Simiduia areninigrae sp. nov., an agarolytic bacterium isolated from sea sand

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During a study intended to screen for agar-degrading bacteria, strain M2-5T was isolated from black sand off the shore of Jeju Island, Republic of Korea. Strain M2-5T exhibited agarase activity; the β-agarase gene of the isolate had 62% amino acid sequence identity to the β-agarase gene of Microbulbifer thermotolerans JAMB A94T. The isolate was closely related to members of the genus Simiduia but was clearly discernible from reported Simiduia species, based on a polyphasic analysis. Cells of strain M2-5T were Gram-negative, catalase- and oxidase-positive, motile rods. The DNA G+C content was 53.3 mol%. The predominant isoprenoid quinone was Q-8. The major cellular fatty acids were C17:1ω8c (25.9%), summed feature 3 (iso-C15:0 2-OH and/or C16:1ω7c; 17.2%) and C17:0 (15.0%). Phylogenetic analysis using 16S rRNA gene sequences showed that strain M2-5T had 96.6% gene sequence similarity to Simiduia agarivorans SA1T, the most closely related type strain of the genus Simiduia. These results suggest that strain M2-5T represents a novel species in the genus Simiduia, for which the name Simiduia areninigrae sp. nov. is proposed; the type strain is M2-5T (=KCTC 23293T=NCAIM B 02424T).

The genus Simiduia was proposed by Shieh et al. (2008) for an agarolytic bacterium isolated during a study of heterotrophic marine bacteria. Members of the genus Simiduia are oxidase- and catalase-positive, and contain C17:1ω8c and summed feature 3 (iso-C15:0 2-OH and/or C16:1ω7c) as predominant fatty acids. At the time of writing, the genus contained only one species, Simiduia agarivorans SA1T (Shieh et al., 2008).

Agar, a polysaccharide extracted from the cell wall of marine red algae (Rhodophyceae), has a wide variety of uses, e.g. as a food additive and in microbiological media, due to its stabilizing and gelling characteristics (Armišen et al., 2000; Fu & Kim, 2010). Diverse bacteria, isolated from marine environments, freshwater and soil, have been reported to have the ability to degrade agar, e.g. members of the genera Acinetobacter (Lakshmikanth et al., 2006), Agarivorans (Kurahashi & Yokota, 2004), Alteromonas (Potin et al., 1993), Bacillus (Suzuki et al., 2003), Gilvimarinus (Du et al., 2009), Maribacter (Nedashkovskaya et al., 2004), Marinomonas (Lim et al., 2006), Microbulbifer (Miyazaki et al., 2008), Pseudoalteromonas (Vera et al., 1998), Pseudomonas (Morrice et al., 1983), Pseudozobellia (Nedashkovskaya et al., 2009), Saccharophagus (Ekborg et al., 2005), Simiduia (Shieh et al., 2008), Thalassomonas (Jean et al., 2006), Vibrio (Aoki et al., 1990) and Zobellia (Barbeyron et al., 2001).

Recently, a number of bacterial strains were isolated in a study intended to screen for agar-degrading bacteria. Among them, strain M2-5T, which degraded agar and carboxymethyl cellulose (CMC) and possessed a β-agarase gene, was identified as a member of the genus Simiduia, but it could be clearly differentiated from other Simiduia species on the basis of 16S rRNA gene sequence analysis. Results of a polyphasic analysis demonstrated that strain M2-5T represents a novel species in the genus Simiduia.

Agarolytic bacteria were isolated from samples collected from near the seashore. Collected samples were serially diluted with sterile 0.85% (w/v) NaCl solution and these dilutions were plated onto marine agar 2216 (MA; BD) plates. After cultivation for 6 days at 25 °C, single colonies from these plates were selected and transferred onto MA plates for three consecutive incubations. Agar-degrading
bacteria were identified by the formation of clear hollows around the colonies after staining with Lugol’s iodine solution. Strain M2-5T was isolated as an agarolytic bacterium from black sand collected on Jeju Island, Republic of Korea (33° 14’ 39” N 126° 34’ 29” E). Strain M2-5T was routinely cultured on MA plates or PY medium (Shieh et al., 2000) and stored frozen in a glycerol suspension (20%, w/v) at −70 °C. Escherichia coli KCTC 2441T was obtained from the KCTC and used as a reference strain for DNA G+C content analysis. A closely related reference Simiduia strain, Simiduia agarivorans SA1T (= JCM 13881T) was obtained from JCM for phenotypic comparison.

