Planococcus versutus sp. nov., isolated from soil

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Abstract

A taxonomic study was performed on a novel Gram-stain-positive, coccus-shaped, orange-pigmented motile bacterium, designated as strain L10.15¹. The organism was isolated from a soil sample collected in Lagoon Island (close to Adelaide Island, western Antarctic Peninsula) using a quorum-quenching enrichment medium. Growth occurred at 4–30 °C, pH 6–11 and at moderately high salinity (0–15 %, w/v, NaCl), with optimal growth at 26 °C, at pH 7–8 and with 6 % (w/v) NaCl. 16S rRNA gene sequence analysis showed that strain L10.15¹ belonged to the genus Planococcus and was closely related to Planococcus halocryophilus Or1¹ (99.3 % similarity), Planococcus donghaensis JH1¹ (99.0 %), Planococcus antarcticus DSM 14505¹ (98.3 %), Planococcus plakortidis AS/ASP6 (III)¹ (97.6 %), Planococcus maritimus TF-9¹ (97.5 %), Planococcus salinarum ISL-6¹ (97.5 %) and Planococcus kocurii NCIMB 629¹ (97.5 %). However, the average nucleotide identity-MUMmer analysis showed low genomic relatedness values of 71.1–81.7 % to the type strains of these closely related species of the genus Planococcus. The principal fatty acids were anteiso-C₁₅:₀, C₁₆:₁ω7c and anteiso-C₁₇:₀, and the major menaquinones of strain L10.15¹ were MK-5 (48 %), MK-6 (6 %) and MK-7 (44 %). Polar lipid analysis revealed the presence of phosphatidylethanolamine, phosphatidyldiglycerol, diphosphatidylglycerol and aminophospholipid. The DNA G+C content was 39.4 mol%. The phenotypic and genotypic data indicate that strain L10.15¹ represents a novel species of the genus Planococcus, for which the name Planococcus versutus sp. nov. is proposed. The type strain is L10.15¹ (=DSM 101994¹ =KACC 18918¹).

The genus Planococcus was proposed by Migula [1] to accommodate aerobic, Gram-stain-positive, motile, coccus- or rod-shaped bacteria. In 2001, five Planococcus species were transferred to the newly proposed genus Planomicrobium to differentiate rod-shaped, motile, non-sporogenous and low G+C content bacterial species within the original genus Planococcus [2]. These two genera can be differentiated through their 16S rRNA gene sequences, which were shown to have sequence signatures at positions 183 (T for Planococcus and C for Planomicrobium) and 190 (A for Planococcus and G for Planomicrobium), following the 16S rRNA gene sequence numbering of Escherichia coli. To date, according to the List of Prokaryotic Names with Standing in Nomenclature (LPSN) (www.bacterio.net/planococcus.html), there are 12 species described in the genus Planococcus. Although 18 species are cited in the files of the genus Planococcus in LPSN, six of these have been reclassified to the genera Planomicrobium or Marinococcus.

Members of Planococcaceae are able to survive extreme environments having been isolated from a wide range of sources, including deep-sea sediments, marine solar salt-erns, glaciers, permafrost, Antarctic deserts, faeces, cyanobacterial mats and sea ice brine [3–6]. All members of the genus Planococcus are able to grow at moderately low temperatures (psychrotrophic) and are moderately halotolerant (halophilic). The type strain of Planococcus halocryophilus, which was isolated from Artic permafrost, was reported to grow and divide even at extremely low temperature (–15 °C) [7]. Members of the genus Planococcus can be exploited in the field of biotechnological and industrial applications.
applications, for instance through their production of carotenoids, thermophilic and alkaline/salt-tolerant xylanases and biosynthesis of butanol [3, 8–10]. Here, we provide a detailed taxonomic characterization of a novel member of the genus Planococcus, strain L10.15T, which was recently isolated from Antarctic soil samples.

