environmental microbiology

Environmental Microbiology (2014) **16**(12), 3743–3752 doi:10.1111/1462-2920.12658

Microbial cargo: do bacteria on symbiotic propagules reinforce the microbiome of lichens?

Ines Aline Aschenbrenner,[1,2] Massimiliano Cardinale,[1,2] Gabriele Berg[1] and Martin Grube[2]*

[1]*Institute of Environmental Biotechnology, Graz University of Technology, Petersgasse 12, 8010 Graz, Austria.*

[2]*Institute of Plant Sciences, University of Graz, Holteigasse 6, 8010 Graz, Austria.*

Summary

According to recent research, bacteria contribute as recurrent associates to the lichen symbiosis. Yet, the variation of the microbiomes within species and across geographically separated populations remained largely elusive. As a quite common dispersal mode, lichens evolved vertical transmission of both fungal and algal partners in specifically designed mitotic propagules. Bacteria, if co-transmitted with these symbiotic propagules, could contribute to a geographical structure of lichen-associated microbiomes.

The lung lichen was sampled from three localities in eastern Austria to analyse their associated bacterial communities by bar-coded pyrosequencing, network analysis and fluorescence in situ hybridization. For the first time, bacteria were documented to colonize symbiotic propagules of lichens developed for short-distance transmission of the symbionts. The propagules share the overall bacterial community structure with the thalli at class level, except for filamentous *Cyanobacteria* (*Nostocophycideae*), and with *Alphaproteobacteria* as predominant group. All three sampling sites share a core fraction of the microbiome. Bacterial communities of lichen thalli from the same sampling site showed higher similarity than those of distant populations. This variation and the potential co-dispersal of a microbiome fraction with structures of the host organism contribute new aspects to the 'everything is everywhere' hypothesis.

Received 14 April, 2014; revised 30 September, 2014; accepted 1 October, 2014. *For correspondence. E-mail martin.grube @uni-graz.at; Tel. (+43) 316 380 5655; Fax (+43) 316 380 9880.

Introduction

Lichens are known as ecologically diversified symbioses that colonize almost all climate zones and occur on a wide range of substrates (Nash, 2008). In the symbiotic stage, the microscopic fungi (mycobionts) produce macroscopic thalli of characteristic appearance. They provide a long-living habitat for the photoautotrophic partners (alga and/or cyanobacterium), which are sheltered between peripheral fungal layers (Honegger, 2012). Despite a general ubiquity of lichens, individual species of lichen-forming fungi may be highly specialized for their ecological niches. One prominent example for ecological specialization is the lung lichen, a conspicuous foliose species adapted to old-growth forests and highly sensitive to air pollution (Scheidegger, 1995; Scheidegger and Werth, 2009). This species is among the most intensely studied lichens because of its importance as indicator species (Rose, 1992). Detailed studies have assessed chemical, ecological and ecophysiological aspects in lung lichens (e.g. McEvoy *et al*., 2007; Bidussi *et al*., 2013), as well as in population genetics, phylogeography and reproductive biology (e.g., Jüriado *et al*., 2011; Widmer *et al*., 2012). The lung lichen represents a tripartite lichen as it hosts additionally to the mycobiont (*Lobaria pulmonaria*) two distinct photobionts internally. The green-algae *Dictyochloropsis reticulata* is found beneath more than 90% of the lichen surface and strains of cyanobacterium *Nostoc* are locally restricted to gall-like colonies, in so-called internal cephalodia (Myllys *et al*., 2007). These *Nostoc* strains are generally captured from the surface to become gradually integrated into the lichen thallus, where they primarily contribute to the symbiotic system by nitrogen fixation (Jordan, 1970; Cornejo and Scheidegger, 2013).

