Arenimonas aestuarii sp. nov., isolated from estuary sediment

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A novel species of the genus Arenimonas, represented by strain S2-21T, was isolated from an estuary of Asan in South Korea. Cells of strain S2-21T were Gram-stain-negative, aerobic, non-motile rods that were oxidase- and catalase-positive. Growth of strain S2-21T was observed at 15–40 °C (optimum, 25–30 °C), at pH 7.0–8.0 (optimum, pH 7.0) and in the presence of 0–2.0 % (w/v) NaCl (optimum, 0 %). The major cellular fatty acids were iso-C15:0, C11:0 3-OH, iso-C16:0, summed feature 9 (comprising iso-C17:0 9c and/or C16:0 10-methyl), anteiso-C17:0 and iso-C11:0. The only respiratory quinone detected was ubiquinone-8 (Q-8) and the major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylmethyllethanol and two unknown phospholipids. The G+C content of the genomic DNA was 62.2 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain S2-21T formed a tight phyletic lineage with Arenimonas donghaensis HO3-R19T within the genus Arenimonas. Strain S2-21T was related most closely to A. donghaensis HO3-R19T at 98.1 % 16S rRNA gene sequence similarity and the mean DNA–DNA relatedness value between strain S2-21T and the type strain of A. donghaensis was 23.6 ± 2.2 %. On the basis of phenotypic, chemotaxonomic and molecular features, strain S2-21T is considered to represent a novel species of the genus Arenimonas, for which the name Arenimonas aestuarii sp. nov. is proposed. The type strain is S2-21T (=KACC 18504T=JCM 31129T).

The genus Arenimonas was first proposed by Kwon et al. (2007) with the single species Arenimonas donghaensis isolated from seashore sand as a member of the family Xanthomonadaceae within the class Gammaproteobacteria. The genus Arenimonas contains aerobic, Gram-negative and rod-shaped bacteria containing ubiquinone 8 (Q-8) as the major isoprenoid quinone and iso-C16:0, iso-C15:0 and iso-C17:0 9c as the predominant fatty acids. Members of the genus Arenimonas have been isolated from various environmental habitats including seashore sand, compost, an oil-contaminated site, rice paddy water and freshwater, suggesting that their physiological properties may be diverse. At the time of writing, the genus Arenimonas comprises nine recognized species, A. donghaensis (Kwon et al., 2007), A. malthae (Young et al., 2007), A. oryziterrae (Aslam et al., 2009), A. composti (Jin et al., 2007; Aslam et al., 2009), A. daechungensis (Huy et al., 2013), A. daeungvensis (Huy et al., 2013), A. maotaiensis (Yuan et al., 2014) and A. subflava (Makk et al., 2015), plus ‘A. taoyuanensis’ (Zhang et al., 2015). In this study, an additional new member of the genus Arenimonas, designated strain S2-21T, was isolated from estuary sediment and its taxonomic characterization was carried out using a polyphasic approach.

Strain S2-21T was isolated from an estuary of Asan (36° 56' 41.61" N 126° 59' 27.12" E), South Korea. Briefly, a sediment sample obtained from the Asan estuary was serially diluted in 0.85 % (w/v) saline and aliquots of each serial dilution were spread on R2A agar (Difco). The 16S rRNA genes of colonies grown on R2A agar after 5 days of incubation at 30 °C were PCR-amplified using primers F1 and R13 and the PCR amplicons were double-digested with HaeIII and HhaI as described previously (Jeong et al., 2013). Representative PCR products showing unique fragment patterns were sequenced using the F1 primer and their resulting sequences were compared with the 16S rRNA gene sequences of all reported type strains using the Nucleotide Similarity Search program in the EzTaxon-e server (http://www.ezbiocloud.net/eztaxon;
Kim et al., 2012). A putative novel isolate belonging to the genus Arenimonas, designated strain S2-21T, was selected for further taxonomic characterization. Strain S2-21T was grown on R2A agar at 30 °C for 2 days, except where indicated otherwise, and stored at −80 °C in R2A broth supplemented with 15 % (v/v) glycerol for long-term preservation. A. donghaensis KACC 11381T (=HO3-R19T), A. daejeonensis KCTC 12667T (=T7-07T) and A. malthae DSM 21305T (=CC-JY-1T) were used as reference strains for comparisons of phenotypic properties, fatty acid compositions and DNA–DNA homology.

