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A microbiotic survey of lichen-associated bacteria reveals a new lineage from the Rhizobiales

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Abstract
This study uses a set of PCR-based methods to examine the putative microbiota associated with lichen thalli. In initial experiments, generalized oligonucleotide-primers for the 16S rRNA gene resulted in amplicon pools populated almost exclusively with fragments derived from lichen photobionts (i.e., Cyanobacteria or chloroplasts of algae). This effectively masked the presence of other lichen-associated prokaryotes. In order to facilitate the study of the lichen microbiota, 16S ribosomal oligonucleotide-primers were developed to target Bacteria, but exclude sequences derived from chloroplasts and Cyanobacteria. A preliminary microbiotic survey of lichen thalli using these new primers has revealed the identity of several bacterial associates, including representatives of the extremophilic Acidobacteria, bacteria in the families Acetobacteraceae and Brucellaceae, strains belonging to the genus *Methylobacterium*, and members of an undescribed lineage in the Rhizobiales. This new lineage was investigated and characterized through molecular cloning, and was found to be present in all examined lichens that are associated with green algae. There is evidence to suggest that members of this lineage may both account for a large proportion of the lichen-associated bacterial community and assist in providing the lichen thallus with crucial nutrients such as fixed nitrogen.

Keywords: 16S, Alphaproteobacteria, backbone constraint, LAR1, lichens, maximum likelihood, Bayesian inference, *Methylobacterium*, microbiota, nitrogen fixation, non-photosynthetic bacteria, primers, Rhizobiales

1. Introduction

Non-photobiont bacteria have never been accepted as an essential part of the lichen symbiosis. Yet lichens are known for being able to grow on extremely nutrient-poor substrates (Brodo, 1973), such as rock outcrops. Without access to sufficient amounts of nitrogen, it seems that lichens (especially when forming large thalli) would need to associate in some way with bacteria that are able to fix nitrogen (Liba et al., 2006). About 10% of lichen-forming fungi are associated with nitrogen-fixing Cyanobacteria (e.g., Peltigerales and Lichinomycetes); however, the remaining 90% of lichen-forming fungi are not known to be intimately associated with any bacteria (Richardson and Cameron, 2004). The absence of certain microorganisms could explain why algae and fungi in axenic culture seem to exhibit symbiotic interactions but will only rarely form a structure resembling a stratified lichen thallus (Stocker-Wörgötter, 2001). When growth of stratified lichen thalli has been successful in culture, the cultures have generally been derived from pieces of lichens that have had their symbiosis established in nature (Schuster et al., 1985). This evidence supports the notion that there may be microorganisms helping to establish and facilitate the lichen symbiosis.

Previous studies have characterized lichen-associated bacterial communities through culture-based experiments (Cardinale et al., 2006; Cengia-Sambo, 1923, 1925; González et al., 2005; Grube et al., 2009; Henckel, 1938; Henckel and Plotnikova, 1973; Henckel and Yuzhakova, 1936; Hodkinson et al., 2006; Iskina, 1938; Krasil'nikov, 1949; Lambright and Kapustka, 1981; Liba et al., 2006; Zakharova, 1938; Zook, 1983). However, since the vast majority of microorganisms remain unculturable in the laboratory (Amann et al., 1995), this method can easily lead to false conclusions about the abundance and importance of certain bacteria in nature (for an example and discussion of the biases inherent in culture- versus PCR-based methods used in microbial diversity studies, see Arnold et al., 2007). Indeed, the pool of unculturable bacteria is most likely to include bacteria that are obligate symbionts or intrinsic to

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symbioses and, in this regard, the most interesting for studies focusing on the biological role of bacteria in symbiotic systems. As a result of this problem, culture-based experiments alone may not provide the data necessary for reliable extrapolation about biochemical activities and overall community composition.

Culture-independent molecular methods for studying bacterial diversity have transformed the field of microbial ecology in recent decades (DeLong and Pace, 2001; Jensen et al., 1993; Rappe and Giovannoni, 2003). The first PCR-based studies of lichen-associated bacteria were conducted by Cardinale et al. (2006) and Hodkinson et al. (2006). The former authors showed that a great diversity of bacteria was present in each lichen thallus that they sampled; however, no clear trends were detectible (geographical, taxonomical, or otherwise) when comparing the bacterial community profiles. The latter authors demonstrated that some lichen-associated bacterial lineages have affinities to documented symbiotic bacteria. Subsequent *in situ* hybridization experiments by Cardinale et al. (2008) and Grube et al. (2009) have revealed that bacteria forming biofilm-like coatings on lichen surfaces are predominantly Alphaproteo-bacteria. Grube et al. (2009) additionally demonstrated through SSCP analyses that three very different lichen species with green algal photobionts each have specific bacterial communities. The preliminary results of Hodkinson et al. (2009; using PCO analyses of bacterial community 16S rRNA gene sequence data) corroborated this notion, but showed that these trends are not generally 'species-specific', and that photobiont-type can be strongly correlated with trends in bacterial community composition.

Because the nucleotide sequence encoding the prokaryotic small subunit rRNA (SSU or 16S rRNA) is particularly useful for inferring phylogenetic relationships at the genus and higher taxonomic levels, it has become the standard for studies of bacterial diversity (Rosselló-Mora and Amann, 2001; Stackebrandt and Rainey, 1995; Woese, 1987). When PCR is performed on DNA derived directly from an environmental sample, the result is a pool of amplicons containing sequences from many different organisms that were in the original sample. To identify specific organisms from the sample, this pool of amplicons can be dissected using molecular cloning (De la Torre et al., 2003; Rondon et al., 1999; Zhiyong et al., 2007) or high-throughput metagenomic methods (Edwards et al., 2006; Ley et al., 2006; Liu et al., 2007; Margulies et al., 2005; Roesch et al., 2007; Turnbaugh et al., 2006, 2007). For this study, preliminary microbiotic data was collected using ribosomal heterogeneous amplicon pool sequence analysis with degeneracy ('RHAPSA-D') to test selective PCR primers and perform rapid microbiotic surveys on multiple samples characterized separately. Subsequently, molecular cloning of 16S rRNA gene sequences was conducted to identify specific members of the lichen-associated bacterial community and infer their phylogenetic relationships.

2. Materials and Methods

Primer design

Degenerate 16S rDNA PCR primers that exclude cyanobacterial and/or chloroplast-derived DNA from amplification (Table 1) were designed using 16S sequences from plastids (of both algae and higher plants) and over one hundred sequences (see Table S1 in the supplemental material) representing a wide diversity of Bacteria from the Ribosomal Database Project-II (RDP-II; http://rdp.cme.msu.edu; Cole et al., 2005). Sequences were aligned by hand using MacClade v4.07 (Maddison and Maddison, 2002). Primer design involved identifying the relatively conserved sites across the eubacteria and targeting sites shared only by chloroplasts and Cyanobacteria for exclusion, leading to the development of three degenerate primers (895F, 902R, and 904R) to accommodate the remaining eubacterial diversity (Table 1). One primer (1185mR) was designed to target a diversity of Bacteria, but exclude 16S rRNA gene sequences derived only from plastids. A modified version of this primer (1185aR) was produced to target only lichen-associated Rhizobiales bacteria. Two additional primers (1381R and 1381bR; Table 1), were designed to target a wider array of 16S sequences, and are intended to exclude only sequences derived from *Asterochloris* sp., the primary algal partner in *Cladonia* sp. thalli (DePriest, 2004; Miadlikowska et al., 2006). Primers were named for the corresponding position of the 3' end on the *E. coli* 16S rRNA molecule. RDP-II Probe Match (Cole et al., 2005) was used to determine the approximate percentage of bacteria with sequences matching each of the primers (Table 1).

Specimen collection and storage

The following ten specimens (representing eight different species) were collected in the Appomattox-Buckingham State Forest of Virginia: *Cladonia cristatella* (Hodkinson 5005, 5033), *C. cryptochlorophaea* (5018), *C.* cf. *sobolescens* (5015), *C. peziziformis* (5006), *C. subtenuis* (5026), *Flavoparmelia caperata* (5012), *Parmotrema perforatum* (5027, 5028), and *Peltigera phyllidiosa* (5025). A second collection site was located in the area of Hanging Rock State Park in North Carolina, at which two more specimens were collected for this study: *Lasallia pensylvanica* (5036) and *Umbilicaria mammulata* (5038). Specimens were stored at -20°C shortly after collection, and have been deposited at the Duke Cryptogamic Herbarium (DUKE). Detailed label data for all vouchers can be found in the DUKE Catalog of Lichens (http://www.biology.duke.edu/herbarium/lichendata.html).