Although the lung lichen is among the fastest growing lichens, it is only fertile under rare optimal habitat conditions. Apothecia are usually only found on older and sometimes even senescing thallus lobes. On the other hand, mitotic propagules for simultaneous dispersal of both symbionts (fungus and alga), a common propagation strategy of many lichens, are almost generally present in this species. Their function as dispersal units has also been shown experimentally (Scheidegger, 1995), and a recent microsatellite analysis revealed co-dispersal of

both symbionts as one of the key factors shaping the genetic structure of this species (Werth and Scheidegger, 2012). The symbiotic propagules of the lung lichen can be called isidioid soredia, which are developed at margins and ridges of the upper thallus surfaces as dust-like particles comprising a few algal and fungal cells, as typical for soredia structures. Before the propagules detach, they normally develop a peripheral cortex to resemble isidia (stratified propagules). These symbiotic propagules of the lung lichen are much larger and heavier than the sexual fungal spores of this species. Therefore, the propagules appear to be less suitable for long-distance dispersal, compared with the much smaller meiotically produced fungal ascospores. Previous work underlined that symbiotic dispersal with isidioid soredia is rather local in this species (Walser, 2004). More recently, Dal Grande and colleagues (2012) provided a quantitative assessment of both propagation strategies and showed that vertical transmission decreases significantly within short distance (approximately cut in half over distances of 10 m).

Long-living lichen thalli provide various niches for associated microorganisms, of which colonizing fungi have been well studied (Lawrey and Diederich, 2003; Werth *et al*., 2013). Even though earlier culture-dependent search for microorganisms revealed the presence of lichen-associated bacteria (Grube and Berg, 2009), the molecular characterization has only just started in the last few years. According to fluorescence in situ hybridization and confocal laser scanning microscopy (FISH-CLSM), bacteria colonize lichens in a biofilm-like manner (Cardinale *et al*., 2008). The composition of these communities is host specific (Grube *et al*., 2009; Bates *et al*., 2011), with *Alphaproteobacteria* as the prominent bacterial class on many lichens (e.g. Grube *et al*., 2009; Schneider *et al*., 2011). Other lineages may also be detected at considerable relative abundances, including *Acidobacteria*, *Actinobacteria*, *Betaproteobacteria*, *Spartobacteria* and *Sphingobacteria*, among others (Bjelland *et al*., 2010; Mushegian *et al*., 2011; Grube *et al*., 2012; Hodkinson *et al*., 2012). These surface-colonizing bacteria of lichens are supposed to play a beneficial role for lichens by contributing to the lichen metabolism with release of nitrogen compounds like amino acids, vitamins and phytohormones, or by solubilization of phosphate (Liba *et al*., 2006; Grube *et al*., 2009; 2014; Schneider *et al*., 2011).

Despite new insights into the structure and function of lichen-associated bacteria, little is known so far about the intraspecific variation of microbiome composition, and also, how lichens acquire their specific bacterial communities. In the present study, we set out to study whether symbiotic propagules of lichens could contribute to a co-dispersal of lichen-associated bacteria. Multiple symbiont dispersal has not been addressed before. We assume that this process could reinforce the composition of the lichen-associated microbiome and contribute to its geographical structure. We studied this possibility by microcopy to assess bacterial colonization on the propagules and by analyses of pyrosequencing data and community fingerprinting patterns.

Results

First insights into the bacterial communities on the lichen thalli and symbiotic propagules

Single strand conformation analysis (SSCP) of 16S rRNA gene fragments obtained from three sampling sites (Tamischbachgraben, St. Oswald/Eibiswald, Johnsbach in Styria, Austria) revealed generally high numbers of bands (> 20) in samples of both thalli and vegetative propagules (isidioid soredia). The entire thalli were characterized by several additional strong bands, which were not present in the samples of the symbiotic propagules (Fig. S2).

Microbial community composition of lichen thalli and symbiotic propagules

A comparison of the lichen-associated bacterial community compositions on both thallus and isidioid soredia by amplicon sequencing showed that the lichen propagules shared most of the dominant taxa (at class level) with entire thalli except for the filamentous *Nostocophycideae* (*Cyanobacteria*), which were only present on thalli (Fig. 1A). *Alphaproteobacteria* were the predominant taxon on both lichen parts [on average: soredia 33%, standard deviation (SD) 6.7%; thalli 26%, SD 4.7%] followed by *Sphingobacteria* (on average: soredia 23%, SD 8.9%; thalli 14%, SD 2.8%). The relative abundance of *Betaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria* as well as *Actinobacteria* on thalli was at least twofold higher compared with that on the propagules, and in contrast, the relative abundance of *Spartobacteria* was twofold higher on the propagules than on thalli (Fig. 1A). The lichen-associated communities on thalli and symbiotic propagules were high in alpha diversity and richness indices (Table S1).

