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ORIGINAL PAPER

Lichenibacterium ramalinae gen. nov, sp. nov., *Lichenibacterium minor* sp. nov., the first endophytic, *beta*-carotene producing bacterial representatives from lichen thalli and the proposal of the new family *Lichenibacteriaceae* within the order *Rhizobiales*

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Abstract This study of lichens in the subarctic zone of the northern hemisphere has resulted in the detection of new representatives of the order *Rhizobiales*. The16S rRNA gene sequence phylogeny placed the strains as a separate branch inside the *Rhizobiales* clade. Strain RmlP001^T exhibits 91.85% similarity to *Roseiarcus fermentans* strain Pf56^T and 91.76% to *Beijerinckia doebereinerae* strain LMG 2819^T, whilst strain RmlP026^T is closely related to *B. doebereinerae* strain LMG 2819^T (91.85%) and *Microvirga pakistanensis* strain NCCP-1258^T (91.39%). A whole-genome phylogeny of the strains confirmed their taxonomic positions. The cells of both strains were observed to be Gram-negative, motile rods that multiplied by binary fission. The cells were found to contain poly-β-hydroxybutyrate and polyphosphate, to grow at pH 3.5–8.0 and 10–30 °C, and could not fix atmospheric nitrogen. Their major cellular fatty acid identified was $C_{18:1}\omega7c$ (68–71%) and their DNA G + C contents determined to be 70.5–70.8%. Beta-carotene was identified as their major carotenoid pigment; Q-10 was the only ubiquinone detected. Strains RmlP001^T and RmlP026^T are distinguishable from related species by the presence of β-carotene, the absence of C1 metabolism and the ability to grow in the presence of 3.5% NaCl. Based on their phylogenetic, phenotypic and chemotaxonomic features, we propose a novel genus *Lichenibacterium* and two novel species, *Lichenibacterium ramalinae* (the type species of the genus) and *Lichenibacterium minor*, to accommodate these bacteria within the family *Lichenibacteriaceae* fam. nov. of the order

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Rhizobiales. The *L. ramalinae* type strain is RmlP001^T (= KCTC 72076^T = VKM B-3263^T) and the *L. minor* type strain is RmlP026^T (= KCTC 72077^T = VKM B-3277^T).

Keywords *Lichenibacterium* · LAR1 · Lichen endophytes · *Rhizobiales* · Beta-carotene · Phylogenetic analysis · Symbiotic bacteria

Introduction

Since the beginning of this century, a large amount of data has been obtained regarding the microbial communities of lichens. The bacteria that inhabit lichens form unique communities inside the lichen thalli and carry out highly diverse functions, such as phosphate solubilisation, nitrogen fixation and biomass mobilisation, while influencing growth through hormone production (Grube et al. 2009). Independent methods of investigation–by molecular analysis and culturing–have identified a variety of different taxa dwelling within lichen thalli (Grube and Berg 2009). At least seven new species have been described and validly named from lichen thalli in the past decade (Cardinale et al. 2011; Lee et al. 2013; Männistö et al. 2010; Phongsopitanun et al. 2016; Yamamura et al. 2011).

Recently, new genera of the families *Isosphaeraceae* (Kulichevskaya et al. 2017) and *Acidobacteriaceae* (Belova et al. 2018) have been described for isolates from lichen-dominated environments. The typical bacterial inhabitants of lichens are members of the class *Alphaproteobacteria* (Cardinale et al. 2008, 2012; Grube et al. 2009). The 16S rRNA gene sequences of that group were obtained from foliose and fruticose lichens, such as *Cladonia* spp. (Hodkinson and Lutzoni 2009), *Lobaria pulmonaria* (Schneider et al. 2011) and *Cetraria aculeata* (Printzen et al. 2012), as well as from crustose lichens (Hodkinson et al. 2012). The most abundant group within the class *Alphaproteobacteria* is the order *Rhizobiales* (Bates et al. 2011; Erlacher et al. 2015). Among representative strains and numerous clones, LAR1 (Lichen Associated *Rhizobiales*) is the most specific for lichens. LAR1 is a complex group of clones first discovered in lichens (Bates et al. 2011; Hodkinson and Lutzoni 2009; Hodkinson et al. 2012) and similar clones have also been detected in human specimens (Oh et al. 2013), in the microbiota associated with representatives of the *Hymenoptera* (Martinson et al. 2011) and in house dust (Täubel et al. 2009). Recently, a few LAR1 strains have been isolated from lichens (Jiang et al. 2017), but their taxonomic descriptions have not been published.

The lichen genus *Ramalina* is ubiquitous in terrestrial ecosystems and is represented by several fruticose species, with *Ramalina farinacea* and *Ramalina pollinaria* being the most common. Recently, an unexpected associated microalgal diversity has been found in the epiphytic species *R. farinacea* (Moya et al. 2017). *R. pollinaria* has not been previously investigated as a source of bacteria, fungi or algae.

The present work reveals new *Rhizobiales* bacterial strains obtained from the widespread *R. pollinaria* lichen and presents the first taxonomic description of new representatives of the order *Rhizobiales* from that lichen.

Materials and methods

Isolation and purification of strains

Samples of *R. pollinaria* thalli were collected in 2016 from birch trunks (*Betula pubescens* Ehrh.) at the 'Belomorskaya' biological station of M.V. Lomonosov Moscow State University (66°33′ N, 33°06′ E). To separate epiphytic and endophytic microbial communities, the method of preliminary washing of thalli was used (Pankratov 2018). Washed thalli fragments (200 mg) were cut with scissors and homogenised using an ULTRA-TURRAX® Tube Drive homogenizer with DT-20 tube (rotor–stator element, volume 5–15 ml) in 10 ml of PBS for 5 min at 6000 rpm. The suspension was diluted 10^2–10^4 times and plated on agar plates. The isolation was performed using basal MM1 medium containing (g l^{-1}, distilled water): KH_2PO_4 (0.1); NaCl (0.2); $MgSO_4{\cdot}7H_2O$ (0.04); $Ca(NO_3)_2{\cdot}5H_2O$ (0.04); NH_4NO_3 (0.1) and yeast extract (0.1). Pectin, sucrose, arabinose, xylose, maltose, mannitol, fructose, rhamnose, arbutin, xylan and inositol were used as carbon sources as a mixed solution (0.025%, w/v each component). The pH was 5.0–5.5. The solidifying agent used for the medium preparation was agar–agar (1.5 g l^{-1}). Purification and control of axenity were carried out by plating the

inocula from dilution of the bacterial suspensions. Individual colonies were picked, resuspended in water and the bacterial suspensions were checked for contamination via light microscopy.

