The genus *Roseovarius* belongs to the family *Rhodobacteraceae* within the class *Alphaproteobacteria* and it was established by Labrenz *et al.* [1]. At the time of writing, the genus comprises 20 species.

The genus *Roseovarius* accommodates Gram-stain-negative, aerobic, ovoid- or rod-shaped members characterized as being bacteriochlorophyll a (BChla)-positive, oxidase- and catalase-positive, and requiring Na⁺ for growth. Most of the bacteria of this genus have been isolated from marine environments including seawater or tidal flats [2–6].

In this study we describe strain D15T, a moderately halophilic bacterium isolated from saline soil in Rambla Salada (Murcia).

Strain D15T was isolated from a sample of soil taken from Rambla Salada (Murcia) south-eastern Spain, 38° 07‘ 27.1” N 1° 07‘ 01.4” W, a hypersaline rambla (a steep-sided river bed, normally dry but subject to flash flooding). The culture medium used for the isolation of the bacteria was S3, a low-nutrient medium [7] supplemented with 3 % (w/v) sea-salt solution [8] and the cultivation method used was the dilution-to-extinction approach.

In this approach serial dilutions from 1 g soil were prepared, previously sonicated for 30 s, in 10 ml supplemented S3 medium. The number of micro-organisms in each dilution was determined using a Petroff Hausser counting chamber using methylene blue as contrast. A 48-well microtiter plate, containing 490 µl supplemented S3 medium, was inoculated with 10 µl dilution containing 100 bacteria per millilitre (approximately 1 bacterium per well). The extinction cultures were then incubated at 25°C for 30 days. The growth wells were then re-isolated in Reasoner’s 2A agar (R2A) medium plates [9]. The pure strain was maintained and grown routinely in R2A [9] at 30°C with 3 % (w/v) sea-salt solution [8] as well as in marine agar (MA: 2216, Difco).

The advantage of this method is the improvement of strain recovery, especially from species that are apparently uncultivable or from slow-growing species [10–13].

Optimum growth conditions were evaluated by growing the strain in R2A medium at 0.5, 1, 3, 5, 7.5, 10, 15, 20, 25 and 30 % (w/v) NaCl concentrations. The effect of different temperatures (4, 15, 20, 25, 30, 32, 35, 40 and 45°C) and

The genus *Roseovarius ramblicola* sp. nov., a moderately halophilic bacterium isolated from saline soil in Spain

David J. Castro,1,2,3 Isabel Cerezo,1,2 Inmaculada Sampedro1,2,* and Fernando Martínez-Checa1,2

**Abstract**

Strain D15T was isolated from a soil sample taken from Rambla Salada (Murcia), south-eastern Spain, by using the dilution-to-extinction method. The strain, a Gram-stain-negative aerobic bacteria, is non-motile, ovoid- or rod-shaped, catalase- and oxidase-positive, and grows at NaCl concentrations within the range 0.5–10 % (w/v) [optimum 3 % (w/v)], at 5–30°C (optimum 28°C) and at pH 6–9 (optimum pH 7.0). The 16S rRNA gene sequence indicates that it belongs to the genus *Roseovarius* in the class *Alphaproteobacteria*. Its closest relatives are *Roseovarius tolerans* EL-172T and *Roseovarius azorensis* SSW084T, to which the strain shows 16S rRNA gene-sequence similarity values of 96.1 and 95.3 %, respectively. The DNA G+C content is 63 mol%. The major fatty acids (>5 % of the total fatty acids) of strain D15T are C_{18:1ω7c}, C_{16:0} and C_{12:0}. The only detected isoprenoid quinone of strain D15T is ubiquinone 10 (Q-10). The polar lipid profile contains phosphatidylethanolamine, phosphatidylglycerol, aminolipid and three polar lipids. Based on the phylogenetic, genotypic, phenotypic and chemotaxonomic data, the strain represents a novel species of the genus *Roseovarius*, for which the name *Roseovarius ramblicola* sp. nov. is proposed. Strain D15T (=CECT 9424=LMG 30322) is the type strain. In this study we describe strain D15T, a moderately halophilic bacterium isolated from saline soil in Rambla Salada (Murcia).

