Pseudidiomarina aestuarii sp. nov., a marine bacterium isolated from shallow coastal seawater

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A non-motile, rod-shaped and aerobic marine bacterium, designated strain KYW314^T, was isolated from seawater collected from the South Sea, Republic of Korea. Cells were Gram-negative, catalase- and oxidase-positive and had proteolytic activity. The major fatty acids were iso-C_{15:0} (17.2%), C_{16:0} (11.1%), iso-C_{17:0} (10.5%) and C_{18:1ω7c} (10.0%). The DNA G + C content was 56.4 mol%. A phylogenetic tree based on 16S rRNA gene sequences showed that strain KYW314^T formed a lineage within the genus *Pseudidiomarina* (95.4–97.3% sequence similarity) and a distinct branch within the clade containing *Pseudidiomarina taiwanensis* PIT1^T and *Pseudidiomarina sediminum* c121^T. Phenotypic characteristics could distinguish strain KYW314^T from members of the genus *Pseudidiomarina*. On the basis of the data presented, strain KYW314^T represents a novel species, for which the name *Pseudidiomarina aestuarii* sp. nov. is proposed. The type strain is KYW314^T (=KCTC 22740^T =JCM 16344^T).

A study of *Alteromonas*-like bacteria by Ivanova *et al.* (2004) resulted in the proposal of the family *Idiomarinaceae*, on the basis of phylogeny and signature nucleotide positions in the 16S rRNA gene. Members of the family *Idiomarinaceae* are unique among *Alteromonas*-like bacteria in possessing a high content of iso-branched fatty acids (Ivanova *et al.*, 2004). The family *Idiomarinaceae* comprises two recognized genera: *Idiomarina* (Ivanova *et al.*, 2000) as the type genus and *Pseudidiomarina* (Jean *et al.*, 2006). Several species of the genus *Pseudidiomarina* are non-motile, while all species of the genus *Idiomarina* are motile by means of flagella.

At the time of writing, the genus *Pseudidiomarina* comprises eight species, with *Pseudidiomarina taiwanensis* as the type species (Jean *et al.*, 2006, 2009; Hu & Li, 2007; Wu *et al.*, 2009). Members of the genus *Pseudidiomarina* have been isolated from saline habitats with a wide range of salinities such as coastal and oceanic waters, seashore sands, coastal sediment and solar salterns (Jean *et al.*, 2006, 2009; Kwon *et al.*, 2006; Yoon *et al.*, 2007; Hu & Li, 2007; Wu *et al.*, 2009). During a survey of the diversity of heterotrophic marine bacteria, a bacterial strain, KYW314^T, was recovered from seawater samples collected from the shallow coastal region of Gwangyang Bay in the south of the Republic of Korea (34° 51' N 128° 47' E) in July 2008.

Strain KYW314^T was isolated from seawater using the standard dilution-plating technique. Isolation was achieved using marine agar 2216 (MA; BD) (Yang *et al.*, 2006) at 30 °C for 7 days. The isolate was routinely cultured on MA and maintained at –80 °C as a suspension in marine broth (MB; BD) containing glycerol (20%, w/v).

DNA preparation and PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Chun & Goodfellow (1995). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Related sequences and the novel sequence were aligned using CLUSTAL X (Thompson *et al.*, 1997) and the alignment was refined using PHYDIT (Chun, 1995). The phylogenetic analysis was performed by using PHYLIP (Felsenstein, 1993) and PAUP* version 4.0 (Swofford, 1998). Phylogenetic trees were inferred using the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) algorithms. Distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). The robustness of the topologies from each method was evaluated by bootstrap analyses (Felsenstein, 1985) with 1000 resamplings.

A preliminary sequence comparison with the newly determined 16S rRNA gene sequence from strain KYW314^T and sequences obtained from GenBank indicated that strain

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KYW314^T is FJ768737.

Biolog GN2 MicroPlate results for strain KYW314^T are available as supplementary material with the online version of this paper.

KYW314^T was closely related to the genus *Pseudidiomarina*. The 16S rRNA gene sequence from strain KYW314^T was then aligned manually with those from representatives of the genus *Pseudidiomarina*. Strain KYW314^T had a nucleotide T at position 143, whereas members of the genus *Pseudidiomarina* have a signature nucleotide A at this position (Jean *et al.*, 2006). Strain KYW314^T showed the highest 16S rRNA gene sequence similarity to *Pseudidiomarina salinarum* ISL-52^T (97.3 %), *P. donghaiensis* 908033^T (96.7 %) and other members of the genus *Pseudidiomarina* (96.6–95.4 %). In the neighbour-joining tree, strain KYW314^T formed a distinct branch with the clade comprising *P. taiwanensis* PIT1^T and *Pseudidiomarina sediminum* c121^T and the same topology was seen in trees generated with the Fitch–Margoliash, maximum-parsimony and maximum-likelihood algorithms (Fig. 1).