Phenotypic characterisation of strains

Further experiments were done using the same mineral medium; glucose (1 g l^{-1}) was used as a source of carbon. Vitamin mixture (mg in 100 ml: p-aminobenzoic acid, 1.0; biotin, 0.2; nicotinic acid, 2.0; thiamine-HCl × $2H_2O$, 1.0; Ca-pantothenate, 0.5; pyridoxamine, 5.0; cyanocobalamin, 2.0) (1 ml l^{-1}) was added (medium MM1 V) to improve growth. Better growth was achieved in a liquid medium if the shaking rate was 60–100 rpm. Growth of the novel strains under a variety of conditions, including temperatures of 2–37 °C, pH 2–10 was examined using batch cultures grown in liquid medium MM1 V with glucose. The OD_{600} was measured in a photometer after 1 week of incubation at 23 °C. The range of potential growth substrates of the strains was examined by replacing glucose in medium MM1 V with single carbon sources. Susceptibility to antibiotics was determined on MM1 V agar plates using discs (Oxoid) containing the following antibiotics: penicillin (10 µg), gentamicin (10 µg), kanamycin (30 µg), streptomycin (10 µg), chloramphenicol (30 µg) and erythromycin (10 µg).

The enzymatic profiles were examined with API ZYM (bioMérieux). Urease, β-galactosidase and oxidase activity, acetoin production, indole production from tryptophan, and ability to hydrolyse gelatin were performed according to Gerhardt (1981). Catalase activity was detected by the formation of gas bubbles after dropping 3% (w/v) H_2O_2 onto a fresh culture grown on MM1 V medium with glucose. The ability to grow under anaerobic conditions was tested in Hungate tubes under nitrogen.

Chemotaxonomic analysis

The fatty acid composition of strains was analysed using a gas chromatograph and a Maestro mass spectrometer (Interlab LLC, Russia). The centrifuged biomass samples were dried with three drops of methanol and subjected to acid methanolysis (400 µL of 1.2 M HCl/MeOH, 80 °C, 45 min). The products were extracted with hexane, and the extract was dried and subjected to treatment with N, O-bis (trimethylsilyl) trifluoroacetamide derivatising agent (20 µl, 80 °C, 5 min). The separation of obtained products was carried out on a column of 5% -phenyl-95% -methyl-polysiloxane 25 m × 0.25 mm with a phase thickness of 0.25 µm (Rxi®-5 ms, Restek, USA) using a temperature gradient of 135–320 °C with an ascent rate 7 °C/min and ionization by electron impact with energy of 70 eV detection in full scan mode. Compounds were identified using the NIST14 mass spectra library (https://www.nist.gov).

For lipid identification, the bacterial biomass was homogenised in isopropanol using a pestle and mortar, and incubated at 70 °C for 30 min. Lipids were extracted using the Nichols' method (Nichols 1963) with a few changes involving extraction with isopropanol and an isopropanol–chloroform mixture (1:1 and 1:2) at 70 °C, evaporation in a rotary evaporator and extraction of the residue with chloroform–methanol (1:1) supplemented with a 2.5% sodium chloride solution to remove water-soluble substances. After separating the mixture by allowing it to stand overnight or by centrifugation, the chloroform layer was dried by passing it through water-free sodium sulfate, evaporated, and desiccated with a vacuum pump. The resulting pellet was dissolved in a small amount of chloroform–methanol mixture (2:1) and stored at − 21 °C.

Polar lipids were separated with the previously described solvent systems for two-dimensional TLC (Benning et al. 1995). The lipids (100–200 µg) were applied to a plate. The chromatograms were sprayed with 5% sulfuric acid in ethanol with subsequent heating to 180 °C. Phospholipids were identified using individual markers and qualitative tests for amino groups (with ninhydrin), choline-containing phospholipids (with the Dragendorff reagent) and glycolipids (with α-naphthol) (Kates 1972).

Pigments were identified using HPLC and spectroscopy methodologies. The primary identification of pigments was carried out using a scanning spectrophotometer SF56 (LOMO, Russia) (slit 1 nm) in the range of 350–1000 nm. The analysis of carotenoids was performed according to a previously described method (Ashikhmin et al. 2014).

Microscopy

Morphological observations and cell size measurements were made with a Zeiss Axio Imager 2

microscope and Axiovision 4.2 software (Zeiss, Germany). For preparation of ultrathin sections, cells of an exponentially growing culture were collected by centrifugation and pre-fixed with 1.5% (w/v) glutaraldehyde in a 0.05 M cacodylate buffer (pH 6.5) for 1 h at 4 °C and then fixed with 1% (w/v) OsO_4 in the same buffer for 4 h at 20 °C. After dehydration in an ethanol series, the samples were embedded into Epon 812 epoxy resin. Thin sections were divided with LKB-4800 microtome, stained with 3% (w/v) uranyl acetate in 70% (v/v) ethanol, and then were stained with lead citrate (Reynolds 1963) at 20 °C for four to 5 min. Ultrathin sections were examined with a Libra 120 (Carl Zeiss, Germany) transmission electron microscope at 80 kV.

DNA extraction, PCR, sequencing, genome sequence and accession numbers

Genomic DNA was extracted using the DNA Easy Power Soil Kit (Qiagen) according to the manufacturer's instructions. The 16S rRNA genes were amplified from genomic DNA by PCR with primers 27F and 1492R (Lane 1991). The PCR products were purified via the Wizard PCR Preps Kit (Promega) as recommended by the manufacturer. The 16S rRNA genes were sequenced in both directions with primers 27F, 357F, 1114F and 519R by means of the Big Dye Terminator v.3.1 according to the manufacturer's instructions for use of the ABI PRISM 3730 sequencer (Applied Biosystems). The 16S rRNA gene sequences were aligned with MUSCLE (Edgar 2004) and the maximum-likelihood tree was inferred with the TN + F + I + G4 model, which is recommended when using the Model Finder (Kalyaanamoorthy et al. 2017) in IQ-Tree (Nguyen et al. 2014). Branch supports were obtained with 10,000 ultra-fast bootstraps (Hoang et al. 2017).

