RESEARCH ARTICLE

Bacterial communities in an optional lichen symbiosis are determined by substrate, not algal photobionts

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One sentence summary: In a loose lichen association, algal–fungal interactions do not influence bacterial communities significantly. A more complex morphological structure such as the lichen thallus is likely required for hosting specific microbiota

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ABSTRACT

Borderline lichens are simple mutualistic symbioses between fungi and algae, where the fungi form loose mycelia interweaving algal cells, instead of forming a lichen thallus. Schizoxylon albescens shows two nutritional modes: it can either live as a borderline lichen on Populus tremula bark or as a saprotroph on Populus wood. This enables us to investigate the microbiota diversity in simple fungal–algal associations and to study the impact of lichenization on the structure of bacterial communities. We sampled three areas in Sweden covering the distribution of Schizoxylon, and using high-throughput sequencing of the 16S rRNA gene and fluorescence in situ hybridization we characterized the associated microbiota. Bacterial communities in lichenized and saprotrophic Schizoxylon were clearly distinct, but when comparing the microbiota with the respective substrates, only the fruiting bodies show clear differences in composition and abundance from the communities in the substrates. The colonization by either lichenized or saprotrophic mycelia of Schizoxylon did not significantly influence the microbiota in the substrate. This suggests that in a morphologically simple form of lichenization, as represented by the Schizoxylon–Coccomyxa system, algal–fungal interactions do not significantly influence bacterial communities, but a more complex structure of the lichen thallus is likely required for hosting specific microbiota.

Keywords: Coccomyxa; FISH; fungal life style; metabarcoding; microbiota; Schizoxylon

INTRODUCTION

Lichenization is the symbiotic lifestyle where fungi utilize green algae or cyanobacteria to gain carbohydrates. In a lichen thallus, the photosynthetic partners (the photobionts) usually occur inside a protective structure built by fungal hyphae (Hawksworth and Honegger 1994). In some lichenized associations, however, the fungal partner does not develop a well-differentiated thallus but instead forms simple packages of fungal mycelia interweaving clusters of algal cells. These loose fungal–algal associations have been described as ‘borderline lichens’ (e.g. Lud, Huiskes and Ott 2001; Kohlmeyer, Hawksworth and Volkman-Kohlmeyer 2004; Pérez-Ortega et al. 2016). During a series of studies on the ascomycete family Stictidaceae (Wedin,
Döring and Gildenstam, 2004, 2006), several species growing on Populus were found to be borderline lichens. For these cases, where the spore-producing fungus either is weakly lichenized or saprotrophic depending on the substrate (bark or wood, respectively), Wedin, Döring and Gildenstam (2004) coined the term ‘optional lichenization’.

Among the Stictidaceae exhibiting optional lichenization, Schizoxylon albescens Gildenstam, H. Döring & Wedin is one of the most common and widespread species in northern Sweden, but it was largely overlooked until being described by Wedin, Döring and Gildenstam (2006). The different lifestyles of S. albescens can be easily distinguished in the field, because they develop on different substrates and show morphological differences (Fig. 1). The lichenized morph grows on Populus bark, where it forms white patches (Fig. 1A) containing minute fruiting bodies (∼0.5–1 mm) that are surrounded by lichenized clumps of photobionts (Fig. 1B). In contrast, the saprotrophic morph grows on Populus wood (twigs and branches), producing slightly larger fruiting bodies (∼gal1–2 mm) that emerge from the substrate without creating whitened patches (Fig. 1C). A detailed description of the species was provided by Wedin, Döring and Gildenstam (2006).

Muggia et al. (2011) studied the genetic diversity of the fungal and algal partners of this system, and their haplotype and single-marker phylogenetic analyses revealed two genetically characterized fungal lineages that did not correspond with lifestyle differences. Schizoxylon albescens was concluded to include two cryptic lineages that both express the lichenized and the saprotrophic lifestyles. The chloroplast rbcL sequences suggested that the photobiont isolates all belong to the genus Coccomyxa, a common green algal photobiont of lichens (Gustav et al. 2017). Muggia et al. (2011) also studied the fungal–algal interactions in detail using in vitro cultures. Light microscopy and scanning electron microscopy (SEM) images showed how the Schizoxylon hyphae produced a filamentous matrix when growing with the algal cells in co-cultures, indicative of lichen-like interactions (Honegger 1991). SEM images of environmental samples also revealed bacteria entangled in this fungal matrix, but these were not considered at that time.