The 16S rRNA gene of strain S2-21T was cloned into the pCR2.1 vector using a TOPO cloning kit (Invitrogen) and sequenced using the M13 reverse and T7 primers of the TOPO cloning kit according to the manufacturer’s instructions. The resulting 16S rRNA gene sequence (1493 nt) of strain S2-21T was compared with those of all reported type strains using the Nucleotide Similarity Search program (Kim et al., 2012). The 16S rRNA gene sequences of strain S2-21T and closely related type strains were aligned using the fast secondary-structure aware Infernal aligner available in the Ribosomal Database Project (Nawrocki & Eddy, 2007). Phylogenetic trees were aligned using the fast secondary-structure aware Infernal aligner available in the Ribosomal Database Project (Nawrocki & Eddy, 2007). Phylogenetic trees based on the neighbour-joining and maximum-parsimony algorithms were reconstructed using the PHYLIP software (ver. 3.695; Felsenstein, 2002) and their topologies were evaluated through a bootstrap analysis based on a 1000 resampled dataset. Maximum-likelihood analysis with bootstrap values was conducted using RAxML-HPC Black-Box (version 8.1.24) of the Cyber-Infrastructure for Phylogenetic Research project (www.phylo.org; Stamatakis et al., 2005). DNA–DNA hybridization between strain S2-21T and Arenimonas species showing >97 % 16S rRNA gene sequence similarity was conducted in triplicate to evaluate their DNA–DNA homology as described previously (Lee et al., 2011) using the DIG High Prime DNA Labelling kit (Roche Applied Science) and hybridization signals were analysed using Adobe Photoshop CS6 (version 13.0). Self-hybridization signal of the probes to the homologous target DNA was taken to be 100 %, and self-hybridization signals from serially diluted homologous target DNA were used for the construction of a standard curve. The DNA–DNA hybridization result was confirmed by a reciprocal interchange of probe and target DNA.

Comparative analysis based on 16S rRNA gene sequences revealed that strain S2-21T was related most closely to A. donghaensis HO3-R19T, A. malthae CC-JY-1T and A. daejeonensis T7-07T at similarities of 98.1, 97.2 and 97.2 %, respectively; sequence similarities with other validly reported type strains were less than 97.0 %. Phylogenetic analysis based on the neighbour-joining algorithm showed that strain S2-21T formed a tight phylogenetic lineage with A. donghaensis HO3-R19T with a 99 % bootstrap value within the family Xanthomonadaceae (Fig. 1). Phylogenetic analysis using the Ribosomal Database Project classifier (Wang et al., 2007) also supported strain S2-21T as a member of the genus Arenimonas. In addition, the phylogenetic trees reconstructed based on the maximum-likelihood and maximum-parsimony algorithms also showed that strain S2-21T formed a distinct phylogenetic lineage within the genus Arenimonas (Fig. S1, available in the online Supplementary Material). Mean DNA–DNA relatedness values between strain S2-21T and the type strains of A. donghaensis (KACC 11381T), A. daejeonensis (KCTC 12667T) and A. malthae (DSM 21305T) were 23.6 ± 2.2, 12.5 ± 1.9 and 45.6 ± 2.2 %, respectively, which were clearly lower than the 70 % threshold generally accepted for species delineation (Stackebrandt et al., 2002). These results suggest that strain S2-21T may represent a novel species of the genus Arenimonas.

