ORIGINAL PAPER

A novel bacteriobiont of the Arctic lichen *Flavocetraria nivalis*, *Lichenifustis flavocetrariae* gen. nov, sp. nov. demonstrating hydrolytic properties and containing a full set of the Calvin–Benson–Bassham cycle genes

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Abstract

A Gram-negative, strictly aerobic, chemoorganotrophic, bacteriochlorophyll *a*-containing, slow-growing bacterium was isolated from the lichen *Flavocetraria nivalis* and designated strain BP6-180914[T]. Cells of this strain were large nonmotile rods, which reproduced by binary fission. Cells grew under oxic conditions and were able to utilize sugars and several polysaccharides, including starch and pectin. Strain BP6-180914[T] was psychrotolerant and moderately acidophilic growing at 4–35 °C (optimum 20–28 °C) and between pH 4.0 and 7.5 (optimum 4.5–5.5). The major fatty acids were $C_{18:1}\omega7c$, $C_{19:0}$ *cyclo*, $C_{16:0}$ and $C_{18:0}$. The polar lipids were diphosphatidylglycerols, phosphatidylglycerols, phosphatidylethanolamines, phosphatidylcholines, unidentified aminolipids, and a number of glycolipids, the major one being an unidentified glycolipid. The quinone was Q-10. The DNA G + C content was 63.65%. Comparative 16S rRNA gene sequence analysis revealed that strain BP6-180914[T] was a member of the order *Hyphomicrobiales* and belonged to the family *Lichenihabitantaceae* defined by the lichen-dwelling facultative aerobic chemo-organotroph *Lichenihabitans psoromatis* (92.7% sequence similarity). The results of phylogenomic and genomic relatedness analyses showed that strain BP6-180914[T] could clearly be distinguished from other species in the order *Hyphomicrobiales* with average nucleotide identity values of < 74.05% and genome-to-genome distance values of < 21.1%. The AAI value of 65.9% between strain BP6-180914[T] and *L. psoromatis* allowed us to assign this strain to the novel genus of the family *Lichenihabitantaceae*. Therefore, it is proposed that strain BP6-180914[T] represents a novel species in a new genus, *Lichenifustis flavocetrariae* gen. nov., sp. nov.; strain BP6-180914[T] (= KCTC 92872[T] = VKM B-3641[T] = UQM 41506[T]) is the type strain.

Keywords Lichens · *Hyphomicrobiales* · *Lichenifustis flavocetrariae* · Calvin cycle

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Introduction

The recent studies of the microbial populations of fruticose and foliose lichens have yielded significant results, which have repeatedly confirmed the dominance of two main orders of the class *Alphaproteobacteria*: *Rhodospirillales* and *Hyphomicrobiales* (Printzen et al. 2012; Hodkinson et al. 2012; Park et al. 2016; Sierra et al. 2020).

Phylogenetically, members of the order *Hyphomicrobiales* known so far belong to 40 validly published families under the LPSN (https://lpsn.dsmz.de/order/hyphomicrobiales); their important differentiating features, apart from phylogenetic status, include the ability to fix molecular nitrogen, to utilize methane and methanol, as well as the ability

to form symbiotic or parasitic relationships with eukaryotes (Carvalho et al. 2010; Rosselii et al. 2021). Using a number of diverse methodological approaches, previously unculturable members of the orders *Rhodospirillales* and *Hyphomicrobiales* have been isolated from lichens of various species (Noh et al. 2019, 2020, 2021; Pankratov et al. 2020a, 2020b). These novel bacteria have characteristics that determine their presence in lichens as specific components: growth on polyols, synthesis of polysaccharides, ability to utilize CO_2 under light, urease activity, and ability to utilize products of fungal metabolism (organic acids and polysaccharides).

Three validated species, *Lichenihabitans psoromatis*, *Lichenibacterium ramalinae* and *L. minus* (Noh et al. 2019; Pankratov et al. 2020a, b; https://lpsn.dsmz.de/species/lichenibacterium-minus) of the order *Hyphomicrobiales*, isolated from lichens form a phylogenetically separate cluster within the lichen-associated rhizobiales (LAR) 1 group of this order. *Lichenihabitans psoromatis*, *Lichenibacterium ramalinae*, and *L. minus* are characterized by their inability to fix atmospheric nitrogen and to grow on C1 substrates, which distinguishes them from other, phylogenetically fairly closely related members of this order—bacteria of the families *Beijerinckiaceae*, *Methylocystaceae*, *Methylobacteriaceae*, and *Rhizobiaceae*. Most members of these families are capable of assimilating methanol, methane and/or atmospheric nitrogen (Bowman 2015; Garrity et al. 2015; Kuykendall 2015; Dedysh and Dunfield 2016).

The most important aspect of lichen bacteriology is identification of the functional role of lichenophilic prokaryotes that form microcolonies or micro-biofilms in thalli. A study of the genomes of two lichen bacteriobionts, *Lichenibacterium ramalinae* and *L. minus*, revealed several families of glycoside hydrolases whose activity is phenotypically manifested as the ability to degrade hemicelluloses and starch, as well as laminarin (Pankratov et al. 2020a). Meanwhile, *L. psoromatis* is capable of utilizing a number of carboxylic acids, including succinic, gluconic and lactic acids, the products of metabolic activity of yeast and fungi (Noh et al. 2019).

These findings suggest that bacteriobionts of the lichen cluster LAR1 can be considered as a link in the maintenance of carbon balance in lichen thalli. In this context, the discovery of new bacteria belonging to the LAR1 cluster could broaden our understanding of the role of this group in lichen symbioses. We attempted to find new members of this group in specimens of *Flavocetraria* lichens. Similar to *Cladonia*, these lichens are widely distributed in temperate and Arctic zones of the northern and southern hemispheres and generate significant biomass reserves due to the rapid growth of their thalli (Cornelissen et al. 2007; Nash 2008). An analysis of microbial communities of lichens of the genus *Cetraria* was previously performed by Printzen et al. (2012) and Klarenberg et al. (2020) and it

was shown that the second dominant alphaproteobacterial group after the order *Rhodospirillales* were representatives of the order *Hyphomicrobiales*. The microbial communities of lichens of the genus *Flavocetraria* have been studied previously by pyrosequencing. Sequences belonging to the LAR1 group (about 2–10% of all sequences) were found in two lichen samples of this genus (Hodkinson et al. 2012). In our current study, a novel organism belonging to the order *Hyphomicrobiales* within the phylogenetic cluster LAR1 was isolated and characterized from the lichen thallus of *Flavocetraria nivalis*.

