND
Lecithinase	+	−	+	ND	ND
Hydrolysis of:					
Casein	+	+	+	−	−
DNA	+	+	−*	−	+
Tyrosine	+	−	+	ND	+
Susceptibility to:					
Penicillin	+	+	−	+	−
Polymyxin B	+	+	−	+	+
DNA G+C content (mol%) (by HPLC)	45.5	45.2	50.0*	49.3	53.9

*Data from Hu & Li (2007).

and 908087^T with respect to *P. sediminum* CICC 10319^T were 39.1 and 37.5 %, respectively. A low level of DNA–DNA relatedness (41.3 %) was found between strains 908033^T and 908087^T, suggesting that the two strains represent two species of the genus *Pseudidiomarina* (Stackebrandt & Goebel, 1994). Comparison of phenotypic properties (Table 1 and Supplementary Table S1) indicated differences between strains 908033^T and 908087^T and the type strains of *P. taiwanensis* and *P. sediminum*, such as motility, salt or temperature range for growth, nitrate reduction, H_2S formation, hydrolysis of substrates, susceptibility to antimicrobial compounds and DNA G+C content.

Based on 16S rRNA gene sequence analysis, as well as the DNA–DNA hybridization data and differential phenotypic properties, we suggest that strains 908033^T and 908087^T represent two novel species of the genus *Pseudidiomarina*, for which the names *Pseudidiomarina donghaiensis* sp. nov. and *Pseudidiomarina maritima* sp. nov. are proposed, respectively.

Description of *Pseudidiomarina donghaiensis* sp. nov.

Pseudidiomarina donghaiensis (dong.ha.i.en′sis. N.L. fem. adj. *donghaiensis* pertaining to Donghai, the Chinese name for the East China Sea).

Cells are Gram-negative, slightly curved rods, approximately 0.4–0.6 μm in width and 1.0–1.4 μm in length. Cells are motile by means of peritrichous flagella. No endospores are formed. Colonies on MA plates are 1–2 mm in diameter, non-pigmented, circular and smooth after 48 h at 37 °C. Growth occurs at NaCl concentrations of 0.5–10.0 % (w/v) with optimum growth at 3.0 %. pH and temperature ranges for growth are pH 6.5–10.0 and 15–45 °C (optimum growth at pH 8.0–9.0 and 37 °C). No growth is detected at 10 or 50 °C. Positive for oxidase and catalase. No growth occurs on MacConkey agar or cetrimide agar. Casein, gelatin, DNA, Tweens 20 and 80 and tyrosine are hydrolysed. Aesculin and starch are not

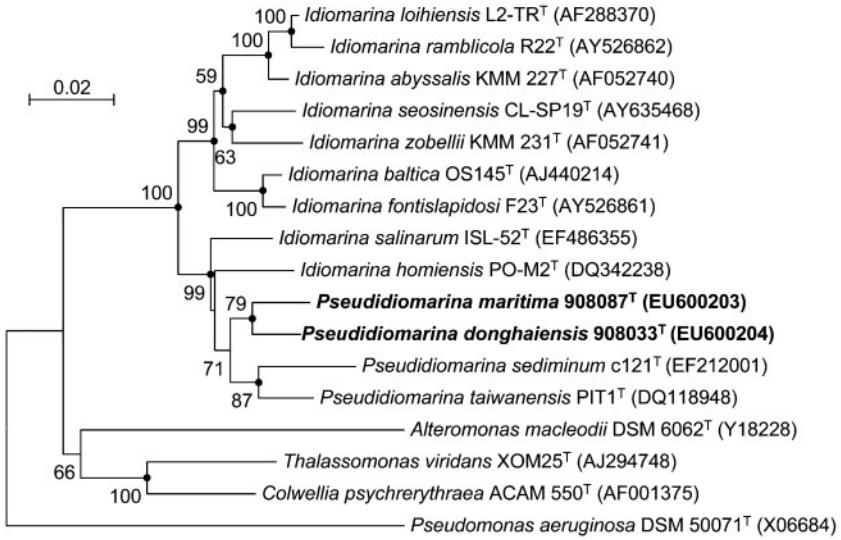

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships among strains 908033^T and 908087^T and related taxa. Bootstrap values are based on 1000 replicates; only values >50 % are shown. Bar, 0.02 substitutions per nucleotide position. Filled circles indicate nodes that were also recovered with bootstrap values >50 % in both the maximum-likelihood and the maximum-parsimony trees (see Supplementary Fig. S2a, b).