The isolate and reference strain (S. agarivorans SA1T) were cultured on MA plates, marine broth 2216 (MB; BD) or PY medium for physiological tests under the same growth conditions, at 25–30 °C and pH 8.0–8.5, unless otherwise stated. Gram staining was performed using a Gram stain set (BD). Oxidase activity was assessed using oxidase reagent (bioMérieux) and catalase activity was determined by bubble production around colonies on MA plates using 3% (v/v) H2O2. Anaerobic growth was evaluated by culturing the organism on MA with or without 0.5–2.0% (w/v) KNO3 under an anaerobic atmosphere that was maintained with the GasPak EZ Anaerobe Pouch System (BD) and an anaerobic chamber (model 1024 anaerobic chamber; Forma Scientific) in an atmosphere comprising 5% H2, 10% CO2 and 85% N2. Colony morphology on MA plates was observed after culturing the strain for 3 days at 30 °C. The morphology and size of the cells were observed using light microscopy (Nikon Eclipse 80i) and transmission electron microscopy (H-7600; Hitachi). For transmission electron microscopy analysis, cells cultured for 2 days at 30 °C on MA plates were stained with 1% (w/v) phosphotungstic acid. Motility was tested by culturing the organism in half-strength MA media that contained 0.4% agar. The salt concentration for growth was determined in PY broth containing 0–10% (w/v) NaCl. The temperature for growth was tested using MA medium incubated at 4, 10, 15, 20, 25, 30, 37, 40 or 45 °C. The initial pH for growth was tested in pH-adjusted PY broth (pH 4.0–10.0 in 1.0 unit increments). Growth in liquid media was assessed by measuring OD660 over a period of 3–6 days using a DU 730 UV/Vis Scanning Spectrophotometer (Beckman Coulter). Growth on MacConkey agar was tested using standard MacConkey agar plates (BD). Alginate hydrolysis was determined by zones of clearance around the colony on LB agar plates (BD) containing 1 mg sodium alginate ml−1 (Kawamoto et al., 2006). Hydrolysis of casein and starch was measured with cells cultured for 6 days using standard microbiological methods (Atlas, 1993). Hydrolysis of l-tyrosine and chitin was indicated by zones of clearance after 6 days of culture on MA containing 5 mg l-tyrosine ml−1 and 5 mg chitin ml−1, respectively. For hydrolysis of CMC, cells were cultured on MA plates containing 5 mg CMC ml−1 for 6 days and the zones of clearance around the colonies were visualized after staining using 1% Congo red solution (Teather & Wood, 1982). Hydrolysis of Tween 80 was measured as described previously (Chakrabarty et al., 1970), Susceptibility to antibiotics was determined by the disc diffusion method (NCCLS, 2003) on PY plates after 3 days incubation at 30 °C using antimicrobial discs (6 mm in diameter; Liofilchem). The effect of antibiotics was measured based on the diameter of the cleared zone. A strain was regarded as susceptible when the diameter was greater than 15 mm, and as resistant when it was less than 15 mm. Enzyme activities of the isolate were measured with API ZYM test strips (bioMérieux). API 20NE test strips (bioMérieux) were used to determine other physiological characteristics of the isolates over a period of 2 days at 30 °C. For assimilation of carbon in API 20NE tests, the NaCl concentration in API AUX medium was adjusted to 2%. Biochemical tests were performed using strain M2-5T and a closely related type strain, S. agarivorans SA1T, in order to compare data.

Isoprenoid quinones from strain M2-5T and S. agarivorans SA1T were extracted from freeze-dried cells previously grown on MB at 30 °C for 2 days. Isoprenoid quinones were purified by preparative TLC (silica gel F254; Merck) and identified by HPLC (Hitachi L-5000) according to the method of Komagata & Suzuki (1987). Polar lipid profiles of strain M2-5T and S. agarivorans SA1T were determined with cells cultured on MA plates for 1 day at 30 °C, according to the method of Minnikin et al. (1984). Lipids were visualized using 5% ethanolic molybdatophosphoric acid. Molybdenum blue and ninhydrin were used for detection of phospholipids and aminolipids, respectively. Fatty acid methyl esters of strain M2-5T and S. agarivorans SA1T were analysed using cells cultured on MA plates for 1 day at 30 °C. Fatty acid methyl esters were extracted according to the standard protocol of the Microbial Identification System (Sasser, 2001), separated by GC (HP 6890N; Agilent) and identified with the Sherlock software package (MIDI Inc., Newark, DE, database TSBA40, version 4.0).

