Analysis of the *Peltigera membranacea* metagenome indicates that lichen-associated bacteria are involved in phosphate solubilization

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Although lichens are generally described as mutualistic symbioses of fungi and photosynthetic partners, they also harbour a diverse non-phototrophic microbiota, which is now regarded as a significant part of the symbiosis. However, the role of the non-phototrophic microbiota within the lichen is still poorly known, although possible functions have been suggested, including phosphate solubilization and various lytic activities. In the present study we focus on the bacterial biota associated with the foliose lichen *Peltigera membranacea*. To address our hypotheses on possible roles of the non-phototrophic microbiota, we used a metagenomic approach. A DNA library of bacterial sequence contigs was constructed from the lichen thallus material and the bacterial microbiota DNA sequence was analysed in terms of phylogenetic diversity and functional gene composition. Analysis of about 30 000 such bacterial contigs from the *P. membranacea* metagenome revealed significant representation of several genes involved in phosphate solubilization and biopolymer degradation.

INTRODUCTION

Lichens are generally characterized as the symbiotic association of a fungal partner (mycobiont), green algae and/or cyanobacteria (Nash, 2008), and together the lichen symbiotic partners form a thallus, which may be able to tolerate and sustain growth in hostile and dynamic environments where they could not survive alone (Bjelland et al., 2011). Lichens generally have high tolerance to ecological extremes in temperature, low nutrient and/or water availability and UV light intensities and can therefore be found in a wide range of habitats, often where few other organisms survive (Beckett et al., 2008; Boddy et al., 2010). Although generally described as the symbiosis of a fungus and a photosynthetic partner, recent studies have revealed a high diversity and abundance of non-phototrophic bacteria present in lichen thalli (Cardinale et al., 2006, 2008; Grube et al., 2009; Hodkinson & Lutzoni, 2009; Bjelland et al., 2011; Mushegian et al., 2011). The presence of this non-phototrophic microbiota has long been known (Henkel & Yuzhakova, 1936; Henkel & Plotnikova, 1973), and members are now suggested to be involved in several important roles within the symbiosis, including iron and phosphate mobilization, hormone production, nitrogen fixation and several lytic activities (Liba et al., 2006; Grube et al., 2009). Since many lichens can thrive on extremely nutrient-poor substrates, it has also been suggested that the non-phototrophic microbiota plays an important role in the lichen thalli by facilitating supply of crucial nutrients (González et al., 2005; Cardinale et al., 2006; Liba et al., 2006). Phosphorus (P) is a primary element involved in all major metabolic pathways (Khan et al., 2010; Zhao et al., 2014; Sharma et al., 2013). In nature, fully oxidized P occurs as phosphate, and the majority is present in insoluble form that cannot be directly utilized by organisms (Richardson et al., 2001). P must therefore be released from organic and inorganic compounds and it is well known that phosphate-solubilizing bacteria can make it available to plants (Chhabra et al., 2013; Richardson et al., 2001; Sashidhar & Podile, 2010; Zhao et al., 2014; Sharma et al., 2013). These pathways are complex and several key enzymes are involved, including alkaline and acid phosphatases, phytases and phosphonatases (Rodriguez et al., 2006; Sharma et al., 2013). A variety of bacteria are known to convert bound phosphorus to a soluble form and the genera *Azospirillum*, *Azotobacter*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* have been reported as the most significant
phosphate-solubilizing bacteria (Sturz & Nowak, 2000; Sudhakar et al., 2000). Proteobacteria, particularly Alphaproteobacteria, form the largest part of the non-phototrophic bacteria in many lichens (Cardinale et al., 2006, 2008; González et al., 2005; Grube et al., 2009; Hodkinson & Lutzoni, 2009; Liba et al., 2006; Muggia et al., 2013; Schneider et al., 2011). Many of the proteobacterial genera listed above are commonly found in lichens, and recent studies have shown that one of the potential roles of the associated microbiota could well be phosphate solubilization (Liba et al., 2006; Grube et al., 2009; Sigurbjörnsdóttir et al., 2014). Thus we hypothesized in the present study that phosphate solubilization is an important trait of the lichen-associated non-phototrophic microbiota.

Several previous studies have characterized lichen-associated microbiota with culture-based experiments (Cardinale et al., 2006; González et al., 2005; Grube et al., 2009; Henkel & Plotníkova, 1973; Liba et al., 2006). However, the vast majority of micro-organisms found in natural habitats remain unculturable in the laboratory (Amano et al., 1995), underscoring the relevance of culture-independent approaches. By using metagenomics, the functional gene composition of microbial communities can be assessed, yielding a more extensive description than phylogenetic methods (Thomas et al., 2012). The first study of lichen-associated bacteria based on metagenomic methods was conducted by Cardinale et al. (2006), where a great diversity of bacteria was shown to be present in the lichen thalli sampled.