Table 1. PCR primers for the 16S rRNA gene used in this study.

Primer	Sequence (5'-3')*	Target group	Probe Match (SC)+	Probe Match (SM)+	Reference
27F	AGAGTTTGATCMTGGCTCAG	Bacteria (universal)	80.59%	93.48%	Lane et al. (1991)
533F	GTGCCAGCAGCCGCGGTAA	Bacteria (universal)	96.18%	99.17%	Weisburg et al. (1991)
895F	CRCCTGGGGAGTRCRG	Bacteria exc. plastids & Cyanobacteria	66.68%	91.07%	This study
902R	GTCAATTCITTTGAGTTTYARYC	Bacteria exc. plastids & Cyanobacteria	75.85%	92.64%	This study
904R	CCCCGTCAATTCITTTGAGTTTYAR	Bacteria exc. plastids & Cyanobacteria	75.66%	94.68%	This study
1185mR	GAYTTGACGTCATCCM	Bacteria exc. plastids	71.17%	95.73%	This study
1185aR	GAYTTGACGTCATCCA	Lichen-associated Rhizobiales	2.49%	72.66%	This study
1381R	CGGTGTGTACAAGRCCYGRGA	Bacteria exc. *Asterochloris* sp. plastids	90.19%	95.50%	This study
1381bR	CGGGCGGTGTGTACAAGRCCYGRGA	Bacteria exc. *Asterochloris* sp. plastids	89.74%	95.26%	This study
1492R	ACCTTGTTACGACTT	Bacteria (universal)	95.54%	97.84%	Lane et al. (1991)

*For each degenerate primer, an equimolar mix of all of the constituent primers implied by the degenerate sequence is recommended, since machine mixes are generally not guaranteed to approximate equimolarity. +The results of RDP-II Probe Match analyses using either a strict consensus (SC) or allowing a single mismatch (SM) are shown with the percentage of matched sequences in the data set of all full 16S rRNA gene sequences found in the RDP-II database.

Molecular methods

DNA was isolated from small fragments (5–50 mg, dry weight) of freshly collected lichen thalli that had minimal contact with the substrate. For samples 5036 and 5038, the algal layer and upper cortex were removed before DNA extraction. DNA isolation was performed using a standard procedure (Zolan and Pukkila, 1986) modified by employing a 2% SDS lysis buffer. Isolated DNA was resuspended in sterile water and stored at -20°C.

PCR amplification was performed using all 16S primer combinations that would result in the amplification of fragments 0.5–1.0 kb in length. All primers used for amplification are listed in Table 1; these primers can also be found on the internet at http://www.lutzonilab.net/primers/. To ensure equimolarity, each of the constituent primers was ordered separately and equimolar mixes were subsequently made by hand. Every experiment conducted on a given lichen thallus was performed using the same DNA extract so that results with different primer combinations would be directly comparable.

Amplification reactions of 25 µl were performed following a modified Vilgalys and Hester (1990) procedure using 0.4 mg μl^{-1} of bovine serum albumin (Hillis et al., 1996), 1.5-3.0 mM $MgCl_2$, 0.03 U μl^{-1} Red Hot® DNA Polymerase (ABgene Inc., Rochester, NY, USA), 0.2 mM of each dNTP, and a 0.5–1.0 µM concentration of each primer (dependent upon the degree of degeneracy). Touchdown PCR conditions consisted of initial denaturation for 3 min at 94°C, followed by 24 cycles (30 s at 94°C; 30 s at 55°C, decreasing by 0.4°C with each cycle; 72°C for 60 s, increasing by 2 s with each cycle) with a subsequent set of 12 cycles (30 s at 94°C; 30 s at 45°C; 72°C for 120 s, increasing by 3 s with each cycle), followed by 10 min of final extension at 72°C. Amplified PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) prior to automated sequencing using Big Dye chemistry with a 3730xl DNA Analyzer (PE Applied Biosystems, Foster City, CA, USA).

A TOPO TA Cloning® Kit (Invitrogen™, life technologies, Carlsbad, CA, USA) was used to separate amplicons from cleaned PCR products amplified using the 533F/1185aR primer pair. Eight clones were selected from each plate for vector amplification using the T7 'sequencing' primer and the reverse M13 primer with thermocycler specifications provided in the TOPO TA Cloning® Kit manual. Purification and sequencing (performed with the T7 and M13R primers) were conducted as previously described.

Sequences were assembled and edited using the software package Sequencher™ v4.8 (Gene Codes Corporation, Ann Arbor, MI, USA). Nearest sequence matches were found through BLASTn searches of the nucleotide collection in GenBank (Altschul et al., 1997). If the top 100 BLASTn hits were predominantly from the 16S rRNA gene, taxon identifications were made using the RDP-II Sequence Match Analysis Tool (SeqMatch v9.42; http://rdp.cme.msu.edu/seqmatch/; for SeqMatch identifications of preliminary 'RHAPSA-D' survey data, see Table S2). Cloned 16S rRNA gene sequences were deposited in GenBank under the accession numbers GU191848–GU191872 (for individual accession numbers, see Table S3).

Phylogenetic analyses

Reference data set assembly and DNA sequence alignment. Reference 16S rRNA gene sequences representing cultured strains from the Rhizobiales were compiled from Gallego et al. (2005), Lee et al. (2005), and the RDP-II database (see Table S3). In order to be included in this set, sequences needed to contain nearly the full-length of the 16S rRNA gene (this criterion was enforced so that a robust phylogeny could be inferred and used as a backbone constraint tree in subsequent analyses with the shorter partial 16S rRNA gene sequences generated as part of this study). Reference sequences were aligned using MacClade v4.08 (Maddison and Maddison, 2002) and were manually adjusted taking into consideration rRNA secondary structure (Kjer, 1995).

Chimera detection. Cloned 16S rRNA gene sequences derived from amplicon pools produced using primer combination 533F/1185aR were aligned to one another in a separate file as previously described and checked for chimeras using Mallard (Ashelford et al., 2006). Once Deviation from Expectation (DE) values were calculated for each sequence (using the Pintail algorithm; Ashelford et al., 2005) and plotted against mean percentage difference values, outlier DE values were identified using a raw data quantile plot (derived from analyses of type strain sequences stored in the RDP-II database) correlated with a 75% cut-off value. This analysis yielded a list of likely anomalous sequences (Thornhill et al., 2007; Wang and Wang, 1997), and sequences classified as 'good' under these criteria were integrated (along with their closest GenBank matches obtained as previously described) into the larger alignment file of cultured reference strains and aligned with those sequences as previously described (all sequences in the final alignment are listed in Table S3).

Building a backbone constraint tree. The first round of phylogenetic analyses was run excluding all sequences from uncultured bacterial strains (i.e., those generated as part of this study and their closest matches from GenBank). This data set, comprised only of nearly full-length 16S rRNA gene sequences from cultured reference strains, was analyzed using maximum parsimony (MP) as the optimality criterion in PAUP* v4.0b10 (Swofford, 2001). Constant sites and ambiguously-aligned sites were excluded from analyses. Ambiguously-aligned regions were recoded using INAASE v3.0 (Lutzoni et al., 2000), and were reintegrated as new characters as outlined by Reeb et al. (2004) and Gaya et al. (2008). Characters recoded using INAASE were subjected to specific step matrices generated using a transition:transversion:gap ratio of 1:1:1, and were adjusted based on hand-alignment of ambiguous regions. Unambiguously-aligned portions were subjected to symmetric step matrices computed in STMatrix v3.0 (available at http://www.lutzonilab.net/downloads/) as outlined by Gaya et al. (2003; 2008). For each data set, a first round of searches was performed with 1,000 random-addition-sequence (RAS) replicates and tree bisection-reconnection (TBR) branch swapping. The MULTREES option was in effect and zero-length branches were collapsed. This revealed five equally most parsimonious trees that were hit in 43.5% of the RAS replicates. Branch support for MP trees was estimated through bootstrap analyses (Felsenstein, 1985) by performing 1,000 bootstrap replicates with 10 RAS per bootstrap replicate, with all other settings as above.