Materials and methods

Isolation and cultivation conditions

Samples of *Flavocetraria nivalis* (L.) Kärnefelt & Thell thalli were collected from the rocky soil of the circumpolar tundra (shore of Providence Bay, 64°25′09.9 "N 173°14′51.8 "E) in 2021 during voyage of the frigate *Pallada* dedicated to the 280th anniversary of Vitus Bering's expedition. After sampling, the lichen samples were stored in sterile paper bags in air-dry state for 2 weeks at + 10–15 °C until they were delivered to the laboratory. The thalli fragments (200 mg) were cut with scissors and homogenized using an ULTRA-TURRAX® Tube Drive homogenizer with DT-20 tube (rotor–stator element, volume 5–15 ml) in 10 ml of MM2 medium containing (g l^{-1}, distilled water): KH_2PO_4 (0.2); $MgSO_4 \cdot 7H_2O$ (0.08); $Ca(NO_3)_2 \cdot 5H_2O$ (0.04); peptone (0.1); tryptone soy broth (0.1); trace elements (μg l^{-1}: $NiCl_2 \cdot 6H_2O$, 200; $CoCl_2 \cdot 2H_2O$, 100; $ZnSO_4 \cdot 7H_2O$, 20; $Na_2MoO_4 \cdot 2H_2O$, 20). The treatment duration was 5 min, speed 6000 rpm. The suspension was diluted 2-, 10-, and 100-fold and used for plating on MM2 medium, to which glucose, rhamnose, maltose, inulin, dulcitol, and galactose were added as carbon sources as a mixed solution (0.005%, w/v each component). The pH was 5.0–5.5. The solidifying agent used for the medium preparation was Phytagel™ (10 g l^{-1}). The duration of incubation at 15 °C was 1 month. Then individual colonies were picked up and tested for contamination by light microscopy. Separation of bacteria in commingled colonies was performed by plating the dilutions of bacterial suspensions. The obtained pure cultures were maintained on MM2 medium with the addition of mannitol, galactose, and glucose (0.025% w/v). All further experiments were done using the same mineral medium; since mannitol and galactose (0.5 g l^{-1}) provided the highest growth rate of strain BP6-180914 ^{T}, they were used as carbon sources for the subsequent experiments. The pure culture suspensions were placed in cryotubes containing

700 μl of 35% glycerol in MM2 medium and further stored at -18 °C and in liquid nitrogen (− 196 °C).

16S rRNA gene phylogeny

Genomic DNA was extracted using the DNeasy PowerSoil kit (Qiagen) according to the manufacturer's instructions. The 16S rRNA gene was amplified from genomic DNA by PCR with the primers 27F and 1492R (Lane 1991). The PCR products were purified using the Wizard PCR Preps kit (Promega) as recommended by the manufacturer. The 16S rRNA genes were sequenced in both directions with the primers 27F, 357F, 1114F and 519R (Lane 1991) by means of Big Dye Terminator v.3.1 (Applied Biosystems) according to the manufacturer's instructions made for the ABI PRISM 3730 sequencer (Applied Biosystems). The 16S rRNA gene sequences were aligned using MUSCLE (Edgar 2004). Evolutionary history was inferred by maximum likelihood and the Tamura-Nei model (Tamura, Nei 1993) using MEGA X program (Kumar et al. 2018). Branch supports were obtained with 1000 standard nonparametric bootstraps.

The nearly complete and complete (genome-extracted) 16S rRNA gene sequences of strain BP6-180914[T] have been deposited in GenBank under accession numbers OP023140 and OQ625528, respectively.

Genome features

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 the SPAdes version 3.11.0 using the default settings (Bankevich et al. 2012). Annotations of the scaffolds was performed 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 laboratory (http://enve-omics.ce.gatech.edu/ani) (Goris et al. 2007) and the GGDC (http://ggdc.dsmz.de/ggdc.php) (Meier-Kolthoff et al. 2022), respectively. The AAI calculator (http://enve-omics.ce.gatech.edu/aai/) was used for the calculation of the AAI values (Rodriguez-R and Konstantinidis 2014).

Core gene sequences were extracted from the whole genome of isolate BP6-180914[T] and strains of the order *Rhizobiales* were extracted using the USEARCH program (Edgar 2010) with a 50% sequence identity cut-off within the BPGA (Chaudhari et al. 2016). Multiple sequence alignment

of the concatenated 120 ubiquitous single-copy proteins with those of closely related species in the order *Hyphomicrobiales* was performed using GTDB-Tk (Chaumeil et al. 2020). Phylogenomic trees were reconstructed using the MEGA X program (Kumar et al. 2018). The robustness of the tree topologies was assessed by bootstrap analyses of 1000 replications.

Initial genome annotation was performed using the RAST server (Aziz et al. 2008). The NCBI automated annotation (assembly number GCA_026130545) was also used for genome analysis.

Draft genome of strain BP6-180914[T] was deposited in GenBank under the accession number JAMOIM000000000.

Morphology and physiology

Growth of the novel strain under a variety of conditions, including temperatures of 2, 4, 9, 15, 18, 20, 22, 25, 28, 34, 37, 42, 44 °C and pH 2.0–10.0 (in increments of 0.5), was examined using batch cultures grown in liquid MM2 medium supplemented with mannitol and galactose (both 0.5 g l^{-1}), in small volumes (3–4 ml in standard 15 ml tubes). The OD_{600} was measured on a spectrophotometer (Jenway 7315) after ten days of incubation at 24 °C. The range of potential growth substrates of strain BP6-180914[T] was examined by replacing mannitol and galactose in medium MM2 with respective carbon sources (1 g l^{-1}). The negative control was MM2 medium without a carbon source. Na$^+$ ion sensitivity tests were performed under aerobic conditions in liquid MM2 medium supplemented with mannitol and galactose. NaCl concentrations ranging from 0 to 1% were tested.

Growth on polysaccharides was assessed by gas chromatography. A solution or suspension of polysaccharide in sugar-free MM2 medium (10 ml at 0.4 mg/ml) was dispensed into 100-ml serum vials. After sealing the vials with rubber stoppers and aluminum caps, the medium was inoculated until the relative optical density reached 0.05. The rate of respiration was assessed by the increase in CO_2 concentration compared with a blank test (medium without substrate) and a positive control with mannose as a carbon source.

