*Palleronia marisminoris* gen. nov., sp. nov., a moderately halophilic exopolysaccharide-producing bacterium belonging to the *α*-Proteobacteria, isolated from a saline soil.

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The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain B33^T^ is AY926462.
**Summary**

*Palleronia marisminoris* gen. nov., sp. nov. is a moderately halophilic, exopolysaccharide-producing, Gram-negative, non-motile rod isolated from a hypersaline soil bordering a saline saltern on the Mediterranean seaboard in Murcia (Spain). The bacterium is chemoheterotrophic and strictly aerobic. It contains a pink pigment but does not synthesise bacteriochlorophyll *a*. It requires 0.66 M of Na⁺, 0.1 M of Mg²⁺ and 0.1 M of K⁺ for optimum growth. It does not produce acid from carbohydrates. It cannot grow with carbohydrates, organic acids, sugars alcohols or amino acids as sole sources of carbon and energy. Its major fatty-acids are 18:1 ω7c (68.9%) and 19:0 cyclo ω8c (12.8%). The sole respiratory lipoquinone found in strain B33ᵀ is ubiquinone-10. The G+C content is 64.2 mol%. 16S rRNA gene sequence comparisons show that the isolate is a member of the *Roseobacter* clade within the class of α-Proteobacteria. The similarity values with *Roseivivax halodurans* and *Roseivivax halotolerans* are 88.2% and 88.0% respectively and 92.2% with *Salipiger mucosus*. DNA-DNA hybridization values with these species are < 30%. In the light of the polyphasic evidence gathered in this study it is proposed that the isolate be classified as a new genus and species with the name *Palleronia marisminoris* gen. nov., sp. nov. The proposed type strain is strain B33ᵀ (=CECT 7066ᵀ = LMG 22959ᵀ).
Moderately halophilic bacteria require from 3% to 15% w/v NaCl for satisfactory growth (Kushner & Kamekura, 1988). They are widely distributed among many hypersaline habitats. Taxonomically the majority of Gram-negative halophilic bacteria belong to the $\gamma$-Proteobacteria class but they can also be found in other bacterial phyla (Ventosa et al., 1998). Some halophilic microorganisms, such as those which produce exopolysaccharides, have interesting industrial applications (Quesada et al., 2004). Microbial exopolysaccharides have a potentially wide range of applications in such fields as pharmacy, foodstuffs, cosmetics, and the petroleum industry, where emulsifying, viscosifying, suspending, and chelating agents are required (Sutherland, 1990). During an extensive search of 18 hypersaline habitats in Spain and Morocco, designed to obtain new exopolysaccharides, we discovered that the commonest halophilic exopolysaccharide producers were various species of the genus *Halomonas*, most importantly *Halomonas maura* and *Halomonas eurihalina* (Quesada et al., 1990; Bouchotroch et al., 2001; Quesada et al., 2004; Martínez-Cánovas et al., 2004d). As a result of these searches we have described the first moderately halophilic exopolysaccharide-producing microorganism belonging to the $\alpha$-Proteobacteria, *Salipiger mucosus* (Martínez-Cánovas et al., 2004e), three new *Halomonas* species, *H. ventosae* (Martínez-Cánovas et al., 2004c), *H. anticariensis* (Martínez-Cánovas et al., 2004a) and *H. almeriensis* (Martínez-Checa et al., 2005b), two new *Idiomarina* species *I. fontislapidosi* and *I. ramblicola* (Martínez-Cánovas et al., 2004b) and a new *Alteromonas* species, *A. hispanica* (Martínez-Checa et al., 2005a) all of which produce exopolysaccharides (EPS’s). We describe and classify here an unassigned halophilic EPS-producing strain also isolated in these studies and propose it as a novel genus and species belonging to the $\alpha$-Proteobacteria class with the name *Palleronia marisminoris*.