The same data set (excluding sequences from uncultured organisms) was analyzed phylogenetically with maximum likelihood as the optimality criterion. MrModeltest (Nylander, 2004) was run using input files specifying likelihood scores generated by PAUP* v4.0b10 (derived from alignments with ambiguous regions excluded). The results, evaluated using AIC, indicated that a general time-reversible (GTR) model (+I+gamma) would be optimal. Maximum likelihood (ML) searches were conducted using RAxML-VI-HPC (Stamatakis, 2006) with the GTRMIX setting and 1,000 replicates. Ambiguously-aligned regions were excluded and the job was distributed across 50 processors at the Duke Shared Cluster Resource (DSCR) center. ML bootstrap analyses were performed with 1,000 resamplings, and run as previously outlined for the ML topology search. ML and MP 70% majority-rule bootstrap consensus trees were calculated using PAUP* v4.0b10. The ML consensus tree was imported into MacClade v4.08 (Maddison and Maddison, 2002) and all nodes not supported by both MP- and ML-BP (Bootstrap Proportion) values $\geq$70% were collapsed. The resulting tree (see Fig. S1) was used as a backbone constraint in subsequent analyses.

Topological and support inferences. The full aligned sequence data set (i.e., the data set used to generate a backbone constraint tree and the 16S sequences from uncultured strains) was analyzed phylogenetically for both topology and BP support under the ML criterion as previously described (but restricted to the 533–1185 region; *E. coli* numbering). Each ML analysis was run both with and without the aforementioned backbone constraint tree (comprised of cultured reference strains represented by nearly full-length 16S rRNA gene sequences; Fig. S1) enforced. Bayesian analyses were run on this same smaller region of the data set with MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). Model parameters for GTR+I+gamma were specified and a dirichlet (1,1,1,1) prior was set (as recommended by MrModeltest). Markov chain Monte Carlo parameters consisted of 10,000,000 generations, with four chains, and a tree sampled every 1,000 generations. The first 1,000 trees were discarded as burnin (in accordance with an analysis by Tracer v1.4; Rambaut and Drummond, 2007), and results were summarized in the form of a 50% majority-rule consensus tree using the 'sumt' option in MrBayes. The finalized NEXUS file with

specifications for all described analyses is available at http://www.lutzonilab.net/publications/.

3. Results

PCR with new primers

RDP-II Probe Match demonstrated that all of the primers (with the exception of 1185aR), in a strict consensus (SC) analysis, match >66% of full 16S sequences found in the RDP-II database (a data set that includes Cyanobacteria and Chloroplast sequences), and that the primers match >91% of the same set of sequences if a single mismatch (SM) is allowed (Table 1). When preliminary experiments were run with lichen thalli using universal 16S primers (533F/1492R), eight of the ten samples from which the photobiont layer was not removed had a population of amplicons dominated by sequences derived from algal plastids or Cyanobacteria (sequences derived from samples 5027 and 5028 were identifiable only as Alphaproteobacteria; Table S2). The results of preliminary 'RHAPSA-D' experiments on these eight samples were used to evaluate the effectiveness of the primers in avoiding plastid and *Nostoc* genomes. For each of the primer pairs, there is a clear difference from the control that is in accordance with the design of each new primer (Table S2).

Lichen-associated non-photobiont bacteria

A preliminary survey of lichen-associated bacterial diversity revealed the presence of organisms that were assigned to the following taxonomic groups: Acetobacteraceae, Acidobacteriaceae, Alphaproteobacteria, Bacteria, Brucellaceae, Gammaproteobacteria, *Methylobacterium*, Proteobacteria, Rhizobiales, and Rhodospirillales (Table S2). One clear overall trend is the difference in the results obtained for the one cyanolichen, *Peltigera phyllidiosa* (sample 5025). With nearly every pair of primers used in the preliminary 'RHAPSA-D' screening experiment, different bacteria were detected in association with the cyanolichen *P. phyllidiosa* than with each of the other lichens sampled (each of which has a green-algal photobiont instead of *Nostoc*; Table S2). In a separate set of experiments, microscopy revealed numerous bacterial cells on the lower cortices of lichens, including aggregates of cells that are covered by a polysaccharide-like substance, and which seem to be in close association with the lichen thallus (Fig. 1).

Phylogenetic relationships

An undescribed lineage in the Rhizobiales (Lichen-Associated Rhizobiales-1 or LAR1; Fig. 2) was detected with high support in all analyses. This lineage contains

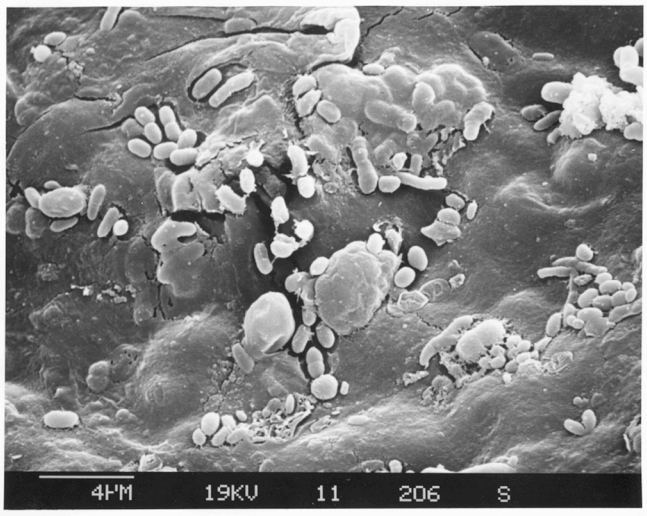

Figure 1. Scanning electron micrograph of the lower cortex of a foliose lichen (*Punctelia rudecta*) with numerous bacteria present. Some of these bacteria form cell aggregates that are sometimes covered with what appears to be a polysaccharide. These results are consistent with the small colonies of bacterial cells previously reported in a biofilm-like layer in the inner chamber of a fruticose lichen, *Cladonia arbuscula*, and the underside of an umbilicate lichen, *Umbilicaria cylindrica* (Cardinale et al., 2008; Grube et al. 2009).

sequences cloned from all green-algal lichen thalli tested. While LAR1 was well-supported as a monophyletic group, its position within the Rhizobiales remains uncertain, though backbone-constrained ML and Bayesian analyses both show a weakly-supported affinity to Beijerinckiaceae. Within the group of cloned sequences from the Rhizobiales, the one cyanolichen, *Peltigera phyllidiosa*, was found to have only sequences representing *Methylobacterium*, a genus also found occasionally in other lichens (Fig. 2). The backbone tree (Fig. S1) used to constrain the ML search (resulting in the topology displayed in Fig. 2) contained a total of 27 nodes that were well-supported by both MP and ML-BP analyses. Of the various topology searches performed as part of this study on the 533–1185 (*E. coli* numbering) portion of the data set (backbone-constrained ML: Fig. 2; unconstrained ML: Fig. S2; Bayesian: Fig. S3), the results of the backbone-constrained ML analysis were the most similar to previously-published phylogenetic analyses (e.g., Lee et al., 2005, which displays a tree inferred from an ML analysis of nearly full 16S rRNA gene sequences derived from cultured strains of Alphaproteobacteria). The Bayesian 50% majority-rule tree (Fig. S3) also showed a significant degree of similarity to the constrained ML tree (Fig. 2) and previous studies. However, certain nodes in the Bayesian tree do not exist in the constrained ML topology (Bayesian posterior probability (B-PP) values in brackets in Fig. S3), even though two of these nodes are highly-supported (B-PP =