Morphological observations and cell size measurements were performed using a Zeiss Axio Imager 2 microscope and Axiovision 4.2 software (Zeiss, Germany). Type of fission was observed using light microscopy. For preparation of ultrathin sections, cells of an exponentially growing culture were collected by centrifugation and pre-fixed with 2.5% (w/v) glutaraldehyde in 0.05 M cacodylate buffer (0.05 M sodium cacodylate solution, pH 7.0–7.5) for 1 h at 4 °C and then fixed with 1% (w/v) OsO4 in the same buffer for 4 h at 4 °C. After fixation, the samples were embedded in 2% agar–agar, stained with a 3% uranyl acetate solution in 30% ethanol for 4 h, and dehydrated in 70% ethanol for 12 h at
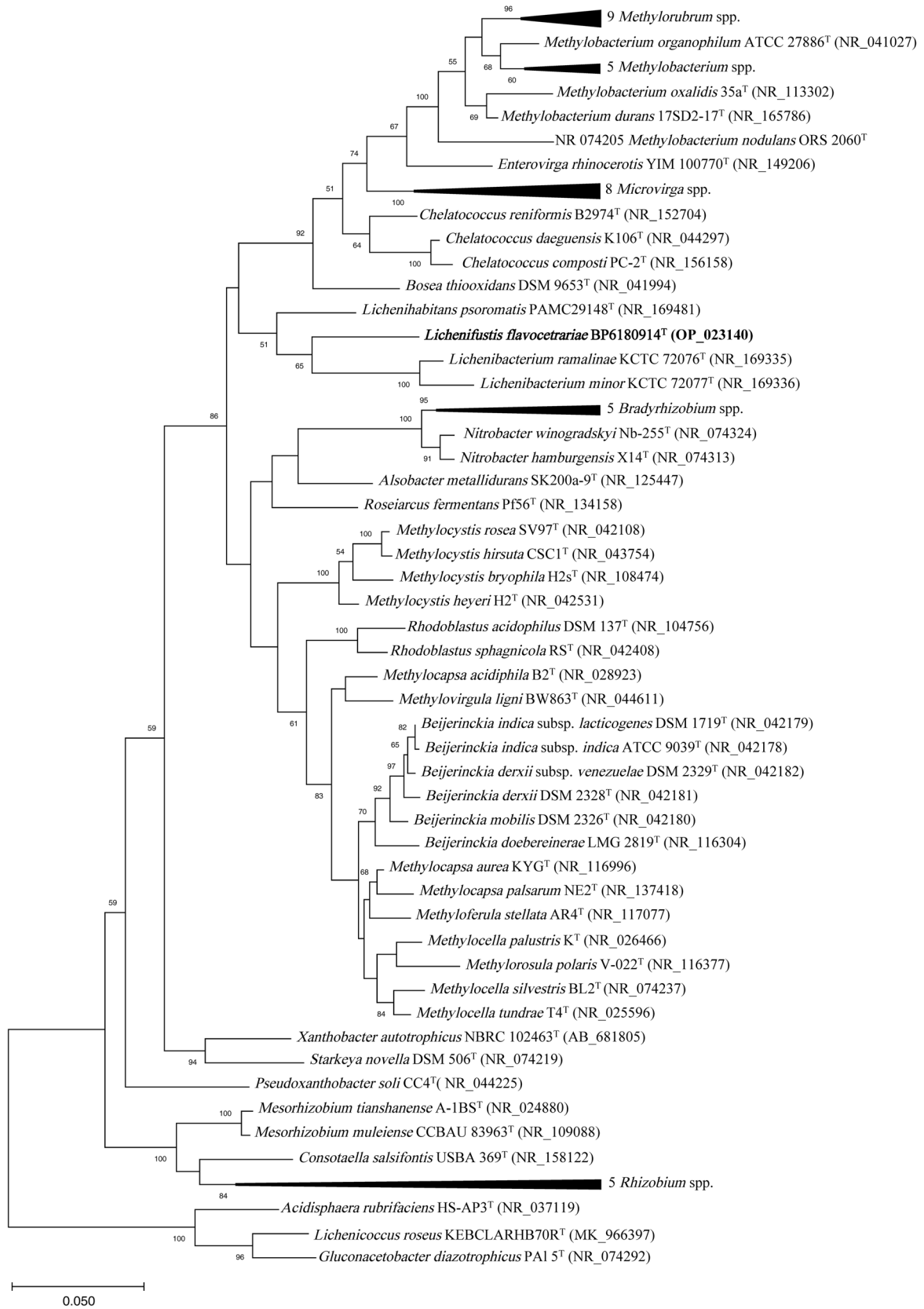
◄**Fig. 1** Maximum-likelihood phylogenetic trees based on 16S rRNA gene sequences (1403 nucleotide sites) reconstructed with Tamura-Nei evolutionary model showing the phylogenetic position of strain BP6-180914 [T] in relation to taxonomically characterized members of the order *Hyphomicrobiales*. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Numbers before genus names indicate numbers of type strains included in the clusters. Bootstrap values (>50%) based on 1000 resamplings are showing at the branch. Sequences of *Lichenicoccus roseus* strain KEBCLAR70R[T](GenBank accession no. MK_966397), *Acidisphaera rubrifaciens* strain HS-AP3[T] (NR_037119) and *Gluconacetobacter diazotrophicus* PAl 5 [T] (NR_074292) were used as an outgroup. Bar, 0.050 substitutions per nucleotide position

4 °C, 96% ethanol (2 times for 15 min), and in absolute acetone (3 times for 10 min). The samples were impregnated with EPON-812 (Epoxy Embedding Medium Epon® 812, "Sigma-Aldrich", USA) keeping in a mixture of epoxy: acetone at a ratio of 1: 1 (1 h), then in a mixture of epoxy: acetone in a ratio of 2: 1 (1 h). The resulting material was dispensed into capsules with epoxy and polymerized at 37 °C for 1 day, then at 60 °C for 1 day. Ultrathin sections were obtained on an LKB-III microtome ("LKB", Sweden) and contrasted in an aqueous solution of 3% uranyl acetate (30 min), then in an aqueous solution of 4% lead citrate (30 min). The obtained preparations were analyzed using a JEM 100CXII electron microscope ("Jeol", Japan) at an accelerating voltage of 80 kV and a working magnification of 5000–50,000×. Photo documentation of the materials was carried out using a Morada G2 digital optical image output system.