The strain in question, B33$^T$, was isolated from a saline soil bordering a saltern on the Mediterranean seaboard at Marchamalo (Murcia, SE. Spain) (Martínez-Cánovas et al., 2004d). It was routinely grown at 32ºC in MY medium (Moraine and Rogovin, 1966) supplemented with a 5% w/v sea-salt solution (Rodríguez-Valera et al., 1981). Its phenotype was studied with 135 tests by Martínez-Cánovas et al., (2004d) and it was included in phenon E along with other unidentified strains. The procedures we followed for its phenotypic characterization have been described by Ventosa et al., (1982), Quesada et al., (1983) and Mata et al., (2002). Salt requirements and optimum salt concentration were determined in MY medium (Moraine and Rogovin, 1966). The salt concentrations assayed ranged from 0.5% to 30% w/v and were prepared from a mixture of sea salts according to Rodríguez-Valera et al., (1981). We also tested to see whether strain B33$^T$ could survive with NaCl alone or whether it required other sea salts. To determine its nutritional requirements we also assayed its growth in Koser medium supplemented with yeast extract (0.1 to 3% w/v), malt extract (1 to 3% w/v) or proteose peptone (1 to 5% w/v). The presence of bacteriochlorophyll $a$ was analysed spectrophotometrically
using Cohen-Bazire and colleagues’ procedure (1957), following the recommendations of Allgaier et al., (2003). DNA was purified using the technique of Marmur (1961). The guanine-plus-cytosine (G+C) content of the DNA was estimated from the midpoint value ($T_m$) of the thermal denaturation profile (Marmur and Doty, 1962). $T_m$ was determined by the graphic method described by Ferragut and Leclerc (1976) and the G+C content was calculated from this temperature using Owen and Hill’s equation (1979). The $T_m$ value of reference DNA from *Escherichia coli* NCTC 9001 was taken to be 74.6°C in 0.1x SSC (Owen and Pitcher, 1985).

The phenotypic characteristics and G+C content are given in the species description. Phenotypic features distinguishing between strain B33$^T$, *Salipiger mucosus* and the two species of *Roseivivax* are available in Table 1, where it can be seen that strain B33$^T$ is phenotypically most closely related to *S. mucosus*. Both species are Gram-negative, non-motile, moderately halophilic rods. They are chemoheterotrophic, strictly aerobic and produce exopolysaccharides. They do not produce bacteriochlorophyll α. They do not produce acids from sugars and have low nutritional versatility as they cannot grow with any of the carbohydrates, alcohols, organic acids or amino acids tested as sole sources of carbon and energy. For optimum growth it requires yeast extract (0.3% w/v), malt extract (0.3% w/v) and proteose peptone (0.5% w/v) together with Na$^+$ (0.66 M), Mg$^{2+}$ (0.1M) and K$^+$ (0.01M), and thus it flourishes in an MY complex medium (Moraine and Rogovin, 1966) supplemented with 5% w/v sea salts. The most important phenotypic tests distinguishing between *Palleronia marisminoris* and *Salipiger mucosus* are pigment production, a negative reaction for oxidase, urease and gluconate oxidation, positive reaction for ONPG, and an inability to grow with NaCl alone. The G+C (mol%) content of strain B-33$^T$ (64.2) is very similar to those of *S. mucosus* and *R. halodurans* (64.5 and 64.4 mol% respectively).

Fatty acids and quinones were identified by high-resolution GLC and HPLC respectively at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (cf. Table 1). Strain B33$^T$ contains a large quantity of cis-11 octadecenoic acid (18:1 $\omega 7c$) (68.9 %) together with 19:0 cyclo $\omega 8c$, 3-hydroxy 10:0, 16:0 and 18:0 (12.8%, 5%, 4.2% and 3.4% respectively). It also has 2.3% of an unknown component at a retention time of 4.870 min. The presence of 18:1 $\omega 7c$ as predominant fatty acid is a feature characteristic of taxa within the α-Proteobacteria. Nevertheless, the cyclo-substituted fatty acid (19:0 cyclo $\omega 8c$) is not widely present in the *Rhodobacteraceae* family; though it has been described in lesser quantities (2.2 %) in *Salipiger mucosus*. The only respiratory lipoquinone detected was ubiquinone-10. The presence of ubiquinone 10 as the dominant respiratory lipoquinone is characteristic of members of the α-class of the *Proteobacteria*. 
The transmission electron micrograph (Fig. 1), made using the methods described by Bouchotroch et al., (2001), shows the cell morphology of strain B33<sup>T</sup>. Thin sections reveal a typical Gram-negative cell-envelope profile; the cell contains poly-β-hydroxyalkanoate (PHA) granules. EPS appears in the external medium.

