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

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Strain B33^T 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 synthesize bacteriochlorophyll *a*. It requires 0.66 M Na⁺, 0.1 M Mg²⁺ and 0.1 M 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 ω 7c (68.9%) and 19:0 cyclo ω 8c (12.8%). The sole respiratory lipoquinone found in strain B33^T is ubiquinone-10. The DNA 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 'Alphaproteobacteria'. 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 novel genus and species with the name *Palleronia marisminoris* gen. nov., sp. nov. The proposed type strain is strain B33^T (=CECT 7066^T=LMG 22959^T).

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 class 'Gammaproteobacteria' but they can also be found in other bacterial phyla (Ventosa *et al.*, 1998). Some halophilic micro-organisms, such as those which produce exopolysaccharides (EPS), have interesting industrial applications (Quesada *et al.*, 2004). Microbial EPS 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 EPS, we discovered that

the commonest halophilic EPS-producers were various species of the genus *Halomonas*, most importantly *Halomonas maura* and *Halomonas eurihalina* (Quesada *et al.*, 1990, 2004; Bouchotroch *et al.*, 2001; Martínez-Cánovas *et al.*, 2004d). As a result of these searches we have described the first moderately halophilic EPS-producing micro-organism belonging to the 'Alphaproteobacteria', *Salipiger mucosus* (Martínez-Cánovas *et al.*, 2004e), three novel *Halomonas* species, *Halomonas ventosae* (Martínez-Cánovas *et al.*, 2004c), *Halomonas anticariensis* (Martínez-Cánovas *et al.*, 2004a) and *Halomonas almeriensis* (Martínez-Checa *et al.*, 2005a), two new *Idiomarina* species, *Idiomarina fontislapidosi* and *Idiomarina ramblicola* (Martínez-Cánovas *et al.*, 2004b), and a new *Alteromonas* species, *Alteromonas hispanica* (Martínez-Checa *et al.*, 2005b), all of which produce EPS. 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 class 'Alphaproteobacteria' 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, south-eastern Spain) (Martínez-Cánovas *et al.*, 2004d). It was routinely grown at 32 °C in MY

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Abbreviations: EPS, exopolysaccharide; PHA, poly- β -hydroxyalkanoate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Palleronia marisminoris* B33^T is AY926462.

A more detailed phylogenetic tree based on 16S rRNA gene sequences is available as a supplementary figure in IJSEM Online.

medium (Moraine & 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 & 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 (0.1–3 % w/v), malt extract (1–3 % w/v) or proteose peptone (1–5 % w/v). The presence of bacteriochlorophyll *a* was analysed spectrophotometrically using the procedure of Cohen-Bazire *et al.* (1957), following the recommendations of Allgaier *et al.* (2003). DNA was purified using the technique of Marmur (1961). The G+C content of the DNA was estimated from the midpoint value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962). T_m was determined by the graphic method described by Ferragut & Leclerc (1976) and the DNA G+C content was calculated from this temperature using Owen and Hill's equation (Owen & Hill, 1979). The T_m value of reference DNA from *Escherichia coli* NCTC 9001^T was taken to be 74.6 °C in 0.1 × SSC (Owen & Pitcher, 1985).

Table 1. Characteristics that distinguish *Palleronia marisminoris* B33^T from related members of the family 'Rhodobacteraceae'

Taxa: 1, *Palleronia marisminoris* B33^T; 2, *Salipiger mucosus* CECT 5855^T (Martínez-Cánovas *et al.*, 2004e); 3, *Roseivivax halodurans* JCM 10272^T; 4, *Roseivivax halotolerans* JCM 10271^T (Suzuki *et al.*, 1999; Nishimura *et al.*, 1994). +, Positive; –, negative; ND, no data available; RT, retention time; S, single; SP, subpolar.

Characteristic	1	2	3	4
Source	Hypersaline soil	Hypersaline soil	Charophyte sp. in a saline lake	Cyanobacterial mat in a saline lake
Pigment	Pink	–	Pink	Pink
Flagella	–	–	S, SP	S, SP
PHA	+	+	ND	ND
Oxidase	–	+	+	+
EPS production	+	+	–	–
Bacteriochlorophyll <i>a</i>	–	–	+	+
Na ⁺ requirement	+	+	–	+
Salt growth range (% w/v)	0.5–15	0.5–20	0–20	0.5–20
Optimum salt concentration (% w/v)	5	3–6	ND	ND
NO ₃ [–] to NO ₂ [–]	–	–	+	–
Acid from glucose	–	–	+	+
Growth on glucose*	–	–	+	+
Indole	–	–	+	+
ONPG	+	–	+	+
Urease	–	+	–	+
Phosphatase	+	+	+	–
Gluconate oxidation	–	+	ND	ND
Fatty acid content (%)†				
Major fatty acids	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)	18:1 (not quantified)	18:1 (not quantified)
3-OH fatty acids	10:0 (5.0)	12:1 (2.3)	–	–
Methyl fatty acids	–	11-methyl 18:1ω7c (1.9)	–	–
Cyclo-substituted fatty acids	19:0 cyclo ω8c (12.8)	19:0 cyclo ω8c (2.3)	–	–
Unidentified fatty acids	4.870 RT (2.3)	–	–	–
DNA G+C content (mol%)	64.2	64.5	64.4	59.7

*Growth on minimum medium.

†Only percentages higher than 1 % are shown.

