Pseudoruegeria aquimaris gen. nov., sp. nov., isolated from seawater of the East Sea in Korea

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A Gram-negative, non-motile, rod-shaped bacterial strain, SW-255^T, was isolated from seawater from Hwajinpo, on the coast of the East Sea, Korea, and subjected to a polyphasic taxonomic study. Strain SW-255^T grew optimally at pH 7.0–8.0 and 37 °C in the presence of 2 % (w/v) NaCl. It contained Q-10 as the predominant ubiquinone and $C_{18:1}\omega7c$ as the major fatty acid. The DNA G+C content was 67.0 mol%. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showed that strain SW-255^T is phylogenetically closely related to the genera *Ruegeria* and *Silicibacter* of the *Alphaproteobacteria*. The levels of 16S rRNA gene sequence similarity between strain SW-255^T and the type strains of *Ruegeria atlantica* and two *Silicibacter* species were in the range 95.8–96.2 %. A phylogenetic tree based on *gyrB* sequences showed that strain SW-255^T forms a distinct evolutionary lineage within the *Alphaproteobacteria*. Differential phenotypic properties, polar lipid profiles and DNA G+C contents, together with the phylogenetic distinctiveness, suggest that strain SW-255^T should be distinguished from the members of the genera *Ruegeria* and *Silicibacter*. On the basis of the phenotypic, chemotaxonomic and phylogenetic data, strain SW-255^T represents a novel genus and species, for which the name *Pseudoruegeria aquimaris* gen. nov., sp. nov. is proposed. The type strain of *Pseudoruegeria aquimaris* is SW-255^T (=KCTC 12737^T=JCM 13603^T).

The genus *Ruegeria* was created from the reclassification of *Agrobacterium atlanticum*, *Agrobacterium gelatinovorum* and *Roseobacter algicola* (Uchino *et al.*, 1998). 16S rRNA gene sequence analyses have since shown that *Ruegeria gelatinovorans* and *Ruegeria algicola* have phylogenetic positions that are independent of *Ruegeria atlantica*, the type species of the genus (González *et al.*, 2003; Lee *et al.*, 2005). *Ruegeria gelatinovorans* and *Ruegeria algicola* have subsequently been reclassified as *Thalassobius gelatinovorus* (Arahal *et al.*, 2005) and *Marinovum algicola* (i.e. in a novel genus; Martens *et al.*, 2006). The related genus *Silicibacter* was proposed by Petursdottir & Kristjansson (1997) with a single species, *Silicibacter lacuscaerulensis*; another *Silicibacter* species, *Silicibacter pomeroyi*, has since been described (González *et al.*, 2003). Here we describe a bacterial strain, SW-255^T, which is phylogenetically closely related to *Ruegeria atlantica*, *S. lacuscaerulensis* and *S. pomeroyi*. The aim of the present study was to determine the exact taxonomic position of strain SW-255^T by means of a polyphasic characterization that included determination of phenotypic and chemotaxonomic properties and a detailed phylogenetic analysis based on 16S rRNA gene sequences.

Strain SW-255^T was isolated from seawater from Hwajinpo on the coast of the East Sea, Korea, by means of the standard dilution plating technique at 25 °C on marine agar 2216 (MA; Difco). The type strains of *Ruegeria atlantica*, *S. lacuscaerulensis* and *S. pomeroyi* were used as reference strains for the phenotypic and fatty acid analyses; *Ruegeria atlantica* KCTC 12424^T and *S. lacuscaerulensis* KCTC 2953^T were obtained from the Korean Collection for Type Cultures, Taejon, Korea. *S. pomeroyi* DSM 15171^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). The morphological, physiological and biochemical characteristics of strain SW-255^T were investigated using routine cultivation on MA at 37 °C. The cell morphology was examined by using light microscopy (E600; Nikon) and transmission electron microscopy. The presence of flagella was determined using transmission electron microscopy (CM-20; Philips) with cells from cultures growing exponentially. For transmission electron microscopic observation, cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after air drying. Growth under anaerobic conditions was determined after incubation in a Forma

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SW-255^T is DQ675021, and those for the *gyrB* sequences of strain SW-255^T, *S. lacuscaerulensis* KCTC 2953^T, *S. pomeroyi* DSM 15171^T and *Ruegeria atlantica* KCTC 12424^T are EF010915–EF010918, respectively.