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**Keywords:** *Roseovarius ramblicola* sp. nov.; polyphasic taxonomy; phylogenetic; chemotaxonomy.

**Abbreviations:** A, aminolipid; DPG, diphosphatidylglycerol; L, unidentified polar lipid; MA, marine agar; PC, phosphatidylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; R2A, Reasoner’s 2A agar.

The GenBank/EMBL/DDBJ accession number for 16S rRNA gene sequence of strain D15T is MF527111. The GenBank/EMBL/DDBJ accession number for pufM and pufL gene sequences of strain D15T is MG831321.

Three supplementary figures are available with the online version of this article.
different pH values (4, 5, 6, 7, 8, 9, 10 and 11) were assessed in R2A medium at 3% w/v NaCl.

Gram staining was performed according to the method of [14]. Catalase activity was determined by bubble production in 3% (v/v) \( \text{H}_2\text{O}_2 \) solution. Oxidase activity was examined using 1% (v/v) tetramethyl-p-phenylenediamine [15]. Growth under anaerobic conditions was determined in an anaerobic jar using AnaeroGen (Oxoid) and an anaerobic indicator (Oxoid) on R2A medium supplemented with 3% (v/v) sea-salt solution [8]. Phenotypic characterization, including biochemical characters, sugar fermentation and enzymatic tests, were carried out by using API 20NE, API 50CH and API ZYM strips (bioMérieux) following the manufacturer’s instructions. Sensitivity to antimicrobial compounds was assayed according to the conventional Kirby–Bauer method [16].

Transmission electron micrographs of strain D15 were available as supplementary material (Fig. S1, available in the online version of this article). The results obtained are detailed in the species description.

The cells of strain D15 were non motile, ovoid- or rod-shaped, Gram-stain-negative, catalase- and oxidase-positive, and strictly aerobic. They could grow at 0.5–10% (w/v) NaCl, optimally at 3% (w/v) NaCl. The temperature range for growth was 5–30 °C, with the optimum being 28 °C. The pH range for growth was 6–9 with optimum growth at pH 7.0. Other characteristics of strain D15 are given in the species description and those that differ with the type strains in the genus Roseovarius are shown in Table 1.

The DNA G+C content of strain D15 was estimated from the midpoint value (\( T_m \)) of their DNA [17]. \( T_m \) was determined by the graphic method [18] and the DNA G+C content was calculated by using the equation described by [19]. The G+C content of reference DNA of *Escherichia coli* NCTC 9001\(^T\) was 50.9 mol% [20]. The DNA G+C content was 63 mol%, a similar value to members of the genus *Roseovarius* [5, 21] (Table 1).

Phylogenetic analyses based on the 16S rRNA gene were performed as described elsewhere [22]. In this case, the PCR product was cloned into the pGEM-T cloning vector (Promega) according to the manufacturer’s recommendations, and transformed into *Escherichia coli* DH5-\( \alpha \). The identification of phylogenetic neighbours was initially carried out by the BLASTN [23] program against the GenBank/EMBL/DDBJ database containing type strains with validly
published prokaryotic names and representatives of uncultured phylotypes. The 16S rRNA sequence of strain D15\(^T\) was also implemented into the current release of the LTP ARB database 'All-species Living Tree Project' [24]. We also carried out the identification of phylogenetic neighbours and a calculation of pairwise 16S rRNA gene sequence similarity by using the EzTaxon server (www.ezbiocloud.net; [25]). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7 [26]. Clustering was determined using the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms and the evolutionary distances were computed using the Jukes–Cantor method [27]. The analysis involved 29 nucleotide sequences and the stability of the clusters was ascertained by performing a bootstrap analysis (1000 replications).

