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TAXONOMIC DESCRIPTION
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Lichenihabitans psoromatis gen. nov., sp. nov., a member of a novel lineage (*Lichenihabitantaceae* fam. nov.) within the order of *Rhizobiales* isolated from Antarctic lichen

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Abstract

Two Gram-stain-negative, facultative anaerobic chemoheterotrophic, pink-coloured, rod-shaped and non-motile bacterial strains, PAMC 29128 and PAMC 29148^T, were isolated from lichen. Phylogenetic analysis based on the 16S rRNA gene sequences revealed that strains PAMC 29128 and PAMC 29148^T belong to lichen-associated *Rhizobiales*-1 (LAR1), an uncultured phylogenetic lineage of the order *Rhizobiales* and the most closely related genera were *Methylocapsa* (<93.9 %) and *Methylosinus* (<93.8 %). The results of phylogenomic and genomic relatedness analyses also showed that strains PAMC 29128 and PAMC 29148^T were clearly distinguished from other species in the order *Rhizobiales* with average nucleotide identity values of <71.4 % and genome-to-genome distance values of <22.7 %. Genomic analysis revealed that strains PAMC 29128 and PAMC 29148^T did not contain genes involved in atmospheric nitrogen fixation or utilization of carbon compounds such as methane and methanol. Strains PAMC 29128 and PAMC 29148^T were able to utilize certain monosaccharides, disaccharides, sugar alcohols and other organic compounds as a sole carbon source. The major fatty acids (>10 %) were summed feature 8 ($C_{18:1}$ ω7*c* and/or $C_{18:1}$ ω6*c*; 33.7–39.7 %), summed feature 3 ($C_{16:1}$ ω7*c* and/or $C_{16:1}$ ω6*c*; 25.2–25.4 %) and $C_{19:0}$ cyclo ω8*c* (11.9–15.4 %). The major respiratory quinone was Q-10. The genomic DNA G+C contents of PAMC 29128 and PAMC 29148^T were 63.0 and 63.1 mol%, respectively. Their distinct phylogenetic position and some physiological characteristics support the proposal of *Lichenihabitans* gen. nov., with the type species *Lichenihabitans psoromatis* sp. nov. (type strain, PAMC 29148^T=KCCM 43293^T=JCM 33311^T). *Lichenihabitantaceae* fam. nov. is also proposed.

The order *Rhizobiales* of the class *Alphaproteobacteria* is known to be one of the most predominant bacterial groups in lichens that grow through the symbiotic relationship among lichenized fungi, green algae and/or cyanobacteria, and bacteria [1–6]. Since the first study on lichen-associated bacteria by using culture-independent molecular approaches, the lichen-associated *Rhizobiales*-1 (LAR1) lineage of the order *Rhizobiales*, has been known to be predominant in diverse lichens across diverse geographical areas [2, 4, 6–9]. The most closely related known taxa of LAR1 are the families *Beijerinkiaceae* and *Methylocystaceae*, which include cultured representatives of nitrogen-fixers. Based on these findings, it was suggested that LAR1 may supply lichen thalli with crucial nutrients such as fixed nitrogen [2, 4, 7]. Cultivation of lichen-associated bacteria has been performed [3, 9–12]. Isolation of LAR1 strains has been reported and metabolic potential as nitrogen-fixing bacteria was determined by the presence of the *nifH* gene [10]. However, detailed determination of physiological characteristics of LAR1 strains with appropriate nomenclature has not been performed yet. In this study, two strains, PAMC 29128 and PAMC 29148^T, that belong to LAR1 lineage were isolated from Antarctic lichen and subjected to initial characterization as the representatives of the LAR1 lineage.