Growth of strain S2-21T was tested on R2A agar (BD), laboratory-prepared Luria–Bertani (LB) agar, nutrient agar (NA; BD), marine agar (MA; BD) and trypticase soy agar (TSA; BD) at 30 °C for 2 days. Growth of strain S2-21T at different temperatures (4, 10, 15, 20, 25, 30, 35, 37, 40 and 45 °C) and pH values (6.0–10.0 at 0.5 pH unit intervals) was evaluated in R2A medium for 2 days. R2A broth with pH values below 8.0 and pH 8.0–10.0 was prepared using the Na2HPO4–NaH2PO4 and Tris/HCl buffers, respectively (Gomori, 1955) and the pH values were adjusted again if necessary after sterilization (121 °C for 15 min). Growth at different NaCl concentrations (0–5 %, w/v, NaCl, 0.5 % intervals) was assessed in laboratory-prepared R2A broth. Gram staining was tested using the Gram stain kit (bioMérieux) according to the manufacturer’s instructions. Anaerobic growth was assessed on R2A agar and R2A agar containing nitrate (0.2 % NaNO3) or nitrite (0.2 % KNO2) at 30 °C for 20 days under anaerobic conditions (with 4–10 % CO2).
using the GasPak Plus system (BBL). Cell morphology and motility of strain S2-21<sup>T</sup> were investigated using transmission electron microscopy (JEM-1010; JEOL) and phase-contrast microscopy (Axioimager AX10; Carl Zeiss) with cells grown on R2A agar at 30 °C for 2 days. Nitrate reduction was tested according to the method of Lányi (1987). Catalase and oxidase activities of strain S2-21<sup>T</sup> were tested by the production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution and the oxidation of 1 % (w/v) tetramethyl-p-phenylenediamine (Merck), respectively (Smibert & Krieg, 1994). The following properties of strain S2-21<sup>T</sup> and reference strains were investigated in parallel under the same conditions at 30 °C. Hydrolysis of Tween 20, Tween 80, casein, starch and tyrosine was tested on R2A agar according to methods described previously (Lányi, 1987; Smibert & Krieg, 1994). Additional enzyme activities, biochemical features and oxidation of carbon sources were tested using the API ZYM and API 20NE kits (bioMérieux) and GN2 MicroPlate system (Biolog), respectively, according to the instructions of the manufacturers, except that cells were resuspended in 0.85 % NaCl for inocula. Strain S2-21<sup>T</sup> grew on R2A agar, LB agar, NA, MA and TSA (best growth was observed on R2A agar). Cells were Gram-stain-negative, non-motile rods (0.3–0.4 μm in length) without flagellum (Fig. S2). Anaerobic growth was not observed after 20 days of incubation at 30 °C. Phenotypic characteristics of strain S2-21<sup>T</sup> are presented in the species description and compared with those of the closely related type strains of the genus Arenimonas in Tables 1 and S1. Many phenotypic properties, including strictly aerobic growth, activities of catalase and oxidase, and hydrolysis of gelatin, casein and trypsin, were in common with those of closely related Arenimonas species, but some other phenotypic properties such as colony colour, flagellum motility and activities of lipase (C14) and cystine arylamidase differentiated strain S2-21<sup>T</sup> from closely related Arenimonas species (Table 1). The isoprenoid quinone of strain S2-21<sup>T</sup> was analysed with an HPLC (model LC-20A; Shimadzu) system equipped with a reversed-phase column (250 × 4.6 mm, Kromasil; Akzo Nobel) and a diode array detector (SPD-M20A; Shimadzu) using methanol/2-propanol (2:1, v/v) as an eluent (1 ml min<sup>−1</sup>) as described previously (Komagata & Suzuki, 1987). For cellular fatty acid analysis, strain S2-21<sup>T</sup> and the three reference Arenimonas type strains were cultivated in R2A broth at 30 °C and microbial cells were harvested after approximately 2 days showing the same growth phase (exponential phase, OD<sub>600</sub> of 0.8). The cellular fatty acids were saponified and methylated using the standard MIDI protocol. Fatty acid methyl esters were analysed by GC (Hewlett Packard 6890 chromatograph) and identified by using the TSBBA6 database of the Microbial Identification System (Sherlock ver. 6.0B; Sasser, 1990). The DNA G+C content of strain S2-21<sup>T</sup> was determined by the fluorometric method (Gonzalez & Saiz-Jimenez, 2002) using SYBR Green I and a real-time PCR thermocycler (Bio-Rad). The polar lipids of strain S2-21<sup>T</sup> and the three reference Arenimonas type strains were analysed by TLC using cells harvested during the exponential growth phase according to the procedure described by Minnikin et al. (1977). The following reagents were used to detect different polar lipids: 10 % ethanolic molybdophosphoric acid (for total polar lipids), ninhydrin (for aminolipids) and Dittmer–Lester reagent (for phospholipids). The only respiratory quinone detected from strain S2-21<sup>T</sup> was Q-8. The predominant cellular fatty acids (>5 %) were iso-C<sub>15:0</sub> (29.7 %), C<sub>11:0</sub> 3-OH (14.0 %), iso-C<sub>16:0</sub> (10.7 %), summed feature 9 (comprising iso-C<sub>17:1</sub> 9<sub>c</sub> and/or C<sub>16:0</sub> 10-methyl, 10.2 %), anteiso-C<sub>17:0</sub> (8.9 %) and iso-C<sub>11:0</sub> (6.9 %). The overall fatty acid profile of strain S2-21<sup>T</sup> was similar to those of the reference Arenimonas type strains, but there were some differences in the respective compositions of some fatty acids (Table 2). In particular, the presence of anteiso-C<sub>17:0</sub> as a major fatty acid in strain S2-21<sup>T</sup> clearly allowed the differentiation of strain S2-21<sup>T</sup> from other closely related Arenimonas species. The major polar lipids of strain S2-21<sup>T</sup> were diphasphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylmethylthanol and two unknown phospholipids, a profile that was similar to those of the reference strains of the genus Arenimonas (Fig. S3, Table 1). The DNA G + C content of strain S2-21<sup>T</sup> was 62.2 mol%, which was within the range of the DNA G+C contents of Arenimonas species (52.0–72.3 mol%) (Makk et al., 2015; Zhang et al., 2015). The physiological and chemotaxonomic features and phylogenetic inference supported the proposition that strain S2-21<sup>T</sup> represents a novel species of the genus Arenimonas, for which the name Arenimonas aestuarii sp. nov. is proposed.