Libraries were constructed with the NEBNext DNA Library Prep Reagent Set for Illumina, according to the kit's protocol. Sequencing was undertaken using the Illumina HiSeq 1500 platform with single-end 250-bp reads. Raw reads were checked for quality with FastQC v 0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and low-quality reads were trimmed using Trimmomatic v. 0.36 (Bolger et al. 2014) applying default parameters. The quality-filtered reads were assembled de novo with SPAdes version 3.11.0 using the default settings (Bankevich et al. 2012). Annotations of the scaffolds were carried out using the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al. 2016). Average nucleotide identity (ANI) and in silico DNA–DNA hybridization (dDDH) values were calculated via the ANI calculator from the Kostas lab (http://enve-omics.ce.gatech.edu/ani) (Rodriguez-R and Konstantinidis 2016) and the GGDC (http://ggdc.dsmz.de/ggdc.php) (Auch et al. 2010), respectively.

Core genes from the whole genomes of RmlP001^T, RmlP026^T and strains of *Rhizobiales* were extracted using the USEARCH program (Edgar 2010) with a 50% sequence identity cut-off within the BPGA (Chaudhari et al. 2016). Their concatenated amino acid sequences were aligned using MAFFT (Katoh and Standley 2013). Phylogenetic analysis was performed using the IQ-TREE program (Nguyen et al. 2014) with a selection of an evolutionary model employing Model Finder (Kalyaanamoorthy et al. 2017) and an estimate of branch supports with UFBoot2 (Hoang et al. 2017).

Results and discussion

Two strains were obtained from the homogenised thallus of the lichen *R. pollinaria*. On agar medium, cultures were observed to produce compact orange or red–orange colonies with diameters of 1–3 mm. Colonies of strain RmlP001^T were observed to be rounded, with smooth edges, and to grow slightly raised above the surface, without the formation of "lenses". Colonies of strain RmlP026^T were observed to be smaller (0.5–2 mm), convex, with a peculiar central invagination and red–orange in colour (Supplementary Fig. 1).

The appearance and shape of the bacterial cells suggested an association with the family *Beijerinkiaceae* or *Methylobacteriaceae*. The cells were observed to be large (2–4 μm) and bent in the form of an arch, with inclusions at the poles similar to poly-*β*-hydroxybutyrate (Fig. 1a, b). The cells did not form spores. In aged cultures, cells of both strains lost their original shape. In a liquid medium, cells of both strains were motile in the early stages of growth. Electron microscopy examination showed the presence of one long flagellum on each strain (Supplementary Fig. 2). After 2 days of incubation, the cells lost their flagella and attached to the glass, forming a highly adhesive

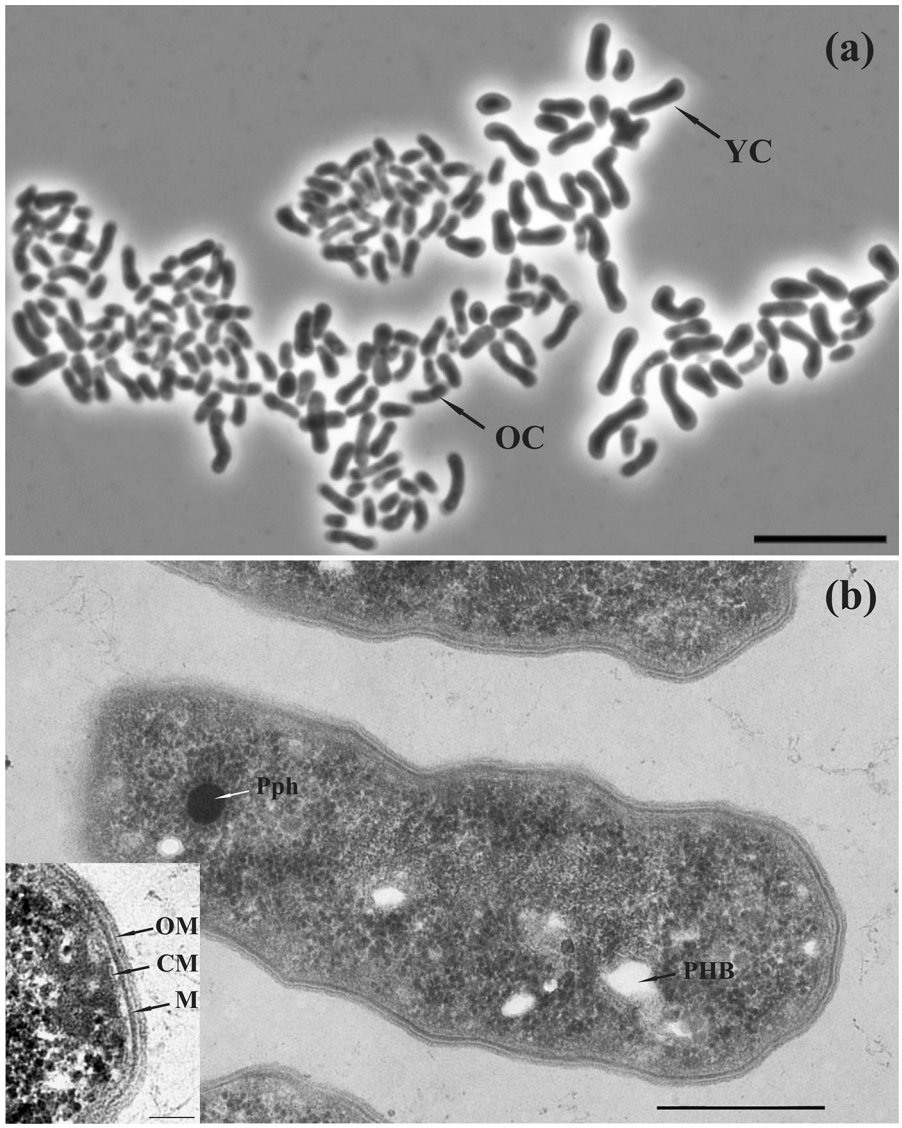


Fig. 1 Morphological observation of strain RmlP001^T. Phase contrast microscope picture **(a)** shows two types of cells: young (YC) and old (OC); bar, 10 µm. Electron micrograph of an ultrathin section **(b)** showing Gram-negative type of the cell wall, poly-β-hydroxybutyrate granules (PHB) and polyphosphate granule (Pph); bar, 0.5 µm. Left insert: OM—outer membrane; CM—cytoplasmic membrane; M—murein; bar 0.1 µm

film. Electron microscopy observation of sections of strain RmlP001^T cells sections showed the presence of a Gram-negative type cell wall, poly-β-hydroxybutyrate granules, polyphosphate and unidentified intracytoplasmic granules (Fig. 1b).