Many studies have shown that bacteria are present and abundant in lichen thalli (e.g. Cardinale, Puglia and Grube 2006; Cardinale et al. 2008; Grube et al. 2009, 2015; Hodkinson and Lutzoni 2009; Sigurbjörnsdóttir et al. 2014) in a biofilm-like manner on the surfaces and in the internal structures. Lichen-associated bacteria are not extensions of the bacterial communities found in the substrate (e.g. rocks, soil, bark, wood), but are structured and specific (Grube et al. 2009; Bates et al. 2011; Maier et al. 2014). The lichen species was suggested to be the strongest predictor of community composition (Grube et al. 2009; Bates et al. 2011; Wedin et al. 2016), although geographic origin, exposure and thallus age are additional factors (Cardinale et al. 2012). Hodkinson et al. (2012) suggested that the photobiont type also contributes to the composition of the bacterial fractions in lichens. All these previous studies focused only on complex lichenized fungi with highly organized thalli that represent long-lived niches for bacterial communities (Grube and Berg 2009). Only recently, we isolated multiple bacterial strains associated with the S. albescens–Coccomyxa system and strains were co-cultured with the fungus and the algae to study the morphological organization of three-partner associations under controlled conditions (Muggia et al. 2016). The bacteria selected for the cultures belonged to Actinobacteria, Firmicutes and Proteobacteria, groups already found among the lichen-associated microbes (Cardinale, Puglia and Grube 2006; Grube et al. 2009, 2015; Hodkinson and Lutzoni 2009; Aschenbrenner et al. 2014).

Morphologically simple symbioses have been interpreted as ‘primitive stages of lichenization and as contemporary analogues of lichen evolution’ (Gostinčar, Muggia and Grube 2012) and, so far, bacterial communities of borderline lichens have never been studied by culture-independent approaches. The S. albescens–Coccomyxa system offers a unique model to explore the bacterial community composition in less organized lichenized associations. Furthermore, the phenomenon of optional lichenization offers a system where it is possible to investigate the effect of the lichen symbiosis on bacterial community structure in a fungus that can also live as saprotroph. To accomplish this, we studied the bacterial microbiota associated with S. albescens under its two lifestyles and compared them with the bacterial communities resident in the Populus substrates (bark and wood). If the microbial communities in the lichenized morphs differ more from those in the bark than the communities in the saprotrophic morphs differ from those in the wood, this would imply that lichenization plays a role in the bacterial composition. If microbial communities associated with the fungus are similar.
to those in the substrata, then there is no support for viewing the lichenization as playing a major role. We characterized the microbial communities using high-throughput sequencing of the 16S rRNA gene, and localizing them with fluorescence in situ hybridization (FISH) and confocal laser scanning microscopy (CLSM).

MATERIALS AND METHODS

Sampling

Samples of S. albescens were collected during autumn 2013 from nine sites located in three areas in the coniferous forest region of northern Sweden (supplementary Table S1 and Fig. S1 available online). These areas were selected to cover representative sites from the distribution area and to enable study of the geographic variation in the bacterial communities associated with Schizoxylon in Scandinavia. They were at least ∼650 km in a transect from north to south. The localities were characterized by mostly mixed forests growing on abandoned agricultural lands. We collected a similar number of specimens per area, with at least six replicates of each lifestyle. The only exception is the Västerbotten area, where only three saprotrophic specimens were found. For the molecular study we included 28 lichenized and 23 saprotrophic specimens, plus 13 samples of each substrate (bark and wood). Sampling tools were sterilized between collections. Samples were kept in Petri dishes on dry ice until arrival at the laboratory, where they were immediately stored at −70°C until further processing. Additional S. albescens samples to be used for the FISH analyses were collected and kept at room temperature.

DNA extraction

For each lichenized (L) and saprotrophic (S) specimen of S. albescens, three different sample types were dissected under the stereomicroscope (outlined in Fig. 1B and C): (i) only fruiting bodies (S–3/specimen), avoiding the substrate (LA, SA); (ii) Schizoxylon-colonized substrate of ∼2 mm radius surrounding the fruiting bodies (LB, SB)—for the lichenized morphs, these areas include the photobiont clumps, visible as greenish patches (Fig. 1B); and (iii) Schizoxylon-colonized substrate (pieces ∼25 mm²) where no apothecia or photobionts were present (LC, SC).