Phylogenetic analyses based on these three methods indicated that strain D15\(^T\) was included in the cluster which

![Phylogenetic Tree](image)

**Fig. 1.** Maximum-likelihood phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between strain D15\(^T\), type species of the genus *Roseovarius* and the closest related species in the family *Rhodobacteraceae*. Filled circles indicate nodes that were also recovered in the maximum-parsimony and neighbour-joining trees based on the same sequences. Numbers at nodes are levels of bootstrap support (percentages) based on analyses of 1000 re-sampled datasets; only values above 50% are shown. Bar, 0.05 nt changes per position. The GenBank/EMBL/DDBJ accession number of each sequence is shown in parentheses.
make up the *Roseovarius* species, resulting in highly similar tree topologies. Fig. 1 shows the tree containing the phylogroup in which our new strain is included according to the maximum-likelihood algorithm.

The most closely related species were *Roseovarius tolerans* EL-172T and *Roseovarius azorensis* SSW084T to which strain D15T showed 16S rRNA gene sequence similarity values of 96.1 and 95.3 %, respectively.

The fatty acids of strain D15T and *R. azorensis* DSM 100674T were analysed at the Spanish Type Culture Collection. Cells were grown on MA for 48 h incubation at 30 °C (stationary growth phase). The whole-cell composition of the fatty acids was determined by gas chromatography using the MIDI Microbial Identification System [28]. The fatty-acid profile was obtained with an Agilent 6850 gas chromatograph using the TSBA6 145 database [29]. Analysis of the polar lipids and respiratory quinones of strain D15T was carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. Polar lipids are extracted from 100 mg freeze dried cell material using a chlorofrom: methanol:0.3 % aqueous NaCl mixture 1 : 2 : 0.8 (v/v/v) [30]. The extraction solvent was stirred overnight and the cell debris pelleted by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the chloroform: methanol:0.3 % aqueous NaCl mixture to a ratio of 1 : 1 : 0.9 (v/v/v).

Polar lipids were separated by two-dimensional silica gel thin-layer chromatography (Macherey–Nagel Art. No. 818 135). The first direction was developed in chloroform–methanol–water (65 : 25 : 4, v/v/v), and the second in chloroform–methanol–acetic acid–water (80 : 12 : 15 : 4, v/v/v/v). Total lipid material was detected using molybdophosphoric acid and specific functional groups detected using spray reagents specific for defined functional groups [31]. The two-stage method described by [32, 33] was used to first extract the respiratory lipoquinones followed by the polar lipids.

The major fatty acids (>5 % of the total fatty acids) of strain D15T were C18:1ω7c (61.5 %), C16:0 (11.9 %) and C12:0 (7.1 %) (Table 2). The fatty-acid profile of strain D15T was similar to the profile described for *R. tolerans* EL-172T [1] and *R. azorensis* DSM 100674T, as shown in Table 2. The only isoprenoid quinone detected was ubiquinone Q10 in accordance with the lipoquinone present in the described species of the genus *Roseovarius*. The polar lipid profile of strain D15T was composed of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, aminolipid and three unidentified polar lipids (Fig. S3). This pattern was different from the reference strains previously described [1, 5, 21]. For example, it had no dipht phosphatidylglycerol compared with *R. tolerans* EL-172T and *R. azorensis* DSM 100674T, but it did have phosphatidylcholine, phosphatidyethanolamine as well as the two type strains of genus *Roseovarius* considered in this study.

