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Arboriscoccus pini gen. nov., sp. nov., an endophyte from a pine tree of the class *Alphaproteobacteria*, emended description of *Geminicoccus roseus*, and proposal of *Geminicoccaceae* fam. nov.☆

Diogo N. Proença[a,*], William B. Whitman[b], Neha Varghese[c], Nicole Shapiro[c], Tanja Woyke[c], Nikos C. Kyrpides[c], Paula V. Morais[a,*]

[a] *Department of Life Sciences and CEMMPRE, FCTUC, University of Coimbra, 3000-456 Coimbra, Portugal*
[b] *Department of Microbiology, 527 Biological Sciences Building, University of Georgia, Athens, GA 30602-2605, USA*
[c] *DOE Joint Genome Institute, 2800 Mitchell Drive, Walnut Creek, CA 94598, USA*

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ABSTRACT

Bacterial strain B29T1^T was isolated from the endophytic microbial community of a *Pinus pinaster* tree trunk and characterized. Strain B29T1^T stained Gram-negative and formed diplococci that grew optimally at 26–30 °C and at pH 6.0–7.0. The G + C content of the DNA was 61.6 mol%. The respiratory quinone was ubiquinone 10 (UK-10), and the major fatty acids were $C_{16:0}$, cyclo-$C_{19:0}$ $\omega 8c$ and $C_{18:0}$ 12-methyl, representing 64% of the total fatty acids. Phylogenetic analyses based on the 16S rRNA gene sequences placed strain B29T1^T within the order *Rhodospirillales* in a distinct lineage that also included the genus *Geminicoccus*. The 16S rRNA gene sequence similarities of B29T1^T to that of *Candidatus* Alysiomicrobium bavaricum, *Geminicoccus roseus* and *Candidatus* Alysiosphaera europeae were 92.6%, 89.9% and 89.2%, respectively. The analysis of the available genomes from the closest families showed 177 core genes that reveals a novel family-level clade including the type strain of the genus *Geminicoccus* and the strain B29T1^T. Analysis of B29T1^T genome revealed all the genes involved in autotrophic carbon dioxide fixation via the reductive pentose phosphate pathway and genes encoding for starch/glycogen and chitin degradation. The phylogenetic, phenotypic and chemotaxonomic data showed that strain B29T1^T (=CIP 110763^T, =LMG 27745^T) represents the type of a novel species and genus, for which we propose the name *Arboriscoccus pini* gen. nov., sp. nov. A new family (*Geminicoccaceae* fam. nov.) is proposed for *Arboriscoccus*, *Geminicoccus*, *Candidatus* Alysiomicrobium and *Candidatus* Alysiosphaera. The description of the *Geminicoccus roseus* DSM 18922^T is also emended.

The class *Alphaproteobacteria* comprises many species isolated from a broad range of environments belonging to 11 orders and 30 families. The order *Rhodospirillales* [24] includes the families *Acetobacteraceae* [15] and *Rhodospirillaceae* [24], and other genera unassigned to a particular family. Currently, a total of 36 genera have been classified within the family *Acetobacteraceae* (http://www.bacterio.net/acetobacteraceae.html). Most of these genera stain Gram-negative and are rod-shaped. All members of the *Acetobacteraceae* are also obligatory aerobic and their metabolism is strictly respiratory with oxygen as the terminal electron acceptor. A common feature of these bacteria, with the exception of *Asaia* spp., is the aerobic oxidation of ethanol to acetic acid, with accumulation of the latter compound in the medium. The major respiratory lipoquinones in *Acetobacter* are ubiquinones 10 and 9. The family *Rhodospirillaceae* includes the so-called purple non-sulfur bacteria. The type genus is *Rhodospirillum*, and the family embraces a total of 39 genera (http://www.bacterio.net/rhodospirillaceae.html). Members stain Gram-negative and form rod- to spirillum-shaped cells. They also possess diverse metabolic and nutritional properties, which include photoheterotrophy, photoautotrophy and chemoheterotrophy. The major respiratory lipoquinones are ubiquinones 9, 10 and 11 and/or menaquinone 10. The genus *Gem-*

☆ *Note:* Nucleotide sequence data for the 16S rRNA gene of strain B29T1^T is available in the GenBank/DDBL/EMBL databases under the accession number KY367357. This Whole Genome Shotgun project has been deposited at ENA under the accession numbers FYEH01000001–FYEH01000028.