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anaerobic chamber on MA and on MA supplemented with nitrate, both of which had been prepared anaerobically using a nitrogen atmosphere. Growth in the absence of NaCl was investigated using trypticase soy broth prepared according to the formula of the Difco medium except that no NaCl was used. Growth at various NaCl concentrations was investigated in marine broth 2216 (Difco) or trypticase soy broth (Difco). Growth at various temperatures (4–55 °C) was measured on MA. Catalase and oxidase activities and the hydrolysis of casein, starch and Tweens 20, 40, 60 and 80 were determined as described by Cowan & Steel (1965). The hydrolysis of hypoxanthine, tyrosine and xanthine was tested on MA using the substrate concentrations described by Cowan & Steel (1965). The hydrolysis of aesculin, gelatin and urea and nitrate reduction were investigated as described previously (Lanyi, 1987), with the modification that artificial seawater was used in the preparation of the media. The artificial seawater contained (l^{-1} distilled water) 23.6 g NaCl, 0.64 g KCl, 4.53 g $MgCl_2.6H_2O$, 5.94 g $MgSO_4.7H_2O$ and 1.3 g $CaCl_2.2H_2O$ (Bruns *et al.*, 2001). H_2S production was tested as described previously (Bruns *et al.*, 2001). Susceptibility to antibiotics was detected on MA plates by using antibiotic discs with the following amounts: 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) and tetracycline (30 μg). Acid production from carbohydrates was determined as described by Leifson (1963). The utilization of various substrates for growth was determined as described by Baumann & Baumann (1981) using supplementation with 2 % (v/v) Hutner's mineral salts solution (Cohen-Bazire *et al.*, 1957) and a 1 % (v/v) vitamin solution (Staley, 1968). Other physiological and biochemical tests were performed with the API 20E and API ZYM systems (bioMérieux).

Cell biomass for respiratory lipoquinone analysis and for DNA extraction was obtained from cultivation in marine broth 2216 at 37 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene was amplified by using a PCR with two universal primers, as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). PCR amplification of the DNA gyrase B subunit gene (*gyrB*) was performed by using two primers, UP-1 and UP-2r, according to a method described previously (Yamamoto & Harayama, 1995). The PCR product was purified with the QIAquick PCR purification kit (Qiagen). Sequencing of the purified PCR product was performed with an Applied Biosystems automatic DNA sequencer (model 3130) using two primers, as described by Yamamoto & Harayama (1995). Alignment of the sequences was carried out with the CLUSTAL W software (Thompson *et al.*, 1994). Gaps at the 5′ and 3′ ends of the alignment were omitted from further analysis. Evolutionary distance matrices were calculated by using the algorithm of Jukes & Cantor (1969) with the DNADIST program. A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) in the PHYLIP package (Felsenstein, 1993). The stability of the relationships was assessed by means of a bootstrap analysis based on 1000 resamplings of the neighbour-joining dataset, using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package.

Respiratory lipoquinones were analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC. For cellular fatty acid analysis, cell mass of strain SW-255^T, *Ruegeria atlantica* KCTC 12424^T, *S. lacuscaerulensis* KCTC 2953^T and *S. pomeroyi* DSM 15171^T was harvested from MA plates after cultivation for 3 days at 37, 20, 45 and 30 °C, respectively. 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 the appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). The presence of phosphatidylcholine was tested by spraying with Dragendorff′s reagent (Sigma). The DNA G+C content was determined by the method of Tamaoka & Komagata (1984), with the modification that the DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC.