Comparative 16S rRNA gene sequence analyses positioned these two novel strains in the order *Rhizobiales,* but as a new independent lineage (Supplementary Fig. 3). Strains RmlP001^T and RmlP026^T were found to possess 98–99% 16S rRNA gene sequence similarity to environmental clones retrieved from lichens and human specimens. Strain Pao5 (GenBank accession no. KJ016001), recently obtained from the lichen *Parmelia omfalodes* (Jiang et al. 2017), and strain M016010 (GenBank accession no. KP185149), isolated from a wetland (unpublished), have 98% similarity to the same group of clones. All the strains formed a common cluster with a number of 16S rRNA gene clones retrieved from lichens (EL, NL, HL, CL, 50**c**), human specimens (ncd, ELU),

the Alaska Byron glacier surface (Bysf-50-Sf10-050), *Hymenoptera* insects (SHN), urban aerosols (AKIW), glacial snow (TP-Snow), tropical soil (HF), rainwater (SH) and house dust (FD, FB). A set of clones included in this cluster was earlier analysed during the studies of lichen microbial communities (Hodkinson and Lutzoni 2009; Hodkinson et al. 2012) and named LAR1 (lichen associated Rhizobiales). The greatest number of these clones have been found in lichens of the genera *Usnea*, *Cladonia* and *Sticta*. Similar clones were amplified from genomic DNA of the lichen genera *Flavoparmelia*, *Umbilicaria* and *Cladonia* (Hodkinson and Lutzoni 2009). Later, the ncd group of clones was obtained from different sources, including a human skin microbiome in patients with primary immunodeficiencies and the microbial community associated with the human ileum (Li et al. 2012; Oh et al. 2013). Afterwards, other clones were retrieved from various samples, including the bacterial community of an urban aerosol, glacial snow, honeybees and soil. The groups of clones represented by strains RmlP001^T, RmlP026^T, Pao5 and M016010 are phylogenetically well isolated from related families in the order *Rhizobiales* and constituted a single coherent group at the family level.

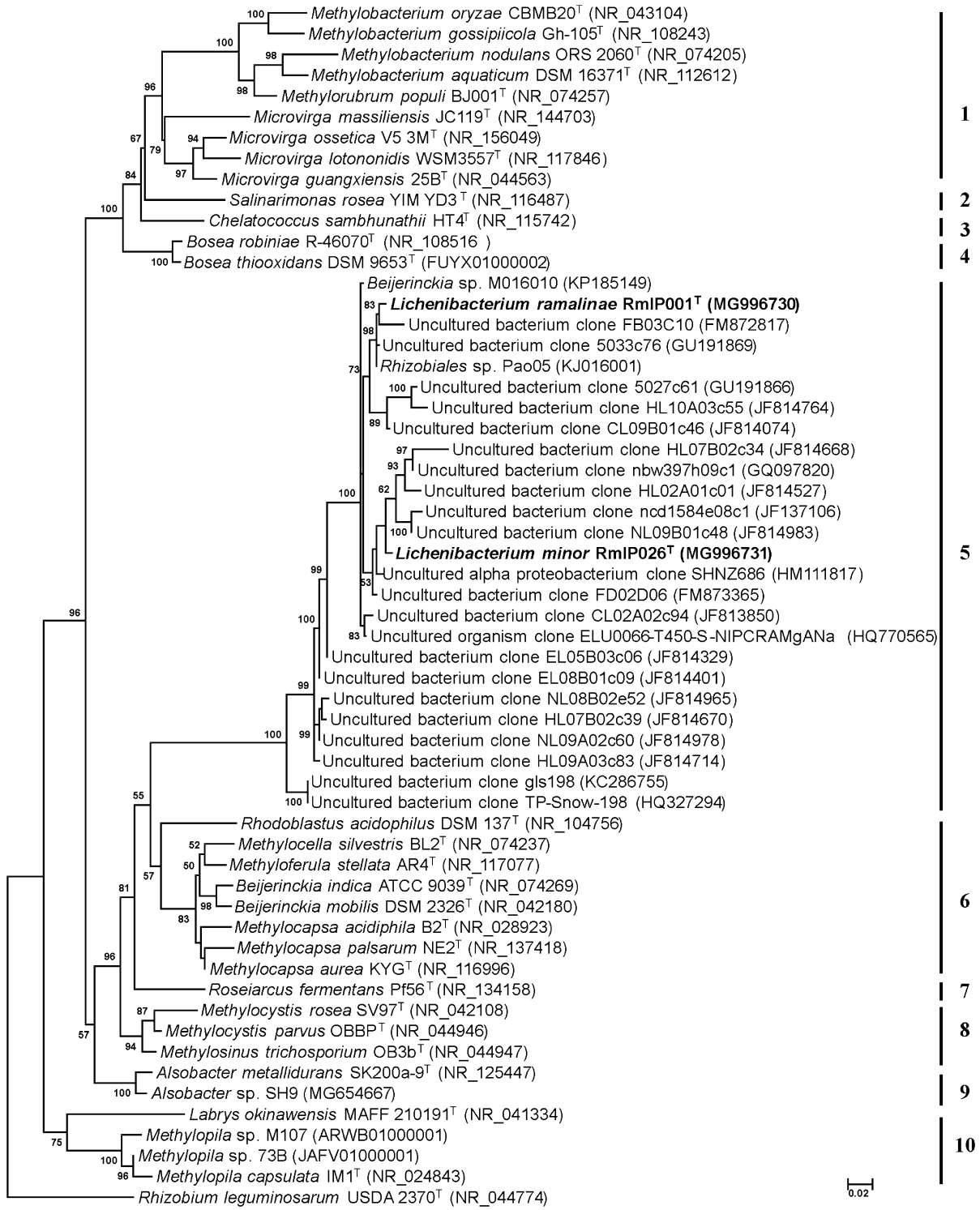
A maximum-likelihood phylogenetic tree inferred from the comparison of concatenated *Rhizobiales* core proteins shows the position of the strains RmlP001^T and RmlP026^T (Supplementary Fig. 4). In accordance with this analysis, both strains form a cluster that is clearly separated from the other branches represented by typical species of the corresponding closely related groups at the family level.