With this sampling strategy, we aimed to characterize the microbiota associated with different parts of the fungus, including the part containing the photobiont in the lichenized morphs. We sampled Populus bark and wood close to the specimens, not colonized by Schizoxylon, to characterize the microbiota in the substrate. All samples were frozen into liquid nitrogen for 30 s and homogenized in a mini-beadbeater (Thomas Scientific, USA) for 30 s twice. DNA was extracted with the DNeasy Plant Mini Kit (Qiagen) following the manufacturer’s instructions except for the incubation step, where the original recommendation of 65°C for 10 min was extended to 30 min to maximize the cell lysis. An extra purification step was included and the DNA was precipitated with ice-cold isopropanol and volumes adjusted to get a final concentration of 5 ng/μl.

Metagenomic sequencing

We targeted the V4 hypervariable region of the 16S ribosomal RNA gene with barcode-tagged primers of F515 and R806 (Caporaso et al. 2011a). We designed a forward blocking primer (CAGTCGCCGTAACACGTGGAAGAC) to minimize the amplification of the S. albescens mitochondrial 12S rRNA, which was added in a proportion of 5:1 compared with the corresponding F515 primer. Polymerase chain reaction (PCR) triplicates were run for each sample; negative blanks were included. The PCR products and the negative controls were purified and concentrations normalized with the SequiPrep Normalization Plate kits (Invitrogen). Three libraries (two with samples and one containing only the negative controls) were constructed with TrueSeq DNA PCRFree (Illumina) as per the manufacturer’s instructions, adding an initial phosphorylation step with T4 Polynucleotide Kinase (Thermo Scientific). Sequencing was run in one lane of the MiSeq 2 × 301. Information on the S. albescens internal transcribed spacer (ITS) amplification and sequencing is given in the supplementary data available online.

Processing of 16S rRNA amplicon data

Raw forward and reverse 16S rRNA gene sequences of each library were merged using PEAR 0.9.5 (Zhang et al. 2014) and stitched reads processed with QIIME 1.9.1. (Caporaso et al. 2011b) to be demultiplexed and quality filtered. The open reference method in QIME was used for operational taxonomic unit (OTU) picking, at a similarity of 97%, using a customized version of the Greengenes dataset as reference. Taxonomy was assigned to sequences using the UCLUST classifier method (Edgar 2010). Chimeras were identified using UCHIME 4.2 (Edgar et al. 2011) and removed. The method outlined in Troccaz et al. (2015) was used to identify the OTUs present in the negative blanks that might be considered potential contaminants and those were also removed. Chloroplast and mitochondria sequences were also excluded to remove amplifications of cyanobacteria and eukaryotes. A final filter was applied to remove reads representing a fraction below 0.0005%. Additional information on 16S rRNA amplicon data processing is given in the supplementary data available online.

Statistical analyses

The alpha diversity was calculated with the Shannon and Faith’s phylogenetic indices and analyses were based on multiple rarefied OTU tables for which a maximum of 7700 reads per sample was set. A two-sample nonparametric t-test (with 999 permutations and a Bonferroni corrected P-value) was applied between (i) lichenized and bark samples and (ii) saprotrophic and wood samples. Multiple rarefactions and alpha diversity analyses were conducted in QIME.

All the beta diversity analyses were performed using the R phyloseq package (McMurdie and Holmes 2013). Samples with fewer than 5000 sequences were removed and the DESeq2’s variance stabilization technique (Love, Huber and Anders 2014) was applied to normalize the OTU table. This because it has been shown that methods based on a statistical mixture model perform better library size normalization (McMurdie and Holmes 2014). Weighted and unweighted UniFrac distance matrices (Lozupone and Knight 2005) were calculated for several sample groups (all samples, only lichenized and bark samples, and only saprotrophic and wood samples), and the distances visualized by Principal Coordinate Analysis (PCoA). The analysis of similarities (ANOSIM) was applied (significance of the R value calculated by 9999 Monte Carlo permutations) to determine the significance of groups by sample type, specimen, geography and Schizoxylon ITS haplotype. The core microbiota of each sample...
type was identified using QIME’s script ‘compute core microbiome’, setting a threshold of presence in at least 50% of samples.