We determined the almost complete 16S rDNA sequence of strain B33<sup>T</sup> (1351 bp) corresponding to positions 46 to 1445 of the *Escherichia coli* 16S rRNA gene using the standard protocols (Saiki et al., 1988). The forward primer was 16F27 (5´-AGAGTTTGATCMTGGCTCAG-3´), annealing at positions 8-27, and the reverse primer was 16R1488 (5´-CGGTTACCTTGTTAGGACTTCACC-3´), annealing at the complement of positions 1511-1488 (*E. coli* numbering according to Brosius et al., 1978.). The PCR products were purified using the Qiaquick spin-gel extraction kit (Qiagen). Direct sequence determinations of PCR-amplified DNA’s were carried out with the ABI PRISM dye-terminator, cycle-sequencing, ready-reaction kit (Perking-Elmer) and an ABI PRISM 377 sequencer (Perking-Elmer) according to the manufacturer’s instructions. The sequences obtained were compared to reference 16S rRNA gene sequences available in the GenBank, EMBL and DDBJ databases obtained from the National Center of Biotechnology Information database using the BLAST search. Phylogenetic analysis was made using the software MEGA (Molecular Evolutionary Genetics Analysis) version 3.0 (Kumar et al., 2004) after multiple alignments of data by CLUSTALX (Thompson et al., 1997). Distances and clustering were determined with the neighbour-joining and maximum-parsimony methods. The stability of the clusters was ascertained by performing a bootstrap analysis (1000 replications). Our phylogenetic analysis with the neighbour-joining method included, along with the sequence of B33<sup>T</sup>, some representatives of the *Rhodobacteraceae* family (Figs 1 and 2, supplementary data). The maximum-parsimony algorithm gave a similar result (data not shown). Strain B33<sup>T</sup> showed 92.2% similarity with *Salipiger mucosus*. Other phylogenetically close species were *Roseivivax halodurans* and *Roseivivax halotolerans*, with which they showed 88.2% and 88.0% sequence similarity respectively. Strain B33<sup>T</sup> is in the same clade as *Roseivivax* and *Salipiger*, belonging to the α-3 group of the α-class of the *Proteobacteria* within the “*Rhodobacteraceae*” family (Garrity, 2002). *Roseivivax* is taxonomically related to the *Roseobacter* clade, a group of genera of the “*Rhodobacteraceae*” family (Allgaier et al., 2003), which makes up the most abundant population in marine habitats (González & Moran, 1997).

DNA-DNA hybridization was done according to Lind & Ursing’s method (1986) with the modifications of Ziemke et al., (1998) and Bouchotroch et al., (2001). DNA-DNA hybridization values of B33<sup>T</sup> with the most phylogenetically related species, *Roseivivax halodurans*, *Roseivivax halotolerans* and *Salipiger mucosus*, are 22, 27.7 and 29.7 respectively.
Thus, on the basis of phylogenetic evidence, DNA-DNA hybridization values, fatty-acid profiles, quinones, differences in phenotypic characteristics and its inability to synthesise bacteriochlorophyll \( a \), we are of the opinion that strain B33\(^T\) should be recognised as the representative species of a novel genus, for which we propose the names *Palleronia* and *Palleronia marisminoris*.

**Description of *Palleronia* gen. nov.**

*Palleronia* (Pall. er. o’nia, N.L. sb. f., in honour of Professor Norberto Palleroni, a pioneer in the use of molecular identification techniques in prokaryote taxonomy).