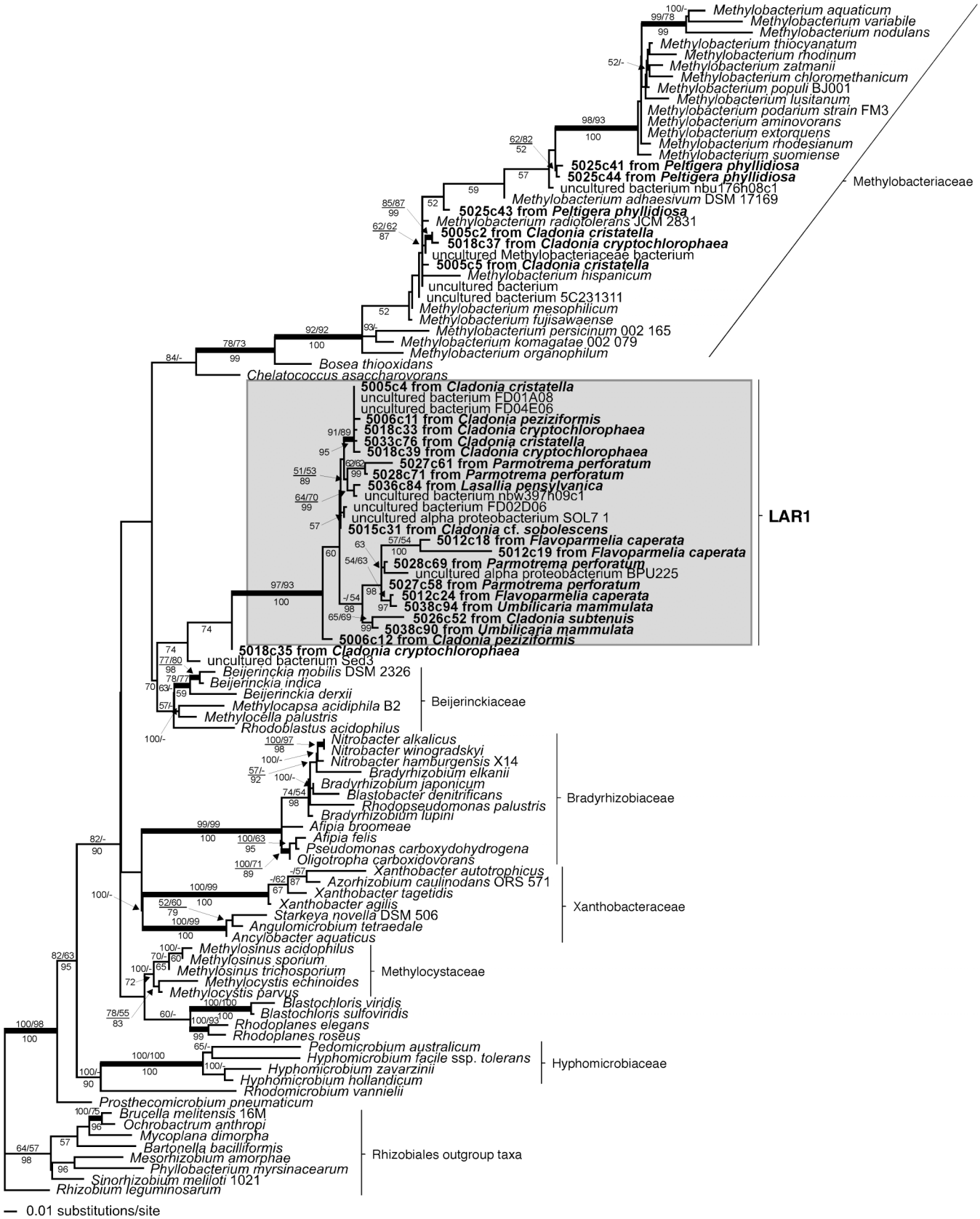

Figure 2. See legend on next page.

99%). These highly-supported nodes not found in the constrained ML topology are also in conflict with the phylogeny presented by Lee et al. (2005).

4. Discussion

A previously undescribed lineage

All phylogenetic analyses revealed high support for a clade of bacteria that is almost exclusively associated with lichen thalli (LAR1 in Fig. 2). Given that these bacteria can be found in a diverse array of lichen thalli, and that previous studies of environmental samples have isolated so few members of this lineage in clone libraries, it remains quite possible that these organisms are specifically adapted to certain conditions in the thalli of lichens (more specifically, lichens with a green alga as the primary photobiont, but this level of specificity needs to be confirmed with data from more cyanolichens). It is also worth mentioning that recent *in situ* analyses have shown that the great majority of bacteria associated with lichens belong to the Alphaproteobacteria (Cardinale et al., 2008), and bacteria from this previously undescribed lineage may well account for a large proportion of these lichen associates.

Symbiosis

Some bacteria found in this study are closely related to organisms that have already been documented to form symbioses with diverse multicellular eukaryotes, such as plant-associated root-nodulating bacteria (e.g., Aceto-bacteraceae, Brucellaceae and *Methylobacterium*; Table S2; Ngom et al., 2004; Saravanan et al., 2008; Sy et al., 2001). Many of the lichen-associated bacteria found in this survey may associate with a wide range of hosts, and perhaps carry some of the well-known 'symbiosis genes' (e.g., *nod, fix, exo*; Niner and Hirsch, 1998). Some of these organisms may even represent bacteria that have been closely associated with lichenized fungi for a long period of evolutionary time, and have had certain lineages switch hosts or substrate to become closely associated with other eukaryotes (for a discussion of lichens as cradles of microbial diversification, see Arnold et al., 2009). LAR1 (Fig. 2) supports the hypothesis that some lineages of bacteria may be specific to the lichen thallus, similar to certain green-algal and cyanobacterial lineages (Helms et al., 2001; Miadlikowska et al., 2006; Lücking et al., 2009).

Diazotrophy

A great number of 16S rRNA gene sequences found in this study belong to taxonomic groups that contain nitrogen-fixing members (e.g., Acetobacteraceae, Brucellaceae, *Methylobacterium*, Rhizobiales; Table S2; Hunter et al., 2006; Ngom et al., 2004; Sy et al., 2001; Young, 1992). Diverse nitrogen-fixers have previously been reported in association with lichens (Cengia-Sambo, 1923, 1925; Grube et al., 2009; Henckel, 1938; Henckel and Plotnikova, 1973; Henckel and Yuzhakova, 1936; Hodkinson et al., 2006; Iskina, 1938; Lambright and Kapustka, 1981; Liba et al., 2006; Zakharova, 1938), and there is good circumstantial evidence that members of LAR1 may fix nitrogen as well. The sequence encoding a *nif*H gene (the gene for dinitrogenase reductase, used in nitrogen fixation) has been directly amplified from the *Umbilicaria mammulata* sample analyzed in this study (Hodkinson and Lutzoni, 2009; Acc. #GU176620). Both this sequence and one amplified from *Cladonia arbuscula* (Grube et al., 2009) have affinities toward *Beijerinckia*, but each sequence remains $\leq$90% similar to any of the sequences stored in GenBank. This evidence from a second locus supports the notion that the new lineage (which shares phylogenetic affinities with *Beijerinckia*, yet is quite distinct) may actually fix nitrogen. Since lichens are notorious for growing on extremely nutrient-poor substrates, it is likely that some of these bacteria are able to provide crucial fixed nitrogen that would otherwise not be available to the lichenized fungi and algae. Some lichens are able to overcome a severe limitation of fixed nitrogen by developing a symbiotic relationship with heterocystous cyanobacteria (Raven, 2002); this same principle may well apply to other nitrogen-fixing bacteria in association with lichens. Symbiotic nitrogen-fixing bacteria could potentially form multiple aggregates in microscopic pockets on the lower cortex of a green-algal lichen (Fig. 1) under dry conditions, but grow to form large populations on the lower surface of lichens when wet conditions are persistent (and the lichen thallus is physiologically most active).

Figure 2. Maximum likelihood (ML) phylogenetic tree showing inferred relationships among 16S rRNA gene sequences obtained from lichen-associated bacteria (in bold), with the most similar GenBank sequences and sequences from cultured reference strains representing the order Rhizobiales. The topology search was conducted using a backbone constraint (Fig. S1; see Materials and Methods for details). ML bootstrap proportions $\geq$50% are written above each node (constrained/unconstrained) and Bayesian posterior probabilities are written below each node. Thicker internodes indicate bootstrap support $\geq$70% in both constrained and unconstrained ML analyses. Several bacterial sequences amplified from lichens with green-algal photobionts fall within a distinct undescribed lineage that is well-supported in all analyses ('LAR1' shaded in grey). Members of this lineage were found in association with specimens from both collection sites and all green-algal lichen samples from which 16S sequences were cloned. In contrast to the bacterial sequences from green-algal lichens, those amplified from the one cyanolichen (*Peltigera phyllidiosa*, 5025) all fit well within the bacterial genus *Methylobacterium*; representatives of this genus were also found in lichens with green algae.