We conducted differentially abundant OTUs tests as outlined by McMurdie and Holmes (2013) to identify OTUs with significant differences in proportions between sample types. As we aimed to evaluate the differences between the microbiota associated with Schizopylon and those in the Populus tremula substrate, we tested the differences between the lichenized samples and the bark, and the saprotrophic samples and the wood, respectively. We also constructed heatmaps of bacterial abundances filtered by the most significantly different OTUs between the fungus and its substrate.

**FISH–CLSM**

FISH with rRNA-targeted oligonucleotide probes was combined with CLSM to obtain information on the spatial location of the bacteria following protocols established already for lichens (Cardinale et al. 2008; Muggia et al. 2013). Details on sample fixation, embedding, sectioning, hybridization protocols and CLSM observation are reported in the supplementary data available online.

**RESULTS**

**Richness and diversity of the microbiota**

From the initial number of 179 DNA extractions (51 fungal samples × 3 sample types and 26 substrate samples), 37 were discarded as they did not amplify, leaving a final number of 142 samples. A total of 5628 427 reads from 16S rRNA gene fragments were obtained for the 142 sample replicates (65 lichenized, 54 saprotrophic and 23 substrate) and 10 blank controls, after merging the raw paired-end reads, removing sequences with tag-switching, demultiplexing with quality filtering and removing chimeras. Remaining sequences clustered into 13 762 OTUs at 97% similarity level (singletons excluded). After additional filtering to remove OTUs identified as contaminants, represented by cyanobacteria or mitochondrion sequences, or representing a very small fraction of the total number of reads, the dataset was reduced to 3 178 846 reads for the 142 samples (the number of reads ranged from 1629 to 80 648 per sample), corresponding to 3546 OTUs. Of these, 721 were found only in the lichenized and 357 only in the saprotrophic samples, while 2468 were shared.

The number of OTUs and Shannon index diversity recovered for the lichenized samples indicated that the bacterial communities in the fruiting bodies (LA) and the whitened patches (LC) were significantly less diverse than the ones found associated with the algal clumps (LB) and the bark (supplementary Fig. S2 available online; t-test, \( P < 0.05 \)). These differences disappeared when applying the Faith’s phylogenetic diversity metric. For the saprobic morphs, the bacterial communities in the fruiting bodies (SA) were also less diverse than the communities associated with the other saprotrophic samples (SB and SC) and the wood (supplementary Fig. S2 available online; t-test, \( P < 0.05 \)).

**Taxonomic coverage**

Proteobacteria was the dominant fraction in all sample types (61.5%; percentages are averages across samples) followed by Bacteroidetes (13.4%; Fig. 2A). Regarding the remaining phyla with global relative abundance higher than 4%, Verrucomicrobia (7.8%), Acidobacteria (5.6%) and Planctomycetes (4.7%) were more abundant in the wood and saprotrophic samples than in the lichenized samples and the bark. Actinobacteria and Armatimonadetes ranged between 1 and 4%. Firmicutes represented 2.7% of the bacterial fraction in the lichenized apothecia but its relative abundance in all other sample types was <1% (range 0.1–0.8%). Only a small proportion remained unclassified at the phylum level (0.9%). At the class level, Alphaproteobacteria (55.6%), followed by Spartobacteria (7.7%), Sphingobacteria (6.2%) and Acidobacteria (5.1%), were the most abundant taxa in all samples. The taxonomy summaries at the phylum and class level showed similar averages between the lichenized samples (LA, LB and LC) and the bark, as between the saprotrophic samples (SA, SB and SC) and the wood. However, for the same comparisons (lichenized vs bark, saprotroph vs wood), differences were observed at the order and family level. The order Rhizobiales was more abundant in the fungal environment of both morphs than in their substrates (Fig. 2B), especially on the fruiting bodies. These differences were due to a larger proportion of Methylobacteriaceae in the lichenized morph, and to a larger proportion of Methylocystaceae in the saprotrophic morph. In the fruiting bodies of both morphs, the proportion of Sphingomonadales was smaller compared with the proportion of this order in the substrates. Also, the presence of Burkholderiales was higher in the fruiting bodies compared with the other sample types, especially in the lichenized ascomata, mostly due to Oxalobacteraceae.