Morphological, cultural, physiological and biochemical characteristics of strain SW-255^T are given in the genus and species descriptions (see below) and in Table 1. The almost-complete 16S rRNA gene sequence of strain SW-255^T determined in this study comprised 1421 nt, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, strain SW-255^T was part of the clade comprising *Ruegeria atlantica* and the two *Silicibacter* species, with a bootstrap resampling value of 53.8 % (Fig. 1). Strain SW-255^T exhibited 16S rRNA gene sequence similarity values of 95.8, 96.0 and 96.2 % with respect to the type strains of *Ruegeria atlantica*, *S. lacuscaerulensis* and *S. pomeroyi*, respectively. The 16S rRNA gene sequence similarity values between strain SW-255^T and other species used in the phylogenetic analysis were below 95.6 %. The *gyrB* sequences of strain SW-255^T, *Ruegeria atlantica* KCTC 12424^T, *S. lacuscaerulensis* KCTC 2953^T and *S. pomeroyi* DSM 15171^T determined in this study each comprised 1146 nt. Strain SW-255^T exhibited *gyrB* sequence similarity values of 76.2, 81.8 and 80.1 % with respect to *Ruegeria atlantica* KCTC 12424^T, *S. lacuscaerulensis* KCTC 2953^T and *S. pomeroyi* DSM 15171^T, respectively. The *gyrB* sequence similarity between *S. lacuscaerulensis* KCTC 2953^T and *S. pomeroyi* DSM 15171^T was 85.4 %.

The predominant respiratory lipoquinone detected in strain SW-255^T was Q-10 (at a peak area ratio of approximately 95 %). The fatty acid profile of strain SW-255^T comprised

Table 1. Differential phenotypic characteristics of strain SW-255^T and members of the genera *Ruegeria* and *Silicibacter*

Taxa: 1, strain SW-255^T; 2, *Ruegeria atlantica* 1480^T [data from Rüger & Höfle (1992) unless indicated]; 3, *S. lacuscaerulensis* [Petursdottir & Kristjansson (1997) unless indicated]; 4, *S. pomeroyi* [González *et al.* (2003) unless indicated]. All four taxa are positive for catalase, oxidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and the utilization of succinate (data for the type strain of *S. lacuscaerulensis* from this study) and pyruvate. All are negative for growth at 4 °C, Gram-staining, indole and H_2S production, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, α-fucosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, hydrolysis of casein (data for the type strains of *S. lacuscaerulensis* and *S. pomeroyi* from this study), starch (data for the type strain of *S. lacuscaerulensis* from this study), Tween 80 (data for the type strains of *Ruegeria atlantica* and *S. lacuscaerulensis* from this study) and urea (data for the type strains of *S. lacuscaerulensis* and *S. pomeroyi* from this study) and acid production from lactose (data for the type strains of *S. lacuscaerulensis* and *S. pomeroyi* from this study). +, Positive; −, negative; W, weak.

Characteristic	1	2	3	4
Motility	−	−	−	+
Optimum temperature for growth (°C)	37	20–30	45	30
Growth at:				
37 °C	+	−	+	+
50 °C	−	−	+	−
Nitrate reduction	−	+	+	−*
Hydrolysis of gelatin	−	−	−	+
Utilization of:				
L-Arabinose	+	−	−*	−*
D-Cellobiose	+	+	+*	−*
D-Galactose	+	+	+*	−*
D-Glucose	+	+	−	+
Maltose	+	+	−*	−*
D-Mannose	+	+	+*	−*
Sucrose	+	+	−*	−*
Salicin	+	−	−*	−*
D-Trehalose	+	+	−*	−*
D-Xylose	+	+	−*	+*
Acetate	+	+	−	+
Citrate	+	+	−	+
L-Glutamate	−	+	−*	−*
Acid production from:				
D-Glucose	+	+	−*	−*
L-Arabinose	+	+	−*	−*
D-Fructose	+	−	+*	−*
Maltose	+	+	−*	−*
D-Mannitol	+	−	−*	−*
Sucrose	+	+	−*	−*
D-Xylose	−	+	−*	−*
Susceptibility to:				
Penicillin G	+	−	+	−*
Chloramphenicol	+	+	+	−*
API ZYM test results*				
Acid phosphatase	+	−	−	−
Naphthol-AS-BI-phosphohydrolase	W	−	W	−
β-Galactosidase	+	−	+	−
N-Acetyl-β-glucosaminidase	+	−	−	−
Major polar lipids*†	PG, DPG, PE, PL, GL	PC, PG, DPG, PE, PL	PG, DPG, PE	PC, PG, PE, AL
DNA G+C content (mol%)	67.0	55	66.2	67.9–68.1

*Data for the type strain from this study.