Among the taxonomically undescribed strains, the strain Pao05 (GenBank accession no. KJ016001; 99.1% similarity to strain RmlP001^T and 97.82% to RmlP026^T, with query cover values of 93% and 94% correspondingly) and strain M016010 (GenBank no. KP185149; 98.31% similarity to RmlP001^T and 98.31% to RmlP026^T) are closely related to strains RmlP001^T and RmlP026^T. Based on 16S rRNA gene sequences, strain RmlP001^T was determined to share high pairwise similarity to *Roseiarcus fermentans* strain Pf56 (91.85%), *Beijerinckia doebereinerae* strain LMG 2819^T (91.76%), *Beijerinckia derxii* subsp. *venezuelae* strain DSM 2329^T (91.73%), *Rhizobium rhizoryzae* strain J3-AN59^T (91.66%), *Beijerinckia indica* subsp. *indica* ATCC 9039^T (91.55%) and *Microvirga lotononidis* strain WSM3557^T (91.61%). Strain RmlP026^T was determined to share a high pairwise similarity to *B. doebereinerae* strain LMG 2819^T (91.85%), *Microvirga pakistanensis* strain NCCP-1258^T (91.39%), *Methylocystis bryophila* strain H2s^T (91.36%), *B. derxii* subsp. *venezuelae* strain DSM 2329^T (91.29%) and *Alsobacter metallidurans* strain SK200a-9^T (91.15%), with lower similarities to members of other genera. Thus, for strains RmlP001^T and RmlP026^T, their levels of homology to all currently described representatives within the families of *Beijerinkiaceae, Chelatococcaceae, Roseiarcaceae* and *Methylobacteriaceae* are lower than 92%. Both strains, together with environmental clones, form a compact group that is well separated from representatives of these families (Fig. 2).

Fig. 2 Maximum-likelihood phylogenetic trees based on 16S rRNA gene sequences (1320 nucleotide sites) reconstructed with evolutionary model TN + F+I + G4 showing the phylogenetic positions of strains RmlP001^T and RmlP026^T, in relation to taxonomically characterised members of the families *Methylobacteriaceae* (1), *Salinarimonadaceae* (2), *Chelatococcaceae* (3), *Bradyrhizobiaceae* (4), *Lichenibacteriaceae* (5), *Beijerinckiaceae* (6), *Roseiarcaceae* (7), *Methylocystaceae* (8), *Alsobacteraceae* (9) and a group of taxa with unidentified positions inside the order *Rhizobiales* (10). Sequence of *Rhizobium leguminosarum* strain USDA 2370^T (GenBank accession no. NR_044774) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position

The phylogenetic distance between the strains RmlP001^T and RmlP026^T is 2.89%. This corresponds to the phylogenetic distance between two separate species.

A total of 1,818,054 and 3,627,973 reads were obtained from draft genome sequencing of strains RmlP001^T and RmlP026^T, respectively. The final assembled genome (5,801,291 bp long) of strain RmlP001^T is comprised of 76 scaffolds, with an N_{50} value of 216,247 bp, an average coverage of 32 times and a G + C content of 70.8%. The final assembled genome (5,916,175 bp long) of strain RmlP026^T is comprised of 199 scaffolds, with an N_{50} value of 127,675 bp, an average coverage of 78 times and a G + C content of 70.5%. Annotation led to the identification of 5404 genes, 5342 coding sequences and 56 tRNA genes for strain RmlP001^T and 5606 genes, 5545 coding sequences and 55 tRNA genes for strain RmlP026^T. The average nucleotide identity (ANI) and digital DNA–DNA hybridisation (dDDH)


Methylobacterium oryzae CBMB20T (NR_043104)
Methylobacterium gossipiicola Gh-105T (NR_108243)
Methylobacterium nodulans ORS 2060T (NR_074205)
Methylobacterium aquaticum DSM 16371T (NR_112612)
Methylorubrum populi BJ001T (NR_074257)
Microvirga massiliensis JC119T (NR_144703)
Microvirga ossetica V5 3MT (NR_156049)
Microvirga lotononidis WSM3557T (NR_117846)
Microvirga guangxiensis 25BT (NR_044563)
Salinarimonas rosea YIM YD3T (NR_116487)
Chelatococcus sambhunathii HT4T (NR_115742)
Bosea robiniae R-46070T (NR_108516)
Bosea thiooxidans DSM 9653T (FUYX01000002)
Beijerinckia sp. M016010 (KP185149)
Lichenibacterium ramalinae RmlP001T (MG996730)
Uncultured bacterium clone FB03C10 (FM872817)
Uncultured bacterium clone 5033c76 (GU191869)
Rhizobiales sp. Pao05 (KJ016001)
Uncultured bacterium clone 5027c61 (GU191866)
Uncultured bacterium clone HL10A03c55 (JF814764)
Uncultured bacterium clone CL09B01c46 (JF814074)
Uncultured bacterium clone HL07B02c34 (JF814668)
Uncultured bacterium clone nbw397h09c1 (GQ097820)
Uncultured bacterium clone HL02A01c01 (JF814527)
Uncultured bacterium clone ncd1584e08c1 (JF137106)
Uncultured bacterium clone NL09B01c48 (JF814983)
Lichenibacterium minor RmlP026T (MG996731)
Uncultured alpha proteobacterium clone SHNZ686 (HM111817)
Uncultured bacterium clone FD02D06 (FM873365)
Uncultured bacterium clone CL02A02c94 (JF813850)
Uncultured organism clone ELU0066-T450-S-NIPCRAMgANa (HQ770565)
Uncultured bacterium clone EL05B03c06 (JF814329)
Uncultured bacterium clone EL08B01c09 (JF814401)
Uncultured bacterium clone NL08B02e52 (JF814965)
Uncultured bacterium clone HL07B02c39 (JF814670)
Uncultured bacterium clone NL09A02c60 (JF814978)
Uncultured bacterium clone HL09A03c83 (JF814714)
Uncultured bacterium clone gls198 (KC286755)
Uncultured bacterium clone TP-Snow-198 (HQ327294)
Rhodoblastus acidophilus DSM 137T (NR_104756)
Methylocella silvestris BL2T (NR_074237)
Methyloferula stellata AR4T (NR_117077)
Beijerinckia indica ATCC 9039T (NR_074269)
Beijerinckia mobilis DSM 2326T (NR_042180)
Methylocapsa acidiphila B2T (NR_028923)
Methylocapsa palsarum NE2T (NR_137418)
Methylocapsa aurea KYGT (NR_116996)
Roseiarcus fermentans Pf56T (NR_134158)
Methylocystis rosea SV97T (NR_042108)
Methylocystis parvus OBBPT (NR_044946)
Methylosinus trichosporium OB3bT (NR_044947)
Alsobacter metallidurans SK200a-9T (NR_125447)
Alsobacter sp. SH9 (MG654667)
Labrys okinawensis MAFF 210191T (NR_041334)
Methylopila sp. M107 (ARWB01000001)
Methylopila sp. 73B (JAFV01000001)
Methylopila capsulata IM1T (NR_024843)
Rhizobium leguminosarum USDA 2370T (NR_044774)
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0.02