The enzymatic profile was examined using MIKRO-LA-TEST® (Erba Lachema, Czech Republic) and API Zym (Biomerieux, France). The tests for microaerobic growth, urease, β-galactosidase and oxidase activity, acetoin production, indole production from tryptophan, and ability to hydrolyze gelatin and Gram staining were performed according to Manual of Methods for General Bacteriology (1981). Nitrate reduction and ketone formation were determined using the LACHEMA DECAFAN express test. Catalase activity was detected by the formation of gas bubbles after dropping 3% (w/v) H_2O_2 onto a fresh culture grown on MM2 agar with mannitol and galactose. The ability to grow under anaerobic conditions was tested in Hungate tubes under nitrogen.

Chemotaxonomic characterization

The fatty acid composition was analyzed using a gas chromatograph-mass spectrometer 7890B + 5977B (Agilent Technologies, USA). The cell biomass was dried, saponificated (3.75 M NaOH/MeOH, 100 °C, 30 min), and subjected to acidic methanolysis (6 N HCl/MeOH, 80 °C, 10 min). The products of methanolysis were extracted with hexane: methyl tert-butyl ether (1: 1 w/w) and processed with alkali

(0.3 M NaOH, 5 min). The obtained products were separated on a 5% phenyl-methyl silicone capillary column HP-5MS (0.25 mm × 30 m) in the temperature gradient from 45 to 300 °C at 40 °C min⁻¹. Fatty acids and other lipid components were ionized by electron impact and analyzed in the scan mode. The compounds were identified using the NIST17 mass spectrometer library. Fatty acid content was determined as the percentage of the total ion current peak area.

Preparation of the polar lipid extract was performed as described previously (Minnikin et al. 1984). After separation of the phases, the chloroform layer was collected and dried by passing it through anhydrous sodium sulfate, evaporated at a rotary evaporator, and dried to constant weight. The resulting residue was dissolved in chloroform: methanol (2: 1) and stored at – 21 ºC. Lipids were separated by two-dimensional TLC on TLC Silica gel 60 glass plates (Merck), in a solvent system chloroform: methanol: water (65: 25: 4)—first direction; chloroform: acetone: methanol: acetic acid: water (50: 20: 10: 10: 5)—second direction (Benning et al. 1995). The lipids (125 μg) were applied to the plate. The components were visualized by spraying the chromatograms with 5% sulfuric acid in ethanol followed by heating for 15 min at 180 ºC (total polar lipid composition). Reactions with Vaskovsky's universal reagent (a modification of molybdenum blue, Dittmer-Lester reagent, as described by Vaskovsky et al. (1975) (for phospholipids); ninhydrin (for lipids containing an amino group); α-naphthol (for glycolipids); and Dragendorf's reagent (for choline), as described by Kates (1972), as well as for individual standard compounds (if required) were carried out.

The pigments were extracted with a mixture of acetone and methanol (3:1 vol/vol; 2 ml) from lyophilized bacterial biomass (~ 50 mg) for 24 h at 5 °C. Pigments were identified by spectroscopy. The primary identification of pigments was carried out using a scanning spectrophotometer Cary100 (Agilent Technologies, USA) (slit 1 nm) in the range from 200 to 900 nm. Quinones were identified by the method described previously (Pankratov et al. 2007).

Results and discussion

Phylogenetic and phylogenomic analysis

Comparative 16S rRNA gene sequence analyses positioned this strain in the family *Lichenihabitantaceae* as a monophyletic clade with *L. psoromatis* (Fig. 1). Based on 16S rRNA gene sequences, strain BP6-180914 [T] was determined to share the highest pairwise similarity with *Lichenihabitans psoromatis* PAMC 29148 [T] (92.7%), *Lichenibacterium ramalinae* RmlP001[T] (92.08%), *Lichenibacterium minus* RmlP026[T] (90.98%), *Beijerinckia doebereinerae* LMG

2819 [T] (93.19%), *Beijerinckia derxii* subsp. *venezuelae* DSM 2329 [T] (92.73%), *Beijerinckia derxii* ATCC 49361 [T] (92.77%), *Methylocystis heyeri* H2[T] (92.96%), and *Methylocystis rosea* SV97[T] (92.08%) (Supplementary Table 1).

Analysis of the relative phylogenetic distance between the 16S rRNA gene sequences of strain BP6-180914 [T] and strains belonging to the families *Lichenibacteriaceae* and *Lichenihabitantaceae* showed its distinct position. The level of homology to taxa with validly described names within the families *Beijerinckiaceae, Rhizobiaceae, Chelatococcaceae, Roseiarcaceae*, and *Methylobacteriaceae* was lower than 93.19% (Fig. 1; Supplementary Table 1).

Analysis of core genes showed the smallest phylogenetic distance between the new isolate and representatives of the families *Lichenibacteriaceae* and *Lichenihabitantaceae* (Fig. 2). In accordance with this analysis, strain BP6-180914 [T] and *L. psoromatis* PAMC 29148 [T] form a cluster that is clearly separated from the other branches represented by typical species of the closely related families in the order *Hyphomicrobiales*. The distance characterizes these two strains as belonging to different genera within the same family *Lichenihabitantaceae*.

Genome characteristics

A total of 3,429,630 reads were obtained from strain BP6-180914 [T]. The final assembled genome (7,523,782 bp long) comprised 181 scaffolds, with an N_{50} value of 180,878 bp, an average coverage of 118× and a G+C content of 63.65%, in which were identified 7,287 genes, 7037 coding sequences, and 48 tRNA genes.

The average amino acid identity (AAI), ortho average nucleotide identity (ANIu) and digital DNA-DNA hybridization (dDDH) values between strain BP6-180914 [T] and its relatives are shown in Supplementary Tables 2, 3, and 4, respectively. The ANI values turned out to be in the range 68.51–74.05% for the compared strains. The AAI values between strain BP6-180914 [T] and two strains of the genus *Lichenibacterium*, *L. minus* RmlP026[T] and *L. ramalinae* RmlP001[T], were 61.60% and 62.93%, respectively, and these values indicated that strain BP6-180914 [T] belonged to the same family according to the criteria for uncultured bacteria and archaea (Konstantinidis et al. 2017). The AAI between strain BP6-180914 [T] and *L. psoromatis* PAMC 29148 [T] was 65.88%, which was higher than the recommended limit of 65% for representatives of different genera (Konstantinidis et al. 2017). Nevertheless, based on the 16S rRNA gene phylogeny (Fig. 1) and phylogenomic distance (Fig. 2), as well as the number of phenotypic differences, strain BP6-180914 [T] must be assigned to the family *Lichenihabitantaceae*.