†AL, Unidentified aminolipid; DPG, diphosphatidylglycerol; GL, unidentified glycolipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, unidentified phospholipid.

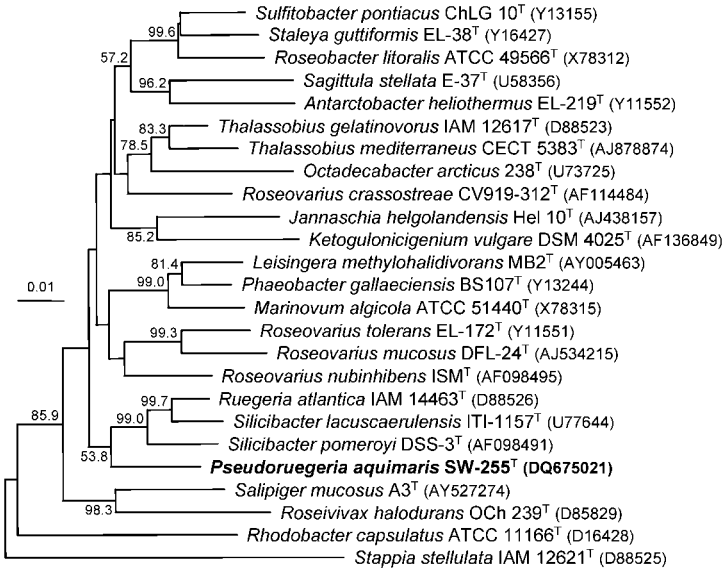

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strain SW-255^T and some related taxa. Bootstrap values (expressed as percentages of 1000 replications) >50 % are shown at branch points. *Stappia stellulata* IAM 12621^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

major amounts of unsaturated, straight-chain and hydroxy fatty acids; the major fatty acid was $C_{18:1}\omega7c$ (72.9 %) (Table 2). This fatty acid profile was similar to those of *Ruegeria atlantica* and the two *Silicibacter* species, although there were differences in the proportions of some fatty acids, perhaps because of differences in cultivation conditions (González *et al.*, 2003; Table 2). The major polar lipids detected in strain SW-255^T were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, an unidentified phospholipid and an unidentified glycolipid (Fig. 2). The major polar lipids detected in *Ruegeria atlantica* KCTC 12424^T, *S. lacuscaerulensis* KCTC 2953^T and *S. pomeroyi* DSM 15171^T are shown in Fig. 2 and Table 1. The DNA G+C content of strain SW-255^T was 67.0 mol%.

In the phylogenetic analysis based on 16S rRNA gene sequences, strain SW-255^T was most closely related to *Ruegeria atlantica* and the two *Silicibacter* species. Strain SW-255^T is relatively similar to *Ruegeria atlantica* in terms of phenotypic properties, as shown in Table 1, but it is clearly distinguishable on the basis of the difference in DNA G+C content. It can also be differentiated from *Ruegeria atlantica* on the basis of the phylogenetic relationships among strain SW-255^T, *Ruegeria atlantica* and the two *Silicibacter* species (Fig. 1). Strain SW-255^T is distinguishable from the two *Silicibacter* species by phenotypic differences (Table 1). The neighbour-joining tree based on *gyrB* sequences showed that strain SW-255^T forms a phylogenetic lineage that is independent of those of *Ruegeria atlantica*, *S. lacuscaerulensis* and *S. pomeroyi* (Fig. 3). Strain SW-255^T is also distinguishable from the type strains of *Ruegeria atlantica*, *S. lacuscaerulensis* and *S. pomeroyi* in terms of the polar lipid patterns (Fig. 2, Table 1). In particular, strain SW-255^T differs from *Ruegeria atlantica* in that phosphatidylcholine is absent, and it differs from *S. pomeroyi* in that diphosphatidylglycerol is present as a major polar lipid and phosphatidylcholine is absent (Fig. 2). Strain SW-255^T is also distinguishable from *S. lacuscaerulensis* by the presence of an unidentified phospholipid and an unidentified glycolipid (Fig. 2). Accordingly, strain SW-