values between strains RmlP001^T and RmlP026^T were 83.6% and 25.9%, respectively. This confirms the difference between the two strains at the species level.

The central carbohydrate metabolism of the strains RmlP001^T and RmlP026^T is supported by 98 and 102 enzymes, respectively, including enzymes involved in the glycolysis and gluconeogenesis pathways (14 enzymes from both pathways). Both genomes are characterised by the presence of a full set of the tricarboxylic acid cycle genes, including the genes *glt*A, *suc*A, *suc*B, *lpd*A, *suc*D and *suc*S. The pentose phosphate pathway was also represented by a complete set of coding genes. Nitrogen metabolism was indicated by the nitrate and nitrite ammonification subsystem, as well as the ammonia assimilation subsystem. The sugar alcohol metabolism pathways included 62 and 51 genes in the strains RmlP001^T and RmlP026^T, respectively, while inositol metabolism, including transporters, was indicated by 39 and 34 genes, respectively. The genomic data indicated that the new strains contain genes encoding trehalose synthesis proteins, including the key trehalose synthase enzyme. Oxidative stress proteins genes were represented almost equivalently in both strains, and included 38–39 proteins associated with oxidative stress (e.g., rubrerythrin and superoxide dismutase), glutathione biosynthesis, the gamma-glutamyl cycle, non-redox reactions, the glutathione redox cycle and glutaredoxins. Polyhydroxybutyrate metabolism genes were found located in different clusters and are represented by several coding areas. The biosynthesis of isoprenoids is controlled by 13 (RmlP001^T) or 17 (RmlP026^T) genes, including lycopene beta-cyclase *crt*Y and beta-carotene hydroxylase. Genome analysis confirmed the absence of specific enzymatic systems providing nitrogen fixation and C1 metabolism.

The major fatty acids of both strains RmlP001^T and RmlP026^T were identified as vaccenic ($C_{18:1}\omega7c$), palmitic and cyclononadecanoic ($C_{19:0}$ cyclo) acids (Table 1). Both strains were found to exhibit similar fatty acid profiles. The polar lipid profiles of strains RmlP001^T and RmlP026^T were found to contain phosphatidylcholine, phosphatidylethanolamines, glycolipids (GL1 and GL2), diphosphatidylglycerol and phosphatidylglycerol as major components (Supplementary Fig. 5). Strain RmlP001^T was found to differ from strain RmlP026^T by the absence of phosphatidic acid and the presence of unidentified polar lipids (PL1 and PL2). Strain RmlP026^T was also

Table 1 Cellular fatty acid compositions (%) of strains RmlP001^T and RmlP026^T

Fatty acid type	RmlP001^T	RmlP026^T
Saturated		
$C_{14:0}$	0.25	0.43
$C_{16:0}$	**15.7**	**15.3**
$C_{18:0}$	0.62	0.58
Unsaturated		
$C_{16:1}\omega7c$	2.5	2.8
$C_{18:1}\omega7c$	**71.2**	**68.8**
$C_{19:0}$ cyclo	**7.4**	**9.9**
Hydroxy		
$C_{14:0}$ 3OH	0.18	0.21

Major fatty acids (> 5%) are shown in bold

enriched with pigments in two fractions (Pig1 and Pig2), which are probably carotenoids.

The spectrometry and liquid chromatography data revealed carotenoids as the dominant pigments in both strains. The main carotenoid identified in strain RmlP001^T was β-carotene (49.7%) and the minor carotene was ζ-carotene (4.7%). Unidentified carotenoids accounted for about 45% of the carotenoid fraction and were represented by four peaks in the chromatogram (Supplementary Fig. 6a). Peaks one and two had identical absorption spectra, while the spectra of peaks three and four differed from each other (Supplementary Fig. 6 b, c). In both strains, the only quinone was found to be ubiquinone (Q-10), which is typical for the majority of representatives of the order *Rhizobiales*.

Under aerobic conditions, both strains were found to be able to grow on sugars, sugar alcohols and some polysaccharides (Supplementary Table 1). The preferred organic carbon sources for both strains were found to be D-galactose, glycerol, D-glucose, *myo*-inositol, mannitol, sorbitol and D-fructose. Carboxylic acids were not metabolised by either strain. Strain RmlP001^T demonstrated good growth on arabitol, dulcitol, melibiose and sodium succinate, while strain RmlP026^T could not use those substrates or grew only weakly when supplied with them. Among the tested polysaccharides, only starch and xylan are capable of supporting the growth of both strains. Strain RmlP001^T can degrade laminarin and pectin. Neither strain described here had specific groups of enzymes that ensured the involvement of C1 compounds (methanol, methylamine, methane or formate) and N, N-dimethylformamide in carbon metabolism.