hydrolysed. Positive for lecithinase. Negative for arginine dihydrolase, gluconate oxidation, indole production, lysine decarboxylase, methyl red, *o*-nitrophenyl β-D-galactopyranosidase, ornithine decarboxylase, selenite reduction and urease. Produces H_2S from thiosulfate. No nitrate or nitrite reduction. The following substrates are not utilized as sole carbon and energy sources: acetate, adonitol, L-alanine, L-arabinose, L-arginine, L-asparagine, L-aspartate, D-cellobiose, citrate, L-cysteine, ethanol, formate, D-fructose, fumarate, D-galactose, glucose, L-glutamate, glycerol, glycine, gluconate, L-glutamine, L-histidine, inositol, isoleucine, lactate, lactose, lysine, malate, malonate, maltose, mannitol, D-mannose, melibiose, L-methionine, L-proline, propionate, pyruvate, D-raffinose, rhamnose, ribose, salicin, L-serine, L-sorbitol, sorbose, succinate, sucrose, trehalose, L-valine, xylitol and xylose. Acid is not produced from adonitol, L-arabinose, D-cellobiose, D-fructose, D-galactose, glucose, inositol, lactose, maltose, mannitol, D-mannose, melibiose, D-raffinose, rhamnose, ribose, L-sorbitol, sorbose, sucrose, trehalose, xylitol or xylose. The following constitutive enzyme activities are detected in API ZYM tests: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase. In API 20NE tests, negative for use of any of the substrates provided with the kit except gelatin. In API 20E tests, produces gelatinase, but cannot ferment glucose or other carbohydrates as substrates. Susceptible to amoxicillin (10 μg), ampicillin (10 μg), carbenicillin (100 μg), cefotaxime (30 μg), cefoxitin (30 μg), chloramphenicol (30 μg), erythromycin (10 μg), minocycline (30 μg), nitrofurantoin (300 μg), novobiocin (30 μg), penicillin (10 IU), polymyxin B (300 IU), rifampicin (5 μg) and tetracycline (30 μg), but resistant to bacitracin (0.04 IU), kanamycin (30 μg), neomycin (30 μg), nystatin (100 μg) and tobramycin (10 μg). The predominant ubiquinone is Q-8. Major fatty acids are iso-$C_{15:0}$, iso-$C_{17:0}$ and iso-$C_{17:1}\omega 9c$. The DNA G+C content of the type strain is 45.5 mol% (determined by HPLC).

The type strain, 908033^T (=CGMCC 1.7284^T=JCM 15533^T), was isolated from a coastal seawater sample collected from the East China Sea, China.

Description of *Pseudidiomarina maritima* sp. nov.

Pseudidiomarina maritima (ma.ri.ti′ma. L. fem. adj. *maritima* inhabiting marine environments).

Cells are Gram-negative, slightly curved rods, approximately 0.4–0.6 μm wide and 1.4–2.0 μm long. Cells are motile by means of peritrichous flagella. Colonies on MA plates are 1–2 mm in diameter, non-pigmented, circular and smooth after 48 h at 37 °C. Growth occurs at NaCl concentrations of 0.5–15.0 % (w/v) with optimum growth at 3.0 %. The pH and temperature ranges for growth are pH 6.5–10.0 and 10–45 °C (optimum growth at pH 8.0–9.0 and 37 °C). No growth is detected at 4 or 50 °C. Positive for oxidase and catalase. No growth occurs on MacConkey agar or cetrimide agar. Casein, DNA, gelatin and Tweens 20 and 80 are hydrolysed. Aesculin, starch and tyrosine are not hydrolysed. Positive for selenite reduction. Negative for arginine dihydrolase, gluconate oxidation, indole production, lecithinase, lysine decarboxylase, methyl red, *o*-nitrophenyl β-D-galactopyranosidase, ornithine decarboxylase and urease. H_2S is not formed from thiosulfate. No nitrate or nitrite reduction. The following substrates are not utilized as sole carbon and energy sources: acetate, adonitol, L-alanine, L-arabinose, L-arginine, L-asparagine, L-aspartate, D-cellobiose, citrate, L-cysteine, ethanol, formate, D-fructose, fumarate, D-galactose, glucose, L-glutamate, glycerol, glycine, gluconate, L-glutamine, L-histidine, inositol, isoleucine, lactate, lactose, lysine, malate, malonate, maltose, mannitol, D-mannose, melibiose, L-methionine, L-proline, propionate, pyruvate, D-raffinose, rhamnose, ribose, salicin, L-serine, L-sorbitol, sorbose, succinate, sucrose, trehalose, L-valine, xylitol and xylose. Acid is not produced from: adonitol, L-arabinose, D-cellobiose, D-fructose, D-galactose, glucose, inositol, lactose, maltose, mannitol, D-mannose, melibiose, D-raffinose, rhamnose, ribose, L-sorbitol, sorbose, sucrose, trehalose, xylitol or xylose. The following constitutive enzyme activities are detected in API ZYM tests: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase. In API 20NE tests, the type strain is not able to use any of the substrates provided with the kit except gelatin. In API 20E tests, the type strain produces gelatinase, but cannot ferment glucose or other carbohydrates as substrates. Susceptible to amoxicillin (10 μg), ampicillin (10 μg), carbenicillin (100 μg), cefotaxime (30 μg), cefoxitin (30 μg), chloramphenicol (30 μg), erythromycin (10 μg), minocycline (30 μg), nitrofurantoin (300 μg), novobiocin (30 μg), penicillin (10 IU), polymyxin B (300 IU), rifampicin (5 μg) and tetracycline (30 μg), but resistant to bacitracin (0.04 IU), kanamycin (30 μg), neomycin (30 μg), nystatin (100 μg) and tobramycin (10 μg). The predominant ubiquinone is Q-8. Major fatty acids are iso-$C_{15:0}$, iso-$C_{17:0}$ and iso-$C_{17:1}\omega 9c$. The DNA G+C content of the type strain is 45.2 mol% (determined by HPLC).

The type strain, 908087^T (=CGMCC 1.7285^T=JCM 15534^T), was isolated from a coastal seawater sample collected from the East China Sea, China.

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