Table 2. Cellular fatty acid compositions (%) of strain SW-255^T and related type strains

Strains: 1, strain SW-255^T; 2, *Ruegeria atlantica* KCTC 12424^T; 3, *S. lacuscaerulensis* KCTC 2953^T; 4, *S. pomeroyi* DSM 15171^T. Fatty acids that constituted <0.5 % in all strains were omitted. ECL, Equivalent chain length; ND, not detected.

Fatty acid	1	2	3	4
Straight-chain fatty acids				
$C_{10:0}$	ND	3.0	3.0	3.1
$C_{12:0}$	0.5	3.7	3.3	1.3
$C_{16:0}$	1.4	3.0	8.2	7.9
$C_{17:0}$	0.9	ND	0.7	0.3
$C_{18:0}$	6.6	0.8	3.7	2.3
Unsaturated fatty acids				
$C_{18:1}\omega5c$	0.8	ND	ND	ND
$C_{18:1}\omega7c$	72.9	53.0	52.8	54.2
$C_{20:1}\omega7c$	0.9	ND	0.5	0.5
Hydroxy fatty acids				
$C_{10:0}$ 3-OH	2.9	0.5	1.0	0.5
$C_{12:0}$ 3-OH	ND	6.5	4.9	5.7
$C_{16:0}$ 2-OH	0.6	10.8	0.4	7.7
$C_{18:1}$ 2-OH	ND	1.6	ND	ND
11-Methyl $C_{18:1}\omega7c$	2.8	15.9	3.4	15.0
cyclo $C_{19:0}\omega8c$	5.9	ND	16.0	ND
Summed feature 3*	0.7	0.2	0.7	0.3
Unknown fatty acid (ECL 11.799)	2.8	0.2	0.3	0.1

*Summed features are groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.

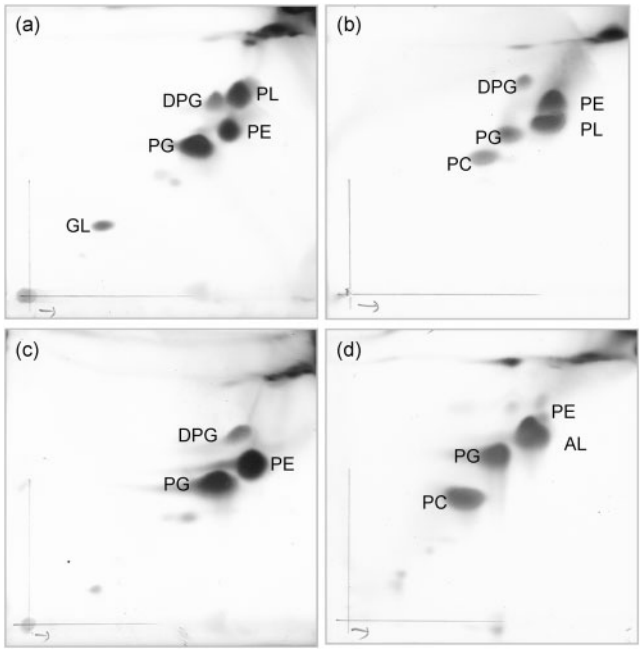

Fig. 2. Two-dimensional thin-layer chromatograms of the polar lipids of strain SW-255^T (a), *Ruegeria atlantica* KCTC 12424^T (b), *S. lacuscaerulensis* KCTC 2953^T (c) and *S. pomeroyi* DSM 15171^T (d). Abbreviations: AL, unidentified aminolipid; DPG, diphosphatidylglycerol; GL, unidentified glycolipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, unidentified phospholipid.