Gram-negative, short non-motile rods, 2-2.5 \( \mu \)m long by 0.75-1 \( \mu \)m wide. Neither flagella nor endospores are present. Bacteriochlorophyll \( a \) is absent. Metabolism is chemoheterotrophic and aerobic, the cells being unable to grow under anaerobic conditions either by fermentation, nitrate or fumarate reduction or photoheterotrophy. PHA and catalase are present. Oxidase is negative. Colonies contain pink pigment. The bacterium cannot produce acids from sugars and has low nutritional and biochemical versatility. It is strictly halophilic, requiring Na\(^+\), Mg\(^{++}\) and K\(^+\) for growth. The principal cellular fatty acids are 18:1 \( \omega 7c \) and 19:0 cyclo \( \omega 8c \). It has ubiquinone with ten isoprene units. The type species is *Palleronia marisminoris*.

**Description of *Palleronia marisminoris* sp. nov.**

*Palleronia marisminoris* (ma’ris, L. sb. n. gen., “of the sea”; minoris, L. adj. n. gen., “smaller”; *marisminoris* of the smaller sea, i.e. from el Mar Menor, a shallow area of sea highly sheltered from the Mediterranean sea on the S. E. coast of Spain, from whence the type strain was isolated).

In addition to the traits reported for the genus, the species grows on MY solid medium in the form of circular, convex, pink, mucoid colonies. In liquid medium its growth pattern is uniform. The cells are encapsulated. It is moderately halophilic, capable of growing in salt concentrations (mixture of sea-salts) from 0.5% to 15% w/v. Optimum growth occurs at 5% w/v sea-salt. It cannot grow with NaCl as sole salt. Minimum salt requirements are 0.66 M Na\(^+\), 0.1 M Mg\(^{++}\) and 0.01 M K\(^+\). It grows within the temperature range of 20\(^\circ\) to 37\(^\circ\)C and at pH values of between 5 and 10. It produces H\(_2\)S from L-cysteine. Selenite reduction and phosphatase are positive. Tween 20 is hydrolysed. It does not produce acids from the following sugars: adonitol, D-cellobiose, D-fructose, D-galactose, D-glucose, *myo*-inositol, lactose, maltose, D-mannitol, D-mannose, D-melezitose, L-rhamnose, sucrose, D-salicin, D-sorbitol, sorbose or D-trehalose.
ONPG is positive. O/F, indol, methyl-red, Voges-Proskauer and gluconate oxidation are negative. Phenylalanine deaminase is not produced. Urea, tyrosine, Tween 80, starch, aesculin, gelatine, DNA, lecithin and casein are not hydrolysed. Growth on either MacConkey or cetrimide agar is inviable. Blood is not lysed. Neither nitrate nor nitrite is reduced. The following compounds are not acceptable as sole carbon and energy sources: L-arabinose, D-cellobiose, aesculin, D-fructose, glucose, galactose, lactose, maltose, D-mannose, D-salicin, trehalose, acetate, citrate, formate, fumarate, gluconate, lactate, malonate, propionate, succinate, adonitol, ethanol, glycerol, inositol, mannitol and sorbose. The following compounds are not used as sole carbon, nitrogen and energy sources: L-alanine, L-cysteine, L-histidine, iso-leucine, L-lysine, L-methionine, L-serine, tryptophan and L-valine. It is susceptible to (μg) amoxicillin (25), ampicillin (10), carbenicillin (100), cefotaxime (30), cefoxitin (30), chloramphenicol (30), erythromycin (15), kanamycin (30), streptomycin (10), nitrofurantoin (300), rifampicin (30), tobramycin (10) and is resistant to nalidixic acid (30), polymyxim B (300) sulfonamide (250) and trimetoprim-sulphametoxazol (1.25-23.7). The major fatty acids are (%): 18:1 ω7c (68.9), 19:0 cyclo ω8c (12.8), 3-hydroxy 10:0 (5), 16:0 (4.2), 18:0 (3.4). Its DNA G+C content is 64.2 mol % (Tm method).