More than 89% of all sequences were shared by both thalli and propagules and represented 28% of all observed operational taxonomic units (OTUs) within this data set (Fig. S3A). Only 9% of the sequences were unique for the thalli and 2% for the propagules. The majority of the OTUs (49%) occurred exclusively on lichen thalli; about 23% were unique on propagules. The distribution of the OTUs between isidioid soredia and thalli was visualized in a Venn diagram (Fig. S3B).

The predominant class within this shared microbiome was *Deltaproteobacteria* (17%; Fig. S4). The most abun-

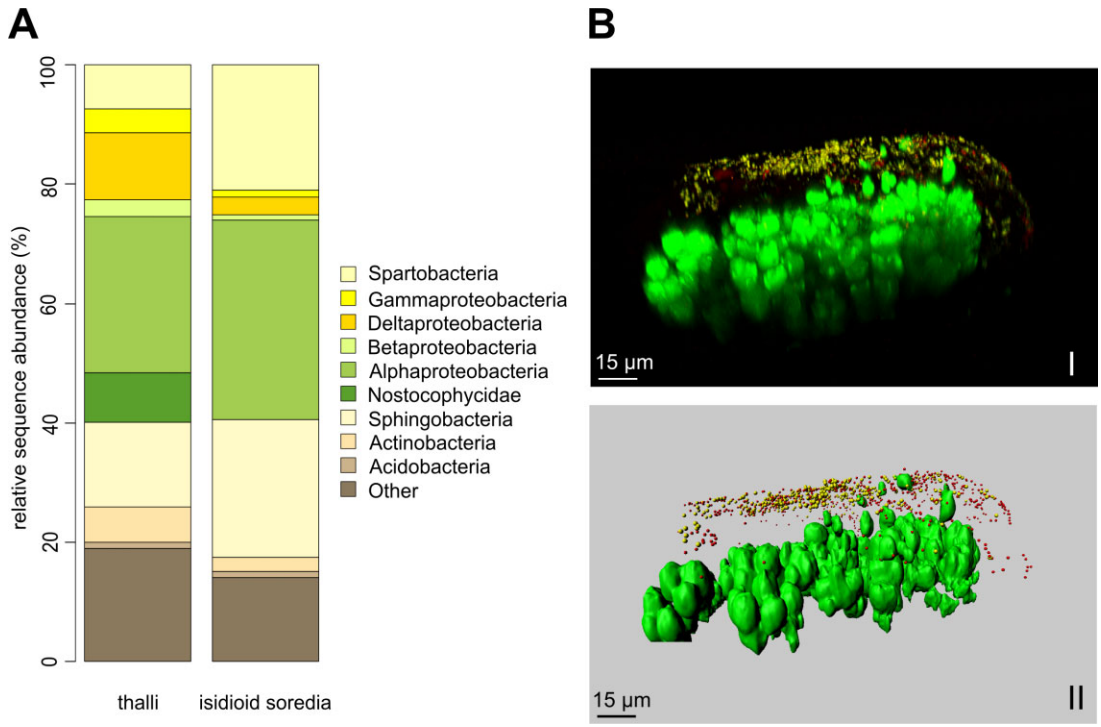

Fig. 1. A. Comparison of microbial community composition of lichen thalli and isidioid soredia at class level sampled in Johnsbach. Besides *Alphaproteobacteria*, thalli were mainly colonized by *Sphingoproteobacteria* and *Deltaproteobacteria* and soredia by *Sphingobacteria* and *Spartobacteria*. *Nostocophycideae* were only present on thalli, but missing on lichen propagules. Taxa representing less than 1% of relative abundance were summarized in the category 'Other'. B. Bacterial colonization on isidioid soredia of *Lobaria pulmonaria* with *Alphaproteobacteria* as dominant group. Volume rendering (I) and 3D reconstruction (II) of confocal stacks were created with IMARIS 7.0; green, algae; yellow, *Alphaproteobacteria*; red, other *Eubacteria* (*Tamischbachgraben*).