Potential effects of acidic secondary compounds

Many lichens produce copious amounts of acidic secondary compounds, such as depsides and depsidones (Culberson et al., 1984). Some of these compounds have antibiotic properties (Huneck and Yoshimura, 1996; Ingolfsdottir et al., 1985; Lawrey, 1989; Müller, 2001; Vartia, 1950). Interestingly, cyanolichens (such as species from the order Peltigerales) usually do not produce the characteristic depsides and depsidones that are typical of most members of the Lecanoromycetidae (Culberson et al., 1984; Miadlikowska et al., 2006). While it may be true that these compounds serve to keep parts of the lichen thallus free of certain harmful bacteria, it seems likely that some bacteria, such as certain members of Acidobacteriaceae, would be able to withstand or thrive in such environments (Barns et al., 1999). The secondary compound composition of lichens might even preferentially select for beneficial bacteria (e.g., nitrogen fixers) and effectively dictate which bacteria could survive and where they could reside in the lichen thallus.

The lichen thallus as a complex microbial community

The results of this study corroborate the notion that lichen thalli commonly host a number of bacterial lineages (e.g., Acidobacteriaceae and Rhizobiales, Table S2). Preliminary analyses reveal the presence of diverse lineages in each sample, indicating that the lichen-associated microbiome may prove to be an excellent microcosm for studying community ecology in environmental microbial systems. Lichen symbioses with the cyanobacterium *Nostoc*, which is concentrated (nearly restricted) to the lichen-forming Peltigerales (Miadlikowska et al., 2006), have previously been suggested as a key innovation in the evolution of lichenized ascomycetes (Miadlikowska and Lutzoni, 2004). Similarly, symbiosis with a certain type of non-photosynthetic bacterium may have been a key innovation that has led to extensive evolutionary radiation in certain groups of lichen-forming fungi (e.g., Parmeliaceae and Umbilicariaceae). Notably, the results obtained from the one cyanolichen in this study were quite different from those obtained from the green-algal lichens, implying that there may be a fundamentally different type of bacterial community in these lichens. Through further research, specific patterns of association between bacteria and lichens should come into better focus. What seems clear at this point is that many lichens exhibit complex interactions (see also Arnold et al., 2009) that are not reflected in the simple 'fungus and alga' model that is widely accepted today.

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Supplemental Material

Table S1. Sequences of the 16S rRNA gene used in primer design; accession numbers in bold represent sequences generated as part of this study.

Organism	GenBank Accession #
Acetobacter aceti	AJ419840
Acetobacter indonesiensis	AB052715
Acetobacter pasteurianus	AJ419834
Acetobacterium halotolerans	AY744449
Acetobacterium sp. TM20-2	AB086092
Acidimicrobium sp. Y0018	AY140240
Acidiphilium sp. NO-14	AF376023
Acidobacteria bacterium Ellin7137	AY673303
Acidobacteriaceae bacterium Ellin6076	AY234728
Acidobacteriaceae bacterium PK35	AY765993
Acidobacteriaceae bacterium TAA43	AY587228
Acidobacteriaceae bacterium TAA48	AY587229
Acidobacteriaceae isolate WJ7	AY096034
Acidomonas methanolica	AB110707
Actinobacterium #59 white	AF423076
Actinomyces georgiae	X80413
Actinomyces naeslundii	AJ234045
Actinomyces oricola	AJ507295
Actinomyces sp. oral strain Hal-1083	AF385522
Agrobacterium tumefaciens	AJ389904
Bacillus licheniformis	AF516177
Bacillus subtilis	AJ276351
Bacterium Ellin337	AF498719
Bacterium Ellin342	AF498724
Bacterium Ellin345	AF498727
Bacterium Ellin347	AF498729
Bacterium Ellin351	AF498733
Bacterium Ellin5227	AY234578
Bacteroidetes bacterium SM26	DQ195837
Beijerinckia derxii	AB119198
Beijerinckia derxii subsp. *derxii*	AJ563933
Beijerinckia indica	AB119197
Bradyrhizobium elkanii	AB072423
Bradyrhizobium genosp. O	Z94823
Burkholderia caledonica	AF215704
Burkholderia caribensis	Y17009
Burkholderia fungorum	AF215706
Burkholderia gladioli	AB021384
Burkholderia vietnamiensis	U96929
Candidatus Nitrospira defluvii	DQ059545
Caulobacter vibrioides	AJ227754
Clostridium acetobutylicum	X68182
Clostridium botulinum	X68171
Clostridium perfringens	AB045290
Clostridium sp. MD3	AY321657
Cryptobacterium curtum	AB019260
Curtobacterium flaccumfaciens pv. *flaccumfaciens*	AF348973
Desulfobacter postgatei	M26633
Enterococcus faecalis	Y18293
Entomoplasma somnilux	AY157871
Escherichia coli	X80727
Fibrobacter intestinalis	M62690
Fibrobacter succinogenes	M62688
Flavobacterium mizutaii	D14024
Frankia sp.	L40610
Frankia sp. M16464	AJ408871
Frankia sp. Sn4-3	AJ408874
Geothermobacterium ferrireducens	AF411013
Gluconacetobacter diazotrophicus	AY230808
Gluconacetobacter hansenii	AB166735
Gluconacetobacter hansenii	X75620
Helicobacter pylori	AY364440
Helicobacter pylori	Z25742
Lactobacillus johnsonii	AJ002515
Legionella jamestowniensis	X73409
Luteimonas mephitis	AJ012228
Methylobacillus sp. 12S	AB027139
Methylobacter sp. BB5.1	AF016981
Methylobacterium podarium	AY468364
Mycobacterium coloregonium	AY624367
Mycobacterium tuberculosis	X52917
Mycobacterium tuberculosis H37Rv	BX842576
Mycoplasma capricolum subsp. *capripneumoniae*	AF009831
Mycoplasma gallisepticum	M22441

Table S1. Continued.

Organism	GenBank Accession #
Myxococcus xanthus	AY724798
Neisseria lactamica	AJ239284
Neisseria perflava	AJ239295
Nitrosomonas communis	AJ298732
Nitrospira cf. *moscoviensis* SBR2016	AF155154
Nitrospira moscoviensis	X82558
Nitrospira sp.	Y14644
Nostoc sp. *Lobaria* cyanobiont 34	AF506259
Nostoc sp. *Lobaria pulmonaria* cyanobiont 36	AF506261
Nostoc sp. Muscicolous cyanobiont 21	AF506246
Nostoc sp. *Nephroma helveticum* cyanobiont 33	AF506258
Nostoc sp. *Nephroma helveticum* cyanobiont 37	AF506262
Nostoc sp. *Nephroma parile* cyanobiont 27	AF506252
Nostoc sp. *Parmeliella triptophylla* cyanobiont 29	AF506254
Nostoc sp. *Peltigera collina* cyanobiont 20	AF506245
Nostoc sp. *Peltigera pruinosa* cyanobiont 14	AF506239
Oxalobacter formigenes	U49753
Paenibacillus apiarius	AJ320492
Paenibacillus naphthalenovorans	AF353703
Paenibacillus sp. L32	DQ196465
Parvularcula bermudensis	AF544015
Phascolarctobacterium faecium	X72866
Plastid of *Arabidopsis thaliana*	NC_000932
Plastid of *Asterochloris* sp. from *Cladonia cristatella* Hodkinson 5005	**GU191846**
Plastid of *Oryza sativa*	NC_001320
Plastid of *Trebouxia* sp. from *Flavoparmelia caperata* Hodkinson 5012	**GU191847**
Plastid of *Zea mays*	NC_001666
Pseudomonas chlororaphis	Z76673
Pseudomonas sp. IrT-R9M1-191	AJ291844
Rhizobium leguminosarum	AF533683
Rhizobium sp. WSM746	AF325771
Rhizobium undicola	ATCC19358
Rickettsia sibirica	D38628
Rubrobacter taiwanensis	AF479791
Sphingobacterium spiritivorum	D14021
Sphingomonas echinoides	AB033944
Sphingomonas pruni	Y09637
Sphingomonas sp. K101	AJ009706
Spirochaeta africana	X93928
Staphylococcus succinus	AJ320272
Stenotrophomonas maltophilia	AJ131914
Stenotrophomonas-like sp. V4.BP.15	AJ244720
Streptococcus uberis	AB023576
Streptomyces griseoruber	AY999723
Streptomyces lavendulae	D85111
Streptomyces sp.	AJ002084
Streptomyces thermosacchari	AF306658
Thermodesulfobacterium commune	AF418169
Thermodesulfobacterium commune	L10662
Uncultured *Acetobacterium* sp.	AY185318
Uncultured *Acetobacterium* sp.	AY185325
Uncultured *Acidisphaera* sp.	AY882809
Uncultured Acidobacteria bacterium	AY395421
Uncultured Acidobacteria bacterium	AY214899
Uncultured Acidobacteria bacterium	AJ582043
Uncultured Acidobacteria bacterium	AF465656
Uncultured Acidobacterium UA1	AF200696

Table S1. Continued.