The present study focuses on the non-phototrophic microbiota associated with the foliose lichen Peltigera membranacea, commonly found in Icelandic vegetation. P. membranacea is a cyanolichen, with Nostoc cyanobacteria as photobiont (Miadlikowska & Lutzoni, 2004). A metagenomic library was constructed from a collection of lichen thalli and the bacterial microbiota were analysed in terms of phylogenetic diversity and functional gene composition. Genes encoding phosphatases as well as other enzymes were identified in order to predict possible roles of the associated non-phototrophic microbiota of the lichen.

**METHODS**

**Lichen samples, DNA extraction, sequencing and assembly.** P. membranacea (accession nos: XBB013, Biology Laboratory, University of Iceland; LA-31632, Icelandic Institute of Natural History) was collected on 21 September 2008 at Keldnagil, Reykjavik, Iceland within a 12 m span (coordinates 64° 64.7’ N, 21° 46.6’ W). At this site P. membranacea thalli are fairly large (mostly 4–8 cm wide and more than 12 cm long), loosely attached to a substrate consisting mainly of the mosses Hylocomium splendens and Pleurozium schreberi, intermingled with Empetrum nigrum, Vaccinium uliginosum and Betula nana, and contain very little soil material. The sample consisted of 8–10 cm long thalli from approximately 20 different individuals collected into a clean aseptic plastic box. The thalli were cleaned under a stereomicroscope until no epiphytes or extraneous material could be seen [analysis of the DNA extracted yielded a low level (<1%) of non-Peltigera eukaryotic sequences] and extensively washed with distilled water before DNA extraction as previously described (Sinnenmann et al., 2000). Dried lichen (1.2 g) was placed in a mortar and cooled with liquid nitrogen before thorough grinding. The powder was incubated at 65°C for 20 min. with 15 ml lysis buffer (10 mM Tris/ClI/EDTA, 100 mM NaCl, 1% SDS and 40 ng RNase A), with occasional gentle mixing. The lysis mixture was divided between 2 ml tubes and centrifuged for 4 min at 20 000 g. Supernatant (1 ml) was transferred to a new 2 ml tube and 428 μl 10.5 M ammonium acetate was added. The mixture was kept on ice for 20 min and centrifuged for 4 min at 20 000 g. Then, 1250 μl supernatant was transferred to a new 2 ml tube, 800 μl 2-propanol was added and the mixture was centrifuged for 4 min at 20 000 g. The supernatant was carefully removed, and 1 ml TE buffer (10 mM Tris/ClI pH 8.0 and 1 mM EDTA) was added. The tubes were incubated at 50°C for 20–60 min with occasional agitation until the pellet was dissolved. The tubes were centrifuged briefly and the supernatant was transferred to a new tube if a pellet was visible. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, and the tubes were vortexed briefly and centrifuged for 4 min at 20 000 g. The aqueous phase was transferred to a fresh 2 ml tube without disturbing the interphase, an equal volume of saturated chloroform was added, and after brief vortexing the tubes were centrifuged for 4 min at 20 000 g. The aqueous phase was again transferred to a fresh tube without disturbing the interphase, 0.1 vol 3 M sodium acetate (pH 5) and 2 vol ethanol were added, and after brief vortexing the tubes were centrifuged for 4 min at 20 000 g. The supernatant was discarded and the pellet washed with ~500 μl 70% EtOH. After air-drying, 100 μl TE buffer was added and the pellet brought into solution at 50°C for 2–5 min with gentle tapping. Approximately 2 mg high molecular weight (>50 kb) DNA was recovered from 4 g dried lichen thalli. This DNA was processed for sequencing at a commercial facility (Microsynth) via Roche 454 technology. Approximately 1.76 Gb of 454 data (~340 base mean read length) was obtained. The 454 reads were assembled into contiguous sequences (contigs) using the Newbler (Roche) software (Chevreux et al., 1999).