* Corresponding authors.
E-mail addresses: diogo.proenca@uc.pt (D.N. Proença), pvmorais@ci.uc.pt (P.V. Morais).

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inicoccus is included in the class *Alphaproteobacteria*, but it has not been unassigned to a family. The single species of this genus, *Geminicoccus roseus*, is a Gram-negative, halophilic, aerobic anoxygenic phototroph.

Strain B29T1^T was isolated from the endophytic microbiome of pine trees in a forest affected with pine wilt disease (PWD). The nematode *Bursaphelenchus xylophilus*, the accepted causal pathogen of PWD, affects the health of the entire tree as well as the endophytic microbiome [27]. The strain was isolated from the trunk of a diseased tree (symptomatic class V of PWD [26]) during the study of the *Pinus pinaster* microbiome [25].

P. pinaster trees in Avô, Oliveira do Hospital, Portugal, were sampled from April to August 2009 [25,26]. Pine bark and sapwood were removed under sterile conditions and each sample consisted of pine wood cross-sections from cut trees or wood obtained by drilling a 5 mm diameter hole to a depth of 10–15 cm with a sterilized hand brace drill (Haglof, Mora, Sweden). Wood chips, 5 g, were suspended in R2A broth, half concentrated, at 25 °C, for 2 h. Strain B29T1^T was isolated from serial dilutions plated on R2A agar (Difco Laboratories, Detroit, USA) and incubated at 25 °C for three days. After subculture and purification, strain B29T1^T was preserved at −80 °C in R2A broth supplemented with 15% (v/v) glycerol.

Geminicoccus roseus DSM 18922^T, the phylogenetic closest isolate related with strain B29T1^T, was obtained from Leibniz-Institut DSMZ, grown under the same conditions as strain B29T1^T and was used as a reference in all tests listed below. Cell morphology was examined by phase-contrast microscopy and by scanning electron microscopy (SEM) after growth on R2A agar at 30 °C for 72 h (Fig. 1). Gliding motility was observed under the microscope in a fresh broth culture as previously described [3]. Growth was tested on tryptic soy agar (TSA, Difco, Laboratories, Detroit, USA), nutrient agar (NA, Difco), MacConkey agar (Difco) and Marine agar (Difco) incubated at 30 °C for 5 days. The temperature range (4, 15, 20, 26, 30, 37, 40, 42, 45 °C) and optimum temperature for growth were examined on R2A agar incubated up to 5 days. Salt tolerance was tested on R2A agar supplemented with 0–4% (w/v) NaCl, in 0.5% increments, at 30 °C up to 5 days. The pH range for growth was examined at 30 °C in R2A broth adjusted by using 50 mM MES (pH 5–7), HEPES (pH 6–8) and TAPS (pH 8–9) over a pH range from 5.0 to 9.0, with intervals of 0.5 pH unit. Gram-staining reaction and the presence of cytochrome oxidase and catalase were determined after 24 h of incubation on R2A agar as previously described [30]. The ability to hydrolyse agar, esculin, casein, xylan, gelatin, arbutin, elastin, starch, DNA, chitin, carboxymethylcellulose, xanthine and Tweens 20, 40, 60 and 80 at concentrations of 1.0% (w/v or v/v) on R2A agar were determined after incubation at 30 °C for up to 5 days as previously described [33]. Other physiological properties and enzyme activities were determined using the API ZYM and API 20NE test strips (bioMérieux), at 37 °C and 30 °C, respectively, according to the manufacturer's instructions. Acid production and single-carbon source assimilation were determined using API 50 CH test strips (bioMérieux) as previously described [23]. The ability to oxidize different carbon sources was assessed using Biolog GN2 MicroPlates incubated at 30 °C. The results were recorded daily for up to 7 days using a MicroPlate reader (Tecan Infinite M200). Growth under anaerobic conditions was assessed on R2A agar incubated in anaerobic chambers (GENbox anaer, bioMérieux). The test for flexirubin type pigments was performed by soaking cells grown on R2A agar at 30 °C for 48 h with 20% (w/v) KOH [11]. Congo red adsorption was tested following growth on R2A-Congo red agar (Congo red 25 mg l^{-1}) incubated at 30 °C for 2 days [14]. Antibiotic-sensitivity tests were performed with Oxoid discs containing 15 μg lyncomycin, 10 μg amplicilin, 30 μg amoxicillin + acid clavulanic, 30 μg gentamicin, 300 U polymyxin B, 100 μg chloramphenicol, 15 μg erythromycin, 30 μg vancomycin, 50 μg streptomycin, 30 μg rifampicin, 30 μg tetracycline, or 30 μg kanamycin.