Genomic characteristics suggest that potentially, strain BP6-180914 [T] can use both readily available organic molecules (heterotrophic mode of nutrition) and carbon dioxide (autotrophic type of nutrition). Central carbohydrate metabolism of strain BP6-180914 [T] is supported with 165 enzymes, including the enzymes of methylglyoxal metabolism, pyruvate metabolism II (acetyl-CoA, acetogenesis from pyruvate), glyoxylate bypass, glycolysis and gluconeogenesis, Entner–Doudoroff pathway, tricarboxylic acid (TCA) cycle, pyruvate metabolism I (anaplerotic reactions, PEP), and pentose phosphate pathway. Carbon dioxide fixation is potentially provided by a full set (eleven) enzymes of the Calvin–Benson–Bassham cycle, including three key enzymes: RuBisCO (both small and large chains), phosphoribulokinase and fructose-1,6-bisphosphatase.

In addition, the presence of genes encoding the light harvesting complex I proteins (alpha and beta subunits), the photosynthetic reaction center of type II photosystem (*puf*L and *puf*M), cytochrome c (*puf*C), as well as the genes encoding chlorophyll synthesis (chlorophyll synthesis pathway protein BchC, 2-vinyl bacteriochlorophyllide hydratase, 3-vinyl bacteriochlorophyllide hydratase, ferredoxin:protochlorophyllide reductase, 3,8-divinyl chlorophyllide *a*/chlorophyllide *a* reductases – *bch*X, *bch*Y, *bch*Z, and chlorophyll synthesis pathway protein BchC) suggest the potential for light-dependent carbon dioxide fixation. Phylogenetic analysis of the evolution of the *puf*L gene showed that the highest level of homology is evident with respect to the *puf*L gene of the anoxygenic phototroph *Rhodospirillum centenum*, but it forms an independent branch (Supplementary Fig. 1). Two *cbb*L gene sequences and one *cbb*M gene sequence were found in the genome of strain BP6-180914 [T]. Phylogenetic analysis showed that one of the *cbb*L genes belongs to type I, and is close to the typical representatives of this RuBisCO type, *Acidisphaera rubrifaciens*, *Rhodospirillum centenum*, and others (Supplementary Fig. 2). The second gene encodes a ribulose bisphosphate carboxylase like enzyme of RuBisCO type IV and is close to those previously isolated from lichen species of the genera *Lichenibacterium* and *Lichenicoccus* (Supplementary Fig. 2).

The pathways for nitrogen metabolism are presented by nitrate and nitrite ammonification subsystem and ammonia assimilation subsystem. No genes encoding nitrogenase have been found. Genomic analysis of strain BP6-180914 [T] confirmed the presence of glycoside hydrolases belonging to five families, namely, glycoside hydrolases of families 15 (glucoamylase; EC 3.2.1.3), 16 (β-1,4 or β-1,3 glycosidases; EC 3.2.1.103, 3.2.1.39, 3.2.1.73 etc.), 25 (β-1,4-N-acetyl- and β-1,4-N,6-O-diacetyl muramidase; EC 3.2.1.17), 37 (α,α-trehalase; EC 3.2.1.28), and 127 (β-L-arabinofuranosidase; EC 3.2.1.185). The detected ability to hydrolyze a number of polysaccharides in vitro
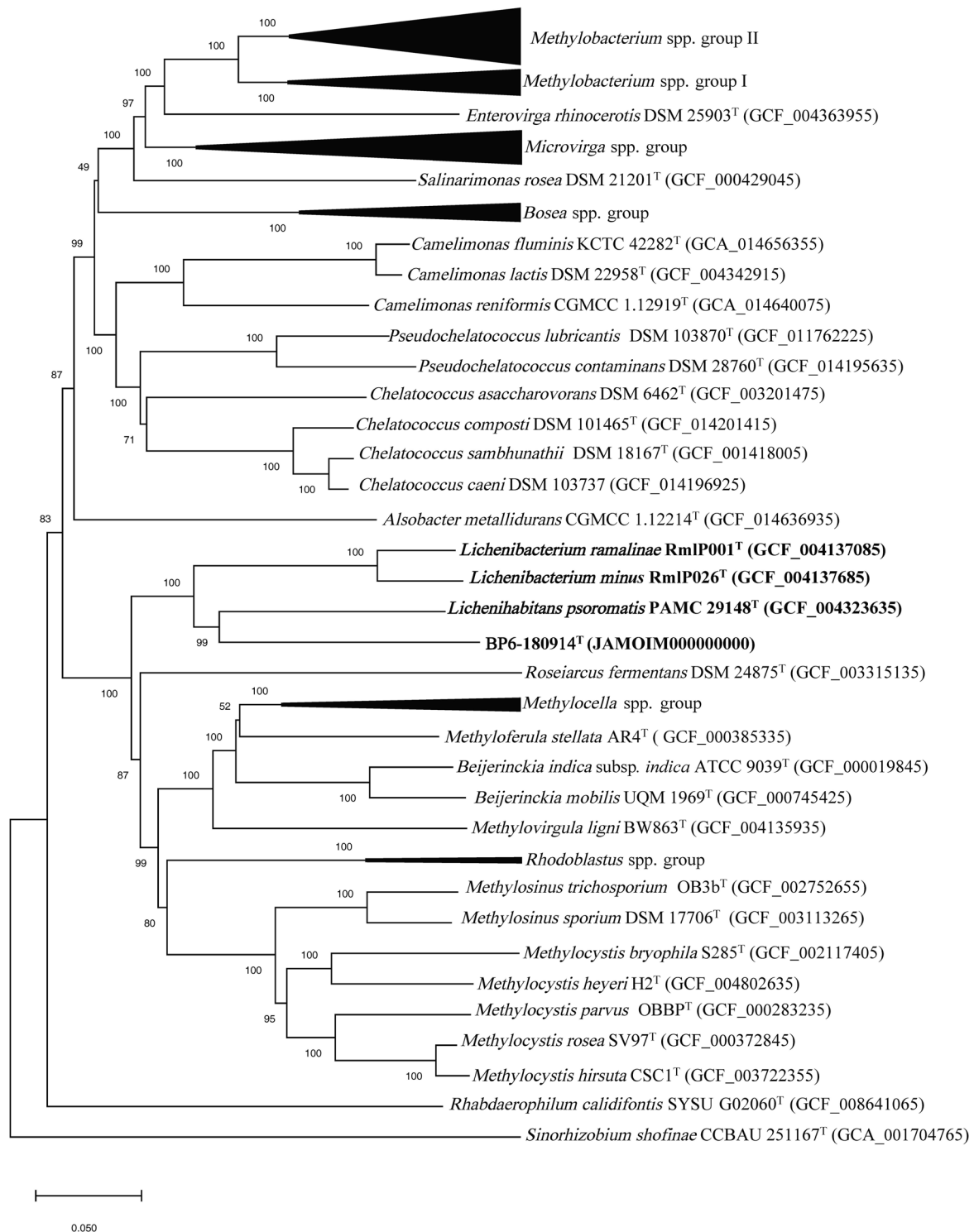
Fig. 2 Maximum-likelihood phylogeny inferred from concatenated alignments of 120 single-copy amino acid sequences retrieved from genomes of type strains of the order *Hyphomicrobiales* identified by the Genome Taxonomy Data Base

confirms the capability of expression of these genes in the presence of specific substrates.