Genomic DNA of strain M2-5T and E. coli KCTC 2441T was extracted according to the method described by Sambrook & Russell (2001). The DNA G+C content of the isolate was determined according to the method described by Mesbah et al. (1989). Genomic DNA extractions were treated with nuclease P1 and alkaline phosphatase, and nucleoside mixtures were analysed by HPLC equipped with a reverse-phase column. The 16S rRNA gene was amplified with universal primers 27F and 1492R by PCR (Lane, 1991). 16S rRNA gene sequencing reactions were performed at SolGent, Daejeon, Republic of Korea, using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit and an ABI 3730XL capillary DNA sequencer (Applied Biosystems). The 16S rRNA gene sequence of strain M2-5T was compared with available 16S rRNA gene sequences from GenBank using the program BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Eztaxon server (http://www.eztaxon.org/; Chun et al., 2007). The 16S rRNA gene sequences of strain M2-5T and closely related type strains were aligned using CLUSTAL_X software (Thompson et al., 1997) and evolutionary distances were computed by the Jukes–Cantor parameter model.
(Jukes & Cantor, 1969). Phylogenetic trees were reconstructed using the neighbour-joining, maximum-parsimony and maximum-likelihood methods implemented in the PHYLI P package (Felsenstein, 2005). Topologies of trees were evaluated by bootstrap analysis (Felsenstein, 1985), based on 1000 resamplings.

The β-agarase gene of strain M2-5T was amplified using the oligonucleotide primers: sense, 5'-CCCTTYGAA(G/G)AGC(T)TACCAGCC-3'; antisense, 5'-TCGTCACGG(A/T)TACCAC(A/T)GCCA-3'. These primers were designed based on the highly similar amino acid sequences among several β-agarase genes identified from *Microbulbifer thermotolerans* JAMB A94T (Ohta et al., 2004), *Saccharophagus degradans* 2-40 T (GenBank accession nos YP-526649.1 and AAT67062.1) and *Pseudomonas* sp. ND137 (GenBank accession no. BAD88713.1). PCR was performed using a Maxime PCR PreMix (iNTrON Biotechnology) with 2.0 pmol of each primer and 50 ng genomic DNA in a 20 µl reaction under the following conditions: 2 min at 95 °C; 35 cycles of 30 s at 94 °C, 30 s at 45 °C, 1 min at 72 °C; 5 min at 72 °C; and held at 4 °C. The PCR product was introduced into a pGEM-T Easy vector (Promega) and sequenced using the same procedure as that described above for 16S rRNA gene sequencing.

The nearly complete 1463 bp 16S rRNA gene sequence of strain M2-5T was determined and analysed comparatively with the 16S rRNA genes of related reference strains. The isolate was assigned to the gammaproteobacteria group and was confirmed to be closely related to strains of the genus *Simiduia*; however, the 16S rRNA gene sequence of the isolate could be clearly distinguished from that of strains of the genus *Simiduia*. Strain M2-5T showed 96.6 % 16S rRNA gene sequence similarity with *S. agarivorans* SA1 T, the only reported type strain in this genus, and formed a monophyletic group with *S. agarivorans* SA1 T in phylogenetic trees based on 16S rRNA gene sequences. The phylogenetic position of strain M2-5T with closely related type strains is shown in a neighbour-joining tree (Fig. 1).

In trees reconstructed with maximum-parsimony and maximum-likelihood methods (Supplementary Figs S1 and S2, available in IJSEM Online), strain M2-5T grouped with *S. agarivorans* SA1 T. Strains with approximately ≥ 70 % DNA–DNA relatedness are generally considered to belong to the same species, whereas organisms with differences in their 16S rRNA gene sequences of more than 3 % will not have DNA–DNA relatedness values more than 60 % (Stackebrandt & Goebel, 1994; Wayne et al., 1987). According to these criteria, strain M2-5T represents a novel species of the genus *Simiduia*.

Strain M2-5T was Gram-negative, strictly aerobic, catalase-positive, oxidase-positive and motile by monochromatic flagella. Single cells of strain M2-5T were rods, 0.5–0.7 µm wide by 1.4–1.8 µm long (Supplementary Fig. S3, available in IJSEM Online). Colonies of strain M2-5T were circular and raised with entire margins on MA plates. Colonies were dark ivory in colour with diameters of 1–3 mm after 3 days cultivation at 30 °C. Strain M2-5T grew at 10–37 °C (optimum 25–30 °C). The initial medium pH range for growth of strain M2-5T was 7.0–9.0 (optimum pH 8.0–9.0). The isolate grew in PY broth that contained 1–6 % (w/v) NaCl [optimum 1–2 % (w/v)], but not in media containing 0 and ≥ 7 % (w/v) NaCl. The isolate could not grow on MacConkey agar. The differential physiological and biochemical properties of strain M2-5T and *S. agarivorans* SA1 T are shown in Table 1.

