Thalassobius maritimus sp. nov., isolated from seawater

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A Gram-stain-negative, aerobic, motile, rod-shaped bacterial strain, GSW-M6^T, was isolated from seawater of Geoje island, Korea, and was subjected to a polyphasic taxonomic study. Strain GSW-M6^T grew optimally at pH 7.0–8.0, at 30 °C and in the presence of 2 % (w/v) NaCl. In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, strain GSW-M6^T clustered with *Thalassobius aestuarii*, *Thalassobius gelatinovorans* and *Thalassobius mediterraneus*. Strain GSW-M6^T exhibited 96.2–96.9 % 16S rRNA gene sequence similarity to the type strains of these three *Thalassobius* species. Strain GSW-M6^T contained Q-10 as the predominant ubiquinone and C_{18:1}ω7c as the major fatty acid. The polar lipid profiles of strain GSW-M6^T and the type strains of the three *Thalassobius* species were similar, with phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and an unidentified lipid as common major components. The DNA G+C content of strain GSW-M6^T was 57 mol%. The mean level of DNA–DNA relatedness between strain GSW-M6^T and the type strain of *Thalassobius gelatinovorans* was 17 %. Differential phenotypic properties, together with the phylogenetic and genetic distinctiveness, enabled strain GSW-M6^T to be differentiated from recognized species of the genus *Thalassobius*. On the basis of the data presented, strain GSW-M6^T is considered to represent a novel species of the genus *Thalassobius*, for which the name *Thalassobius maritimus* sp. nov. is proposed. The type strain is GSW-M6^T (=KCTC 23347^T =CCUG 60021^T).

The genus *Thalassobius* was first proposed by Arahal *et al.* (2005) based on the description of a novel species, *Thalassobius mediterraneus* (the type species), and the reclassification of *Ruegeria gelatinovorans* (Uchino *et al.*, 1998) as *Thalassobius gelatinovorans*. Subsequently, a third species of the genus was described, *Thalassobius aestuarii* (Yi & Chun, 2006). Phylogenetically, the genus *Thalassobius* forms an evolutionary lineage within the *Alphaproteobacteria* (Arahal *et al.*, 2005; Lee *et al.*, 2005). In this study, we report the taxonomic characterization of a *Thalassobius*-like bacterial strain, GSW-M6^T, which was isolated from seawater of the South Sea of Korea. The aim of the present work was to determine the exact taxonomic position of strain GSW-M6^T by using a polyphasic characterization that included determination of phenotypic properties and a detailed phylogenetic investigation based on 16S rRNA gene sequences.

Strain GSW-M6^T was isolated by the dilution plating technique on marine agar 2216 (MA; Difco) at 25 °C. The

type strains of the three recognized *Thalassobius* species were used as reference strains for phenotypic characterization, including analyses of polar lipids and fatty acids, and DNA–DNA hybridization experiments. *T. aestuarii* KCTC 12049^T and *T. gelatinovorans* KCTC 22092^T were obtained from the Korean Collection for Type Cultures, Taejeon, Republic of Korea, and *T. mediterraneus* CCUG 49438^T was obtained from the Culture Collection of the University of Göteborg, Göteborg, Sweden. Cell morphology was examined by using light microscopy (E600; Nikon) and transmission electron microscopy (CM-20; Philips). Flagellation was determined by using transmission electron microscopy on cells from an exponentially growing culture. For this, cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after being air-dried. The Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. The morphological, physiological and biochemical characteristics of strain GSW-M6^T were investigated by using routine cultivation on MA at 30 °C. Growth at 4, 10, 20, 25, 30, 37, 40 and 45 °C was

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain GSW-M6^T is HM748766.