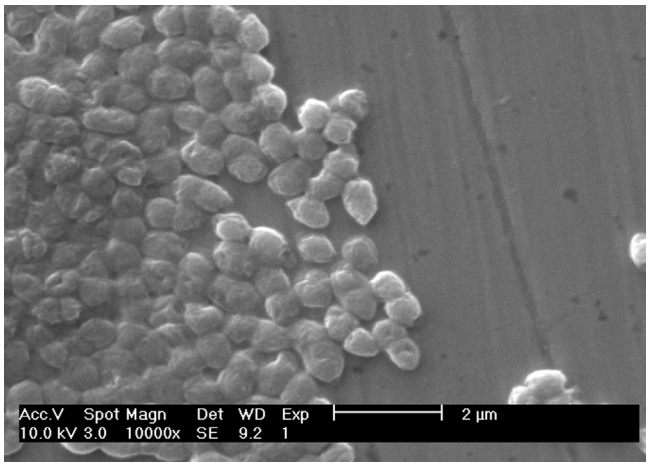

Fig. 1. SEM micrograph showing the morphology of *Arboriscoccus pini* B29T1^T. Scale bar represents 2 μm.

Cells for polar lipid and lipoquinone analyses were grown on R2A agar at 30 °C for 72 h, harvested and lyophilized. Polar lipids were extracted, and two dimensional thin-layer chromatography was performed on silica gel G plates (Merck, 10 × 10 cm, 0.25 mm thickness) using chloroform/methanol/water (65:25:4, by vol.) in the first direction and chloroform/acetic acid/methanol/water (80:15:12:4, by vol.) in the second direction [10]. Lipoquinones were extracted from freeze-dried cells, purified by thin-layer chromatography and separated by high performance liquid chromatography [8]. Cells for fatty acid analysis were grown on R2A agar at 30 °C in sealed plastic plates, for 72 h [23]. The fatty acids profiles of both strain B29T1^T and *G. roseus* DSM 18922^T were determined at pH 7 and pH 8, corresponding the optimum pH of each strain, respectively, and the profiles allowed to differentiate strains at each pH. Fatty acid methyl esters (FAMEs) were obtained from the fresh wet biomass and were separated, identified and quantified using the standard MIS Library Generation Software (Sherlock Microbial ID System, TSBA 6 database, version 6.0) as previously described [9]. The G + C content of the genome was determined by high-performance liquid chromatography as previously described [22].

Biochemical and physiological characteristics of strain B29T1T are summarized in Table 1 and in the species description. The profile of polar lipids of strain B29T1^T differed from that of *Geminicoccus roseus* DSM 18922^T, which consisted of mostly phospholipids (Fig. S1). No glycolipids were detected (Fig. S1).

Ubiquinone 10 (UK-10) was the respiratory quinone of strain B29T1^T, like the most closely related members of the order *Rhodospirillales*. UK-10 was also found to be the respiratory quinone of *G. roseus* DSM 18922^T.

The major fatty acids of strain B29T1^T were saturated $C_{16:0}$, cyclo-$C_{19:0}$ $\omega 8c$ and $C_{18:0}$ 12-methyl, which accounted for 64.1% of the total fatty acids (Table 2). Only strain B29T1^T possessed $C_{14:0}$, $C_{16:0}$ 2-OH and $C_{18:0}$ 12-methyl. Both strains shared the fatty acids $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ $\omega 7c$ 11-methyl, cyclo-$C_{19:0}$ $\omega 8c$ and summed feature 8.

The G + C content of the DNA of strain B29T1^T determined by HPLC was 61.6 mol%, a value within the range for species belonging to the closely related members of the order *Rhodospirillales*.

The 16S rRNA gene was amplified by PCR and sequenced as previously described [23]. The almost complete 16S rRNA gene sequence of strain B29T1^T (1,460 bp) was then aligned with those of the type species of the genera belonging to the order *Rhodospirillales* and other reference sequences obtained from the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; [19]), by SINA

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