Strain M2-5T and *S. agarivorans* SA1 T had Q-8 as major isoprenoid quinone; trace amounts of Q-6 were also detected in both strains, but menaquinones were not present in either strain. The polar lipid components of strain M2-5T were phosphatidylethanolamine, phosphatidylglycerol and three unknown lipids. The major fatty acids of strain M2-5T were C17 : 1ω9c (25.9 %), summed feature 3 (iso-C15 : 0ω2c and/or C16 : 1ω7c, 17.2 %) and C17 : 0 (15.0 %). The major cellular fatty acids of strain M2-5T and *S. agarivorans* SA1 T were C17 : 1ω9c and summed feature 3 (iso-C15 : 0ω2c and/or C16 : 1ω7c) (Supplementary Table S1, available in IJSEM Online). The DNA G + C content of strain M2-5T was 53.3 mol%.

A 735 bp partial fragment of the β-agarase gene of strain M2-5T was sequenced (GenBank accession no. HQ022880), and the expected amino acid sequence of the DNA sequence was compared to reported β-agarase sequences. The most closely related β-agarase amino acid sequences were those of *Microbulbifer thermotolerans* JAMB A94T.
**Table 1. Phenotypic characteristics of S. areninigrae M2-5T and S. agarivorans**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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<tbody>
<tr>
<td>Cell size (μm)</td>
<td>0.5–0.7 x 1.4–1.8</td>
<td>0.4–0.6 x 2.0–5.0</td>
</tr>
<tr>
<td>Motility/flagella</td>
<td>+</td>
<td>–*</td>
</tr>
<tr>
<td>Growth temperature range (°C)</td>
<td>10–37</td>
<td>15–40</td>
</tr>
<tr>
<td>NaCl range for growth (%, w/v)</td>
<td>1–6</td>
<td>0.5–7.0</td>
</tr>
<tr>
<td>Growth pH range (optimum)</td>
<td>7.0–9.0</td>
<td>7.0–10.0</td>
</tr>
<tr>
<td>Activity of:†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine arylamidase</td>
<td>w</td>
<td>+</td>
</tr>
<tr>
<td>Cystine arylamidase</td>
<td>–</td>
<td>w</td>
</tr>
<tr>
<td>Trypsin</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of:†</td>
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<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tween 80</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Assimilation of:†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Glucose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Vancomycin resistance</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>53.3</td>
<td>55.6</td>
</tr>
</tbody>
</table>

*Cells were occasionally positive.
†Data from this study.

(Ohta et al., 2004) and Saccharophagus degradans 2-40T (Ekberg et al., 2006) with 62% and 59% amino acid sequence identities, respectively. This result indicates that the agarolytic activity of strain M2-5T is derived from a novel β-agarase.

The data obtained in this polyphasic study showed that strain M2-5T was a member of the genus *Simiduia*. However, it could be distinguished from reported strains of the genus *Simiduia* in that it had more than 3% 16S rRNA gene sequence difference with other *Simiduia* strains, a different cellular fatty acid composition, a differential carbon source utilization profile and it was unable to hydrolyse gelatin, Tween 80 and L-tyrosine. According to the results of this study, strain M2-5T represents a novel species of the genus *Simiduia*, for which the name *Simiduia areninigrae* sp. nov. is proposed.

**Description of *Simiduia areninigrae* sp. nov.**

*Simiduia areninigrae* (a.re.ni.ni’gra.e. L. fem. n. arena sand; L. adj. niger -grå -grum black; N.L. gen. n. areninigrae of black sand).

Cells are Gram-negative, strictly aerobic, catalase-positive, oxidase-positive, motile rods (approximately 0.5–0.7 x 1.4–1.8 μm). Colonies are circular, raised, entire and dark ivory in colour. Grows at 10–37°C (optimum 25–30°C), at initial pH 7.0–9.0 (optimum at pH 8.0–9.0) and in the presence of 1–6% NaCl, but not at 0% and ≥7% NaCl. Alginase, starch, CMC, casein and DNA are hydrolysed, but chitin, gelatin, Tween 80, urea and L-tyrosine are not. In API ZYM tests, positive for alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase, weakly positive for esterase (C4), esterase lipase (C8) and x-glucosidase, and negative for arginine dihydrolase, urease, lipase (C14), β-chymotrypsin, x-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, z-mannosidase and z-fucosidase. Both strains are negative for chitin hydrolysis, indole production, glucose fermentation, and assimilation of L-arabinose, d-mannose, d-mannitol, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. Both strains are susceptible to ampicillin (10 μg), carbenicillin (100 μg), chloramphenicol (30 μg), erythromycin (15 μg), kanamycin (30 μg), lincomycin (15 μg), nalidixic acid (30 μg), neomycin (30 μg), sulfamethoxazole (50 μg), tetracycline (30 μg) and trimethoprim (5 μg).

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**References**


