**Rufibacter ruber** sp. nov., isolated from fragmentary rock


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A red-pigmented, Gram-stain-negative, rod-shaped, aerobic bacterium, designated strain CCM 8646\textsuperscript{T}, was isolated from stone fragments in James Ross Island, Antarctica. Strain CCM 8646\textsuperscript{T} was able to grow from 10 to 40 °C, in the presence of up to 1 % (w/v) NaCl and at pH 7.0–11.0. Analysis of the 16S rRNA gene sequence placed strain CCM 8646\textsuperscript{T} in the genus *Rufibacter* with the closest relative being *Rufibacter roseus* H359\textsuperscript{T} (97.07 % 16S rRNA gene sequence similarity). The digital DNA–DNA hybridization values between strain CCM 8646\textsuperscript{T} and *R. roseus* H359\textsuperscript{T} were low (21.30±2.34 %). The major quinone was menaquinone MK-7. The polar lipids comprised phosphatidylethanolamine, an unknown aminoglycolipid and six unknown polar lipids. The G+C content of strain CCM 8646\textsuperscript{T} was 51.54 mol%. On the basis of phenotypic, chemotaxonomic and genotyping results, strain CCM 8646\textsuperscript{T} is considered to represent a novel species within the genus *Rufibacter*, for which the name *Rufibacter ruber* sp. nov. is proposed. The type strain is CCM 8646\textsuperscript{T} (=LMG 29438\textsuperscript{T}).

The genus *Rufibacter* is a member of the family Cytophaga-<em>aceae</em> and comprises four species with validly published names. *Rufibacter tibetensis* isolated from soil was described as the type species of the genus (Abaydulla <em>et al.</em>, 2012). Later, *Rufibacter roseus* from radiation-polluted soil (Zhang <em>et al.</em>, 2015), *Rufibacter immobilis* from saline lake (Polkade <em>et al.</em>, 2015) and *Rufibacter glacialis* from glacier soil (Liu <em>et al.</em>, 2016) were assigned to the genus. Bacteria of the genus *Rufibacter* are aerobic, psychrotolerant, Gram-stain-negative, catalase-positive and non-spore-forming with red carotenoid pigments. The major respiratory quinone is menaquinone 7 (MK-7) and phosphatidylethanolamine is the predominant polar lipid of the genus *Rufibacter*.

Strain CCM 8646\textsuperscript{T} was isolated from fragmentary rock in James Ross Island, Antarctica (57° 48′ 32.036″ W 63° 47′ 34.888″ S). Phenotypic classification of CCM 8646\textsuperscript{T} was performed using key tests applicable for Gram-negative rods, such as those for oxidase, catalase and urease, nitrate reduction, oxidation–fermentation (OF), arginine dihydrolase, ornithine and lysine decarboxylase, and production of hydrolytic enzymes (Atlas, 2010; Barrow & Feltham, 1993; Kosina <em>et al.</em>, 2013). The temperature range for growth (1–40 °C in increments of 5.0 °C) and NaCl concentration tolerance (0, 1, 2 and 3 %, w/v) were tested on R2A agar (Oxoid) adjusted accordingly. The pH range for growth was...
tested on R2A agar adjusted to pH 6.0–11.0 (in increments of 1 pH unit) by using the buffer system (pH 6.0–8.0, 0.1 M KH₂PO₄/0.1 M NaOH; pH 9.0–10.0, 0.1 M NaHCO₃/0.1 M Na₂CO₃; pH 11.0, 0.05 M Na₂HPO₄/0.1 M NaOH) as described by Da et al. (2015). Further extended phenotyping was achieved by using GN2 MicroPlates (Biolog) and API ZYM (bioMérieux) identification test kits. The morphology of the isolate was observed by Gram staining and transmission electron microscopy (Morgagni 268D Philips; FEI) using samples stained with 2% ammonium molybdate (Fig. S1, available in the online Supplementary Material). Differences in the antibiotic resistance patterns were tested by the disc diffusion method on R2A agar (Oxoid). Sixteen antibiotic discs generally used for Gram-negative rods (EUCAST v5.0, 2015; CLSI, 2015) were chosen: ampicillin (10 µg), aztreonam (30 µg), carbenicillin (100 µg), cefixim (5 µg), ceftazidim (10 µg), cefalothin (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), chloramphenicol (30 µg), imipenem (10 µg), kanamycin (30 µg), cefotaxim (25 µg), piperacillin (30 µg), polymyxin B (300 U), streptomycin (10 µg), aztreonam (30 µg), carbenicillin (100 µg), cefixim (5 µg), gentamicin (10 µg), chloramphenicol (30 µg), imipenem (10 µg), kanamycin (30 µg), cefotaxim (25 µg), piperacillin (30 µg), polymyxin B (300 U), streptomycin (10 µg) and tetracycline (30 µg). EUCAST/CLSI standards were strictly followed for cultivation and inhibition zone diameter reading. Strain CCM 8646T was susceptible to the majority of tested antibiotics. It was resistant to aztreonam, gentamicin and polymyxin B only. The biochemical/physiological data of CCM 8646T are presented in the species description. The characterization of CCM 8646T using GN2 MicroPlates is detailed in Table S1. The strain was grown at 20°C for 48–72 h on R2A medium (Oxoid) during all experiments. Reference strain R. roseus CCM 8621T was obtained from the Czech Collection of Microorganisms (http://www.sci.muni.cz/ccm/).