### Table 2. Fatty-acid profile of the strain D15T and the type strains of the genus *Roseovarius*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12:0 2-OH</td>
<td>0.9</td>
<td>0.3</td>
<td>2.4</td>
</tr>
<tr>
<td>C12:0 3-OH</td>
<td>−</td>
<td>6.0</td>
<td>−</td>
</tr>
<tr>
<td>C12:1 3-OH</td>
<td>3.6</td>
<td>0.2</td>
<td>3.6</td>
</tr>
<tr>
<td>C12:0</td>
<td>7.1</td>
<td>2.9</td>
<td>−</td>
</tr>
<tr>
<td>C16:0</td>
<td>11.9</td>
<td>13.4</td>
<td>6.2</td>
</tr>
<tr>
<td>C18:0</td>
<td>1.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>C16:1</td>
<td>−</td>
<td>−</td>
<td>0.8</td>
</tr>
<tr>
<td>C18:2</td>
<td>−</td>
<td>−</td>
<td>s</td>
</tr>
<tr>
<td>C16:0 2-OH</td>
<td>2.5</td>
<td>2.3</td>
<td>−</td>
</tr>
<tr>
<td>C18:ω9c</td>
<td>0.7</td>
<td>2.4</td>
<td>−</td>
</tr>
<tr>
<td>C18:ω7c 11-methyl</td>
<td>2.3</td>
<td>5.9</td>
<td>−</td>
</tr>
<tr>
<td>Cyclo-C19:ω8c</td>
<td>0.9</td>
<td>4.0</td>
<td>−</td>
</tr>
<tr>
<td>Summed features</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.8</td>
<td>0.8</td>
<td>−</td>
</tr>
<tr>
<td>8</td>
<td>61.5</td>
<td>56.9</td>
<td>70.2</td>
</tr>
</tbody>
</table>

*Summed feature 3: C16:1ω7c/C16:0ω6c, summed feature 8: C18:ω7c.

To analyse the presence of bacteriochlorophyll *a*, *puf* genes (photosynthetic reaction centre genes *pufL* and *pufM*) were amplified by PCR using *pufL* (5'–CTKTTCGACT TCTGGGTSGG–3') and *pufMt* (5'–CATSGTCCAGCCG–CATGAA–3') as the pair of primers, following the protocol described by [34]. The PCR product of about 1.5 kb was cloned into the pGEM-T cloning vector (Promega) according to the manufacturer’s recommendations, transformed into *Escherichia coli* DH5–α and sequenced with T7 and SP6 primers. The *pufL* and *pufM* gene sequences of strain D15T showed 89 and 87 % similarity to the sequences of *pufL* and *pufM* genes of *R. tolerans* EL-172T (DDBJ/EMBL/GenBank accession number DQ915720) and *R. mucosus* DFL-24T (DDBJ/EMBL/GenBank accession number CP020474) respectively, and 81 % similarity to the sequences of *pufL* and *pufM* genes of *R. denitrificans* Och 114T (DDBJ/EMBL/GenBank accession number DQ915721) and *R. halotolerans* Och 210T (DDBJ/EMBL/GenBank accession number DQ915719).

Fig. S2 shows the PCR products of the *pufLM* genes amplified from total DNA of strain D15T and the absence of these genes in another type strain of genus *Roseovarius* like *Roseovarius pacificus* LMG 24575T.

Accordingly, on the basis of differences in phenotypic and chemotaxonomic characteristics, and genetic distinctiveness, strain D15T should be recognized as representing a novel species of the genus *Roseovarius*, for which we propose the name *Roseovarius ramblicola*.
DESCRIPTION OF ROSEOVARIUS RAMBLICOLA SP. NOV.

Roseovarius ramblica [ram.blí’co.la. Spanish fem. n. ramb-lba sandy ground; L. suff. -cola (from L. masc. or fem. n. incola) inhabitant; N.L. n. ramblicola inhabitant of a rambla].

Cells are ovoid- or rod-shaped, Gram-stain-negative, non-motile, 0.7–1.2 x 2.1–3.2 µm in size and reproduce by budding or asymmetrical division. Cell colonies are white, circular, convex and opaque when grown on MA and R2A media. The growth pattern is uniform in a liquid medium. Capable of growing in NaCl concentrations of 0.5 to 10% (w/v), with optimum growth occurring at 3%. Grows within a temperature range of 5–30°C at pH values of between 6 and 9, the optimum values being 28°C and pH 7. Catalase and oxidase are produced. Nitrate reduction, nitrite reduction, indol production, hydrolysis of arginine, aesculin and 9, the optimum values being 28°C and pH 7. Catalase and oxidase are produced. Nitrate reduction, nitrite reduction, indol production, hydrolysis of arginine, aesculin and 9, the optimum values being 28°C and pH 7.

The authors declare that there are no conflicts of interest.

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