The type strain is strain B33T (=CECT 7066T = LMG 22959T), isolated from a hypersaline soil bordering a solar saltern in Marchamalo (Murcia, S.E. Spain).

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REFERENCES


Table 1. Characteristics that distinguish *Palleronia marisminoris* from related members of the family “*Rhodobacteraceae*”.

1, *Salipiger mucosus* CECT 5855<sup>T</sup> (Martínez-Cánovas et al., 2004e); 2 and 3, *Roseivivax halodurans* JCM 10272<sup>T</sup> and *Roseivivax halotolerans* JCM 10271<sup>T</sup> (Suzuki et al., 1999; Nishimura et al., 1994).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>P. marisminoris</em></th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source of isolation</td>
<td>Hypersaline soil</td>
<td>Hypersaline soil</td>
<td>Charophyte sp. in a saline lake</td>
<td>Cyanobacterial mat in a saline lake</td>
</tr>
<tr>
<td>Pigment</td>
<td>Pink</td>
<td>-</td>
<td>Pink</td>
<td>-</td>
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<tr>
<td>Flagella*</td>
<td>-</td>
<td>-</td>
<td>S, SP</td>
<td>S, SP</td>
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<tr>
<td>PHA</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Oxidase</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>EPS production</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Bacteriochlorophyll a</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt; requirement</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Salt growth range (%) w/v</td>
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<td>0.5-20</td>
<td>0-20</td>
<td>0.5-20</td>
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<tr>
<td>Optimum salt concentration (% w/v)</td>
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<td>3-6</td>
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<td>ND</td>
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<td>NO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt; to NO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Acid from glucose</td>
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<tr>
<td>Growth on glucose†</td>
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<tr>
<td>Indol</td>
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<td>ONPG</td>
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<tr>
<td>Urease</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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<td>Phosphatase</td>
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<td>-</td>
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<td>Gluconate oxidation</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Major fatty acids (%)‡</td>
<td>18:1ω7c (68.9) 16:0 (4.3) 18:0 (3.4) 18:1ω7c (78.0) 16:0 (12.4) 18:0 (2.0) 16:1ω7c (1.3)</td>
<td>18:1ω7c (68.9) 18:0 (4.3) 18:0 (3.4) 18:1ω7c (78.0) 16:0 (12.4) 18:0 (2.0) 16:1ω7c (1.3)</td>
<td>(not quantified) (not quantified)</td>
<td></td>
</tr>
<tr>
<td>3-OH fatty acids</td>
<td>10:0 (5.0)</td>
<td>12:1 (2.3)</td>
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<td>Methyl fatty acids</td>
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<tr>
<td>Cyclo-substituted fatty acids</td>
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<td>19:0 cyclo ω8c (12.8)</td>
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<tr>
<td>Unidentified fatty acids</td>
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<td>4.870 RT (2.3)</td>
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<tr>
<td>G+C content (mol %)</td>
<td>64.2</td>
<td>64.5</td>
<td>64.4</td>
<td>59.7</td>
</tr>
</tbody>
</table>

*<sup>S</sup>, single; SP, subpolar; †Growth on minimum medium; ‡Only percentages higher than 1% are shown.
*+, positive; -, negative; ND, no data available; RT, retention time.
Fig. 1. Transmission electron micrograph of *Palleronia marisminoris* strain B33<sup>T</sup> cells stained with ruthenium red. Bar, 1 μm.

Fig. 2. Phylogenetic relationships between *Palleronia marisminoris* strain B33<sup>T</sup> and other genera of the "Rhodobacteraceae" family. The tree was constructed using the neighbour-joining algorithm. Only bootstrap values above 50% are shown (1000 replications). Bar, 1% estimated sequence divergence.

Fig. S1. Phylogenetic relationships between *Palleronia marisminoris* strain B33<sup>T</sup> and other genera of the "Rhodobacteraceae" family plus other taxa of Gram-negative halophilic bacteria. The tree was constructed using the neighbour-joining algorithm. Only bootstrap values above 50% are shown (1000 replications). Bar, 2% estimated sequence divergence.