Genomic DNA was extracted for phylogenetic analysis using a FastPrep Lysing Matrix type B and FastPrep Homogenizer (MP Biomedicals) and purified using a High Pure PCR Template Preparation Kit (Roche Diagnostics). A fragment of the 16S rRNA gene corresponding to positions 8–1542 (Escherichia coli nomenclature) was amplified by PCR with FastStart PCR Master (Roche Diagnostics) and using conserved primers forward pA (AGAGTTTGATCTTGCTCAG) and reverse pH (AAGGAGGTGATCCAGCGCA) (Edwards et al., 1989) and purified using a QIA quick PCR Purification Kit (Qiagen). Sequencing was performed using PCR primers and custom primers F1 (GTGGGGACRAACAGAGATTAG), F2 (CGTARGTCTCATGGCCCTT), R1 (ATACCCGCGCTGTGGCAC) and R2 (CACATSMTCMCCRC TTGT) in the Eurofins MWG Operon sequencing facility. The obtained partial 16S rRNA gene sequence of strain CCM 8646T was compared with corresponding sequences of type strains using the EzTaxon database (Kim et al., 2012). Preliminary 16S rRNA gene sequence analysis identified R. roseus H359T as its closest relative (97.07% similarity). To determine the taxonomic position of this novel strain in more detail, whole genome sequencing was performed.

The purified genomic DNA of strain CCM 8646T and R. roseus CCM 8621T was used for 400 bp sequencing library preparation as described previously (Sedláček et al., 2016). The sample was loaded on a 316 v2 chip and sequenced using the Ion PGM Hi-Q sequencing kit (Life Technologies) on an Ion PGM system (Life Technologies). Quality trimming and error correction of the reads were performed with the Ion Torrent Suite Software (version 5.0.2). The assembly computation was performed using the plug-in Assembler SPAdes (v5.0.0). The total length of the assembly comprised 5 502 314 bp (CCM 8646T) and 5 117 794 bp (R. roseus CCM 8621T). Assembled contigs larger than 200 bp were used for subsequent analysis.

The complete 16S rRNA gene sequence extracted from whole genome sequence data using the RNAmer 1.2 server (Lagesen et al., 2007) showed similarity to that obtained by Sanger sequencing and therefore was used for further phylogenetic analysis. Pairwise similarities were calculated with the BioNumerics version 7.5 software (Applied Maths) and showed that strain CCM 8646T shared 95.4–97.1% 16S rRNA gene sequence similarity with other members of the genus Rufibacter. Phylogenetic analysis was performed using MEGA version 6 software (Tamura et al., 2013). Genetic distances were corrected using Kimura’s two-parameter model (Kimura, 1980) and the evolutionary history was inferred using the maximum-likelihood (Fig. 1) and neighbour-joining algorithms using a bootstrap test based on 5000 replications. Neighbour-joining clustering confirmed the tree topology obtained by the maximum-likelihood analysis (data not shown).

To evaluate the mean level of nucleotide sequence similarity at the genome level among strain CCM 8646T and R. roseus CCM 8621T, average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) values were determined. The dDDH values were calculated using the web-based genome-to-genome distance calculator (GGDC) version 2.1. (Meier-Kolthoff et al., 2013) and the recommended formula 2 was taken into account to interpret the results. To calculate the ANI value, the algorithm implemented at the EzGenome server was used (http://www.ezbiocloud.net/ezgenome; Goris et al., 2007). The dDDH values (21.30 ±2.34%) and ANI (74.5465%; reciprocal value 74.5491%) were well below the cut-off values recommended for delineation of species (70% and 95–96%, respectively) (Richter & Rossello-Mora, 2009; Meier-Kolthoff et al., 2013).