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Keywords: *Lichenihabitans psoromatis*; polyphasic taxonomy, lichen; Antarctica; new species.
Abbreviations: LAR1, lichen-associated Rhizobiales-1; R2A, Reasoner's 2A; NJ, neighbour-joining; ML, maximum-likelihood; COGs, clusters of orthologous gropus of proteins; KAAS, KEGG automatic annotation server; ANI, average nucleotide identity; DDH, DNA-DNA hybridization; GGDC, genome-to-genome distance calculation.
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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the complete genome sequence of strain PAMC 29148^T are MK344722 and CP036515–CP036517 under BioProject number PRJNA523865, respectively. Those for the 16S rRNA gene sequence and the draft genome sequence of strain PAMC 29128 are MK344721 and SJAT00000000 under BioProject number PRJNA523866, respectively.
Two supplementary figures and two supplementary tables are available with the online version of this article.

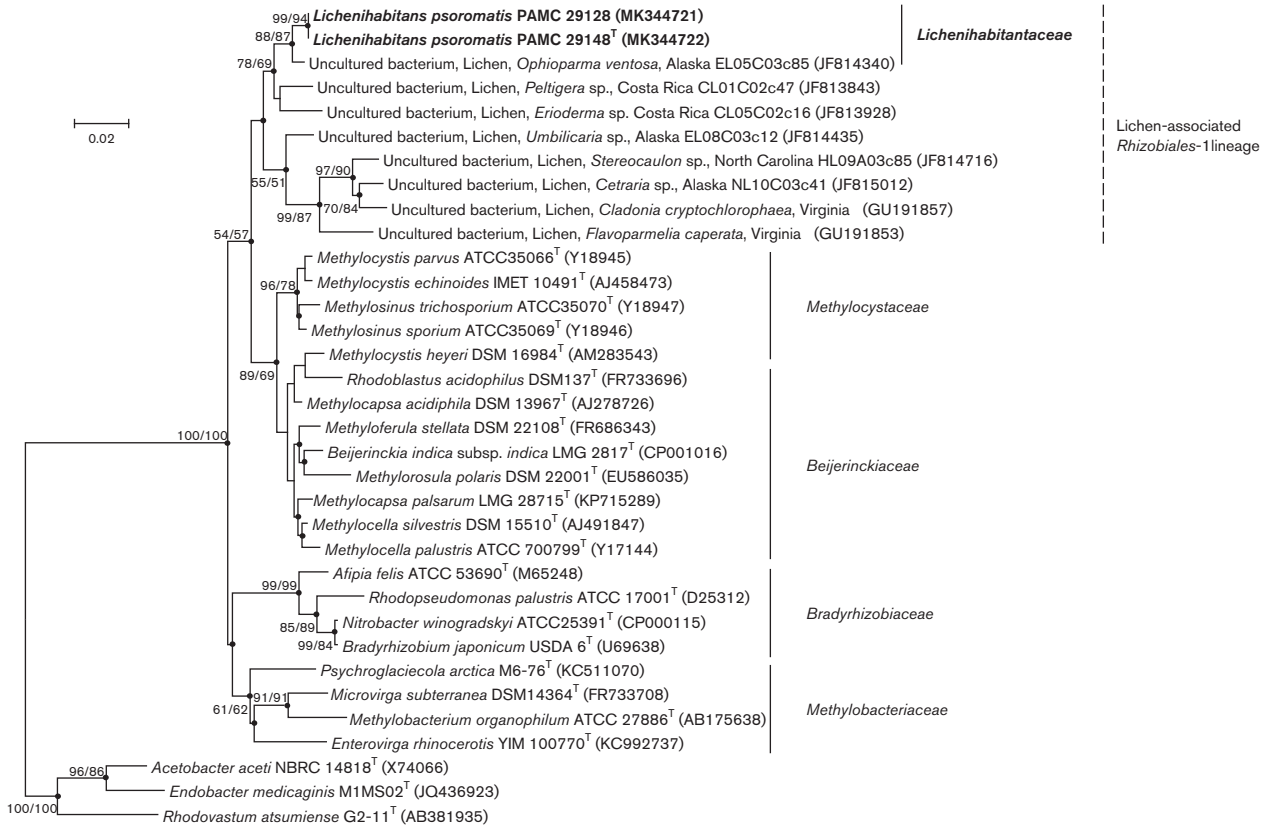

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences of strains PAMC 29128 and PAMC 29148^T, members of related families of the order *Rhizobiales*, and uncultured bacterial clones of lichen-associated *Rhizobiales*-1 lineage of *Alphaproteobacteria*. Bootstrap values (>50 %) based on 1000 replications were shown on corresponding branches (neighbour-joining/maximum likelihood). Filled circles indicate conserved nodes in both neighbour-joining and maximum-likelihood trees. Sequences from uncultured bacterial clones are indicated with the name and location of the organisms from which the sequences were obtained. *Rhodovastum atsumiense* G2-11^T, *Endobacter medicaginis* M1MS02^T and *Acetobacter aceti* NBRC 14818^T of the order *Rhodospirillales* were used as outgroups. Bar, 0.02 substitutions per nucleotide position.