**Construction and screening of the metagenomic library.** Contigs were sorted into three bins based on significant scores with tblastn (Altschul et al., 1990) against protein databases of filamentous ascomycetes (P < 10^-5) and of Nostoc (P < 10^-5). The remaining contigs were mainly bacterial and were used for the construction of the metagenomic library used in this study. The non-Nostoc contigs were first assigned to taxonomic groups by Infernal and SeqMatch matching against the RDP database (Wang et al., 2007). Based on the assignments, 15 reference genomes were selected for extraction of the non-Nostoc bacterial metagenome: Acidiphilium cryptum JF-5, Acidobacterium capsulatum ATCC 51196, Akkermansia muciniphila ATCC BAA-835, Cytophaga hutchinsonii ATCC 33406, Frankia alni ACN14a, Methylobacterium chloromethanicum CM4, Nocardia farcinica IFM10152, Opitutus terrae PB901_1, Oxalobacter formigenes HOxRLS uid32497, Pedobacter heparginus DSM 2366, Phenylbacterium zunicum HLK1, Rubrobacter xylanophilus DSM 9941, Sinorhizobium melloti 1021, Sorangium cellulosum So_ce_56 and Sphingomonas wittichii RW1. The BLASTN algorithm was used to compare the non-Nostoc bacterial community with the 15 reference genomes and resulted in 150 573 contigs, composing the metagenomic library. Contigs that returned results with E-values below 10^-5 from the BLASTN search were extracted from the library and imported into CLC Genomic Workbench (CLC Bio). The number of actual contigs used for further analysis in this study was 30 033. The BLASTX suite was run via CLC against the ‘non-redundant protein sequence’ database from NCBI for assembly-based functional analysis and an overview of the taxonomic profiles. Based on the BLASTX results, the contigs were mapped manually to metabolic pathways using KEGG orthology groups and Cluster of Orthologous Groups (COG) categories (Tatusov et al., 2000). The bacterial contigs were deposited in GenBank under accession numbers KP422466–KP452497.
RESULTS AND DISCUSSION

Taxonomic diversity of total DNA from *P. membranacea*

In the present study, the non-*Nostoc* bacterial metagenome of the cyanolichen *P. membranacea* was studied. An initial extraction of the contigs generated using 454 technology based on 16S rRNA homology yielded 518 contigs, whereof 14 were determined to be of non-bacterial origin or of insufficient quality for classification. The remaining 504 contigs were classified into 64 operational taxonomic units, which, while not composing a fully rarefied collection (analysis not shown), were deemed likely to contain representatives of the most abundant taxa present. The majority of contigs (381) were found to belong to the *Cyanobacteria*, presumably mostly to the *Nostoc* photobiont, but the remaining 123 contigs were found to represent a population dominated by *Proteobacteria* (58%), followed by *Bacteroidetes* (14%), *Actinobacteria* (11%) and *Verrucomicrobia* (8%) (Fig. 1).

The proteobacterial population is strongly dominated by *Alphaproteobacteria* (70%), followed by *Betaproteobacteria* (15%), *Gammaproteobacteria* (8%) and *Deltaproteobacteria* (6%). This is in good accordance with several recent studies on lichen-associated bacteria, which have shown that the most common taxa in the growing parts of lichens belong to *Alphaproteobacteria* (Bates et al., 2011; Bjelland et al., 2011; Hodkinson et al., 2012; Mushegian et al., 2011; Grube et al., 2012). Of the orders within *Alphaproteobacteria*, we found *Rhizobiales* to be dominant among the microbiota associated with *P. membranacea* (42% of alphaproteobacterial sequences), although the *Rhodospirillales* (32%), *Caulobacterales* (14%) and *Sphingomonadales* (10%) are also well represented. The betaproteobacterial fraction, on the other hand, appears quite homogeneous and is almost exclusively made up of members of the *Burkholderiales*. Hodkinson et al. (2012) reported that the *Rhizobiales* was generally dominant in the bacterial part within nine different lichens, including *Peltigera*, which also agrees well with our results. Our results are thus in general agreement with other recent studies (Cardinale et al., 2006, 2008; González et al., 2005; Grube et al., 2009; Hodkinson & Lutzoni, 2009; Hodkinson et al., 2012; Liba et al., 2006; Muggia et al., 2013; Schneider et al., 2011).