To estimate the DNA G+C content, the draft genome sequence was used. The G+C content was 51.54 mol%, which falls in the range 43.9–52.8% observed in other Rufibacter species (Abaydulla et al., 2012; Zhang et al., 2015; Polkade et al., 2015; Liu et al., 2016).

Colonies of strain CCM 8646T exhibited a pink–red colour and the whole absorbance spectra with three maxima (458, 487 and 518 nm; Fig. S2) suggested the presence of a red-shifted carotenoid. Pigments were extracted with an excess of methanol and the extract was injected into an Agilent-1200 HPLC system. Separation was performed on a reversed-phase column (Kinexent C8, 2.6 µm particle size, 3.9×150 mm; Phenomenex) with 35% methanol and 15% acetonitrile in 0.25 M pyridine (solvent A) and 20%
The analysis of fatty acid methyl esters (FAMEs) was performed with cells grown on R2A agar (Oxoid) incubated at 20 ± 2 °C for 72 h (late log-phase). Extraction of FAMEs was performed according to the standard protocol of the Sherlock Microbial Identification System (Sasser, 1990). Cellular fatty acid extracts were analysed by GC (model 7890B; Agilent) by the Microbial Identification System (Sasser, 1990). Cellular fatty acids were eluted with a linear gradient of solvent B (30 to 95 % in 25 min) followed by 95 % of solvent B at a flow rate of 0.8 ml min⁻¹ at 40 °C.

As shown by HPLC separation of extracted pigments, cells of strain CCM 8646ᵀ contained three major and several minor pigments (Fig. S3a). All major pigment absorbances resembled those of carotenoids; the spectrum of the most abundant pigment is shown in Fig. S3(b). It is of note that this red-shifted spectrum did not correspond to any typical carotenoid and it might represent a new, relatively polar carotenoid.

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Based on 16S rRNA gene sequence similarities, strain CCM 8646ᵀ was related most closely to members of the genus Rufibacter within the family Cytophagaceae. Further investigation by a polyphasic taxonomic approach differentiated strain CCM 8646ᵀ from the type strains of recognized Rufibacter species and revealed strain CCM 8646ᵀ as a representative of a novel species of the genus Rufibacter, for which the name Rufibacter ruber sp. nov. is proposed.

**Description of Rufibacter ruber sp. nov.**

*Rufibacter ruber* (ru’ber. L. masc. adj. ruber red, referring to the colour of the colonies).

Cells are Gram-stain-negative, short rods, occurring predominantly in pairs or in irregular clusters and are non-spore-forming. Colonies on R2A agar are circular, with whole margin, slightly convex, smooth, glistening and 1–2 mm in diameter. Aerobic; non-haemolytic on sheep blood agar. Grows at 10–37 °C but not at 5 or 42 °C. Growth is observed in the presence of up to 1 % (w/v) NaCl and at pH 7.0–11.0 on R2A medium. Most abundant growth is observed on R2A agar without NaCl, at pH 8.0 and at 25 °C. No fluorescein pigment on King B medium. Grows on plate count agar (Oxoid), tryptone soya agar (Oxoid) and brain infusion agar (Oxoid) but not on MacConkey agar (Oxoid) or CM03 agar (Oxoid). No anaerobic growth is observed on R2A agar. OF test is negative. Positive for catalase, DNase, esterase lipase (C8), leucine arylamidase, valine arylamidase, α-glucosidase, α-galactosidase (weak), N-acetyl-β-glucosaminidase, alkaline phosphatase and acid phosphatase.
Negative for urease, oxidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, esterase (C4), lipase (C14), cystine arylamidase, trypsin, chymotrypsin, naphthol-AS-BI-phosphohydrolase, \(C_14\), cystine arylamidase, trypsin, chymotrypsin, naphthol-AS-BI-phosphohydrolase, \(C_19\), cystine arylamidase, trypsin, chymotrypsin, naphthol-AS-BI-phosphohydrolase, \(C_20\), cystine arylamidase, trypsin, chymotrypsin, naphthol-AS-BI-phosphohydrolase.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CCM 8646 (^T)</th>
<th>(R.) roseus CCM 8621 (^T)</th>
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<tbody>
<tr>
<td>Growth in the presence of 3 %</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>((w/v)) NaCl</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Growth at 5 °C</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>–</td>
<td>+</td>
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<td>Enzyme activity of (API ZYM):</td>
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<tr>
<td>(\alpha)-Galactosidase</td>
<td>(w)</td>
<td>–</td>
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<tr>
<td>(\beta)-Galactosidase</td>
<td>(w)</td>
<td>–</td>
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All presented data were obtained in this study. +, Positive; –, negative; \(w\), weakly positive.

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References