Morphology and physiological characteristics

Strain BP6-180914 ^{T} grew better on solid medium containing Phytagel™ than agar as a solidifying agent.

Pale pink colonies were obtained on solid medium with Phytagel™ from the homogenized lichen thallus of *Flavocetraria nivalis*. The size of the colonies was 1–3 mm. They were slightly submerged in the solid medium. The colonies were rounded, smooth, slightly raised above the surface, without 'lenses', smooth and of a firm, gelatinous consistency.

Bacterial cells were large (1.2–1.6 × 1.5–8 µm), straight or irregularly and disproportionately curved, with dark inclusions (Fig. 3a, b). Life-cycle analysis of strain BP6-180914[T] carried out by light and electron microscopy showed that the cells did not form endospores or exospores. In aging cultures, the cells became pleomorphic and could lose their original shape; the structure of the cytoplasm also changed. The cells were nonmotile at all stages of culture growth. Electron microscopy of cell sections of strain BP6-180914[T] showed a Gram-negative cell wall (Gram staining confirmed the electron microscopy data) and unidentified granules (Fig. 3b).
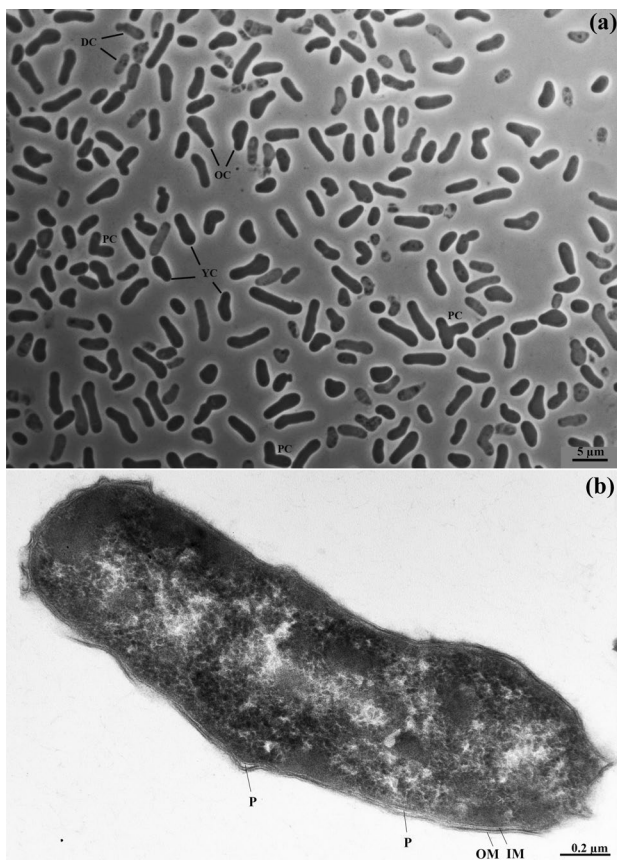
Fig. 3 Morphological observation of the cells of strain BP6-180914[T]. Phase contrast microscopic image **a** shows four types of cells: young (YC), old (OC), dead (DC) and pleomorphic (PC); bar, 5 µm. Electron micrograph of an ultrathin section **b** showing the gram-negative type of the cell wall; *OM* outer membrane, *IM* inner membrane, *P* periplasm, bar, 0.2 µm

The cells also showed internal membranes, vesicles surrounded by a double membrane, and other membrane structures (Supplementary Fig. 3a, b, c). Vesicles localized in the bacterial cytoplasm and surrounded by a double membrane were especially visible in dead cells (Supplementary Fig. 3c, light insert).

Morphologically, strain BP6-180914[T] differs from *L. psoromatis* PAMC 29148[T] by its longer (3.4 µm vs. 8 µm) pleomorphic cells, the presence of a dense (rather than pourable as in *L. psoromatis*) polysaccharide matrix, and the color of the colony (slightly pink or beige vs. pink-colored).

Under aerobic conditions strain BP6-180914[T] was able to grow on sugars, sugar alcohols and some polysaccharides (Supplementary Table 5). The best culture growth was induced when mannitol, L-arabinose, D-mannose, D-galactose, D-glucose, D-fructose, or sodium malate were used as carbon sources. Good growth was observed on sorbitol, inositol, adonitol, erythritol, arabitol, D-xylose, D-maltose, sodium pyruvate, and melibiose. A slight optical density increase was observed when growing on ethanol, D-lactose, trehalose, raffinose, *iso*-propanol, and amygdalin. N-acetyl glucosamine, D-cellobiose, methanol, sodium acetate, sodium butyrate, sodium citrate, sodium propionate, sodium glucuronate, sodium oxalate, sodium succinate were not used by strain BP6-180914[T]. Among the polysaccharides tested (inulin, starch, Phytagel™, chitin, carboxymethylcellulose, sodium alginate, xylan and pectin), starch, Phytagel™ and pectin were able to support the growth activity of strain BP6-180914[T]. Yeast extract, casamino acids, peptone, ammonium, and nitrate, as well as L-arginine, DL-lysine, and L-alpha-alanine were the sources of nitrogen. No growth occurred when the only sources of carbon and nitrogen were yeast extract, casamino acid, peptone, DL-threonine, L-histidine hydrochloride, DL-serine, DL-phenyl-β-alanine, DL-methionine, DL-aspartic acid, DL-cysteine hydrochloride monohydrate or DL-tryptophan. L-arginine, DL-lysine, L-alpha-alanine could be the only sources of nitrogen and carbon.