Organism	GenBank Accession #
Uncultured bacterium	AF523898
Uncultured bacterium	AY850302
Uncultured bacterium	AY328760
Uncultured bacterium	DQ058675
Uncultured bacterium	AJ576372
Uncultured *Cryptobacterium*	AB189698
Uncultured *Cytophaga* sp.	AB015550
Uncultured delta proteobacterium	AJ567598
Uncultured eubacterium WD243	AJ292579
Uncultured Fibrobacteres bacterium	AB192086
Uncultured forest soil bacterium	AY913273
Uncultured *Geothermobacterium* sp.	AY882738
Uncultured Green Bay ferromanganous micronodule bacterium MNC2	AF293010
Uncultured *Holophaga* sp.	AJ519373
Uncultured *Mycobacterium* sp.	AY911428
Uncultured Thermodesulfobacteriaceae bacterium	AY082369
Uncultured *Thermodesulfobacterium* sp.	AY862037
Vibrio fischeri	X74702
Xanthomonas axonopodis pv. *citri* str. 306	AE012039
Xanthomonas campestris	X99297
Xanthomonas hortorum	Y10759

Table S2. Preliminary 'RHAPSA-D' screening for all samples and primer combinations.

Lichen host*	Primer combination 533F/1492R (control)	895F/1492R	27F/902R	27F/904R	533F/1185mR	533F/1185aR	533F/1381R	533F/1381bR
Cladonia cristatella 5005†	Chloroplast	Bacteria	Acetobacteraceae	Alphaproteobacteria	Acetobacteraceae	Rhizobiales	Acetobacteraceae	Alphaproteobacteria
Cladonia peziziformis 5006†	Chloroplast	Gp1 (Acidobacteriaceae)	Gp1 (Acidobacteriaceae)	Bacteria	Gp1 (Acidobacteriaceae)	Rhizobiales	Bacteria	Bacteria
Flavoparmelia caperata 5012†	Chloroplast	Gp1 (Acidobacteriaceae)	Gp1 (Acidobacteriaceae)	Alphaproteobacteria	Alphaproteobacteria	Rhizobiales	Chloroplast	Chloroplast
Cladonia cf. *sobolescens* 5015†	Chloroplast	Gp1 (Acidobacteriaceae)	Gp1 (Acidobacteriaceae)	Bacteria	Alphaproteobacteria	Rhizobiales	Alphaproteobacteria	Bacteria
Cladonia cryptochlorophaea 5018†	Chloroplast	Proteobacteria	Gp1 (Acidobacteriaceae)	Bacteria	Alphaproteobacteria	Rhizobiales	Bacteria	Bacteria
Peltigera phyllidiosa 5025†	*Nostoc* sp.	Gammaproteobacteria	Brucellaceae	Brucellaceae	*Nostoc* sp.	*Methylobacterium* sp.	Bacteria	Bacteria
Cladonia subtenuis 5026†	Chloroplast	Bacteria	Rhodospirillales	Alphaproteobacteria	Bacteria	Rhizobiales	Bacteria	Bacteria
Parmotrema perforatum 5027	Alphaproteobacteria	Bacteria	Acetobacteraceae	Bacteria	Bacteria	Rhizobiales	Bacteria	Bacteria
Parmotrema perforatum 5028	Alphaproteobacteria	Bacteria	Alphaproteobacteria	Bacteria	Alphaproteobacteria	Rhizobiales	Bacteria	Bacteria
Cladonia cristatella 5033†	Chloroplast	Bacteria	Alphaproteobacteria	Alphaproteobacteria	Acetobacteraceae	Rhizobiales	Bacteria	Bacteria
Lasallia pensylvanica 5036‡	Acidobacteriaceae	Acidobacteriaceae	Gp3 (Acidobacteriaceae)	Bacteria	Bacteria	Rhizobiales	Bacteria	Bacteria
Umbilicaria mammulata 5038‡	Acetobacteraceae	Acidobacteriaceae	Gp1 (Acidobacteriaceae)	Alphaproteobacteria	Bacteria	Rhizobiales	Alphaproteobacteria	Bacteria

*For each lichen sample, species name is followed by the primary author's collection number.
†Denotes samples that demonstrate the effectiveness of the newly designed primers. PCR reactions with DNA extracted from these eight samples produced amplicon pools with a clear overabundance of plastid or cyanobacterial sequences in the control experiment, but reactions with new primers did not produce a detectable amount of the plastid or cyanobacterial amplicons targeted for exclusion in primer design.
‡Denotes samples for which the algal layer was mechanically removed prior to DNA extraction (5036 and 5038).

Table S3. Sequences of the 16S rRNA gene from members of the order Rhizobiales used in phylogenetic analyses; accession numbers are given for sequences generated as part of this study, while GenInfo Identifier (GI) numbers are given for all others.

Organism	GenBank Accession/GenInfo Identifier #
5005c2 from *Cladonia cristatella* Hodkinson 5005	GU191848
5005c4 from *Cladonia cristatella* Hodkinson 5005	GU191849
5005c5 from *Cladonia cristatella* Hodkinson 5005	GU191850
5006c11 from *Cladonia peziziformis* Hodkinson 5006	GU191851
5006c12 from *Cladonia peziziformis* Hodkinson 5006	GU191852
5012c18 from *Flavoparmelia caperata* Hodkinson 5012	GU191853
5012c19 from *Flavoparmelia caperata* Hodkinson 5012	GU191854
5012c24 from *Flavoparmelia caperata* Hodkinson 5012	GU191855
5015c31 from *Cladonia* cf. *sobolescens* Hodkinson 5015	GU191856
5018c33 from *Cladonia cryptochlorophaea* Hodkinson 5018	GU191857
5018c35 from *Cladonia cryptochlorophaea* Hodkinson 5018	GU191858
5018c37 from *Cladonia cryptochlorophaea* Hodkinson 5018	GU191859
5018c39 from *Cladonia cryptochlorophaea* Hodkinson 5018	GU191860
5025c41 from *Peltigera phyllidiosa* Hodkinson 5025	GU191861
5025c43 from *Peltigera phyllidiosa* Hodkinson 5025	GU191862
5025c44 from *Peltigera phyllidiosa* Hodkinson 5025	GU191863
5026c52 from *Cladonia subtenuis* Hodkinson 5026	GU191864
5027c58 from *Parmotrema perforatum* Hodkinson 5027	GU191865
5027c61 from *Parmotrema perforatum* Hodkinson 5027	GU191866
5028c69 from *Parmotrema perforatum* Hodkinson 5028	GU191867
5028c71 from *Parmotrema perforatum* Hodkinson 5028	GU191868
5033c76 from *Cladonia cristatella* Hodkinson 5033	GU191869
5036c84 from *Lasallia pensylvanica* Hodkinson 5036	GU191870
5038c90 from *Umbilicaria mammulata* Hodkinson 5038	GU191871
5038c94 from *Umbilicaria mammulata* Hodkinson 5038	GU191872
Afipia broomeae	2290233*
Afipia felis	28436388*
Ancylobacter aquaticus	173723*
Angulomicrobium tetraedale	40241924*
Azorhizobium caulinodans ORS 571	464201*
Bartonella bacilliformis	39345*
Beijerinckia derxii	49532707*
Beijerinckia indica	173853*
Blastobacter denitrificans	257965*
Blastochloris sulfoviridis DSM 729	1483161*
Blastochloris viridis DSM 133	3549120*
Bosea thiooxidans	6273424*
Bradyrhizobium elkanii	28436387*
Bradyrhizobium japonicum	1165003*
Bradyrhizobium lupini	1165004*
Brucella melitensis 16M	17986284*
Chelatococcus asaccharovorans	9886695*
Hyphomicrobium facile subsp. *tolerans* IFAM I-551	3646013*
Hyphomicrobium hollandicum IFAM KB-677	3646003*
Hyphomicrobium zavarzinii IFAM ZV-622	3646314*
Mesorhizobium amorphae	2801558*
Methylobacterium adhaesivum DSM 17169	157073830*
Methylobacterium aminovorans	59668401*
Methylobacterium aquaticum	51997099*
Methylobacterium chloromethanicum CM4	18026765*
Methylobacterium extorquens	514403*
Methylobacterium fujisawaense DSM 5686	6273567*
Methylobacterium hispanicum	51997100*
Methylobacterium komagatae 002-079	157073772*
Methylobacterium lusitanum	11245771*
Methylobacterium mesophilicum	514404*
Methylobacterium nodulans ORS 2060	12239376*
Methylobacterium organophilum	514405*
Methylobacterium persicinum 002-165	157073773*

