Roseovarius lutimaris sp. nov., isolated from a marine tidal flat

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A novel Gram-staining-negative, moderately halophilic and strictly aerobic bacterium, designated strain 112T, was isolated from a tidal flat at Taean, Korea. Cells were catalase- and oxidase-positive ovoids to rods and did not produce bacteriochlorophyll a. Optimum growth of strain 112T was observed at 30 °C, at pH 6.5–7.5 and in the presence of 2–4 % (w/v) NaCl. The major cellular fatty acids were summed feature 8 (C18:1ω7c and/or C18:1ω6c) and C16:0 and Q-10 was detected as the predominant ubiquinone. The major polar lipids were phosphatidylethanolamine, phosphatidylglycerol, phosphatidylycholine, an unidentified aminolipid and three unidentified lipids. The genomic DNA G+C content of strain 112T was 58.2 mol%.

Phylogenetic analysis based on 16S rRNA gene sequences showed that strain 112T formed a phyletic lineage within the genus Roseovarius of the class Alphaproteobacteria. Strain 112T was most closely related to Roseovarius mucosus DFL-24T, with 96.52 % similarity. On the basis of phenotypic, chemotaxonomic and molecular properties, strain 112T represents a novel species of the genus Roseovarius, for which the name Roseovarius lutimaris sp. nov. is proposed. The type strain is 112T (=KACC 16185T=JCM 17743T).

The genus Roseovarius, a member of the family Rhodobacteraceae of the Alphaproteobacteria (Garrity et al., 2005), was proposed by Labrenz et al. (1999) with a single species, Roseovarius tolerans, containing bacteriochlorophyll (BChl) a, isolated from Ekh Lake in Antarctica. The genus Roseovarius accommodates catalase-positive, ovoid or rod-shaped, obligately aerobic bacteria and, to date, all Roseovarius type strains have been retrieved from marine environments such as tidal flats, seawater, sea squirts and oysters. At the time of writing, the genus Roseovarius included 13 species with validly published names: R. tolerans (Labrenz et al., 1999), R. niobithiens (González et al., 2003), R. mucosus (Biebl et al., 2005), R. crassostreae (Boettcher et al., 2005), R. aestuarii (Yoon et al., 2008), R. halotolerans (Oh et al., 2009), R. pacificus (Wang et al., 2009), R. nanhaiticus (Wang et al., 2010), R. indicus (Lai et al., 2011), R. marinus (Jung et al., 2011), R. halocynthiae (Kim et al., 2012b), R. litoreus (Jung et al., 2012) and R. sediminilitoris (Park & Yoon, 2013). Tidal flats, called getbol in Korean, are broad, low-gradient, coastal muddy marshes that play important roles in habitat restoration and nutrient recycling (Winberg et al., 2007; Math et al., 2012). In this study, we describe the taxonomic characterization of a novel Roseovarius strain, designated 112T, isolated from a tidal flat of the Yellow Sea in South Korea.

Strain 112T was isolated from a surface tidal flat (less than 5 cm depth) in the Taean area (36° 48’ 50.82” N 126° 11’ 09.56” E), using a previously described procedure (Jin et al., 2011) with some modifications. Briefly, the tidal flat sample was serially diluted with artificial seawater (ASW; Jeong et al., 2013), spread on marine agar 2216 (MA; BD) and incubated at 25 °C for 3 days. The 16S rRNA genes of isolates that grew on MA were amplified by PCR using the universal primers F1 and R13 (Jin et al., 2011). The amplicons were double-digested with HaeIII and Hhal and the restriction fragment patterns were used as a guide to classify the colonies, as described previously (Jin et al., 2012). Based on restriction fragment patterns, PCR products with unique patterns were selected and sequenced using the F1 primer. The resulting 16S rRNA gene sequences were analysed using the Nucleotide Similarity Search program (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012a). From this analysis, a novel strain belonging to the genus Roseovarius, designated strain 112T, was selected for further phenotypic and phylogenetic analysis. Strain 112T was routinely grown aerobically on MA at 30 °C for 2 days, except where indicated otherwise. Strain 112T was stored at –80 °C in marine broth (MB; BD) supplemented with 10 % (v/v) glycerol. The type strains R. mucosus KACC 12996T, R. tolerans KACC 13027T and R. nanhaiticus KACC 12561T were used as reference strains for phenotypic comparisons and fatty acid analysis.
The 16S rRNA gene amplicon of strain 112\textsuperscript{T} was ligated into the pCR2.1 vector using a TOPO cloning kit (Invitrogen) according to the manufacturer’s instructions in order to obtain the almost-complete 16S rRNA gene sequence. The inserted 16S rRNA gene was sequenced with the M13 reverse and T7 primers of the TOPO cloning kit. The quality of the resulting 16S rRNA gene sequence (1393 nt) was checked manually. Sequence similarity values of strain 112\textsuperscript{T} were evaluated using the Nucleotide Similarity Search program at EzTaxon (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012a) and the sequence was aligned with those of closely related taxa using the greengenes alignment program (http://greengenes.lbl.gov/; DeSantis et al., 2006). Phylogenetic trees were reconstructed by using the neighbour-joining and maximum-parsimony algorithms with the PHYLIP software (version 3.68; Felsenstein, 2002). The resulting tree topologies were constructed by using the neighbour-joining and maximum-likelihood analysis with bootstrap values was performed using the Ribosomal Database Project Infrastructure for Phylogenetic Research project (http://www.phylo.org; Stamatakis et al., 2005) at the San Diego Supercomputer Center. An additional taxonomic assignment was performed using the Ribosomal Database Project naive Bayesian rRNA Classifier tool (http://rdp.cme.msu.edu/classifier; Wang et al., 2007).