Based on these initial results, 15 reference genomes were selected for BLASTN binning into a putative non-*Nostoc* bacterial metagenome based on E-values below $10^{-3}$. This resulted in a 95 Mb metagenome composed of 30 033 contigs, which were then compared with protein databases using the BLASTX algorithm. The taxonomic analysis, based on the highest similarity to known or predicted proteins and a low E-value from the BLASTX search, largely agreed with the previous 16S rRNA analysis, although agreement between the taxonomic identities of the BLASTN and BLASTX hits was in many cases poor. Of the coding sequences identified, the predominant fraction (86%) of the total metagenome is from *Proteobacteria*, with *Actinobacteria* and *Bacteroidetes* less common (8 and 3%, respectively). Only 1% belong to *Firmicutes*, *Acidobacteria* and *Verrucomicrobia*, and other phyla are present at lower abundances (Fig. 2a). The extracted metagenome is thus expected to contain genes from all major groups present in the non-cyanobacterial bacteriome, with *Proteobacteria* being particularly well represented. The proteobacterial coding sequences identified are from the *Alphaproteobacteria* (55%), *Betaproteobacteria* (31%) and *Gammaproteobacteria* (13%). Representatives from *Epsilon-, Delta-* and *Zetaproteobacteria* are few (Fig. 2b). Among the *Alphaproteobacteria*, *Rhizobiales* and *Sphingomonadales* are the most frequent orders. Within *Rhizobiales*, *Bradyrhizobiaceae* and *Methyllobacteriaceae* are prominent, but members of *Beijerinckiacaeae*, *Phyllobacteriaceae*, *Rhizobiacaeae* and *Xanthobacteriaceae* are also present in lower quantities. The majority of the *Sphingomonadales* belong to *Sphingomonadaecaeae*. Genes presumed to be from members of the order *Burkholderiales* are dominant among the taxon *Betaproteobacteria*, the majority belonging to the family *Comamonadaceae*, and this agrees well with a recent metagenomic study on the associated microbiota of lichens (Grube et al., 2014).

Functional gene composition and key enzymes

Functionalities encoded in the bacterial contigs were mapped to KEGG orthologue groups and COG categories. As can be seen from Fig. 3, nearly half of the sequences (14 362 contigs) could be mapped to metabolic categories. A total of about 10 000 contigs were classified as enzymes participating in cellular processes and signalling or information storage and processing. The remaining contigs (6275) were poorly characterized.
Genes contributing to phosphate solubilization. Several strains isolated from various lichen thalli have been reported as phosphate-solubilizing (Liba et al., 2006; Grube et al., 2009, 2014; Sigurbjörnsdóttir et al., 2014). Given that lichens often thrive under extreme ecological conditions, we hypothesized that phosphate solubilization could be an important role of the lichen-associated non-phototrophic microbiota. About 1335 contigs suggesting the presence of enzymes involved in phosphate metabolism were found in our dataset. Phosphatases that participate in the release of inorganic P were particularly prominent (588 contigs), but organic, alkaline and transport phosphatases are also present, although in lower numbers. We estimate there are more than 20 gene copies encoding alkaline phosphatase per ~5 Mb genome equivalent of the non-phototrophic bacterial metagenome. In a recent metagenomic study...
Grube et al., 2014) on the associated microbiota of the lung lichen Lobaria pulmonaria, phosphatases were also well represented, supporting our primary hypothesis.

The process of mineral phosphate solubilization (MPS) is complex and involves several gene products (Chhabra et al., 2013; Rodríguez et al., 2006; Zhao et al., 2013; Apel et al., 2007). Acidification is a key step in the dissolution of many poorly soluble mineral phosphates, and hence, as proposed by Goldstein (1996), the direct oxidation of glucose to gluconic acid is a key step in some of the major mechanisms for MPS in Gram-negative bacteria, which are suggested to be more efficient at MPS than Gram-positive bacteria owing to secretion of organic acids from sugar metabolism (Sashidhar & Podile, 2009). The pathway is mediated by a membrane-bound glucose dehydrogenase and requires pyrroloquinoline quinone (PQQ) as a cofactor (Goldstein, 1995). Bacteria belonging to the genera Achromobacter, Agrobacterium, Bacillus, Enterobacter, Erwinia, Escherichia, Flavobacterium, Mycobacterium, Pseudomonas and Serratia have been found to be efficient in phosphate solubilization (Goldstein, 2001). Genes encoding PQQ-associated proteins were present in 27 contigs in our dataset. Thereof, PQQ-dependent dehydrogenases were encoded in 13 contigs. The remaining 14 contigs contained members of the PQQ biosynthesis operon: pqqB (two contigs), pqqC (five contigs), pqqD (three contigs) and pqqE (four contigs). All PQQ-associated quinoproteins and PQQ biosynthesis proteins found in the metagenome belong to Gram-negative Proteobacteria, showing greatest similarity to the corresponding genes from members of the Rhizobiaceae, Sphingomonadaceae, Burkholderiaceae, Xanthomonadaceae and Myxococcaceae. PQQ-associated proteins have previously been proposed to be among required factors in mineral phosphate metabolism. Thus, finding these genes among key members of the Peltigera-associated microbiome further supports our hypothesis that phosphate solubilization by the associate members plays an important role in the lichen symbiosis. Further supporting that conclusion is the comparatively large number of contigs encoding phosphatases (Fig. 4). Not surprisingly, the majority of phosphatase-encoding contigs in our dataset belong to Proteobacteria, particularly Alphaproteobacteria.