In this study, strain L10.15T was isolated during an ecological survey of the quorum-quenching (QQ) soil bacteria in Antarctic soil samples using QQ bacteria enrichment medium [11]. The soil sample was collected from an elephant seal wallow in Lagoon Island, close to Adelaide Island, off the west coast of the Antarctic Peninsula (67° 35.689’S 068° 14.495’E). Briefly, around 1 g of soil sample and 5 ml of sterile QQ bacteria enrichment medium with the sole carbon source of 100 µg synthetic N-hexanoyl-L-homoserine lactone (C6-HSL) were added to a sterile 50 ml polypropylene conical tube and incubated at 4°C with agitation at 150 r.p.m. A total of 100 µl of 10% bacterial suspension was transferred into new QQ bacteria enrichment medium, including C6-HSL, after 1 week of incubation. This step was repeated three times, and finally, 100 µl of bacterial suspension was plated onto Luria–Bertani (LB) agar. An orange-pigmented isolate, strain L10.15T, was recovered. The cell suspensions were kept in 20% (w/v) glycerol stock for long-term storage at −80°C. Strain L10.15T was then routinely cultured aerobically in LB broth or on LB agar at 26°C (optimum growth temperature). As this is the first reported Planococcus species with QQ activity, we sequenced its complete genome using Pacific Biosciences RSII to facilitate our investigation.

Colonies of strain L10.15T were orange-pigmented, circular, entire, smooth, convex and 1–2 mm in size on LB agar after 48 h of incubation at 26°C. Gram staining was performed using the Difco Gram stain set and observed using a Leica DM 750 microscope (Leica Microsystems). Cells of strain L10.15T were Gram-stain-positive with no spore formation. Electron micrographs were obtained using a table top scanning electron microscope (TM3030; Hitachi) and a scanning transmission electron microscope (LIBRA 120; Carl Zeiss). For scanning electron microscopy, a sample was prepared as described by Vali et al. [12]. For scanning transmission electron microscopy, an overnight suspension of cells was stained using 1% phosphotungstic acid on a Formvar grid and observed at an operating voltage of 80 kV. Cells of strain L10.15T were coccoid, typically 1.0–1.5 µm in diameter, mostly arranged as diplococci, but single cells or tetrads were also observed (Fig. 1). A catalase test was conducted using 3% (v/v) H2O2 and determined by observing the production of copious bubbles. Oxidase activity was determined using 1% (w/v) N,N,N’,N’-tetramethyl 1,4-phenylenediamine (bioMérieux) as described by Smibert and Krieg [13]. API ZYM and Biolog GEN III microplates were prepared according to the manufacturers’ instructions. The activities of various enzymes were determined by using the API ZYM system after incubation for 24 h. Antibiotic susceptibility was tested by using ATB PSE 5 strips (bioMérieux) and the disc diffusion assay following the manufacturer’s instructions. All tests were performed at 26°C and in triplicate. The temperature range for growth was determined by plating on LB agar and by incubating at 4–37°C with increments of 1 or 2°C for 14 days. The pH range for growth of strain L10.15T was determined on LB agar plates adjusted to various pH values between 4 and 12 with 1 pH unit increments. Salt tolerance was determined by growing on LB agar media supplemented with 0–25% (w/v) NaCl at increments of 1%. Both salt tolerance and pH range tests were conducted by incubating the LB agar plates at 26°C for up to 14 days. The results of physiological tests of strain L10.15T as compared with closely related species are presented in Table 1.

Genomic DNA of L10.15T was extracted from an overnight cell suspension culture using the MasterPure Gram-positive DNA purification kit (Epicentre Technologies). A 20 kb SMRTbell template library was then constructed using the extracted genomic DNA. The whole genome sequencing was performed using Pacific Biosciences RSII sequencing platform with C4 chemistry in two single-molecule real-time cells. The complete genome of strain L10.15T has been sequenced, enabling the discovery of the gene responsible for QQ activity [8]. To determine the identity of strain L10.15T, the 16S rRNA partial gene sequence was amplified from the extracted DNA obtained as described above by using primers 27F and 1492R [14] and analysed using the Ex-Taxon database [15]. Pairwise similarity analysis demonstrated that strain L10.15T is a member of the genus Planococcus, with P. halocryophilus Or1T (99.3%), Planococcus donghaensis JH1T (99.0%), Planococcus antarcticus DSM 14505T (98.3%), Planococcus plakortidis AS/ASP6 (II)T (97.6%), Planococcus maritimus TF-9T (97.5%), Planococcus salinarum ISL-6T (97.5%) and Planococcus kocurii NCIMB 629T (97.5%) as the closest relatives present in the database. Phylogenetic analyses were conducted using the full 16S rRNA gene sequence (1538 bp) retrieved from the complete genome sequence. The MEGA 6.0 software [16] was
Table 1. Differential phenotypic characteristics between strain L10.15^T and its phylogenetically closest related species in the genus *Planococcus*