Yeast extract and ammonium were found to be the optimal sources of nitrogen for both strains. The strains were distinguishable by their abilities to assimilate amino acids as a source of nitrogen and/or carbon (Supplementary Table 1).

The enzymatic profiles of the strains are similar, but RmlP026^T was found to be positive and RmlP001^T negative for alkaline phosphatase and valine arylamidase. Both strains are negative for cytochrome oxidase and positive for catalase. Urease was detected in strain RmlP001^T only. Both strains are negative for protease activity (gelatin liquefaction) under aerobic conditions. Parameters such as the salinity, pH and growth temperature ranges were found to be similar for both strains; the differences were not statistically significant (Supplementary Table 1). Cumulatively the genetic, physiological and biochemical tests confirmed that the two strains belong to different species of the same genus.

We compared the novel isolates to the closely related members of the families *Beijerinckiaceae*, *Roseiarcaceae* and *Methylobacteriaceae* (Table 2). Their most distinctive phenotypic features were nitrogen fixation, ability to produce *beta*-carotene, C1 compound utilisation, and the ability to grow in a 3% sodium chloride solution. Strains RmlP026^T and RmlP001^T cannot metabolise methanol or methane, grow well in the presence of 3% sodium chloride and contain *beta*-carotene as a major carotenoid pigment. Representatives of the new family are distinguished from the strain Pf56^T (*Roseiarcaceae*) by their inability to grow under micro-oxic conditions by means of fermentation and by their set of carotenoid pigments. The strains differed from the closely related organisms (i.e. *B. indica, R. rhizoryzae,* and *Methylobacterium persicinum*) (Kato et al. 2008; Oggerin et al. 2009; Zhang et al. 2014) by a slightly larger content of palmitic acid ($C_{16:0}$) and the presence of cyclononadecanoic acid. *R. fermentans* synthesises more $C_{19:0}$ cyclo when compared with strains RmlP001^T and RmlP026^T. The characteristics of the genus *Methylobacterium* indicate that some species can produce small amounts of $C_{19:0}$ cyclo (Green 2015).

The wide representation of the LAR1 group in lichens of numerous species and from different geographic regions is clearly related to their parasymbiont or symbiotic interrelationships. However, dependence on sugar alcohols, as well as the absence of the utilisation of CO_2 and other C1 compounds, suggests endoparasitism as a strategy for their survival in lichens. Conversely, the presence of superoxide dismutase genes, carotenoid pigments, a significant amount of ubiquinone Q-10, and the TIGR02302 protein homologues may indicate a mutually beneficial integration of bacteria into the lichen thallus. Previously, the TIGR02302 family of proteins was shown to contribute to the integration of bacteria into the roots of plants (Sato et al. 2016). Strain RmlP026^T is able to synthesise notable amounts of *beta*-carotene and ubiquinone Q-10, suggesting potential biotechnological applications of this strain.

Based on the differences in the phylogenetic, physiological and biochemical properties of the strains RmlP001^T and RmlP026^T from those of the members of the families *Methylobacteriaceae*, *Salinarimonadaceae*, *Chelatococcaceae*, *Bradyrhizobiaceae*, *Roseiarcaceae*, *Beijerinckiaceae* and *Methylocystaceae*, we propose the new family *Lichenibacteriaceae* fam. nov. with the type genus *Lichenibacterium* gen. nov.

Description *of Lichenibacterium* gen. nov

Lichenibacterium (Li.che.ni.bac.te'ri.um. L. masc. n. *lichen* a lichen; N.L. neut. n. *bacterium* a rod; N.L. neut. n. *Lichenibacterium* a rod from a lichen).

Gram-negative, asporogenous, motile rods that occur singly. Young cultures contain motile cells (1.5–4 μm). On ageing, cells become non-motile and form aggregates. Colonies are small (0.5–3 mm in diameter), convex, circular and opaque. Carotene-type pigments are present. Oxidase negative and catalase positive. Chemo-organotrophic aerobes. Does not produce H_2S from thiosulfate or peptone; does not produce indole from tryptophan. NaCl inhibits growth at concentrations above 3.5% (w/v). The polar lipids consist of phosphatidylcholine, phosphatidylethanolamine, glycolipids, diphosphatidylglycerol and phosphatidylglycerol. Major fatty acids are $C_{18:1}\omega7c$ and $C_{16:0}$; the quinone is Q-10. The G + C content of the DNA is ca. 70–71%. Member of the family *Lichenibacteriaceae*. The type species is *Lichenibacterium ramalinae*.

Description of *Lichenibacterium ramalinae* sp. nov

*Lichenibacterium ramalinae (*ra.ma.li'nae. N.L. gen. n. *ramalinae* from/of the lichen genus *Ramalina).*

Table 2 Comparison of morphological, physiological and molecular properties among the families *Lichenibacteriaceae*, *Beijerinckiaceae*, *Methylobacteriaceae*, and *Roseiarcaceae*

Characteristic	*Lichenibacteriaceae*	*Beijerinckiaceae*[1]	*Methylobacteriaceae*[2]	*Roseiarcaceae*[3]
Cell morphology	Rods	Rods	Rods	Curved rods
Motility	+	v	v	–
Capsule	–	v	–	–
Type of metabolism	Chemo-organotrophy	Chemo-organotrophy, obligate methanotrophy or facultative methylotrophy	Chemo-organotrophy, methylotrophy	Chemo-organotrophy
Growth on methanol	–	v	+	–
Growth on methane	–	v	–	–
C1-utilization	–	+	+	–
N_2 fixation	–*	+	+	+
Fermentative metabolism	–	–	–	+
Growth at				
35 °C	–	v	v	–
pH 7.0	+	v	+	+
pH 3.5	+	v	v	–
0.5% NaCl	+	v	v	+
3% NaCl	+	–	–	–
Major fatty acids	$C_{16:0}$	$C_{18:1}\omega7c$	$C_{18:1}\omega7c$	$C_{18:1}\omega7c$
	$C_{18:1}\omega7c$	$C_{16:1}\omega7c$		$C_{19:0}$ cyclo
	$C_{19:0}$ cyclo			
Major quinone	Ubiquinone Q-10	Ubiquinone Q-10	Ubiquinone Q-10 and Q-9	Ubiquinone Q-10
Carotenoid pigments	Beta- and zeta carotene	Present, but not identified	Present, but not identified	Spirilloxanthin, rhodopin and 3,4-didehydrorhodopin
Beta-carotene	+	v	–	–
Oscilloxanthin	–	–	+	–
G + C %	70.5–70.8	54.7–62.8	68–72.4	70.0