255^T should be classified within a novel genus and species distinct from the genera *Ruegeria* and *Silicibacter*. On the basis of the data presented, strain SW-255^T represents a novel genus and species within the class *Alphaproteobacteria*, for which the name *Pseudoruegeria aquimaris* gen. nov., sp. nov. is proposed.

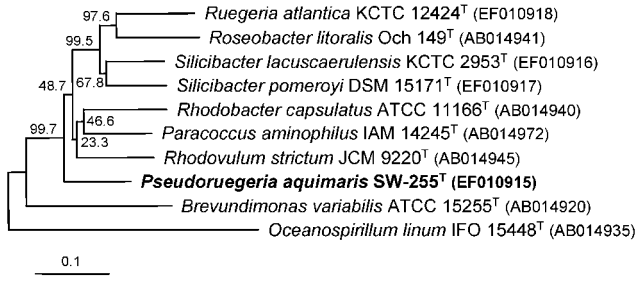

Fig. 3. Neighbour-joining phylogenetic tree, based on *gyrB* sequences, showing the positions of strain SW-255^T and some related taxa. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. *Oceanospirillum linum* IFO 15448^T was used as an outgroup. Bar, 0.1 substitutions per nucleotide position.

Description of *Pseudoruegeria* gen. nov.

Pseudoruegeria (Pseu.do.rue.ge′ri.a. Gr. adj. *pseudes* false; N.L. fem. n. *Ruegeria* a bacterial generic name; N.L. fem. n. *Pseudoruegeria* false *Ruegeria*).

Cells are Gram-negative, aerobic rods (0.3–0.8 × 1.0–8.0 μm). The predominant ubiquinone is Q-10. The major fatty acid is $C_{18:1}\omega7c$. The DNA G+C content is 67.0 mol% (HPLC). The type species is *Pseudoruegeria aquimaris*.

Description of *Pseudoruegeria aquimaris* sp. nov.

Pseudoruegeria aquimaris (a.qui.ma′ris. L. n. *aqua* water; L. gen. n. *maris* of the sea; N.L. gen. n. *aquimaris* of the water of the sea).

Cells are Gram-negative rods (0.3–0.8 × 1.0–8.0 μm). Colonies on MA are circular to slightly irregular, raised, smooth, greyish yellow in colour and 2.0–3.0 mm in diameter after 3 days incubation at 37 °C. Growth occurs at 15 and 49 °C, but not at 10 or 50 °C. Optimal pH for growth is between 7.0 and 8.0; growth occurs at pH 5.5, but not at pH 5.0. Growth occurs in the presence of 8 % (w/v) NaCl, but not in the absence of NaCl or in the presence of more than 9 % (w/v) NaCl. Anaerobic growth does not occur on MA or on MA supplemented with nitrate. Aesculin and hypoxanthine are hydrolysed, but xanthine, L-tyrosine and Tweens 40 and 60 are not. D-Fructose and L-malate are utilized as carbon and energy sources, but benzoate and formate are not. Acid is produced from D-cellobiose, D-galactose, D-mannose, melibiose, L-rhamnose, D-trehalose and *myo*-inositol, but not from D-melezitose, D-raffinose, D-ribose or D-sorbitol. Susceptible to ampicillin, carbenicillin, cephalothin, gentamicin, kanamycin, neomycin, novobiocin, oleandomycin, polymyxin B, streptomycin and tetracycline, but not to lincomycin. The predominant ubiquinone is Q-10. The major fatty acid (>10 % of total fatty acids) is $C_{18:1}\omega7c$. The major polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, an unidentified phospholipid and an unidentified glycolipid. The DNA G+C content of the type strain is 67.0 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, SW-255^T (=KCTC 12737^T=JCM 13603^T), was isolated from seawater from the beach at Hwajinpo, East Sea, Korea.

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