measured on MA. Growth in the absence of NaCl and at 0.5 % and 1.0–10.0 % (w/v) NaCl (in increments of 1.0 %) was investigated in trypticase soy broth (TSB) prepared according to the formula of the Difco medium except that NaCl was excluded. The requirement for Mg^{2+} was tested by using TSB in the presence and absence of 0.45 % (w/v) $MgCl_2 \cdot 6H_2O$. The pH range for growth was determined in marine broth (MB) adjusted to pH 4.5–10.5 (increments of 0.5 pH units) by the addition of HCl or Na_2CO_3 . Growth under anaerobic conditions was determined after incubation in a Forma anaerobic chamber on MA and on MA supplemented with 0.1 % (w/v) potassium nitrate, both of which had been prepared anaerobically under a nitrogen atmosphere. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). Hydrolysis of casein, hypoxanthine, starch, tyrosine, Tweens 20, 40, 60 and 80 and xanthine was tested on MA by using the substrate concentrations described by Cowan & Steel (1965). Nitrate reduction and hydrolysis of aesculin, gelatin and urea were investigated as described by Lányi (1987), with the modification that artificial seawater was used in preparation of the media. The artificial seawater contained (per litre distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g $MgCl_2 \cdot 6H_2O$, 5.94 g $MgSO_4 \cdot 7H_2O$ and 1.3 g $CaCl_2 \cdot 2H_2O$ (Bruns *et al.*, 2001). Acid production from carbohydrates was determined as described by Leifson (1963). Utilization of substrates as sole carbon and energy sources was tested as described by Baumann & Baumann (1981), by using supplementation with 2 % (v/v) Hutner's mineral base (Cohen-Bazire *et al.*, 1957) and 1 % (v/v) vitamin solution (Staley, 1968). H_2S production was tested as described by Bruns *et al.* (2001). Susceptibility to antibiotics was investigated on MA plates by using antibiotic discs (Advantec) containing the following: polymyxin B, 100 U; streptomycin, 50 µg; penicillin G, 20 U; chloramphenicol, 100 µg; ampicillin, 10 µg; cephalothin, 30 µg; gentamicin, 30 µg; novobiocin, 5 µg; tetracycline, 30 µg; kanamycin, 30 µg; lincomycin, 15 µg; oleandomycin, 15 µg; neomycin, 30 µg; and carbenicillin, 100 µg. Enzyme activities were determined, after incubation for 4 h at 30 °C, by using the API ZYM system (bioMérieux). Other physiological and biochemical tests were performed with the API 20E system (bioMérieux) according to the manufacturer's instructions.

Chromosomal DNA was extracted and purified according to the procedure described by Yoon *et al.* (1996), with the modification that RNase T1 was used in combination with RNase A to minimize contamination by RNA. Amplification of the 16S rRNA gene was performed by using two universal primers as described previously (Yoon *et al.*, 1998) and the PCR products were purified by using a QIAquick PCR purification kit (Qiagen). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). Isoprenoid quinones were analysed according to Komagata & Suzuki (1987), by using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. For cellular fatty acid analysis, cell

mass of strain GSW-M6^T and the three reference strains was harvested from MA plates after cultivation for 4 days at 30 °C. The fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and were identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). The presence of phosphatidylcholine was identified by spraying with Dragendorff's reagent. The DNA G + C content was determined according to the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed with nuclease P1 (Sigma) and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically according to the method of Ezaki *et al.* (1989) by using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded, and the means of the remaining three values were quoted as DNA–DNA relatedness values.

The morphological, cultural, physiological and biochemical characteristics of strain GSW-M6^T are given in the species description and in Table 1. Strain GSW-M6^T did not grow in TSB without $MgCl_2$, indicating that Mg^{2+} ions are required for growth. Strain GSW-M6^T could be differentiated from recognized *Thalassobius* species based on differences in phenotypic properties, including motility, temperature range for growth, hydrolysis of several substrates and nitrate reduction, as shown in Table 1. The almost-complete 16S rRNA gene sequence of strain GSW-M6^T, comprising 1384 nt (approx. 96 % of the *Escherichia coli* 16S rRNA gene sequence), was determined in this study. Comparative 16S rRNA gene sequence analysis showed that strain GSW-M6^T was phylogenetically most closely related to the genus *Thalassobius*. In the phylogenetic tree reconstructed with the neighbour-joining algorithm, strain GSW-M6^T joined the cluster comprising *Thalassobius* species (Fig. 1). The phylogenetic affiliation of strain GSW-M6^T to members of the genus *Thalassobius* was also maintained in the tree based on the maximum-likelihood algorithm. Strain GSW-M6^T exhibited 16S rRNA gene sequence similarity of 96.2, 96.6 and 96.9 % to the type strains of *T. mediterraneus*, *T. aestuarii* and *T. gelatinovor*, respectively.