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Strains: 1, L10.15^T; 2, *P. donghaensis* JH1^T; 3, *P. halocryophilus* Orl^T; 4, *P. antarcticus* DSM 14505^T; 5, *P. kocurii* DSM 20747^T; 6, *P. maritimus* JCM 11543^T; 7, *P. piakortidis* DSM 23997^T; 8, *P. salinarum* ISL-16^T. All strains are positive for the utilization of dextrin, D-fructose, D-glucose 6-phosphate, L-alanine, L-glutamic acid, pectin, D-galacturonic acid and glucuronamide. All strains are negative for utilization of turanose, stachyose, D-mannose, 3-methyl glucose, D-sorbitol, citric acid and bromosuccinic acid. In chemical sensitivity assays, all strains are able to grow with 1 % sodium lactate, aztreonam and sodium butyrate, but not with vancomycin, niaproof 4, troleandomycin, rifamycin SV or minocycline. All data were obtained in this study. NA, Not applicable.
used to perform the alignment using the MUSCLE algorithm [17], and the phylogenies were reconstructed using default settings of neighbour-joining (Fig. 2), maximum-likelihood (Fig. S1, available in the online Supplementary Material) and maximum-parsimony (Fig. S2) algorithms. The 16S rRNA gene sequence of L10.15T contained the signature nucleotides of Planococcus, T and A, respectively, at positions 183 and 190 (E. coli 16S rRNA gene sequence numbering) and thus clustered separately from the related genus Planomicrobium [18]. All 16S rRNA gene phylogenies concordantly demonstrated that strain L10.15T clustered within Planococcus but formed a distinct branch separate from P. halocryophilus Or1T, P. donghaensis JH1T, P. antarcticus DSM 14505T, P. plakortidis AS/ASP6 (II)T, P. maritimus TF-9T, P. salinarum ISL-6T and P. kocurii NCIMB 629T. The G+C content of strain L10.15T was 39.4 mol% as determined from the complete genome sequence.

Average nucleotide identity (ANI) analysis was performed using the JSpecies Web Service (JSpeciesWS; http://jspecies.ribohost.com/jspeciesws/) [19] in which strain L10.15T demonstrated ANI-MUMmer values of between 71 and

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82% similarity against all close relatives \([P. \text{ halocryophilus} \text{ Ort}^T (81.2 \%), P. \text{ donghaensis} \text{ JH1}^T (80.8 \%), P. \text{ antarcticus} \text{ DSM 14505}^T (79.6 \%), P. \text{ plakortidis} \text{ AS/ASP6 (II)}^T (71.1 \%), P. \text{ maritimus} \text{ TF-9}^T (72.0 \%), P. \text{ salinarum} \text{ ISL-6}^T (73.0 \%) and P. \text{ kocurii} \text{ NCIMB 629}^T (81.7 \%)]\) (Table S1). ANI-Blast values in comparison with all close relatives indicated 84–88% similarity \([P. \text{ halocryophilus} \text{ Ort}^T (84.8 \%), P. \text{ donghaensis} \text{ JH1}^T (84.8 \%), P. \text{ antarcticus} \text{ DSM 14505}^T (84.3 \%), P. \text{ plakortidis} \text{ AS/ASP6 (II)}^T (85.0 \%), P. \text{ maritimus} \text{ TF-9}^T (84.6 \%), P. \text{ salinarum} \text{ ISL-6}^T (88.2 \%) and P. \text{ kocurii} \text{ NCIMB 629}^T (86.1 \%)]\) (Table S2). The results were similar to those from OrthoANI analysis [20], in which OrthoANI values ranged from 71.5 to 82.2% \([P. \text{ halocryophilus} \text{ Ort}^T (81.4 \%), P. \text{ donghaensis} \text{ JH1}^T (81.3 \%), P. \text{ antarcticus} \text{ DSM 14505}^T (79.9 \%), P. \text{ plakortidis} \text{ AS/ASP6 (II)}^T (72.9 \%), P. \text{ maritimus} \text{ TF-9}^T (72.0 \%), P. \text{ salinarum} \text{ ISL-6}^T (71.5 \%) and P. \text{ kocurii} \text{ NCIMB 629}^T (82.2 \%)]\) (Fig. S3). Richter et al. [21] proposed a threshold of 94–96% for species delimitation, with our analyses therefore indicating that strain L10.15\(^T\) does not belong to any of these related species.