dant family within *Deltaproteobacteria* was *Cystobacterineae* (42%), which belongs to the order *Myxococcales*. *Alphaproteobacteria* were only reaching 11%. Other dominant classes were *Sphingobacteria* (14%), *Actinobacteria* (14%), *Phycisphaerae* (9%), *Spartobacteria* (4%) and *Acidobacteria* (2%). The beta diversity of the microbial communities of lichen thalli and propagules showed also a difference between those lichen parts (Anosim; $P < 0.022$; Fig. S5A), and one of the propagule samples was clearly separated from the others of that kind but had closer similarity with the thallus samples.

Colonization patterns of bacterial communities on propagules

The predominance of *Alphaproteobacteria* on lichen propagules was confirmed by FISH-CLSM experiments (Fig. 1B). By using taxon-specific probes for *Alphaproteobacteria* in combination with universal probes for eubacteria, we could show that bacteria form a cover of evenly distributed *Alphaproteobacteria*, intermingled by other eubacteria. The three-dimensional (3D) reconstruction (Fig. 1B-II) of the volume rendered confocal stack (Fig. 1B-I) showed that bacteria colonize the surfaces of the propagules, whereas no bacterial signals were detected in the internal parts of the isidioid soredia.

Sphingomonadaceae was the most dominant family of *Alphaproteobacteria* on symbiotic propagules, comprising more than 65% of alphaproteobacterial reads (Fig. S6). Besides alphaproteobacterial sequences, which could not be assigned to a family (denoted as 'other'), the remaining 8% of the sequences represented diverse families, including *Acetobacteraceae*, *Caulobacteraceae*, *Methylobacteriaceae*, *Bradyrhizobiaceae*, *Rhizobiaceae*, *Rickettsiaceae*, *Hyphomicrobiaceae*, *Hyphomonadaceae*, *Rhodospirillaceae* and *Aurantimonadaceae*.

Composition and diversity of microbial communities within sampling sites

At phylum level, the only difference in composition and relative abundance within all three sampling sites was found for *Actinobacteria* and *Deltaproteobacteria*. *Actinobacteria* showed a threefold higher relative abundance at one site (St. Oswald/Eibiswald), whereas *Deltaproteobacteria* was two times more abundant at another site (Johnsbach; Fig. 2A). The microbial