The profile of enzyme activities of strain BP6-180914[T] was characterized by the presence of phosphatase, β-glucosidase, *N*-acetyl-*β*-D-glucosaminidase, and urease. The tests for α-galactosidase, β-galactosidase and *γ*-glutamyltransferase, as well as acetamide and Simmons' citrate were negative (Table 1). Strain BP6-180914[T] was cytochrome oxidase-negative and catalase-positive. Protease test (gelatin liquefaction) and growth under anoxic conditions were negative. In relation to parameters such as salinity (up to 4 g l^{-1} of NaCl; optimum 0 g l^{-1}), pH (4.0–7.0; optimum 4.5–5.5), growth temperature range (4–35 °C; optimum 20–28 °C), the strain showed moderate values specific for psychrotolerant, moderately acidophilic microorganisms, and was confined to an environment with low or medium salinity (Table 1).

Table 1 The main characteristics distinguishing the four strains on the basis of morphological, physiological signs

Characteristic	1	2	3	4
Isolation source	Lichen *Flavocetraria nivalis*	Lichen *Psoroma antarcticum*	Lichen *Ramalina pollinaria*	Lichen *Ramalina pollinaria*
Colony diameter (mm)	1–3	ND	1.5–2.0	0.6–1.5
Colony pigmentation	Pale pink, creamy	Pink	Pale orange	Bright orange
Cell width (μm)	1.2–1.6	0.9–1.6	1.0–1.2	1.2–1.5
Cell length (μm)	1.5–8	0.9–3.4	2–4	2–8
Growth temperature (°C)				
Range	4–35	4–20	10–30	10–30
Optimum	20–28	15	23–28	20–26
pH range	4.0–7.5	5.5–7.0	4–8	3.5–8
pH optimum	4.5–5.5	6.5	5.6–6.4	5.0–6.0
NaCl tolerance (g l^{-1})	< 4	0	< 3.5	< 3.5
Fermentative reactions				
Alkaline phosphatase	+	+	−	+
Valine arylamidase	+	+	w	+
Carbon substrates utilization				
Adonitol	+	+	w	+
Arabitol	+	+	+	w
Dulcitol	−	+	+	−
Ethanol	w	ND	w	+
Melibiose	+	ND	+	w
Na-succinate	−	+	+	w
Na-malate	+	+	−	−
Na-pyruvate	+	+	+	+
Trehalose	−	ND	+	+
Rhamnose	+	+	−	+
N-acetyl glucosamine	−	ND	+	+
Starch	+	ND	+	+
Phytagel™	+	ND	w	w
Pectin	+	ND	+	−
Growth on nitrogen sources:				
DL-proline	−	ND	−	+
Asparagine	−	ND	−	+
L-arginine	+	ND	−	−
Tryptophan	−	ND	w	+

1, BP6-180914[T]; 2, *L. psoromatis* PAMC 29148[T] (data from Noh et al. 2019); 3, *L. ramalinae* RmlP001[T] (data from Pankratov et al. 2020a); 4, *L. minus* RmlP026[T] (data from Pankratov et al. 2020a)

+ positive, − negative, *w* weak, *ND* no data available.

Unlike *L. psoromatis* PAMC 29148[T], strain BP6-180914[T] does not grow on dulcitol and Na-succinate and is a more acidophilic and less psychrophilic microorganism. In addition, it is a strict aerobe, whereas *L. psoromatis* is a facultative anaerobe. Also, strain BP6-180914[T] is more halotolerant than *L. psoromatis* PAMC 29148[T] (Table 1).

Chemotaxonomic characterization

The major fatty acids of strain BP6-180914[T] were palmitic acid (C$_{16:0}$), stearic acid (C$_{18:0}$), vaccenic (C$_{18:1}ω7c$), and cyclo-nonadecanoic acids (C$_{19:0}$ *cyclo*) (Table 2). The strain had the fatty acid profile of cell membranes which differed from that of the nearest related organisms, i.e. *Lichenihabitans psoromatis* PAMC 29148[T], *Lichenibacterium ramalinae* RmlP001[T], *L. minus* RmlP006[T] by larger content of C$_{19:0}$

Table 2 Cellular fatty acid composition (%)

Fatty acid type	1	2	3	4
Saturated				
$C_{12:0}$	0	4.4	0	0
$C_{14:0}$	0	0.8	0.2	0.4
$C_{15:0}$	0	0	0	0
$C_{16:0}$	12.1	3.8	15.7	15.3
$C_{17:0}$	0	0	0	0
$C_{18:0}$	9.7	4.7	0.6	0.6
Unsaturated				
$C_{14:1}\omega5c$	0	0	0	0
$C_{15:1}\omega6c$	0	0	0	0
$C_{16:1}\omega7c$	4.2	25.2 SF3	2.5	2.8
$C_{17:1}\omega8c$	0	0	0	0
$C_{18:1}\omega7c$	44.6	39.7 SF8	71.2	68.8
$C_{18:1}\omega9c$	0	0	0	0
$C_{18:1}\omega5c$	0	0	0	0
$C_{18:1}\omega11$	0	0	0	0
$C_{19:0}$ cyclo	29.4	11.9	7.4	9.9
$C_{20:2}\omega6,9c$	0	0	0	0
Methyl-branched				
Anteiso-$C_{15:0}$	0	0	0	0
Iso-$C_{16:0}$	0	0	0	0
Trans-$C_{18:1}\omega7c$	0	3.3	0	0
Hydroxy				
$C_{14:0}$ 3OH	0	2.9 SF2	0.2	0.2
$C_{17:0}$ 3OH	0	0	0	0
$C_{18:0}$ 3OH	0	3.34	0	0

Strains: 1, BP6-180914 [T]; 2, *Lichenihabitans psoromatis* PAMC 29148 [T] (data from Noh et al. 2019); 3, *Lichenibacterium ramalinae* RmlP001[T] (data from Pankratov et al. 2020a); 4, *Lichenibacterium minus* RmlP026[T] (data from Pankratov et al. 2020a). SF—as a part of summed feature

cyclo (29.42% against 11.9, 7.4, and 9.9% respectively) and $C_{18:0}$ (9.7% against 4.7, 0.62, and 0.58% respectively).