A lichen specimen of *Psoroma antarcticum* was collected from King George Island, Antarctica (62° 14′ 23.99″ S, 58° 44′ 35.98″ W) [13]. The specimen was washed for 10 min in 1 ml of 0.85 % NaCl by vortexing in a Multi-EP tube vortexer (FinePCR) followed by centrifugation at 12 000 r.p.m. (Eppendorf) for 2 min and discarding the supernatant. The process was repeated four times. After the final wash, the sample was crushed in TissuLyzer II apparatus containing steel beads (Qiagen) twice for 2 min. One hundred microlitres of the final suspension were spread on 1/10 diluted Reasoner's 2A (R2A) solid medium (BD Difco) and incubated at 10 °C for 19 days. Strains PAMC 29128 and PAMC 29148^T were isolated and subsequently streaked on R2A agar plate three times to obtain pure cultures. These strains were maintained on R2A agar at 15 °C after the determination of optimal temperature and preserved as 20 % (v/v) glycerol at −80 °C.

Genomic DNA was extracted by using the Mini Tissue DNA kit (Cosmo Genetech Inc.) according to the manufacturer's instructions. The 16S rRNA gene was amplified with two universal primers (27F and 1492R) [14]. PCR products were purified using LaboPass PCR purification kit (Cosmo Genetech Inc.) and sequenced using primers, 27F, 518F, 800R and 1492R [14]. The 16S rRNA gene sequences (1413 nt) were compared with those of all type strains in the EzBioCloud database [15] and aligned with their closely related type strains in the order *Rhizobiales* using jPhydit [16]. Phylogenetic trees of the 16S rRNA gene sequences were reconstructed by using the neighbour-joining (NJ) [17] and maximum-likelihood (ML) [18] methods in the MEGA X program [19]. The robustness of the tree topologies was assessed by bootstrap analyses based on 1000 replications of the sequences. Comparison of 16S rRNA gene sequences showed that strains PAMC 29128 and PAMC 29148^T were closely related to *Methylocapsa palsarum* (93.8–93.9% sequence similarity) and *Methylosinus trichosporium* (93.8 %). In phylogenetic trees inferred from the two algorithms, PAMC 29128 and PAMC 29148^T

Table 1. Results of genomic relatedness analyses based on the average nucleotide identity (ANI, Yoon *et al.* 2017 [25]) and *in silico* DNA–DNA hybridization (DDH) inferred by the genome-to-genome distance (Auch *et al.* 2010 [26]). Genome sequences of strains PAMC 29128 and PAMC 29148^T are available under the accession numbers of PRJNA523866 and PRJNA523865, respectively, at the BioProject in NCBI