**Structure of the bacterial communities associated with Schizopylon lifestyles**

The PCoA representation of the weighted and unweighted UniFrac distances (Fig. 3A and supplementary Fig. S3A available online) showed that the lichenized and bark samples had a clear separation from the saprotrophic and wood samples. The ANOSIM results based on the UniFrac distances including all replicates, showed that the most significant factors clustering them were the ‘specimen’ (R-value = 0.468, P-value = 0.0001; all R-values specified in the text will refer to the weighted UniFrac distances, see Table 1 for the unweighted UniFrac R-values) and the ‘sample type’ (R-value = 0.461, P-value = 0.0001). In order to analyse in more detail whether those two factors had the same influence for each of the fungal lifestyles, UniFrac distances were calculated separately for the lichenized and the bark samples and for the saprotroph and the wood samples. The weighted UniFrac-PCoA for the lichenized-bark samples (supplementary Fig. S3B available online) showed that the bark and the lichenized samples containing the photobionts (LB) clustered with replicates from the same sample type but with substantial overlap between them. The remaining lichenized samples (LA, LC) showed a less clear grouping pattern but were distinct from the bark, especially the fruiting bodies (LA). The ANOSIM results showed that the factor ‘sample type’ (R-value = 0.34, P-value = 0.0001) had a greater influence than the ‘specimen’ (R-value = 0.15, P-value = 0.0014) for the lichenized-bark samples. On the contrary, the ANOSIM results for saprotroph-wood samples showed that the factor ‘specimen’ (R-value = 0.51, P-value = 0.0001) had considerably greater influence than the ‘sample type’ (R-value = 0.11, P-value = 0.0010). These results confirmed the weighted UniFrac-PCoA representation (supplementary Fig. S3C available online) showing no clear separation of replicates of the same sample type for saprotroph-wood samples.

Other factors analysed were ‘geography’, referring to the three areas where samples were collected, and ‘haplotype’, referring to the two distinct ITS haplotypes of Schizopylon.
Figure 2. Bar charts showing bacterial community composition among sample types as relative abundances. (A) Phylum level. (B) Order level. Only taxonomic levels representing at least 0.1% relative abundance of all reads are detailed in the legends. Sample codes: LA: lichenized fruiting bodies; LB: *Schizzyxylon*-colonized *Populus* bark surrounding the lichenized fruiting bodies; LC: *Schizzyxylon*-colonized *Populus* bark without apothecia or photobionts present; Bark: *Populus* bark without colonization of lichenized *Schizzyxylon*; SA: saprotrophic fruiting bodies; SB: *Schizzyxylon*-colonized *Populus* wood surrounding the saprotrophic fruiting bodies; SC: *Schizzyxylon*-colonized *Populus* wood without apothecia present; Wood: *Populus* wood without colonization of saprotrophic *Schizzyxylon*.

Table 1. Results of ANOSIM evaluating variation of bacterial communities based on weighted and unweighted UniFrac distances with four categorizations (sample type, specimen, geography, haplotype) with three sets of samples: all replicates, only lichenized and bark replicates, and only saprotrophic and wood replicates. Only to test the factor haplotype, the substrate samples were removed from the dataset. The ANOSIM R-values range from 0 to 1 and indicate the fraction of the variation in distances is explained by the grouping being tested. P-values were calculated with 9999 permutations and significance was assumed when \( P < 0.001 \).

<table>
<thead>
<tr>
<th></th>
<th>All samples</th>
<th>Lichenized-bark samples</th>
<th>Saprotrophic-wood samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R-value</td>
<td>P-value</td>
<td>R-value</td>
</tr>
<tr>
<td>Sample type (weighted UniFrac)</td>
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<td>0.350</td>
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<td>0.0001</td>
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<tr>
<td>Specimen (unweighted UniFrac)</td>
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</tr>
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<tr>
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<td>–</td>
</tr>
<tr>
<td>Haplotype (unweighted UniFrac)</td>
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</tr>
</tbody>
</table>

Sample replicates did not show grouping based on geography (Fig. 3A), as confirmed by the low ANOSIM values (R-value = 0.09, P-value = 0.0001). Finally, weighted UniFrac distances were calculated for the fungal samples only (excluding bark and wood) and replicates were plotted by the two *Schizzyxylon* ITS haplotypes (Fig. 3B). No clustering pattern was evident and ANOSIM R-values showed no significance for this factor (R-value = 0.005, P-value = 0.2525).