**Description of Arenimonas aestuarii** sp. nov. Arenimonas aestuarii (a.es.tu.a’ri.i. L. gen. n. aestuarii of estuary sediment, from where the type strain was isolated).

Colonies on R2A agar are white to translucent with circular and convex entire edges. Cells are strictly aerobic, Gram-stain-negative, non-motile rods (0.3–0.4 μm wide and 1.0–1.7 μm in length) without flagellum. Growth occurs at 15–40 °C (optimum, 25–30 °C), at pH 7.0–8.0 (optimum, pH 7.0) and with 0–2.0 % (w/v) NaCl (optimum, 0%). Oxidase- and catalase-positive. Hydrolyses casein, gelatin and trypsin, but not Tween 20, Tween 80, tyrosine, starch, urea or aesculin. Nitrate is not reduced to nitrite. Activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, x-chymotrypsin, naphthol-AS-BI-phosphohydrolase and acid phosphatase are positive, but activities of x-galactosidase, β-glucosidase, β-galactosidase, β-glucuronidase, x-glucosidase, N-acetyl-β-glucosaminidase, x-mannosidase and x-fucosidase are negative. Negative for indole production, activity of arginine dihydrolase, and assimilation of D-glucose, D-arabinose, D-mannose, D-mannitol, N-acetylglucosamine,
Table 1. Phenotypic characteristics of strain S2-21T and related type strains of the genus Arenimonas

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1*</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony colour</td>
<td>White to translucent</td>
<td>Yellowish white</td>
<td>Creamy white to yellowish</td>
<td>Transparent to brownish</td>
</tr>
<tr>
<td>Cell length (µm)</td>
<td>1.0–1.7</td>
<td>1.3–3.0</td>
<td>1.0–1.5</td>
<td>1.6–2.7</td>
</tr>
<tr>
<td>Cell width (µm)</td>
<td>0.3–0.4</td>
<td>0.4–0.6</td>
<td>0.4–0.5</td>
<td>0.3–0.4</td>
</tr>
<tr>
<td>Flagellum motility</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth:</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>15–40</td>
<td>4–37†</td>
<td>15–37†</td>
<td>15–37†</td>
</tr>
<tr>
<td>pH</td>
<td>7–8</td>
<td>7–9</td>
<td>6–9</td>
<td>NA</td>
</tr>
<tr>
<td>NaCl (%)</td>
<td>0–2.0</td>
<td>0–3.0†</td>
<td>0–3.0†</td>
<td>0–2.0†</td>
</tr>
<tr>
<td>Growth on TSA</td>
<td>+</td>
<td>NA</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Enzyme activity (API ZYM)* of:</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lipase (C14), cystine arylamidase</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Valine arylamidase</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>N-Acetyl-β-glucosaminidase</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Assimilation (API 20NE)* of:</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>d-Glucose, N-acetylgalactosamine</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>d-Mannitol, maltose, malic acid, trisodium citrate</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>d-Mannose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Major polar lipids*</td>
<td>DPG, PE, PG, PME, PL</td>
<td>DPG, PE, PG, PME</td>
<td>DPG, PE, PG, PME</td>
<td>DPG, PE, PG, PME</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>62.2</td>
<td>65.0</td>
<td>68.3</td>
<td>70.4</td>
</tr>
</tbody>
</table>