Comparative analysis of 16S rRNA gene sequences showed that strain 112\textsuperscript{T} was most closely related to \textit{R. mucosus} DFL-24\textsuperscript{T} (96.52 %), \textit{Donghicola eburneus} SW-277\textsuperscript{T} (96.09 %), \textit{R. tolerans} EL-172\textsuperscript{T} (95.94 %) and \textit{R. nanhaiticus} NH52\textsuperscript{T} (95.54 %). Phylogenetic analysis based on 16S rRNA gene sequences using the neighbour-joining algorithm showed that strain 112\textsuperscript{T} formed a phylogenetic lineage with \textit{R. tolerans} (the type species of the genus \textit{Roseovarius}) and \textit{R. mucosus} within the genus \textit{Roseovarius} (Fig. 1). Phylogenetic trees reconstructed using the maximum-likelihood and maximum-parsimony algorithms also supported the conclusion that strain 112\textsuperscript{T} represented a member of the genus \textit{Roseovarius} (not shown), which was confirmed by the RDP Classifier program. The phylogenetic tree also showed that members of the genus \textit{Roseovarius} were clustered into two phylogenetic clades, which suggests that the genus \textit{Roseovarius} may be split into two or more genera.

Growth was tested on R2A agar (BD), laboratory-prepared Luria–Bertani (LB) agar, tryptic soy agar (TSA; BD) and nutrient agar (NA; BD) supplemented to 2 % NaCl and MA at 30 °C. Growth was assessed on MA at 0–45 °C (at 5 °C intervals) and in MB adjusted to pH 4.5–10.0 (at 0.5 pH unit intervals) prior to sterilization using Na\textsubscript{2}HPO\textsubscript{4}/NaH\textsubscript{2}PO\textsubscript{4} and Na\textsubscript{2}CO\textsubscript{3}/NaHCO\textsubscript{3} for below pH 8.0 and pH 8.0–10.0, respectively (Gomori, 1955). The pH was verified after sterilization (121 °C, 15 min) and adjusted again when necessary. Growth at NaCl concentrations ranging from 0 to 10 % (w/v, at 1 % intervals) was investigated using MB prepared in the laboratory according to the formula of the medium. Gram staining was performed using the bioMérieux Gram stain kit according to the manufacturer’s instructions. Cell morphology, the presence of flagella and motility were investigated using

\begin{figure}
\centering
\includegraphics[width=\textwidth]{phylogenetic_tree}
\caption{Neighbour-joining tree based on 16S rRNA gene sequences showing phylogenetic relationships of strain 112\textsuperscript{T} with related taxa. Bootstrap values are shown as percentages of 1000 replicates, when greater than 70 %. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. The type strain of the type species of the genus \textit{Roseovarius} is indicated by an asterisk. The sequence of \textit{Burkholderia cepacia} ATCC 25416\textsuperscript{T} (GenBank accession no. U96927) was used as an outgroup (not shown). Bar, 0.01 changes per nucleotide position.}
\end{figure}

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transmission electron microscopy (JEM-1010; JEOL) with cells from an exponentially growing culture in MB at 30 °C, as described previously (Bernardet et al., 2002; Jeon et al., 2004). Oxidase activity was tested by oxidation of 1% (w/v) tetramethyl p-phenylenediamine (Merck) and catalase activity was evaluated by the production of oxygen bubbles in 3% (v/v) aqueous hydrogen peroxide solution (Smibert & Krieg, 1994). Anaerobic growth was assessed on MA under anaerobic conditions (with 4–10% CO2) using the GasPak Plus system (BBL) at 30 °C for 20 days. Nitrate reduction was assessed according to the method of Lányi (1987). Production of BChl a was determined by spectrophotometric analysis as described by Martens et al. (2006). The presence of the pufLM gene of the bacterial photosynthesis reaction centre was assessed by PCR using the pufLM gene primer set (Allgaier et al., 2003). Phenotypic analysis of strain 112T and the reference strains was performed in parallel as described below. Hydrolysis of casein, starch, tyrosine and Tweens 20 and 80 was investigated on MA according to methods described previously (Lányi, 1987; Smibert & Krieg, 1994). Acid production from carbon sources was determined as described by Leifson (1963). Utilization of various substrates for growth was determined as described by Yurkov et al. (1994). Additional enzyme activities and biochemical features of strain 112T and the reference strains were determined by using the API ZYM and API 20E kits (bioMérieux) and the GN2 MicroPlate system (Biolog) according to the manufacturers’ instructions, except that inocula were prepared by suspending cells in ASW. Antibiotic susceptibility tests were performed according to a previously described method (Jeong et al., 2013).