The chemotaxonomic properties of strain GSW-M6^T confirmed the result of phylogenetic analysis, namely that strain GSW-M6^T is affiliated to the genus *Thalassobius*. The predominant isoprenoid quinone detected in strain GSW-M6^T was ubiquinone-10 (Q-10), consistent with data for *Thalassobius* species (Uchino *et al.*, 1998; Yi & Chun, 2006). The only major fatty acid (>10 % of the total) found in strain GSW-M6^T was $C_{18:1}\omega 7c$ (71.8 %). The fatty acid profiles of strain GSW-M6^T and the type strains of the three recognized *Thalassobius* species were similar,

Table 1. Differential phenotypic characteristics between strain GSW-M6^T and type strains of *Thalassobius* species

Strains: 1, GSW-M6^T; 2, *T. aestuarii* KCTC 12049^T; 3, *T. gelatinovorius* KCTC 22092^T; 4, *T. mediterraneus* CCUG 49438^T. Data are from the present study except those for Gram staining, motility, temperature range for growth, nitrate reduction, hydrolysis of gelatin and DNA G + C content of the reference strains, which are from Arahal *et al.* (2005) and Yi & Chun (2006). All strains are Gram-stain-negative. All are positive for catalase and oxidase activities, utilization of acetate, pyruvate, succinate and L-malate and susceptibility to ampicillin, carbenicillin, cephalothin, chloramphenicol, gentamicin, kanamycin, neomycin, novobiocin, oleandomycin, penicillin G, streptomycin and tetracycline. All strains are negative for production of indole* and H₂S*, activities of arginine dihydrolase*, lysine decarboxylase*, ornithine decarboxylase* and tryptophan deaminase*, hydrolysis of aesculin, starch, Tween 60 and xanthine, utilization of D-galactose, trehalose, L-arabinose, sucrose, maltose, benzoate, formate and L-glutamate, acid production from cellobiose, D-fructose, D-galactose, D-glucose, myo-inositol, lactose, maltose, D-mannitol, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose and D-xylose and susceptibility to lincomycin. All strains contain phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol. w, Weakly positive.

Characteristic	1	2	3	4
Motility	+	–	+	–
Temperature range for growth (°C)	10–37	15–35	4–37	13–37
Nitrate reduction	+	–	+	–
Hydrolysis of:				
Casein	+	–	–	–
Gelatin	–	+	+	–
Hypoxanthine	+	+	+	–
Tween 20	–	+	+	+
Tween 40	–	+	–	–
Tween 80	–	w	–	–
Tyrosine	+	+	+	–
Urea	–	–	+	–
Utilization of:				
Cellobiose	+	–	–	–
D-Fructose	+	–	–	+
D-Glucose	+	+	–	+
D-Mannose	+	–	–	–
D-Xylose	–	+	–	–
Citrate	+	+	–	+
Salicin	+	–	–	–
Acid production from:				
L-Arabinose	+	–	–	–
D-Ribose	–	+	–	–
Susceptibility to polymyxin B	+	–	–	–
Additional major polar lipid(s)†	L	AL, Ls	Ls	L
DNA G + C content (mol%)	57	61	59	57

*Data obtained using the API 20E system.

†AL, Unidentified aminolipid; L(s), unidentified lipid(s).

although there were differences in the proportions of some components (Table 2). Major polar lipids found in strain GSW-M6^T were phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and an unidentified lipid (Fig. 2, Table 1), a profile that was similar to those of the type strains of the three *Thalassobius* species. However, the polar lipid profile of strain GSW-M6^T was distinguishable from those of *T. aestuarii* KCTC 12049^T and *T. gelatinovorius* KCTC 22092^T in that an additional unidentified lipid

and/or an amino lipid were also present (Fig. 2, Table 1). The DNA G + C content of strain GSW-M6^T was 57 mol%.