Table S3. Continued.

Organism	GenBank Accession/GenInfo Identifier #
Methylobacterium podarium strain FM3	40716503*
Methylobacterium populi BJ001	134133397*
Methylobacterium radiotolerans JCM 2831	514406*
Methylobacterium rhodesianum	514407*
Methylobacterium rhodinum	514408*
Methylobacterium suomiense	16554668*
Methylobacterium thiocyanatum	3241964*
Methylobacterium variabile	59668402*
Methylobacterium zatmanii	388906*
Methylocapsa acidiphila B2	15072624*
Methylocella palustris	3805791*
Methylocystis echinoides	21685003*
Methylocystis parvus	7529616*
Methylosinus acidophilus	67942373*
Methylosinus sporium	7529617*
Methylosinus trichosporium	175489*
Mycoplana dimorpha IAM 13154	303639*
Nitrobacter alkalicus	6650226*
Nitrobacter hamburgensis	530890*
Nitrobacter winogradskyi	402722*
Ochrobactrum anthropi IAM 14119	303715*
Oligotropha carboxidovorans	27597202*
Pedomicrobium australicum IFAM ST1306	1314051*
Phyllobacterium myrsinacearum	303752*
Prosthecomicrobium pneumaticum MBIC3489	4126812*
Pseudomonas carboxydohydrogena	4165399*
Rhizobium leguminosarum	22324902*
Rhodoblastus acidophilus	175822*
Rhodomicrobium vannielii EY33	175865*
Rhodoplanes elegans	529092*
Rhodoplanes roseus	435464*
Rhodopseudomonas palustris	529086*
Sinorhizobium meliloti 1021	15963753*
Starkeya novella	514989*
Uncultured alpha proteobacterium BPU225	29893282†
Uncultured alpha proteobacterium SOL7_1	54610222†
Uncultured bacterium clone 3	62868621†
Uncultured bacterium clone 5C231311	190708627†
Uncultured bacterium clone nbu176h08c1	238404679†
Uncultured bacterium clone Sed3	125660699†
Uncultured bacterium FD01A08	215269506†
Uncultured bacterium FD02D06	215269590†
Uncultured bacterium FD04E06	215270126†
Uncultured bacterium nbw397h09c1	238333997†
Uncultured Methylobacteriaceae 10-3Ba06	38195106†
Xanthobacter agilis	1314197*
Xanthobacter autotrophicus	1314199*
Xanthobacter tagetidis	2108340*

*Sequence used in phylogenetic backbone construction (Fig. S1). †Top BLASTn hit for at least one cloned sequence generated as part of this study (not present in the phylogenetic backbone used in constrained ML analyses).

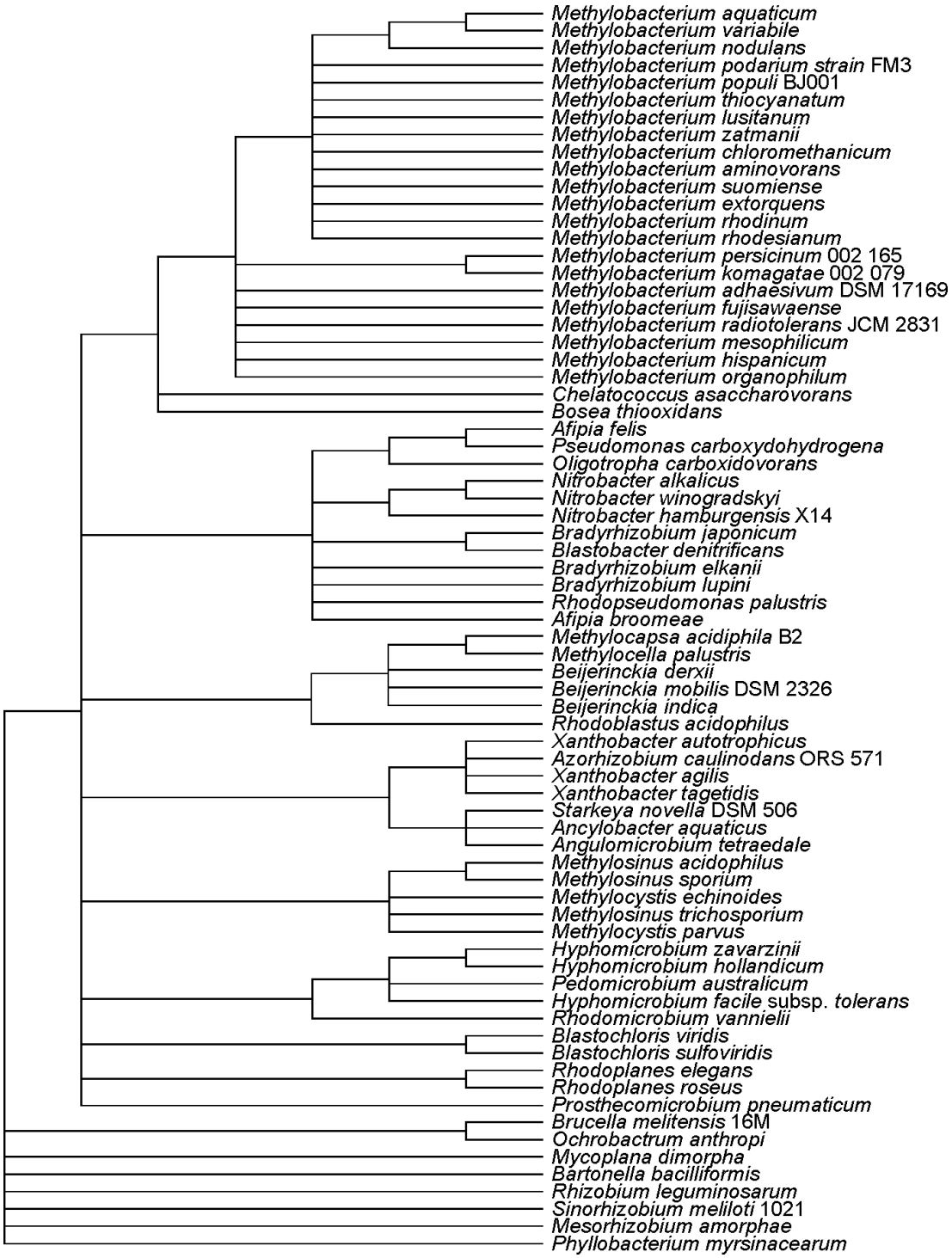

Figure S1. Topology representing the constraint tree enforced on backbone-constrained ML analyses performed as part of this study. The sequences used to generate this tree were all nearly full-length 16S rRNA gene sequences, and the nodes that exist in the tree are supported by BP $\geq$70 in both MP and ML analyses.