Strain 112T was able to grow at 30 °C on R2A agar and TSA supplemented with 2% NaCl and MA, but not on NA or LB agar supplemented with 2% NaCl. Cells of strain 112T were 0.4–0.8 µm wide and 2–3 µm long (Fig. S1, available in IJSEM Online). Strain 112T did not produce BChl a; this result might be supported by the lack of success in PCR amplification of the pufLM gene (not shown). More physiological and biochemical characteristics of strain 112T are presented in Table 1 and in the species description. Some of the characteristics were in agreement with those considered to be characteristic of the genus Roseovarius, whereas others allowed the differentiation of strain 112T from related type strains of the genus (Tables 1 and S1).

Cell mass for analysis of fatty acids and isoprenoid quinones was obtained from cultures in the exponential growth phase. For the analysis of cellular fatty acids, strain 112T and the reference strains were cultivated in MB at 30 °C and microbial cells were harvested at the exponential growth phase (OD600=0.8). Cellular fatty acids were extracted, saponified and methylated using the standard MIDI protocol. Fatty acid methyl esters were separated by gas chromatography (Hewlett Packard 6890) and identified by using the TSBA6 database of the Microbial Identification System (Sherlock version 6.0B) (Sasser, 1990). Isoprenoid quinones of strain 112T were analysed using an HPLC (model LC-20A; Shimadzu) equipped with a diode array detector (SPD-M20A; Shimadzu) and a reversed-phase column (250 × 4.6 mm, Kromasil; Akzo Nobel) as described previously (Komagata & Suzuki, 1987). The genomic DNA G+C content of strain 112T was determined by the fluorometric method (Gonzalez & Saiz-Jimenez, 2002) using SYBR green I and a real-time PCR thermocycler (Bio-Rad). Polar lipids of strain 112T and R. tolerans KACC 13027T were extracted and separated by two-dimensional TLC as described by Minnikin et al. (1977). The following reagents were used to detect the different polar lipids: 10% ethanolic phosphomolybdic acid (for total polar lipids), ninhydrin (for amino lipids), 2-naphthol/sulfuric acid (for glycolipids) and the Dittmer–Lester reagent (for phospholipids). The major cellular fatty acids (>5.0% of the total fatty acids) of strain 112T were summed feature 8 (C18:1ω7c and/or C18:1ω6c) (73.6%) and C16:0 (12.6%) (Table 2). The fatty acid profile of strain 112T was similar to those of the reference strains grown under the same conditions. However, there were some differences in the presence/absence of some fatty acid components, especially in the hydroxylated fatty acids, that might allow the differentiation of strain 112T from other Roseovarius type strains. The predominant respiratory isoprenoid quinone detected in strain 112T was Q-10, as for the other type strains of the genus Roseovarius. The genomic DNA G+C content of strain 112T was 58.2 mol%, which was within the range reported for other type strains of the genus Roseovarius (Wang et al., 2010; Kim et al., 2012b). The major polar lipids detected in strain 112T were phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, an unidentified aminolipid, and three unidentified lipids (Fig. S2). Some other Roseovarius species (R. tolerans and R. mucosus) have been reported to contain diphasatidylglycerol as a major polar lipid, but it was not detected from strain 112T (Table 1). The phenotypic and phylogenetic features of strain 112T support its assignment to a novel species within the genus Roseovarius, for which the name Roseovarius lutimaris sp. nov. is proposed.

**Description of Roseovarius lutimaris** sp. nov. Roseovarius lutimaris (lu.ti.ma´ris. L. n. lutum mud; L. gen. n. mariis of the sea; N.L. gen. n. lutimaris of mud of the sea).