Other enzymes of interest. Sequences encoding proteins responsible for both central and degradative carbohydrate metabolism and transport are well represented, as expected. Furthermore, sequences assigned to proteins involved in energy production, amino acid metabolism and transport of nucleotides, coenzymes and lipids are also well represented. Although many of the enzymes are included in central metabolism, and thus expected in the dataset, the presence of others supports previous findings of amino acid release of the non-phototrophic microbiota of lichens (Schneider et al., 2011; Liba et al., 2006) and that the bacterial microbiota assists in re-allocating resources within the lichen (Schneider et al., 2011).

Further classification of the metagenome into the major COG gene ontology categories can be seen in Fig. 5. A relatively large portion (19.8 %) of the metagenome remained poorly characterized, as BLASTX search resulted in hypothetical proteins with unknown functions. General function could only be predicted for a third of these poorly characterized entities and included many ABC transporter-like proteins that could not be assigned to a particular pathway. BLASTX searches resulted in no significant similarity for 12 % of the putative proteins (Fig. 5).

Recent functional and metagenomics studies have indicated that the bacterial microbiota of lichens possesses a

![Fig. 4. Taxonomic distribution of 1335 contigs containing phosphatase genes as determined by BLASTX against the NCBI nr database.](http://mic.sgmjournals.org)
wide range of lytic activities: chitinolysis, proteolysis and glucanolysis (Cardinale et al., 2006; Grube et al., 2009, 2014; Sigurbjörnsdóttir et al., 2014). Given the cellulose-rich environment of P. membranacea, a terricolous lichen that grows in close proximity to mosses and other vegetation, cellulolysis may be considered likely to be of particular importance for the P. membranacea holobiont. Cellulase systems are known and have been studied for several lichens, including Peltigera canina (de los Ríos et al., 1997), a lichen closely related to P. membranacea used in our study. Thus, we screened our metagenomic dataset for cellulases, yielding several hits (172 contigs). Glycoside hydrolase (GH) gene fragments, especially GH family 5, were particularly abundant. Endocellulases (endoglucanases) belonging to GH family 5 break the internal bonds and disrupt the crystalline structure of cellulose. Although lichens generally obtain their carbon from the photosynthetic partner (Palmqvist, 2000), it has been hypothesized that cellulases found within the lichen are used for saprophytic activity, which could be beneficial for the symbiosis, e.g. when lichens are covered by snow (Beckett et al., 2013). The presence of many cellulases within our dataset suggests that cellulases might help to exploit the substrate when lichens pursue facultative heterotrophy using the substrate matter under snow during long and dark winters and thus supports the previous finding of Beckett et al. (2013). However, cellulases might also help to degrade ageing cells in older parts of the thallus, together with proteases, which are indeed frequently found in our metagenomics dataset (369 contigs).

Enzymes participating in secondary metabolism were found in 654 contigs in our dataset. Among interesting enzymes found are carboxymethylenbutenolidase (EC 3.1.1.45) and phenol 2-monooxygenase (EC 1.14.13.7). Both these enzymes participate in gamma-hexachlorocyclohexane (HCH) degradation. HCH, also known as lindane, is widely used as an insecticide but has now been banned or restricted because of its toxicity and persistence in the environment (Cao et al., 2013). The presence of these enzymes, and other enzymes related to secondary metabolites, in the metagenomic dataset might suggest that the associated microbiota somehow takes part in a defence mechanism within the symbiosis.

CONCLUSIONS

In the present study we have focused on the substantial non-phototrophic microbiota of the lichen P. membranacea, and hypothesized on their possible roles. We used metagenomic methods to elucidate the potential functional roles of endolichenic bacteria and hypothesized that phosphate solubilization is among the most important traits. A phylogenetic analysis of bacterial contigs from the metagenomic database revealed that the major part consists of Proteobacteria, with Alphaproteobacteria being particularly abundant. This supports previously published data on endolichenic bacteria, although metagenomic methods have not, to our knowledge, previously been used to identify the associated microbiota of P. membranacea. A search for genes contributing to phosphate solubilization within the dataset yielded many positive hits (1335 of 30 033 contigs), mainly belonging to Alphaproteobacteria, further supporting our hypothesis of the importance of phosphate solubilization within the symbiosis.

REFERENCES