	ANI (%)									DDH (%)								
Strain	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1. PAMC 29128 (PRJNA523866)																		
2. PAMC 29148^T (PRJNA523865)	98.1									82.6								
3. *Methylocapsa palsarum* LMG 28715^T (PRJEB17422)	70.7	70.7								20.1	20.9							
4. *Methylocapsa acidiphila* DSM 13967^T (PRJNA72841)	71.1	71.2	75.0							21.1	21.5	21.5						
5. *Methylocella silvestris* DSM 15510^T (PRJNA224116)	71.2	71.2	74.1	74.8						19.8	20.9	20.6	20.6					
6. *Methyloferula stellata* DSM 22108^T (PRJNA165575)	70.7	71.4	72.2	73.5	73.2					19.0	19.5	20.4	20.9	20.7				
7. *Beijerinckia indica* LMG 2817^T (PRJNA224116)	70.1	70.2	72.4	72.7	72.1	72.7				21.2	22.7	21.8	22.3	20.6	20.9			
8. *Rhodoblastus acidophilus* ATCC 25092^T (PRJNA224116)	70.9	70.7	71.3	71.8	71.6	70.7	70.6			19.9	19.8	19.2	19.6	19.9	19.7	21.0		
9. *Methylosinus trichosporium* ATCC 35070^T (PRJNA224116)	71.2	71.2	72.1	72.6	72.9	72.2	71.6	72.8		18.9	19.5	20.0	19.8	19.8	19.8	21.2	19.7	
10. *Methylocystis parvus* ATCC 35066^T (PRJNA81429)	70.9	71.2	71.8	72.0	72.3	71.1	70.9	72.3	75.7	20.6	21.0	19.9	20.8	20.3	19.8	22.0	20.1	21.4

belong to the LAR1 lineage and were separated from other families of *Rhizobiales* (Fig. 1).

Genomic sequences of PAMC 29128 were obtained by sequencing with Illumina MiSeq apparatus (LAS Inc.) and assembled with CLC Genomics Workbench (version 8.5.1). Genomic sequences of PAMC 29148^T were obtained by using PacBio RS II apparatus (LabGenomics) and assembled with SMRT Analysis (version 2.3.0) [20]. Genome annotation was performed using the Rapid Annotation using Subsystems Technology (RAST) server [21]. Functional gene annotations were performed using the Clusters of Orthologous Groups of proteins (COGs) database [22] and the KEGG Automatic Annotation Server (KAAS) [23]. The details of genomes of PAMC 29128 and PAMC 29148^T are summarized in Table S1, available in the online version of this article. In brief, the draft genome of PAMC 29128 comprised 54 contigs containing 4673 protein-coding genes, 47 tRNA genes and one rRNA operon (Table S1). The complete genome of PAMC 29148^T comprised three contigs containing 4832 protein-coding genes, 54 tRNA genes and three rRNA operons (Table S1). The results of genomic analysis revealed that strains PAMC 29128 and PAMC 29148^T did not possess genes for nitrogen fixation such as nitrogenase and methane monooxygenases and methanol dehydrogenases (Tables 2 and S2).

The degree of pairwise genome-based relatedness was estimated by both an average nucleotide identity (ANI) value calculated by the orthologous ANI algorithm [24, 25] and the *in silico* DNA–DNA hybridization (DDH) inferred by genome-to-genome distance calculation (GGDC) [26]. The ANI value between PAMC 29128 and PAMC 29148^T was 98.1% (Table 1) and this level is above the ANI cut-off values (95–96 %) to delineate bacterial species [27]. In addition, the DDH value between PAMC 29128 and PAMC 29148^T was 82.6 % (Table 1), indicating that strains PAMC 29128 and PAMC 29148^T are conspecific [28]. In contrast, ANI values between PAMC 29128 and PAMC 29148^T and other related type strains of *Rhizobiales* were below 71.2–71.4 %, and DDH values were below 22.7 % (Table 1). Multiple sequence alignment of the concatenated 120 ubiquitous single-copy proteins [29] for the type strains of the type species in the order *Rhizibiales* was performed by GTDB-Tk (https://github.com/Ecogenomics/GtdbTk). Phylogenomic trees using the NJ and ML algorithms were reconstructed based on 1000 sets of sequence replication using MEGA [18]. Consistent with the phylogenetic tree based on the 16S rRNA sequences, the phylogenomic tree also showed that