The isoprenoid quinones were extracted using petroleum ether as described by Minnikin et al. [22] and subsequently identified by HPLC (Shimadzu; Nexera-X2). The isoprenoid quinone profile of strain L10.15\(^T\) was characterized by the predominance of the menaquinones MK-5 (48%), MK-6 (6%) and MK-7 (44%). The polar lipids of strain L10.15\(^T\) were extracted and analysed by two-dimensional TLC following Embley and Wait [23]. Molybdothiophosphoric acid was used for the detection of total polar lipids, ninhydrin for amino lipids, molybdenum blue for phospholipids, Dragendorff reagent for choline-containing lipids and \(\alpha\)-naphthol/sulphuric acid reagent for glycolipids. Strain L10.15\(^T\) exhibited a complex polar lipid profile consisting of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, an unidentified aminophospholipid, two unidentified lipids and four unidentified aminolipids (Fig. S4). The predominant polar lipids of strain L10.15\(^T\) were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and aminophospholipid. This result is consistent with the description of \(P. \text{ plakortidis}\) [24].

Cellular fatty acid profiles were determined following the standard protocol of the Microbial Identification (MIDI)/Hewlett Packard Microbial Identification System [25]. Fatty acids were extracted, and fatty acid methyl esters were prepared and analysed in the MIDI system. Briefly, overnight cultures of strain L10.15\(^T\) were harvested from LB agar determined previously to be in the mid-exponential growth phase at 26°C. The fatty acids were separated using an Agilent GC device (model 6890N) and were identified using Sherlock version 6.0 via the RTSBA6 database. The fatty acid profile of strain L10.15\(^T\) comprised (each constituting \(\geq 0.5\%\) of the total) saturated fatty acids \(C_{14:0}\) (0.6%), \(C_{15:0}\) (1.5%), \(C_{16:0}\) (4.0%), \(C_{17:0}\) (0.7%) and \(C_{18:0}\) (1.0%); branched fatty acids anteiso-C\(_{13:0}\) (0.6%), anteiso-C\(_{15:0}\) (46.2%), anteiso-C\(_{17:0}\) (10.7%), iso-C\(_{14:0}\) (3.4%), iso-C\(_{15:0}\) (1.9%), iso-C\(_{16:0}\) (5.5%), iso-C\(_{17:0}\) (1.9%), iso-C\(_{17:1}\):\(\omega\)10c (1.3%) and iso-C\(_{18:0}\) (0.7%); unsaturated fatty acids \(C_{16:1}\) \(\omega7c\) alcohol (6.5%), \(C_{16:1}\) \(\omega11c\) alcohol (5.6%), \(C_{17:1}\):\(\omega9c\) alcohol (0.8%) and \(C_{18:1}\):\(\omega9c\) alcohol (0.7%); summed feature 3 (iso-C\(_{15:0}\) \(2\-\text{OH and/or anteiso-C}_{17:1}\) 0.6%) and summed feature 4 (iso-C\(_{17:1}\) and/or \(C_{16:1}\) \(\omega7c\) 6.0%). This profile is similar to those of recognized \(Planococcus\) species, although there were differences in the proportions of some fatty acids. Table 2 presents details of the fatty acids of strain L10.15\(^T\) and closely related species. The fatty acid profile of strain L10.15\(^T\) was similar to those of members of
the genus *Planococcus* and contained anteiso-C<sub>15:0</sub> and anteiso-C<sub>17:0</sub> as the major fatty acids. The distinctive characteristic of strain L10.15<sup>T</sup> compared with other members of the genus *Planococcus* lies in the menaquinone profile, in which the predominant menaquinones are MK-5, MK-6 and MK-7 instead of MK-6, MK-7 and MK-8. Strain L10.15<sup>T</sup> is also the only strain sensitive to fusidic acid of the reference strains tested.