**Differential abundance analysis**

We tested the presence of OTUs showing significant abundance differences by comparing the bacterial communities in the different *Schizzyxylon* sample types with the ones present in the *Populus* substrates. We identified 60 differentially abundant bacterial OTUs, distinguishing communities associated with the lichenized apothecia (LA) from those of the substrate (bark) (Fig. 4A). Differences were detected mainly in Proteobacteria: Rhizobiales and Burkholderiales (15 OTUs) OTUs were more abundant in the fruiting bodies (LA), while Sphingomonadales and Saprospirales OTUs were more abundant in the bark (14 OTUs). Similar results were obtained in the heatmaps (supplementary Fig. S4A available online), suggesting an enrichment of Rhizobiales and Burkholderiales and a loss of Sphingomonadales in the microbiota in the apothecia, compared with in the bark. The tests comparing the microbiota of the two other lichenized sample types (LB and LC) with the one in the bark revealed a number of OTUs showing significantly different abundances (Fig. 4B and C, and supplementary Fig. S4B and C available online). Differences were mostly due to OTUs belonging to Rhizobiales and Sphingomonadales that were more abundant in the lichen samples compared with those in the bark.
When applying the OTU differential abundance testing to compare the three saprotrophic sample types with their substrate (wood), we detected that a higher number of bacterial phylotypes differed in abundance between the fruiting bodies (SA) and the substrate (53 OTUs; Fig. 4D and supplementary Fig. S4D available online) than in the other sample types (SB and SC; Fig. 4E and F, and supplementary Fig. S4E available online). Between the fruiting bodies and the wood, the differences corresponded to 39 OTUs significantly more abundant in the wood (mostly represented by several orders of Actinobacteria and of Sphingomonadales; 23 OTUs), while only 14 OTUs were more abundant in the fruiting bodies.

The same approach was used to compare the three lichenized sample types, to detect OTUs with larger relative abundance in the communities associated with the algal clumps (LB) compared with the two other lichenized sample types (LA and LC). The communities associated with the photobionts had a larger fraction of Sphingomonadales (mainly Sphingomonas), but a smaller fraction of Burkholderiales, compared with the apothecia (supplementary Fig. S4A available online). When compared with the whitened bark patches (LC), the communities associated with the algal clumps (LB) had also a higher abundance of Sphingomonas (supplementary Fig. S5 available online).
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Figure 4. Differential abundance testing plots. (A–C) Comparison of microbiota between the lichenized samples LA, LB, LC and the bark, respectively. (D–F) Comparison of microbiota between the saprotrophic samples SA, SB, SC and the wood, respectively. For all plots, OTUs are represented at the order level, colored by phylum and ranked by log2-fold change (logarithmic scale to base 2). OTUs were considered differentially abundant if their mean proportion was significantly different between the two sample classes. OTUs can have a significantly larger proportion in the fungal samples compared with their substrates (positive abundance values) or have a larger proportion in the substrate compared with the fungal-associated microbiome (negative abundance values). In plots (E) and (F) no OTUs were found to be more abundant in the wood compared with the fungal samples SB and SC.