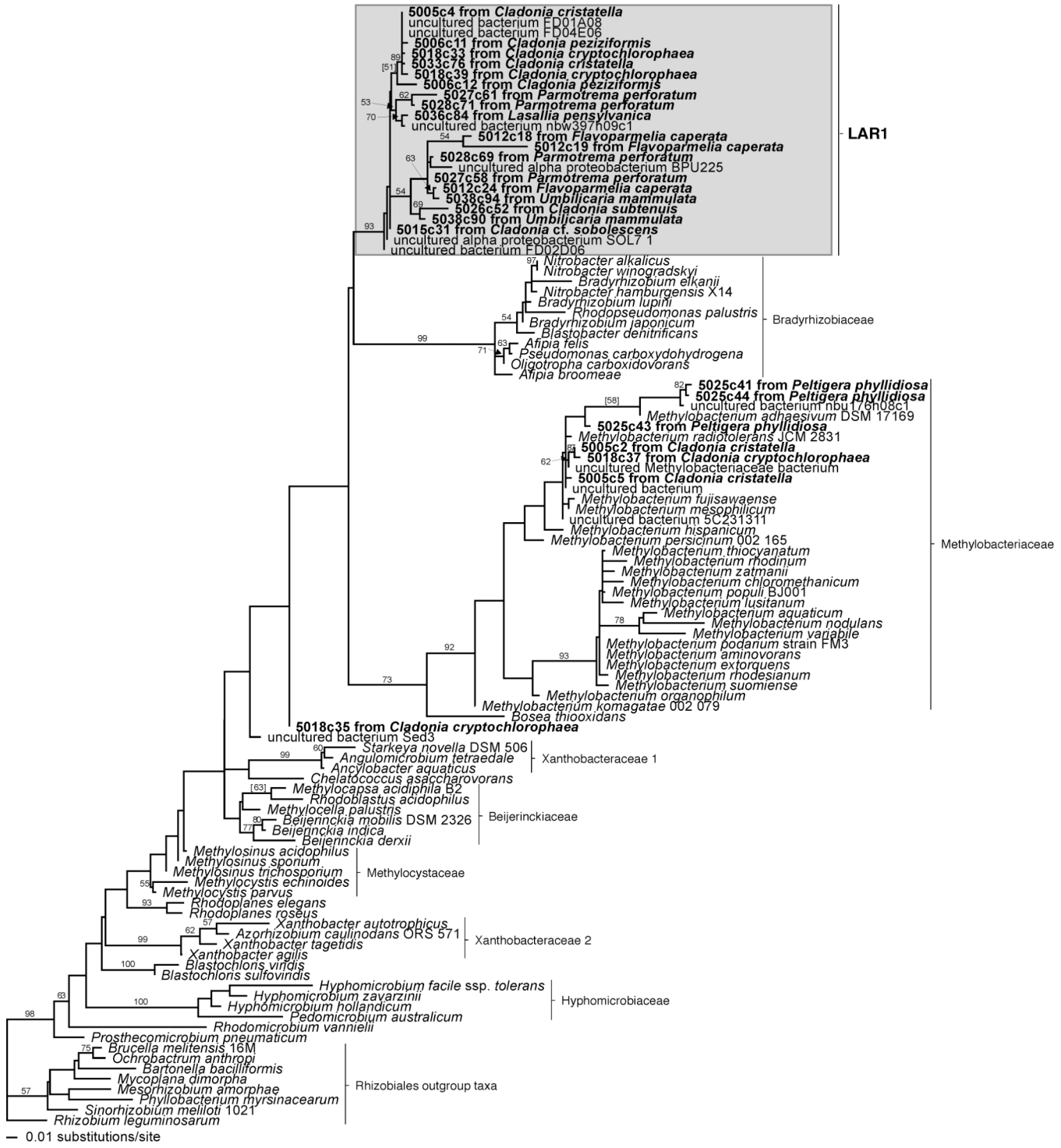

Figure S2. Unconstrained ML phylogenetic tree showing inferred relationships among 16S rRNA gene sequences obtained from lichen-associated bacteria (in bold), with the most similar GenBank sequences and sequences from cultured reference strains representing the order Rhizobiales. ML bootstrap proportions ≥50% are written above each node, with brackets around those that could not be mapped to the backbone-constrained ML topology (Fig. 2). The 'LAR1' lineage is highlighted in grey.

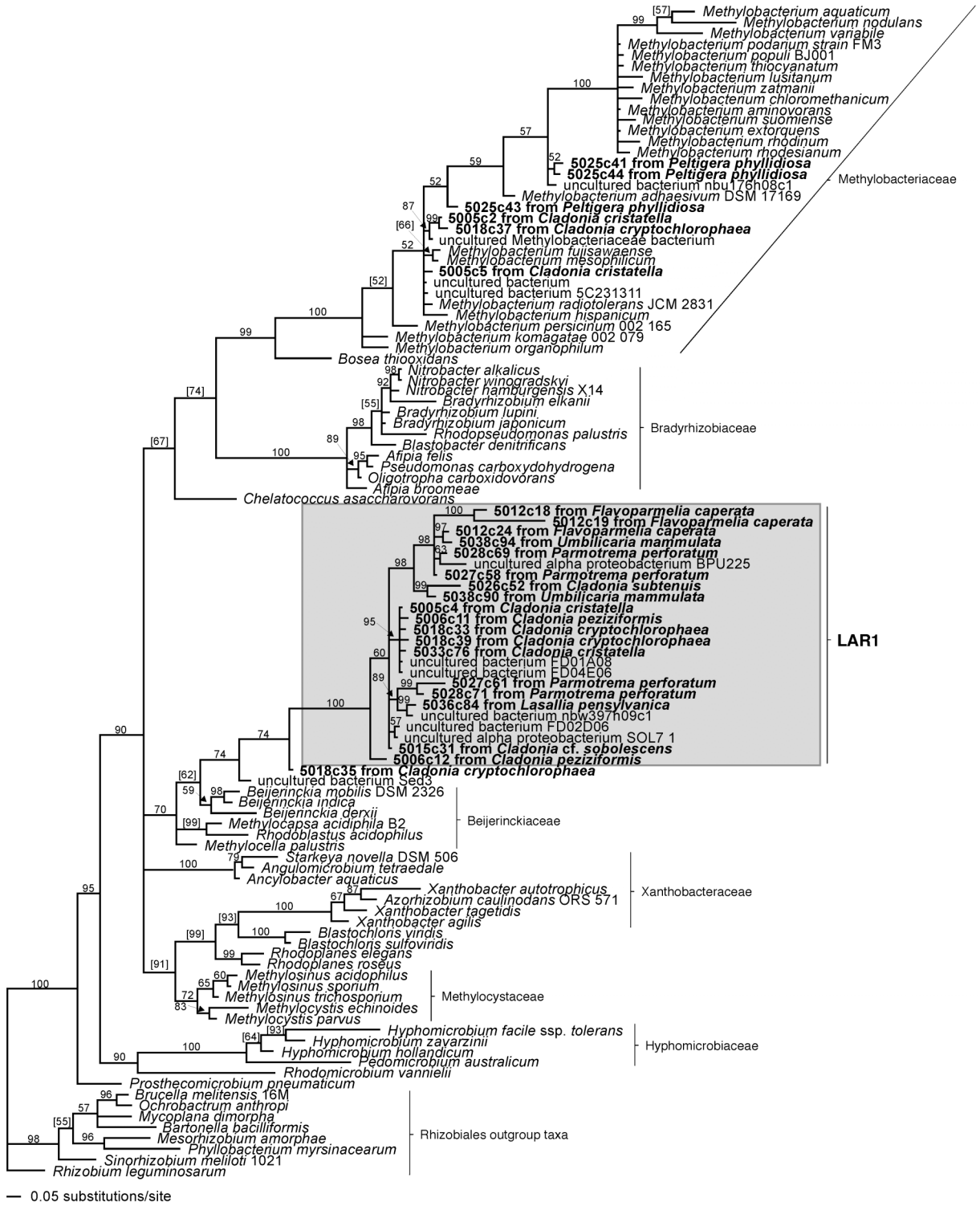

Figure S3. A 50% majority-rule Bayesian phylogenetic tree showing inferred relationships among 16S rRNA gene sequences obtained from lichen-associated bacteria (in bold), with the most similar GenBank sequences and sequences from cultured reference strains representing the order Rhizobiales. Bayesian posterior probability (B-PP) values ≥50% are written above each node, with brackets around those that could not be mapped to the backbone-constrained ML topology (Fig. 2). The 'LAR1' lineage is highlighted in grey.