The taxonomic relationships of strain KYW314^T with *P. salinarum* KCTC 12971^T and *P. taiwanensis* PIT1^T were further examined using DNA–DNA hybridization. Genomic relatedness was determined with a membrane-filter technique (Seldin & Dubnau, 1985) using a DIG High Prime DNA Labeling and Detection starter kit II (Roche) in duplicated reciprocal hybridization experiments. The DNA–DNA relatedness of strain KYW314^T to *P. salinarum* KCTC 12971^T and *P. taiwanensis* PIT1^T was 44.7 and 29.2 %, respectively. It was clear from the 16S rRNA gene sequence analysis and the DNA–DNA hybridization data that strain KYW314^T represents a novel species in the genus *Pseudidiomarina* (Wayne *et al.*, 1987).

Physiological tests with strain KYW314^T and its four closest phylogenetic neighbours were carried out under the same

Fig. 1. Neighbour-joining phylogenetic tree based on almost-complete 16S rRNA gene sequences showing the relationships between strain KYW314^T, members of the genus *Pseudidiomarina* and representatives of related taxa. Bootstrap values (>50 %) based on 1000 resamplings are shown at branch nodes. Solid circles indicate that the corresponding nodes were also recovered in trees generated with the Fitch–Margoliash, maximum-parsimony and maximum-likelihood algorithms. Bar, 0.01 substitutions per nucleotide position.

conditions using cells grown at 30 °C on MA for 2 days or in MB to exponential phase. Growth on various standard bacteriological media was tested by using nutrient agar (NA), tryptic soy agar (TSA), R2A agar (all from BD) and glucose-yeast extract agar. Motility was determined by observing cells grown in wet mounts by phase-contrast microscopy (TMS-F; Nikon). Cell morphology after 30 °C for 2 days in MB was observed by phase-contrast and scanning electron microscopy (S-4800; Hitachi). For scanning electron microscopy, cells were soaked in 0.1 M sodium phosphate buffer (pH 7.4) for several hours, fixed in 0.5 % glutaraldehyde and 0.5 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and dehydrated through an ethanol/isoamyl alcohol series. The sample was subsequently critical-point dried with an SPI dry apparatus (JSM 5310; SPI Supplies) and osmium coater (Hollow Cathode Plasma CVD; Vacuum Device). Gram staining was performed using the method described by Smibert & Krieg (1994). The requirement for NaCl (0.5 % and 1–17 %, in increments of 1 %; Sigma) and sea salts (0–10 %, in increments of 1 %; Sigma) for growth were tested using synthetic ZoBell liquid and agar medium (ZoBell, 1941) for up to 7 days. Growth at pH 3–11, in increments of 1 pH unit, was determined in ZoBell medium supplemented with 4 % (w/v) sea salts and the pH was adjusted by the addition of HCl and NaOH. Growth at 4, 10, 13, 15, 20, 25, 30, 37, 40, 42, 45 and 50 °C and growth in an anaerobic chamber (CO₂/H₂/N₂, 10:10:80; Sheldon Manufacturing) were checked on MA after incubation for up to 1 week. Catalase and oxidase activities were determined using 3 % (v/v) hydrogen peroxide and Kovács' reagent (Kovács, 1956), respectively. Acid production from D-glucose and maltose was tested on modified marine oxidation–fermentation (ZOF) medium (Lemos *et al.*, 1985) supplemented with 0.5 % (w/v) carbohydrate. Nitrate reduction was tested on MB containing 0.2 % KNO₃ (Skerman, 1967). H₂S production was determined on Kligler iron agar (BD) supplemented with 4 % (w/v) sea salts. Hydrolysis of CM-cellulose (1 %, w/v), casein (2 % skimmed milk, w/v), egg yolk (10 %, w/v), starch (0.2 %, w/v) and Tweens 20 and 80 (1 %, w/v) was tested as described by Smibert & Krieg (1994) using MA as the basal medium. Hydrolysis of L-tyrosine (0.5 %, w/v) and xylan (1 %, w/v) was tested as described by Barrow & Feltham (1993) using MA as the basal medium. DNase activity was determined with DNase test agar (BD) supplemented with 4 % (w/v) sea salts. Carbon source utilization and enzymic activities were performed using the GN2 MicroPlate (Biolog) and API ZYM (bioMérieux) kits, respectively, according to the manufacturers' instructions except that bacterial strains were suspended in distilled water supplemented with 4 % (w/v) sea salts. Antibiotic resistance was determined with the disc diffusion method using commercial antibiotic-impregnated discs (BBL Becton Dickinson). After 5 days of incubation at 30 °C on MA, the results were interpreted according to the guidelines set forth by CLSI (2003).