The phenotypic characteristics and DNA G + C content are given in the species description. Phenotypic features that distinguish between strain B33^T, *S. 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 EPS. They do not produce bacteriochlorophyll *a*. 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 strain B33^T 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), Mg²⁺ (0.1 M) and K⁺ (0.01 M), and thus it flourishes in an MY complex medium (Moraine & Rogovin, 1966) supplemented with 5 % w/v sea salts. The most important phenotypic tests distinguishing between *Palleronia marisminoris* B33^T and *S. 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 DNA G + C content of strain B-33^T (64.2 mol%) 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 (Table 1). Strain B33^T contains a large quantity of *cis*-11 octadecenoic acid (18:1 ω 7*c*) (68.9 %) together with 19:0 cyclo ω 8*c*, 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 ω 7*c* as the predominant fatty acid is a feature characteristic of taxa within the 'Alphaproteobacteria'. Nevertheless, the cyclo-substituted fatty acid (19:0 cyclo ω 8*c*) is not widely present in the family 'Rhodobacteraceae'; though it has been described in lesser quantities (2.2 %) in *S. 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 'Alphaproteobacteria'.

The transmission electron micrograph (Fig. 1), prepared using the methods described by Bouchotroch *et al.* (2001), shows the cell morphology of strain B33^T. 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 rRNA gene sequence of strain B33^T (1351 bp) corresponding to positions 46 to 1445 of the *E. coli* 16S rRNA gene using standard protocols (Saiki *et al.*, 1988). The forward primer was 16F27 (5'-AGAGTTTGTGATCMTGGCTCAG-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

Fig. 1. Transmission electron micrograph of *Palleronia marisminoris* B33^T cells stained with ruthenium red. Bar, 1 μ m.

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 DNAs were carried out with the ABI PRISM dye-terminator, cycle-sequencing, ready-reaction kit (Perkin-Elmer) and an ABI PRISM 377 sequencer (Perkin-Elmer) according to the manufacturer's instructions. The sequences obtained were compared with reference 16S rRNA gene sequences available in the GenBank/EMBL/DDJB databases obtained from the National Center of Biotechnology Information database using the BLAST search. Phylogenetic analysis was made using the software MEGA version 3.0 (Kumar *et al.*, 2004) after multiple alignments of data by CLUSTAL_X (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^T, some representatives of the family 'Rhodobacteraceae' (Fig. 2 and Supplementary Fig. S1 in IJSEM Online). The maximum-parsimony algorithm gave a similar result (data not shown). Strain B33^T showed 92.2 % 16S rRNA gene sequence similarity with *S. mucosus*. Other

Fig. 2. Phylogenetic relationships between *Palleronia marisminoris* B33^T and other genera of the family 'Rhodobacteraceae'. The tree was constructed using the neighbour-joining algorithm. Only bootstrap values above 50% are shown (1000 replications). Bar, 1% estimated sequence divergence.

phylogenetically close species were *Roseivivax halodurans* and *Roseivivax halotolerans*, with which it showed 88.2 and 88.0% sequence similarity, respectively. Strain B33^T is in the same clade as *Roseivivax* and *Salipiger*, belonging to the α -3 group of the 'Alphaproteobacteria' within the family 'Rhodobacteraceae' (Garrity *et al.*, 2001). *Roseivivax* is taxonomically related to the *Roseobacter* clade, a group of genera of the family 'Rhodobacteraceae' (Allgaier *et al.*, 2003), which make up the most abundant population in marine habitats (González & Moran, 1997).

DNA–DNA hybridization was done according to Lind and Ursing's method (Lind & Ursing, 1986) with the modifications of Ziemke *et al.* (1998) and Bouchotroch *et al.* (2001). DNA–DNA hybridization values of B33^T with the most phylogenetically related species, *R. halodurans*, *R. halotolerans* and *S. 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 synthesize bacteriochlorophyll *a*, we are of the opinion that strain B33^T should be recognized 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. *Palleronia* 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 μ m long by 0.75–1 μ 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 test is negative. Colonies contain pink pigment. The bacteria cannot produce acids from sugars and have low nutritional and biochemical versatility. They are strictly halophilic, requiring Na⁺, Mg²⁺ and K⁺ for growth. The principal cellular fatty acids are 18:1 ω 7c and 19:0 cyclo ω 8c. They have ubiquinone with ten isoprene units. The type species is *Palleronia marisminoris*.

Description of *Palleronia marisminoris* sp. nov.

Palleronia marisminoris (ma'ris.min.or.is. L. sb. n. gen. *maris* of the sea; L. adj. n. gen. *minoris* smaller; L. adj. *marisminoris* of the smaller sea, i.e. from el Mar Menor, a shallow area of sea highly sheltered from the Mediterranean sea on the south-eastern 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 the sole salt. Minimum salt requirements are 0.66 M Na⁺, 0.1 M Mg²⁺ and 0.01 M K⁺. It grows within the temperature range of 20 to 37 °C and at pH values of between 5 and 10. It produces H₂S from L-cysteine. Selenite reduction and phosphatase tests are positive. Tween 20 is hydrolysed. It does not produce acids from the following sugars and related compounds: 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 and D-trehalose. ONPG is positive. O/F, indole, methyl-red, Voges-Proskauer and gluconate oxidation are negative. Phenylalanine deaminase is not produced. Urea, tyrosine, Tween 80, starch, aesculin, gelatin, 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, D-histidine, isoleucine, 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) and tobramycin (10) and is resistant to nalidixic acid (30), polymyxin B (300) sulfonamide (250) and trimethoprim/sulfamethoxazole (1.25/23.7). The major fatty acids are: 18:1 ω 7c (68.9 %), 19:0 cyclo ω 8c (12.8 %), 3-hydroxy 10:0 (5 %), 16:0 (4.2 %), 18:0 (3.4 %). DNA G+C content is 64.2 mol% (*T*_m method).

The type strain, strain B33^T (=CECT 7066^T=LMG 22959^T), was isolated from a hypersaline soil bordering a solar saltern in Marchamalo (Murcia, south-eastern Spain).

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