Cells are Gram-staining-negative, obligately aerobic ovoids to rods, 0.4–0.8 µm wide and 2–3 µm long. Cells show a predominantly tumbling motion. Flagella are not found. Colonies on MA are beige, circular and convex with smooth edges. Growth occurs at 15–40 °C (optimum, 30 °C), at pH 5.0–9.0 (optimum, pH 6.5–7.5) and in the presence of 1.0–6.0% (w/v) NaCl (optimum, 2.0–4.0%). BChl a is not produced. Anaerobic growth is not observed after 20 days at 30 °C on MA or on MA supplemented with nitrate. Tests for oxidase and catalase are positive. Tween 20 and starch (weakly) are hydrolysed, while Tween 80,
Table 1. Differential phenotypic characteristics of strain 112T and related Roseovarius type strains

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain 112T</th>
<th>Strain 2</th>
<th>Strain 3</th>
<th>Strain 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Rod/ovoid</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod/ovoid</td>
</tr>
<tr>
<td>Colony colour</td>
<td>Beige</td>
<td>Whitish to faintly pink</td>
<td>Red</td>
<td>Beige pink</td>
</tr>
<tr>
<td>Motility</td>
<td>+ (tumbling)</td>
<td>–</td>
<td>+</td>
<td>+ (tumbling)</td>
</tr>
<tr>
<td>BChl a</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ranges for growth</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>15–40</td>
<td>20–40</td>
<td>&lt;3–43.5</td>
<td>4–45</td>
</tr>
<tr>
<td>NaCl (% w/v)</td>
<td>1–6</td>
<td>1–7</td>
<td>&lt;1–10</td>
<td>0.5–10</td>
</tr>
<tr>
<td>pH</td>
<td>5–9</td>
<td>6–8.8</td>
<td>5.3–9</td>
<td>3–10</td>
</tr>
<tr>
<td>Enzyme activities (API ZYM)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystine arylamidase</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trypsin</td>
<td>–</td>
<td>–</td>
<td>W</td>
<td>W</td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Susceptibility to:*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Acid production from:*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Glucose</td>
<td>w</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-Sorbitol</td>
<td>w</td>
<td>+</td>
<td>–</td>
<td>W</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Polar lipids†</td>
<td>PG, PE, PC, AL, Ls</td>
<td>PG, DPG, PE, PC, ALs, Ls*</td>
<td>PG, DPG, PE, PC</td>
<td>NA</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>58.2</td>
<td>62.9</td>
<td>63.3–63.4</td>
<td>62</td>
</tr>
</tbody>
</table>

*Data from this study from the strains indicated in the text.
†DPG, Diphasphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL, unidentified aminolipid; L, unidentified lipid.
Table 2. Cellular fatty acid compositions of strain 112T and related Roseovarius type strains

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12:0</td>
<td>TR</td>
<td>7.9</td>
<td>TR</td>
<td>7.6</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.6</td>
<td>TR</td>
<td>TR</td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>12.6</td>
<td>7.4</td>
<td>11.0</td>
<td>18.1</td>
</tr>
<tr>
<td>C17:0</td>
<td>0.8</td>
<td>TR</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>C18:0</td>
<td>TR</td>
<td>1.2</td>
<td>1.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Unsaturated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C17:0:1o8c</td>
<td>TR</td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>11-Methyl C18:1o7c</td>
<td>2.8</td>
<td>0.8</td>
<td>4.8</td>
<td>12.2</td>
</tr>
<tr>
<td>C19:0: cyclo o8c</td>
<td>0.8</td>
<td>4.7</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Hydroxy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C10:0:3-OH</td>
<td></td>
<td>1.4</td>
<td>TR</td>
<td>0.6</td>
</tr>
<tr>
<td>C12:0:2-OH</td>
<td></td>
<td>0.8</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>C12:0:3-OH</td>
<td></td>
<td>TR</td>
<td>TR</td>
<td>4.4</td>
</tr>
<tr>
<td>C12:1:3-OH</td>
<td></td>
<td>3.5</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>C16:0:2-OH</td>
<td></td>
<td>7.6</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>C18:1:2-OH</td>
<td>4.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summed features*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.4</td>
<td>0.9</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>1.5</td>
<td>TR</td>
<td>0.6</td>
</tr>
<tr>
<td>8</td>
<td>73.6</td>
<td>60.8</td>
<td>67.6</td>
<td>53.0</td>
</tr>
</tbody>
</table>

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained C16:1o7c and/or C18:1o6c. Summed feature 7 contained an unknown fatty acid with an equivalent chain-length of 18.846 and/or C19:0:1o6c. Summed feature 8 contained C18:1o7c and/or C18:1o6c.

References to the text:

**References**


**Acknowledgements**

These efforts were supported by grants from the National Research Foundation of Korea (grant 2012R1A1B) of the Korean Government (MEST) and the Program for Collection of Domestic Biological Resources from the National Institute of Biological Resources (NIBR), Korea.

**References**

**Acknowledgements**

These efforts were supported by grants from the National Research Foundation of Korea (grant 2012R1A1B) of the Korean Government (MEST) and the Program for Collection of Domestic Biological Resources from the National Institute of Biological Resources (NIBR), Korea.