**DISCUSSION**

We characterized for the first time the bacterial communities associated with an optional lichen symbiosis. We found differences in community composition at high taxonomic levels between the microbiota associated with the lichenized and saprotrophic morphs of *S. albescens*. For all samples studied, the main phyla present were Proteobacteria, followed by Bacteroidetes. The predominance of Proteobacteria has been repeatedly reported in lichen symbioses (Bates et al. 2011; Bjelland et al. 2011; Aschenbrenner et al. 2014; Wedin et al. 2016) and it is also dominant in some associations in non-lichenized fungi (Bertaux et al. 2005; Splivallo et al. 2015). Bacteroidetes has also been reported as an important component of lichen-associated microbiota (Bjelland et al. 2011; Park et al. 2016). In our study, the main differences between the microbiota at the phylum level involved other groups: Verrucomicrobia, Acidobacteria and Planctomycetes, which were more abundant in the saprotrophic *Schizoxylon*, whereas Actinobacteria were represented in...
higher proportions in the lichenized thalli. At the order level, Rhizobiales was a larger fraction in the Schizoxylon-associated bacterial communities than in the Populus communities, and the fungal fruiting bodies of both morphs showed a lower proportion of Sphingomonadales than in the substrates. The larger proportion of Rhizobiales showed differences between the two fungal morphs at the family level: in the lichenized morph, there was a larger presence of Methylobacteriaceae, while in the saprotrophs this was due to a larger proportion of Methylocystaceae. Another difference was the higher amount of Oxalobacteraceae in the fruiting bodies than in the surrounding areas, and much more so in the lichenized samples than in the saprotrophs (7% and 1.3%, respectively). If the pruina on the fruiting bodies contains calcium oxalate, this could explain the presence of Oxalobacteraceae, which may indeed play this metabolic role, as suggested in other lichens (Lang et al. 2007; Wedin et al. 2016).

In this study, we wanted to investigate whether lichenization (a symbiotic relationship between one fungus and its algal counterparts) plays a key role influencing bacterial community composition, by studying one fungal species that lives either as lichenized or as a saprotroph. For this purpose, we have focused on comparing the microbiota associated to the fungus of each lifestyle with the microbiota of its substrate. Regarding the bacterial communities associated with the lichenized specimens, the weighted UniFrac-PCoA (Fig. 3A and supplementary Fig. S3A available online) and the ANOSIM R-values (Table 1) indicate a certain degree of influence of the fungus on its associated microbiota, mostly on the fruiting bodies. The bacterial communities in the lichen apothecia clustered separately from those in the bark and the other lichenized sample types (Fig. 3A and supplementary Fig. S3A available online). These differences in the microbial composition in the apothecia suggest that the microhabitat in lichenized ascomata is particularly different. We recovered a large number of OTUs showing significant differences in abundance between apothecia and the Populus bark (Fig. 4A and supplementary Fig. S4A available online): some that were OTUs present in the bark were almost absent in the apothecia and vice versa. The great majority of these OTUs belonged to
Alphaproteobacteria and Betaproteobacteria, and confirmed the taxonomy summaries that there were fewer Sphingomonadales but more Rhizobiales and Burkholderiales in the fruiting bodies. Among these Rhizobiales we found several representatives of the Lichen-Associated Rhizobiales-1, an undescribed bacterial lineage first found in green-algal lichen thalli (Hodkinson and Lutzoni 2009), later detected in thalli from Antarctica and dry arid regions (Bates et al. 2011; Park et al. 2016; Wedin et al. 2016). Among the Burckholderiales that were more abundant in the apothecia, several corresponded to OTUs previously found in lichens from cold regions (Grube et al. 2009; Lee et al. 2014). Bacterial diversity in the lichenized apothecia showed a significantly lower number of OTUs compared with the surrounding area containing the photobionts and the bark. We have observed that the Schizoxylon fruiting bodies have highly hydrophilic surfaces and that could explain their poor bacterial colonization; previous studies have shown that moisture plays an important role in bacterial richness in plant microbiota (Turner, James and Poole 2013; Leff et al. 2015).