Polar lipid profiles of strain BP6-180914 [T] contained diphosphatidylglycerols (DPG), phosphatidylglycerols (PG), phosphatidylethanolamines (PE), phosphatidylcholines (PC), unidentified aminolipids (AL1), and a number of glycolipids, the major one being an unidentified glycolipid GL2 (Supplementary Fig. 4). PG, PE, and PC were common in strains of *Lichenibacterium* and strain BP6-180914 [T]. However, unlike strains of the genus *Lichenibacterium*, strain BP6-180914 [T] was found to have diphosphatidylglycerols (DPG) and unidentified aminolipids (AL1). However, strain BP6-180914 [T] lacked cardiolipin (diphosphatidylglycerol) and phosphatidic acid (PA).

Spectral analysis of cell extracts showed the presence of carotenoids and bacteriochlorophyll *a*. (Supplementary Fig. 5). This trait distinguishes strain BP6-180914 [T] from *L. psoromatis*, which does not synthesize bacteriochlorophyll *a*. The main and only quinone was ubiquinone (Q-10), which is typical of most members of the order *Hyphomicrobiales*.

Conclusions

Strain BP6-180914 T is characterized by the presence of genes encoding photosystem II proteins, bacteriochlorophyll a synthesis protein and genes determining the expression of Calvin-Benson- Bassham cycle enzymes. This suggests that it is an anoxygenic phototroph. An anoxygenic phototroph previously isolated from lichens (Pankratov et al. 2020b) belonged to the order *Rhodospirillales*, whereas the new strain belonged to the order *Hyphomicrobiales*.

The 16S rRNA gene sequence revealed that strain BP6-180914 [T] belongs to the order *Hyphomicrobiales*. Although the phylogenetic distance (according to the 16S rRNA gene) between strain BP6-180914 [T] and members of the families *Beijerinckiaceae*, *Lichenibacteriaceae* and *Lichenihabitantaceae* is less than 93.2%, which would allow the identification of this culture as belonging to the same family according to the criteria for uncultivated bacteria and archaea, the data from the comparative analysis of the main amino acid sequences of the genome within the order *Hyphomicrobiales* (Fig. 2) and the AAI analysis (Supplementary Table 2), as well as the number of phenotypic characteristics, allow us to describe strain BP6-180914 [T] as a novel genus in the family *Lichenihabitantaceae*. Here we propose a novel genus and species within the family *Lichenihabitantaceae*, *Lichenifustis flavocetrariae*, with type strain BP6-180914 [T].

Description of *Lichenifustis* gen. nov.

Lichenifustis (Li.che.ni.fus'tis. L. masc. n. *lichen*, a lichen; L. masc. n. *fustis*, stick; N.L. masc. n. *Lichenifustis*, a lichen-inhabiting rod-shaped bacterium).

It is Gram-negative, non-spore-forming, nonmotile rods that occur singly. On aging, cells become pleomorphic and cytologically heterogeneous. Colonies are small (1–3 mm in diameter), convex, circular, opaque. The colony color on medium MM2 varies from creamy to pale pink. Oxidase-negative and catalase-positive. They are chemo-organotrophic aerobes. Sugars and sugar alcohols are the preferred growth substrates. *Lichenifustis* is unable to ferment glucose and sucrose. *Lichenifustis* cannot utilize cellulose, chitin, methanol, methane, acetate; does not produce H_2S from thiosulfate or peptone and indole from tryptophan.

Lichenifustis are capable of growth at pH 4.0–7.5 and at 4–35 °C. The polar lipids consist of diphosphatidylglycerols, phosphatidylglycerols, phosphatidylethanolamines, phosphatidylcholines, unidentified aminolipids. Major fatty acids are $C_{18:1\omega7c}$ and $C_{19:0}$ *cyclo*; the quinone is Q-10. Member of the family *Lichenihabitantaceae*. Strain has been isolated from lichen *Flavocetraria nivalis*. The type species is *Lichenifustis flavocetrariae*.

Description of *Lichenifustis flavocetrariae* sp. nov.

*Lichenifustis flavocetrariae (*fla.vo.ce.tra'ri.ae N.L. gen. n. *flavocetrariae*, of the lichen *Flavocetraria*).

The description is as for the genus but with the following additional traits. Cells are 1.2–1.6 µm wide and 1.5–8 µm long. Colony color is light orange to pale pink. Carbon sources (0.1%, w/v) utilized include mannitol, L-arabinose, D-mannose, D-galactose, D-glucose, D-fructose, sodium malate, sorbitol, inositol, adonitol, erythritol, arabitol, D-xylose, D-maltose, sodium pyruvate and melibiose. Weak growth on ethanol, D-lactose, trehalose, sucrose, raffinose, iso-propanol, amygdalin. Does not utilize N-acetyl glucosamine, D-cellobiose, methanol, sodium acetate, sodium butyrate, sodium citrate, sodium propionate, sodium glucuronate, sodium oxalate, sodium succinate. Starch, pectin, and Phytagel™ are able to support growth, but not inulin, chitin, carboxymethylcellulose, sodium alginate, and xylans. Nitrogen sources utilized are ammonium, casamino acids, peptone, yeast extract, L-arginine, DL-lysine, L-alpha-alanine but not nitrate, nitrite, DL-threonine, L-histidine hydrochloride, DL-serine, DL-phenyl-β-alanine, DL-methionine, DL-aspartic acid, DL-Cysteine hydrochloride monohydrate or DL-tryptophan. The following enzyme activities are present: alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *β*-galactosidase, *β*-glucosidase, N-acetyl-*β*-D-glucosaminidase, *α*-mannosidase, *α*-fucosidase, urease. Lipase, cystine arylamidase, *α*-chymotrypsin, *α*-galactosidase, *β*-glucuronidase, *α*-glucosidase (API ZYM test) are negative. Oxidase and indol negative. Reduces nitrate to nitrite. Catalase activity is positive. Capable of growth at pH 4.0–7.5 (optimum pH 4.5–5.5) and at 4–35 °C (optimum at 20–28 °C). Sensitive to sodium chloride at concentrations above 0.4% (w/v) with the optimum at 0%. Potassium dihydrophosphate stimulates growth up to a concentration of 0.5%. Anaerobic or microaerobic growth does not occur.

The type strain is BP6-180914[T] (= KCTC 92872[T] = VKM B-3641[T] = UQM 41506[T]) isolated from lichen *Flavocetraria nivalis* thallus, Providence Cove, Russia. The DNA G + C content of the type strain is 63.65 mol%.

The nearly complete and complete (genome-extracted) 16S rRNA gene sequences and draft genome of strain BP6-180914 T have been deposited in GenBank under accession numbers OP023140, OQ625528 and JAMOIM000000000, respectively.

Data availability The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. 16S rRNA gene sequence and draft genome are available in National Center for Biotechnology Information.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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