Table 2. Major characteristics that distinguish PAMC 29148^T and PAMC 29128 and other genera of *Beijerinckiaceae* and *Methylocystaceae*

Strains: 1, *Lichenihabitans psoromatis* PAMC 29148^T; 2, *Methylocapsa palsarum* LMG 287152 ^{T}; 3, *Methylocapsa acidiphila* DSM 13967^T; 4, *Methylocella silvestris* DSM 15510^T; 5, *Methyloferula stellata* DSM 22108^T; 6, *Beijerinckia indica* subsp. *indica* LMG 2817^T; 7, *Rhodoblastus acidophilus* ATCC 25092^T; 8, *Methylosinus trichosporium* ATCC 35070^T; 9, *Methylocystis parvus* ATCC 35066^T. Data for strain PAMC 29148^T and PAMC 29128 were obtained from the present study and other data were from previously published sources as indicated. ND, Not determined.

Characteristic	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	8[h]	9[h]
Oxygen requirement	Facultative anaerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic in the dark/ anaerobic in the light	Aerobic	Aerobic
Quinones	Q-10	ND	ND	ND	Q-10	ND	Q-10 MK-10 RQ-10	Q-8	Q-8
G+C content (mol%)	63.1*	61.7	63.1	60	55.6–57.5	54.7–58.5	62.2–66.8	63	64–67
Nitrogen fixation (*nif*) gene	−†	+	+†	+†	+†	+	+†	+	+
Utilization of:									
Methane	−†	+	+	+	+	+†	−†	+	+
Methylamine	−	ND	−	+	−	ND	ND	−	−
Methanol	−	+	+	+	+	+†	+	+	+
Glucose	+	−	−	−	−	+	−	−	−

*All characteristics of strain PAMC 29128 were identical to those of strain PAMC 29148^T except for the DNA G+C content (63.0 mol%). The G+C content was calculated on the basis of the nucleotide content of the genome sequence.

†Physiological characteristics were inferred from genome sequences.

‡Data from: *a*, this study; *b*, Dedysh *et al.* 2015 [34]; *c*, Dedysh *et al.* 2002 [35]; *d*, Dunfield *et al.* 2003 [36]; *e*, Vorobev *et al.* 2011 [37]; *f*, Kennedy 2005 [38]; *g*, Pfenning 1969 [39]; *h*, Bowman *et al.* 1993 [40].

strains PAMC 29128 and PAMC 29148^T formed the distinct clade from other families in the order *Rhizobiales* (Fig. S1). The genomic DNA G+C content of strains PAMC 29128 and PAMC 29148^T, which were calculated from genome sequences, were 63.0 and 63.1 mol%, respectively (Table 2).

The temperature range and optimal temperature for growth were determined by culturing strains on R2A solid medium at different temperatures (0, 4, 10, 15, 20, 25, 30 and 37 °C) for 14 days. Strains PAMC 29128 and PAMC 29148^T grew at 4–20 °C (optimally at 15 °C). The pH range and optimal pH for growth were determined in R2A liquid medium. The pH was adjusted using the following buffering systems: Na_2HPO_4 -buffered citric acid; pH 4.0–5.0, MES; pH 5.5–6.0, MOPS; pH 6.5–7.0, AMPD; pH 8.0–9.5, CAPS; pH 10.0. The growth of each culture was assessed by measuring optical density at 600 nm (EnVision plate reader; PerkinElmer) every day for up to 14 days. NaCl tolerance test was carried on R2A solid medium supplemented with 0, 0.5, 1, 2, 3, 4, 5, 7.5, 10 and 15 % of NaCl (w/v). Catalase activity was tested with 3 % H_2O_2 and oxidase activity was determined using tetramethyl-*p*-phenylenediamine as per the methods described by Kovacs *et al.* [30]. Anaerobic growth was tested on R2A and minimal salt solid medium [31] and R2A supplemented with glucose (1 %) and phenol red (0.001 %) in a jar containing an AnaeroPak (Mitsubishi Gas Chemical) for up to 14 days at 15 °C.