The communities found in the algal clumps clustered together in the ordination plot but they mostly overlapped with the bark samples (Fig. 3A), indicating the notable influence from the Populus resident microbiota in their composition. The bacterial communities associated with the photobionts, however, had significantly enriched several OTUs from the Rhizobiales and Sphingomonadales compared with the bark (Fig. 4B). A large number of OTUs formed the core microbiota associated with the photobionts: out of the 1710 OTUs detected in this sample type, 469 OTUs were found in at least 50% of the replicates. At least 15 of these were OTUs previously isolated from lichens (Grube et al. 2009; Lee et al. 2014; Sugirb.jörnsdóttir et al. 2014; Cernava et al. 2015; Parrot et al. 2015) and included Actinobacteria (unidentified genera in the Microbacteriaceae, Nakamurellaceae and Pseudonocardiaceae), Bacteroidetes (Hymenobacter, Pedobacter) and Proteobacteria (Aurantimonadaceae, Methylocystaceae, Phyllobacteriaceae and Sphingomonadaceae). This core microbiota was mostly composed by Alphaproteobacteria and had a high number of OTUs assigned to the genus Sphingomonas (102 OTUs out of 469). Some of the bacterial strains isolated by Muggia et al. (2016) from lichenized Schizoxylon belong to Sphingomonas, and it will be interesting to establish future co-culturing experiments with these and other strains. The presence of this genus in lichens has been reported previously (Grube et al. 2009; Hodkinson et al. 2012; Lee et al. 2014) and it has been suggested that its presence could be linked to recycling nutrient functions in the lichen thallus (Aschenbrenner et al. 2016). The bacterial communities associated with the algal clumps (the lichenized structures) had a higher OTU richness than the other lichen sample types (supplementary Fig. S2 available online). Additionally, the FISH–CSML images (Fig. 5) showed how bacteria in the algal clumps concentrate around the photosynthetic cells. We assume that leaking photosynthetic products (ribofl or other low weight polyols) can favor bacterial diversity and abundance.

Schizoxylon forms two genetically distinct units that cannot be separated based on morphology (Muggia et al. 2011). Still, we investigated whether bacterial communities will differ based on the different genotypes of the fungus, but the associated microbiota showed no difference between the two S. albescens ITS haplotypes (Fig. 3B, Table 1). Likewise, geographic origin of the samples had no major effect (Fig. 3A, Table 1) as replicates of the same sample type collected hundreds of kilometres apart clustered with each other. This is also true for the bark samples, which had similar bacterial communities regardless of geographic distance, suggesting that the bark-associated microbiota is quite stable. Based on the ANOSIM results showing ‘sample type’ as a significant factor clustering the lichenized-bark samples, it seems as though the microbiota associated to different Schizoxylon lichenized parts is slightly different, especially on the lichen reproductive structures. However, the PCoA, differential abundance tests and heat maps show the big influence of the bark microbiota: the samples including the symbiotic structures (i.e. algal clumps), even if they are enriched by a number of OTUs, are still very similar in composition to the bark bacterial communities. This suggests that the fungal–algal physiological interactions could favor the presence of certain bacterial groups, but do not select a highly structured and different microbiota.

In the saprotrophic morphs, the main predictor of bacterial structure was the specimen; bacterial communities from saprotrophic replicates were more similar if they were coming from the same specimen rather than by sample type (Table 1). The microbiota found surrounding the fruiting bodies or in other areas colonized by the fungal mycelia was similar in composition and structure to the microbiota already present in the wood (Figs 3 and 4). Differences were only observed when fruiting bodies were compared with the substrate, with a number of OTUs that were very abundant in the wood but almost absent in the apothecia, where the communities were also less diverse (Fig. 4 and supplementary Fig. S2 available online). As for the lichenized morphs, the specific conditions of the pruinose and hydrophobic periphery of the saprotrophic fruiting bodies could possibly explain these differences in OTUs richness. The overall results, however, suggested that colonization by the saprotrophic mycelium had no significant influence on the microbiota in the wood substrate.

In conclusion, the bacterial communities associated with S. albescens, except those in the fruiting bodies, reflect to a large extent the bacterial communities living in the Populus substrate. The microbiota associated with the lichenized Schizoxylon tend to be more structured than the microbiota in the saprotrophs, although more distinct in composition compared with the communities of the surrounding bark than when the same comparison is made between the free-living saprotrophic Schizoxylon and its wood substrate. The present results show that simple algal–fungal interactions have some influence in the bacterial community composition but do not select a specific microbiota. This contrasts with the earlier studies showing that lichens have their own microbiota, which clearly differs from the microbiota in the substrate around them (e.g. Grube et al. 2009; Hodkinson and Lutzoni 2009; Bates et al. 2011; Cardinale et al. 2012; Aschenbrenner et al. 2014; Wedin et al. 2016). In the light of our results and the above-mentioned studies, we suggest that morphologically complex thallus structures are required for the lichens to provide unique niches to host specific bacterial communities in the lichen symbioses.

Data deposition

Illumina paired-end merged reads are deposited in the NCBI Sequences Read Archive (SRP106873).

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC Journal online.
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Conflict of interest. None declared.

REFERENCES