*These analyses were conducted under the same conditions in this study.
†Data were taken from Jin et al. (2012).

maltose, malic acid, capric acid, adipic acid, trisodium citrate, phenylacetic acid and potassium gluconate. Positive for oxidation of α-cyclodextrin, glycerin, succinic acid monomethyl ester, succinamic acid, hydroxy-L-proline, γ-aminobutyric acid, β-hydroxybutyric acid, L-alaninamide, L-alanine, L-alanyl glycine, glycyll-L-aspartic acid, glycyll-L-glumatic acid and L-proline, but negative for oxidation of dextrin, Tween 40, Tween 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosaminic acid, adonitol, L-arabinose, D-arabitol, D-cellobiose, L-erythritol, D-fructose, L-fructose, D-galactose, gentiobiase, γ-D-glucose, myo-inositol, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, methyl β-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, pyruvic acid methyl ester, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucicolic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenylacetate, itaconic acid, α-ketoisobutyric acid, α-keto glutaric acid, α-ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, glucuronamide, D-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proglutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, urocanic acid, inosine, uridine, thymidine, phenylolamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, DL-α-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. The major polar lipids are diphasphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylmethyl ethanol and two unknown phospholipids. The major cellular fatty acids (>5%) are iso-C15:0, C11:0 3-OH, iso-C16:0, summed feature 9 (comprising...
Table 2. Cellular fatty acid compositions (%) of strain S2-21T and related type strains of the genus Arenimonas

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
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<tr>
<td>Saturated:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>2.1</td>
<td>1.3</td>
<td>TR</td>
<td>–</td>
</tr>
<tr>
<td>C16:0</td>
<td>3.8</td>
<td>2.4</td>
<td>1.2</td>
<td>–</td>
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<tr>
<td>Unsaturated:</td>
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<tr>
<td>C16:1ω7c alcohol</td>
<td>–</td>
<td>–</td>
<td>2.2</td>
<td>–</td>
</tr>
<tr>
<td>C15:1ω9c</td>
<td>–</td>
<td>–</td>
<td>3.4</td>
<td>–</td>
</tr>
<tr>
<td>C17:1ω9c</td>
<td>–</td>
<td>1.4</td>
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<td>Branched:</td>
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<tr>
<td>iso-C10:0</td>
<td>TR</td>
<td>TR</td>
<td>TR</td>
<td>0.6</td>
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<tr>
<td>iso-C11:0</td>
<td>6.9</td>
<td>4.2</td>
<td>4.1</td>
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<tr>
<td>iso-C13:0</td>
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<td>0.7</td>
<td>0.6</td>
<td>0.7</td>
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<tr>
<td>iso-C14:0</td>
<td>4.5</td>
<td>8.1</td>
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<td>iso-C15:0</td>
<td>29.7</td>
<td>30.4</td>
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<td>16.8</td>
<td>8.8</td>
<td>19.7</td>
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<td>2.2</td>
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<tr>
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<td>1.2</td>
<td>1.3</td>
<td>0.5</td>
</tr>
<tr>
<td>anteiso-C17:0</td>
<td>8.9</td>
<td>–</td>
<td>TR</td>
<td>–</td>
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<tr>
<td>Hydroxy:</td>
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<tr>
<td>C12:0 3-OH</td>
<td>14.0</td>
<td>9.2</td>
<td>9.2</td>
<td>8.1</td>
</tr>
<tr>
<td>C12:0 3-0H</td>
<td>TR</td>
<td>0.6</td>
<td>TR</td>
<td>1.1</td>
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<tr>
<td>Summed feature*:</td>
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<td>3</td>
<td>0.6</td>
<td>4.3</td>
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<td>2.7</td>
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<tr>
<td>9</td>
<td>10.2</td>
<td>11.3</td>
<td>18.8</td>
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</tbody>
</table>

*Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 1, iso H-C15:1ω and/or C13:0 3-OH; 2, C12:0 ω aldehyde and/or unknown; 3, C16:1ω7c and/or C16:1ω6c; 9, iso-C17:1ω9c and/or C16:0 10-methyl.

iso-C17:1ω9c and/or C16:0 10-methyl, anteiso-C17:0 and iso-C11:0. Q-8 is the only isoprenoid quinone.

The type strain is S2-21T (=KACC 18504T=JCM 31129T), isolated from the Asan estuary in South Korea. The G+C content of the genomic DNA is 62.2 mol%.

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References