Gram-staining was carried out using a Gram-stain kit (Sigma) according to the manufacturer's instructions. Motility was determined by the observation of growth after inoculation in R2A liquid medium with 0.4 % agar. Morphology of cells was examined by TEM (CM200, Philips). For TEM, cells were negatively stained with 2.0 % uranyl acetate on a carbon-coated copper grid. Biochemical activities were determined by using API 20NE (bioMérieux), API ZYM (bioMérieux) and GN2 Microplates (Biolog) according to manufacturers' instructions. The utilization of methanol, formaldehyde, formate, methylamine and glucose as a sole carbon source was determined by cultivation on minimal salt liquid medium at 0.1 % concentration (v/v) of each substrate with the exception of glucose (0.4 % and 1.0 %) by measuring optical density at 600 nm (EnVision plate reader, PerkinElmer) every 3 days for up to 14 days. The ability of nitrogen fixation was tested for strains PAMC 29128 and PAMC 29148^T, with a strain of the genus *Sinorhizobium* as a positive control by cultivation on the nitrogen-free solid medium [31].

The morphological, physiological and biochemical characteristics of strain PAMC 29128 and PAMC 29148^T are listed in Fig. S2, Table 2 and the species description. Growth through fermentation under anaerobic conditions using the glucose and absence of genes encoding enzymes involved in nitrogen fixation with very weak growth on the nitrogen-free medium distinguished strains PAMC 29128 and PAMC 29148^T from the other genera of *Beijerinckiaceae* and *Methylocystaceae*. In addition, the inability to utilize methane or methanol as a single carbon source differentiated strains PAMC 29128 and PAMC 29148^T from the other genera of *Beijerinckiaceae* and *Methylocystaceae*.

Table 3. Fatty acid profiles of strains PACM 29148^T and PAMC 29128

All strains were grown on R2A plates at 15 °C for 7 days. Results are showed as percentages of the total fatty acids. Fatty acids amounting up to 10 % or more are presented in bold. ND, Not detected. Summed features represent fatty acids that could not be separated by GLC with the MIDI system; summed feature 2 comprises $C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I; summed feature 3 comprises $C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω6c*; and summed feature 8 comprises $C_{18:1}$ *ω7c* and/or $C_{18:1}$ *ω6c*.

Fatty acid	PAMC 29148^T	PAMC 29128
$C_{12:0}$	4.4	3.7
$C_{14:0}$	0.8	1.3
$C_{16:0}$	3.8	4.2
$C_{17:0}$ cyclo	ND	1.4
$C_{18:0}$	4.7	4.5
$C_{18:1}$ *ω7c* 11-CH_3	3.3	3.5
$C_{19:0}$ cyclo *ω8c*	**11.9**	**15.4**
$C_{18:0}$ 3-OH	3.3	3.4
$C_{20:2}$ *ω6,9c*	ND	0.8
Summed feature 2	2.9	2.8
Summed feature 3	**25.2**	**25.4**
Summed feature 8	**39.7**	**33.7**

For cellular fatty acid analysis, strains PAMC 29128 and PAMC 29148^T were grown on R2A at 15 °C for 7 days. Analysis was performed according to the method described b y the Sherlock Microbial Identification System version 6.1 (MIDI) using the TSBA6 database [32]. The major fatty acids (>10 %) of strains PAMC 29128 and PAMC 29148^T were summed feature 8 ($C_{18:1}$ *ω7c* and/or $C_{18:1}$ *ω6c*; 33.7–39.7 %), summed feature 3 ($C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω6c*; 25.2–25.4 %) and $C_{19:0}$ cyclo *ω8c* (11.9–15.4 %; Table 3). Quinones were extracted as described by Collins and Jones [33] and analysed by HPLC at the Korean Culture Centre of Microorganisms (Republic of Korea). Q-10 was the only menaquinone present in strains PAMC 29128 and PAMC 29148^T.