Strain GSW-M6^T could be distinguished from recognized *Thalassobius* species based on differences in several phenotypic characteristics, most of which were determined under the same conditions and methods (Table 1). The mean level of DNA–DNA relatedness between strain GSW-M6^T and *T. gelatinovorius* KCTC 22092^T was 17 %. The

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain GSW-M6^T, the type strains of *Thalassobius* species and some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) >50 % are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. Open circles indicate that the corresponding nodes were also recovered in the tree generated with one of these algorithms. The sequence of *Stappia stellulata* IAM 12621^T (GenBank accession no. D88525) was used as an outgroup (not shown). Bar, 0.01 substitutions per nucleotide position.

phylogenetic distinctiveness of strain GSW-M6^T, together with differential phenotypic properties and genetic distinctiveness, shows that this strain is separate from recognized *Thalassobius* species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Tindall *et al.*, 2010). Therefore, on the basis

of the phenotypic, chemotaxonomic and phylogenetic data presented here, strain GSW-M6^T is considered to represent a novel species of the genus *Thalassobius*, for which the name *Thalassobius maritimus* sp. nov. is proposed.

Description of *Thalassobius maritimus* sp. nov.

Thalassobius maritimus (ma.ri'ti.mus. L. masc. adj. *maritimus* of the sea, referring to the marine environment).

Cells are Gram-stain-negative, aerobic rods, 0.4–0.8 µm in diameter and 1.0–6.0 µm long. Motile by means of a single polar flagellum. Colonies on MA are convex, opaque, circular with entire margins and cream-coloured after incubation for 3 days at 30 °C. Growth does not occur under anaerobic conditions on MA or on MA supplemented with nitrate. Optimal growth temperature is 30 °C; growth occurs at 10 and 37 °C but not at 4 or 40 °C. Optimal growth at pH 7.0–8.0; growth occurs at pH 6.0 but not at pH 5.5. Optimal growth occurs in the presence of approx. 2.0 % (w/v) NaCl; growth occurs in the presence of 0–7.0 % (w/v) NaCl. Mg²⁺ ions are required for growth. In assays with the API ZYM system, positive for alkaline phosphatase and leucine arylamidase activities, weakly positive for esterase lipase (C8), but negative for acid phosphatase, cystine arylamidase, esterase (C4), lipase (C14), naphthol-AS-BI-phosphohydrolase, *N*-acetyl-β-glucosaminidase, trypsin, valine arylamidase, α-chymotrypsin, α-fucosidase, α-galactosidase, α-glucosidase, α-mannosidase, β-galactosidase, β-glucosidase and β-glucuronidase activities. The predominant ubiquinone is Q-10. The major polar lipids are

Table 2. Cellular fatty acid compositions of strain GSW-M6^T and the type strains of recognized *Thalassobius* species

Strains: 1, GSW-M6^T; 2, *T. aestuarii* KCTC 12049^T; 3, *T. gelatinovorans* KCTC 22092^T; 4, *T. mediterraneus* CCUG 49438^T. All data are from the present study. Values are percentages of total fatty acids; fatty acids that represented <1.0 % in all strains were omitted. –, Not detected; ECL, equivalent chain-length.

Fatty acid	1	2	3	4
Straight-chain				
C _{16:0}	8.6	4.6	12.2	8.8
C _{17:0}	1.9	0.6	1.0	0.6
C _{18:0}	3.7	2.3	1.7	3.0
Unsaturated				
C _{18:1ω7c}	71.8	75.1	68.2	78.6
Hydroxy				
C _{10:0} 3-OH	4.4	2.1	—	—
C _{12:1} 3-OH	1.2	—	3.8	—
C _{16:0} 2-OH	—	3.9	2.0	—
11-Methyl C _{18:1ω7c}	2.7	7.3	6.3	4.7
Unknown ECL 11.799	2.8	2.1	2.5	—

Fig. 2. TLC chromatograms of polar lipids of strain GSW-M6^T (a), *T. aestuarii* KCTC 12049^T (b), *T. gelatinovorans* KCTC 22092^T (c) and *T. mediterraneus* CCUG 49438^T (d). PC, Phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL, unidentified aminolipid; L, unidentified lipid.

phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and an unidentified lipid. The major cellular fatty acid (>10% of the total) is C_{18:1}ω7c. The DNA G+C content of the type strain is 57 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, GSW-M6^T (=KCTC 23347^T =CCUG 60021^T), was isolated from seawater of Geoje island, Korea.

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