Based on the phenotypic and genotypic data presented, strain L10.15<sup>T</sup> represents a novel species of the genus *Planococcus*, for which the name *Planococcus versutus* sp. nov. is proposed.

**DESCRIPTION OF PLANOCOCCUS VERSUTUS SP. NOV.**

*Planococcus versutus* (ver.su’tus. L. masc. adj. versutus adroit, shrewd, ingenious).

Cells are aerobic, Gram-stain-positive, motile and non-sporulating cocci. Colonies on LB agar are orange, circular, entire, smooth, convex and 1.0–2.0 mm in diameter. Grows at between 4°C and 30°C (optimum, 25°C) and at pH 6.0–11.0 (optimum, pH 7.0–8.0). Growth is observed between 0% and 14% (w/v) NaCl (optimum, 6%). Positive for catalase but negative for amylase. Positive for assimilation of N-acetyl-D-glucosamine, N-acetyl neuraminic acid, α-D-glucose, inosine, D-mannitol, glycerol, D-fructose 6-phosphate, glycy1 L-proline, L-alanine, L-aspartic acid, L-glutamic acid, L-pyroglutamic acid, L-serine, L-galactonic acid lactone, D-glucionic acid, D-glucuronic acid, mucic acid, D-saccharic acid, D-lactic acid methyl ester, α-ketoglutaric acid, D-malic acid, L-malic acid, Tween 40, β-hydroxy DL-butyric acid, acetoacetic acid, acetic acid, formic acid, dextrin, D-fructose, D-glucose 6-phosphate, L-alanine, L-glutamic acid, pectin, D-galacturonic acid, glucuronamide and dextrin. Negative for assimilation of turanose, stachyose, galacturonic acid, glucuronamide and dextrin. Negative for assimilation of turanose, stachyose, galacturonic acid, glucuronamide and dextrin.

### Table 2. Cellular fatty acid profile of strain L10.15<sup>T</sup> and closely related species of the genus *Planococcus*

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<th>4</th>
<th>5</th>
<th>6</th>
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<td>0.5</td>
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<td>1.2</td>
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<td>–</td>
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<td>–</td>
<td>0.4</td>
<td>–</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td>Summed feature 4†</td>
<td>6.0</td>
<td>3.3</td>
<td>6.1</td>
<td>5.3</td>
<td>6.0</td>
<td>5.4</td>
<td>2.9</td>
<td>8.6</td>
</tr>
</tbody>
</table>

*Summed feature 3 contains C<sub>16:1ω7c</sub> and/or C<sub>16:1ω11c</sub>, which could not be separated by GC with the MIDI system.

†Summed feature 4 contains iso-C<sub>17:1ω9c</sub> and/or anteiso-C<sub>17:0</sub>, which could not be separated by GC with the MIDI system.

The fatty acids in bold are the major cellular fatty acid (10% and above).
hydroxybutyric acid, α-ketobutyric acid and propionic acid. In chemical sensitivity tests, resistant to D-serine, lincomycin, guanidine HCl, tetrazolium blue, potassium tellurite, 1% sodium lactate, azetronam and sodium butyrate, slightly resistant to tetrazolium violet and sodium bromate, and sensitive to fusidic acid, nalidixic acid, lithium chloride, vancomycin, niaprof 4, troleandomycin, rifamycin SV and minocycline. The respiratory menaquinones are MK-5, MK-6 and MK-7. Major fatty acids are anteiso-C₁₅:₀, C₁₆:₁ω7c and anteiso-C₁₇:₀. The predominant polar lipids are phosphatidylethanolamine, phosphatidylglycerol, diphasphatidylglycerol and aminophospholipids.

The type strain, L10.15T (¼DSM 101994T¼KACC 18918T), was isolated from a soil sample collected from an elephant seal wallow in Lagoon Island (close to Adelaide Island, western Antarctic Peninsula). The DNA G+C content of the type strain is 39.4 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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