Based on the clearly separated phylogenetic cluster of PAMC 29128 and PAMC 29148^T belonging to the LAR1 lineage, genomic data, and physiological characteristics that differentiated PAMC 29128 and PAMC 29148^T from known strains of the families of the order *Rhizobiales*, we propose the family *Lichenihabitantaceae* fam. nov.

DESCRIPTION OF *LICHENIHABITANS* GEN. NOV.

Lichenihabitans (Li.che.ni.ha'bi.tans. L. masc. n. *lichen*, lichen; L. pres. part. *habitans*, inhabiting; N.L. masc. n. *Lichenihabitans*, inhabitant of lichens).

Cells are Gram-stain-negative, rod-shaped, non-motile and facultatively anaerobic. Cells produce oxidase and catalase. The dominant fatty acids (>10 %) include summed feature 8 ($C_{18:1}$ *ω7c* and/or $C_{18:1}$ *ω6c*), summed feature 3 ($C_{16:1}$ *ω7c* and/or C $_{16:1}$ *ω6c*) and $C_{19:0}$ cyclo *ω8c*. The major isoprenoid quinone is Q-10. DNA G+C contents are 63.0–63.1 mol%. No genes involved in nitrogen fixation and methane or methanol utilization are present. Contains complete sets of genes involved in glycolysis, fermentation and the tricarboxylic acid cycle. Members of the order *Rhizobiales* within the class *Alphaproteobacteria*. The type species is *Lichenihabitans psoromatis*.

DESCRIPTION OF *LICHENIHABITANS PSOROMATIS* SP. NOV.

Lichenihabitans psoromatis (pso.ro'ma.tis. N.L. gen. n. *psoromatis* of *Psoroma* sp., the generic name of the lichen from which the type strain was isolated).

Gram-stain-negative, rod-shaped, non-motile and facultatively anaerobic. Cells are 0.9–1.6 µm wide and 0.9–3.4 µm long. Colonies are circular, convex, glittering and pink-coloured on R2A plates after 2 weeks incubation at 15 °C. Growth occurs at 4–20 °C (optimum, 15 °C), pH 5.5–7.0 (optimum, pH 6.5) and in the absence of NaCl after 2 weeks of incubation on R2A agar at 15 °C. No capacity to utilize methanol, formaldehyde, formate and methylamine. No genes involved in nitrogen fixation or methane or methanol utilization. In the API 20NE system, urea is hydrolysed. In the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are positive. In the carbon source utilization test using GN2 MicroPlates, adonitol, L-arabinose, D-arabitol, cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, *α*-D-glucose, m-inositol, D-mannitol, D-mannose, D-psicose, L-rhamnose, D-sorbitol, xylitol, pyruvic acid methyl ester, succinic acid mono-methyl ester, D-galactonic acid lactone, D-gluconic acid, D,L-lactic acid, succinic acid, L-alaninamide and glycerol are utilized. The type genus is *Lichenihabitans*. The type strain, PAMC 29148^T (=KCCM 43293^T=JCM 33311^T), was isolated from lichen, *Psoroma antarcticum*, collected from King George Island, Antarctica.

DESCRIPTION OF *LICHENIHABITANTACEAE* FAM. NOV.

Lichenihabitantaceae (Li.che.ni.ha.bi.tan.ta.ce'ae. N.L. masc. n. *Lichenihabitans*, type genus of the family; *-aceae* ending to denote a family; N.L. fem. pl. n. *Lichenihabitantaceae*, the *Lichenihabitans* family).

Cells are Gram-stain-negative, rod-shaped, non-motile, facultative anaerobic and chemoheterotrophs. The type genus is *Lichenihabitans*.

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

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