"v"—varied among species; "+"—presence; "-"—not identified; "*"—based on the lack of growth on a nitrogen-free medium and the absence of the nif genes of the strains RmlP001^T and RmlP026^T

[1]Dedysh and Dunfield (2015)

[2]Green (2015)

[3]Kulichevskaya et al. (2014)

The description is as for the genus, but with the following additional traits. Cells are 1.0–1.2 μm wide and 2.0–4.0 μm long. Colony colour varies from light salmon to light carrot. Cells are motile in the early stages, become non-motile with aging. Carbon sources (0.1%, w/v) utilised for growth include arabitol, dulcitol, D-fructose, D-galactose, glycerol, D-glucose, *iso*-propanol, inositol, mannitol, D-mannose, melibiose, N-acetylglucosamine, sodium pyruvate, sodium succinate, sorbitol, sucrose, trehalose and xylose. Does not utilise L-arabinose, arbutin, cellobiose, lactose, maltose, melezitose, sodium acetate, sodium

butyrate, sodium glucuronate, sodium malate, sodium oxalate, sodium propionate, raffinose, L-rhamnose, fumarate, methanol, methane, *N*,*N*-Dimethylformamide or methylamine. Hydrolyses laminarin, pectin, starch and xylan, but not cellulose, carboxymethyl-cellulose, chitin, chitosan and agar–agar. Weak growth is observed on adonitol, ethanol, sodium citrate, ribose, sucrose, and "Phytagel". Nitrogen sources utilised include ammonium, succinimide, tryptophan but not nitrate, nitrite, arginine monohydrochloride, L-cysteine, glutamic acid, DL-proline and asparagine. DL-tryptophan and succinimide are used weakly. Indole and nitrite tests are negative. Capable of growth at pH 4–8 (optimum pH 4.5–5.5) and at 10–30 °C (optimum at 23–28 °C). Sensitive to sodium chloride at concentrations above 3.5% (w/v). Anaerobic growth does not occur. The DNA G + C content of the type strain is 70.8%.

The type strain, RmlP001^T (= KCTC 72076^T = VKM B-3263^T), was isolated from a lichen *Ramalina pollinaria* thallus, Murmansk region, Russia. GenBank accession numbers for sequences of strain RmlP001^T are QYBC00000000 (genome) and MG996730 (16S rRNA gene nucleotide sequence).

Description of *Lichenibacterium minor* sp. nov

*Lichenibacterium minor (*mi'nor. L. comp. neut. adj. *minor* smaller, referring to the small colonies).

The description is as for the genus but with the following additional traits. Cells are 1.2–1.5 μm wide and 2.0–8.0 μm long. Colony colour is bright orange. Carbon sources (0.1%, w/v) utilised for growth include adonitol, ethanol, D-fructose, D-galactose, glycerol, D-glucose, *iso*-propanol, inositol, mannitol, D-mannose, N-acetylglucosamine, sodium pyruvate, L-rhamnose, ribose, sorbitol, trehalose and xylose. Does not utilise L-arabinose, cellobiose, dulcitol, lactose, maltose, sodium acetate, sodium butyrate, sodium glucuronate, sodium malate, sodium oxalate, sodium propionate, raffinose, fumarate, methanol, methane, *N*,*N*-dimethylformamide or methylamine. Hydrolyses starch and xylan but not cellulose, carboxymethyl-cellulose, chitin, chitosan, agar–agar and pectin. Weak growth is observed on arabitol, arbutin, melibiose, laminarin, melezitose, adonitol, sodium succinate, sucrose, sodium citrate, sucrose and "Phytagel". Nitrogen sources utilised include ammonium, DL-proline, asparagine, succinimide and DL-tryptophan, but not nitrate, nitrite, arginine monohydrochloride, L-cysteine and glutamic acid. Indole and nitrite tests are negative. Capable of growth at pH 3.5–8.0 (optimum pH 4.5–5.0) and at 10–30 °C (optimum at 20–26 °C). Sensitive to sodium chloride at concentrations above 3.5% (w/v). Anaerobic growth does not occur. The DNA G + C content of the type strain is 70.5%.

The type strain, RmlP026^T (= KCTC 72077^T = VKM B-3277^T), was isolated from a lichen *Ramalina pollinaria* thallus, Murmansk region, Russia. GenBank accession numbers for sequences of the strain RmlP026^T are QYBB00000000 (genome) and MG996731 (16S rRNA gene nucleotide sequence).

Description of *Lichenibacteriaceae fam. nov*

Lichenibacteriaceae (Li.che.ni.bac.te.ri.a.ce'ae. N.L. neut. n. *Lichenibacterium* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Lichenibacteriaceae* the *Lichenibacterium* family).

Gram-negative, asporogenous bacteria. Aerobes. Mesophilic and moderately acidophilic. Do not possess C1 metabolism. The family belongs to the class *Alphaproteobacteria*, order *Rhizobiales*. The type genus is *Lichenibacterium*.

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Authors' contributions TP designed the study. TP examined lichen samples, obtained isolates, performed growth experiments; took phase contrast and micro pictures. DG, EP, TK obtained, annotated and analyzed the genome sequences. JB performed growth and physiological experiments. NS prepared ultrathin sections of cells and took pictures. TP and DG wrote the manuscript.

Data availability The DDBJ/ENA/GenBank accession numbers for sequences generated in this study are as follows: QYBC00000000 (genome of the strain RmlP001^T), QYBB00000000 (genome of the strain RmlP026^T); GenBank accession numbers MG996730 and MG996731 (16S rRNA gene nucleotide sequences of the RmlP001^T and RmlP026^T strains respectively).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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