Strain KYW314^T grew well on MA and weakly on R2A, TSA and NA, but did not grow on glucose-yeast extract

agar. There were several phenotypic characters that separated strain KYW314^T from phylogenetically related species, e.g. strain KYW314^T was positive for casein hydrolysis and negative for esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase. The detailed results of the physiological and biochemical analyses are given in Table 1, Supplementary Table S1 (available in IJSEM Online) and the species description.

For the analyses of fatty acid and G + C contents, cells were grown on MA at 30 °C for 2 days. Fatty acids of strain KYW314^T and reference strains were prepared and analysed as methyl esters by GLC according to the instructions of the Sherlock Microbial Identification System (MIDI). For the determination of G + C content, DNA samples were prepared in duplicate and analysed by the thermal denaturation method of Marmur & Doty (1962).

The fatty acid profile of strain KYW314^T is given in Table 2. The proportions of the predominant fatty acids of strain KYW314^T were in the range of those for strains in the genus *Pseudidiomarina*, although the proportions of some straight-chain saturated fatty acids in strain KYW314^T were slightly higher. The DNA G + C content of strain KYW314^T was

56.4 ± 0.4 mol%, which was slightly higher than those reported so far for the genus *Pseudidiomarina*.

The phylogenetic, genomic, chemotaxonomic and phenotypic data clearly indicate that strain KYW314^T represents a novel species within the genus *Pseudidiomarina*, for which the name *Pseudidiomarina aestuarii* sp. nov. is proposed.

Description of *Pseudidiomarina aestuarii* sp. nov.

Pseudidiomarina aestuarii (a.es.tu.ar'i.i. L. gen. n. *aestuarii* of a tract overflowed at high tide, a salt marsh, a bay, referring to the isolation of the type strain from shallow coastal seawater).

Cells are aerobic, non-motile rods, approximately 0.3 µm wide and 0.7–1.4 µm long. Colonies on MA medium are circular, convex, smooth, opaque and non-pigmented with entire margins and approximately 1.0–2.0 mm in diameter after 3 days at 30 °C (pH 7). Growth occurs with 1–10 % (w/v) NaCl (optimum 3 %) and 2–10 % sea salts (optimum 4–5 %). Growth occurs at 10–37 °C (optimum 30 °C) and at pH 5–10 (optimum pH 7). Positive for oxidase, catalase and production of acetoin. Negative for nitrate reduction, citrate utilization, production of H₂S and indole and

Table 1. Characteristics that serve to differentiate strain KYW314^T from the type strains of related *Pseudidiomarina* species

Strains: 1, KYW314^T; 2, *P. homiensis* KACC 11514^T (data in parentheses taken from Kwon *et al.*, 2006); 3, *P. taiwanensis* BCRC 17465^T (Jean *et al.*, 2006); 4, *P. sediminum* LMG 24046^T (Hu & Li, 2007); 5, *P. salinarum* CCUG 54359^T (Yoon *et al.*, 2007). Data were obtained in this study unless indicated otherwise. All strains were non-fermentative, rod-shaped and positive for hydrolysis of Tweens 20 and 80 and tyrosine and activity of oxidase and alkaline phosphatase. All strains were negative for hydrolysis of egg yolk and starch and activity of *N*-acetyl-β-glucosaminidase, α-fucosidase, α-galactosidase, β-galactosidase, β-glucosidase, α-glucosidase, β-glucuronidase and α-mannosidase.

Characteristic	1	2	3	4	5
Motility	–	+	–	–	+
Catalase	+	+	+	–	+
NaCl concentration for growth (% w/v)					
Range	1–10	1–15	0.5–12 (0.5–11)	0.5–15	1–15 (1–14)
Optimum	3	2–5 (3–5)	2–4 (1–4)	1–8	2–3
Temperature range for growth (°C)	10–37	4–45	15–42	10–42 (13–42)	4–42
Acid production from D-glucose	–	–	+	–	–
Nitrate reduction	–	+	+	–	+
H ₂ S production	–	+	–	–	–
Hydrolysis of:					
Aesculin	+	+	+	–	–
Casein	+	–	–	–	–
DNA	+	+	–	+	+
Gelatin	–	+	+	+	–
Enzyme activity (API ZYM)					
Acid phosphatase	–	+	+	+	–
α-Chymotrypsin	+	+	+	–	–
Esterase (C4)	–	+	+	+	+
Esterase lipase (C8)	–	+	+	+	+
Leucine arylamidase	–	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	–	+	+	+	–
Trypsin	–	+	+	–	–
Valine arylamidase	–	– (+)	– (+)	–	+
DNA G + C content (mol%)	56.4	(45.1)	(49.3)	(50.0)	(53.9)

Table 2. Cellular fatty acid contents of strain KYW314^T and the type strains of related *Pseudidiomarina* species

Strains: 1, KYW314^T; 2, *P. homiensis* KACC 11514^T; 3, *P. taiwanensis* BCRC 17465^T; 4, *P. sediminum* LMG 24046^T; 5, *P. salinarum* CCUG 54359^T. Data were obtained in this study from cells grown on MA at 30 °C for 2 days. tr, Trace (<1 %); –, not detected.

Fatty acid (%)	1	2	3	4	5
C _{12:0}	tr	1.4	1.0	—	—
C _{14:0}	1.1	1.3	1.4	1.5	—
C _{16:0}	11.1	8.9	7.5	9.5	5.3
C _{17:0}	1.2	—	—	—	—
C _{18:0}	7.0	3.4	4.2	2.9	4.0
iso-C _{11:0}	3.0	3.1	3.0	1.3	6.6
iso-C _{13:0}	1.1	1.6	5.4	2.0	1.8
iso-C _{15:0}	17.2	20.3	27.8	21.5	12.0
iso-C _{16:0}	—	9.0	—	—	—
iso-C _{17:0}	10.5	7.4	10.1	10.6	14.8
iso-C _{19:0}	—	—	—	—	—
C _{10:0} 3-OH	2.6	2.3	1.3	—	1.2
C _{12:0} 3-OH	2.5	2.5	1.5	2.2	1.7
C _{16:0} 3-OH	1.6	—	—	—	—
iso-C _{11:0} 3-OH	4.9	—	7.0	6.2	9.2
iso-C _{13:0} 3-OH	4.3	5.4	4.7	4.6	6.8
iso-C _{17:0} 3-OH	1.6	—	—	—	—
iso-C _{15:1} F	1.4	2.2	5.6	3.0	3.6
C _{17:1ω8c}	tr	—	—	1.2	—
iso-C _{17:1ω9c}	7.6	11.5	7.6	12.2	21.1
C _{18:1ω7c}	10.0	8.8	4.8	10.1	7.1
C _{18:1ω9c}	1.2	2.1	—	1.7	—
Summed feature 3*	7.5	9.9	7.3	8.0	4.8

*Summed features represent groups of two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consists of C_{16:1ω7c} and/or iso-C_{15:0} 2-OH.

activities of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and urease. Hydrolyses casein, DNA, aesculin, Tweens 20 and 80 and tyrosine, but not CM-cellulose, egg yolk, starch or xylan. With API ZYM, alkaline phosphatase and α-chymotrypsin are positive; other results are negative. With GN2 Micro-Plates, weakly utilizes acetic acid, L-alaninamide, citric acid, i-erythritol, D-galactose, gentiobiose, α-D-glucose, γ-hydroxybutyric acid, myo-inositol, lactose, D-mannose, methyl β-D-glucoside, propionic acid, L-pyrogutamic acid, L-rhamnose, D-sorbitol, thymidine and Tween 80; other substrates are not utilized. Cells are sensitive to (μg per disc unless otherwise indicated): ampicillin (10), chloramphenicol (30), erythromycin (15), nalidixic acid (30), polymyxin B (300 IU) and vancomycin (30), but resistant to amikacin (30), gentamicin (10), kanamycin (30), penicillin (10 IU), streptomycin (10) and tetracycline (30). Major fatty acids (>5% of total fatty acid) are iso-C_{15:0}, C_{16:0}, iso-C_{17:0}, C_{18:1ω7c}, iso-C_{17:1ω9c} and summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH). The DNA G + C content of the type strain is 56.4 mol%.

The type strain is KYW314^T (=KCTC 22740^T =JCM 16344^T), isolated from seawater collected from the South Sea, Republic of Korea.

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