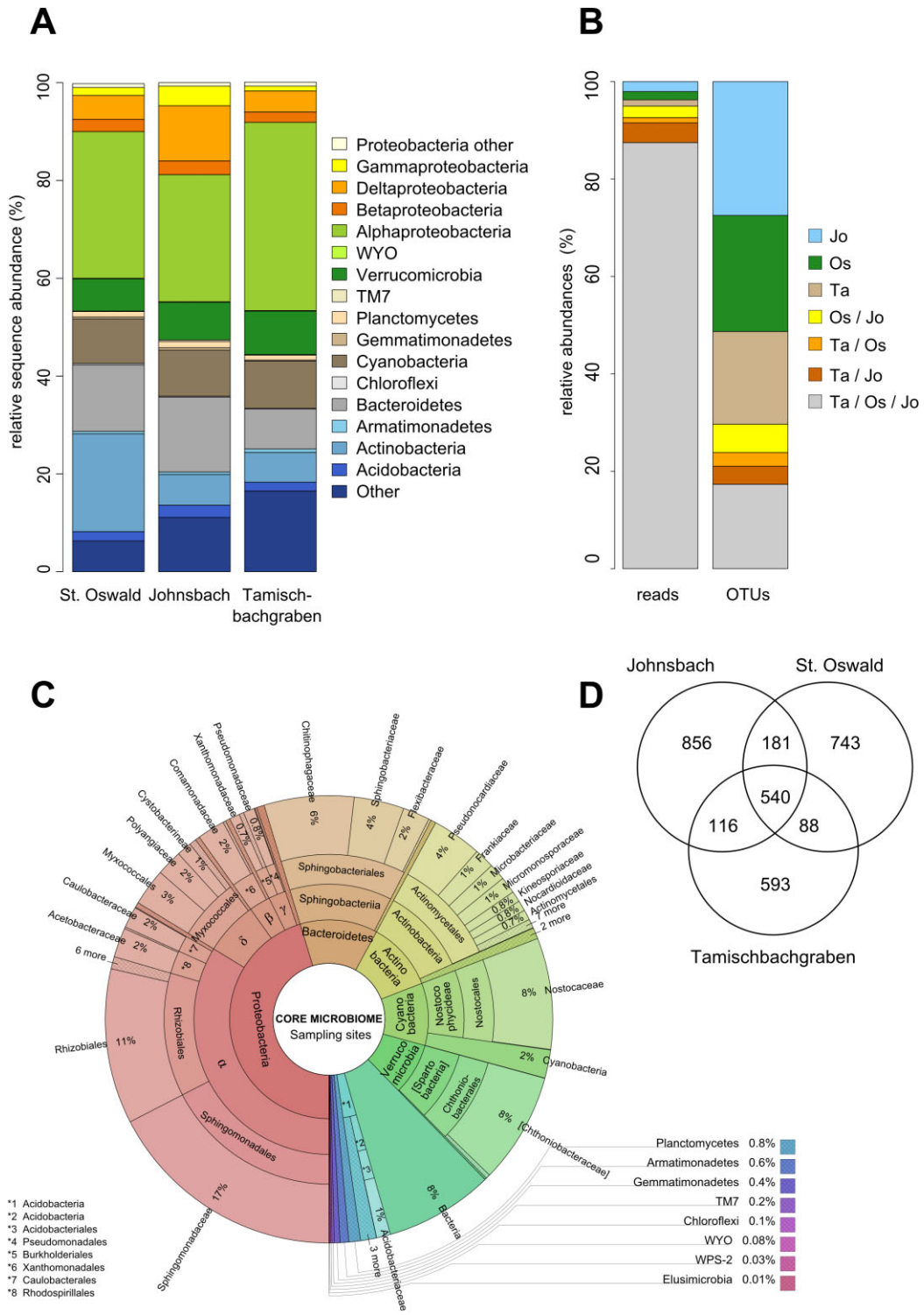

Fig. 2. A. Composition of bacterial communities among the three sampling sites at phylum level. *Proteobacteria* were subdivided to class level in *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Deltaproteobacteria*. *Alphaproteobacteria* was the predominant group followed by *Sphingobacteria* (*Bacteroidetes*) and *Actinobacteria* (*Actinobacteria*). B. Distribution of the relative abundances of either sequence reads or OTUs across sampling sites. Jo, Johnsbach; Os, St. Oswald/Eibiswald; Ta, Tamischbachgraben. C. Composition of the core microbial community among the three sampling sites at phylum, class, order and family level (starting at the inner circle). *Alphaproteobacteria* (34%) was the predominant class followed by *Sphingobacteria* (12%) and *Actinobacteria* (10%). D. Distribution of the microbial communities (3117 observed OTUs) of the lichen thalli within the three locations. Samples of each location were pooled. All locations shared a core microbiome, which represented about 87% of all sequences, but only 16% of all observed OTUs.

community composition of all 15 thallus samples included seven dominant phyla (Fig. 2A shows the taxa summaries for each location; the following data are given as minimum and maximum sequence abundances across the locations): *Proteobacteria* (39.8–44.9%), *Bacteroidetes* (8.2–15.3%), *Actinobacteria* (6.1–20.0%), *Cyanobacteria* (9.1–9.7%), *Verrucomicrobia* (6.6–8.9%), *Acidobacteria* (1.8–2.5%) and *Planctomycetes* (1.0–1.3%), whereas the remaining 6.3–16.5% of the reads could not be assigned to any bacterial phylum (denoted as 'other'). Within the predominant phylum *Proteobacteria*, 58–83% of the sequences were assigned to *Alphaproteobacteria*, followed by *Deltaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*. A comparison of microbial community composition on lichen thalli shown by principal coordinate analysis (PCoA) revealed clear differences among the three sampling sites (Anosim; $P < 0.001$; Fig. S5B).

Alphaproteobacterial families, which were found at all localities, are *Caulobacteraceae*, *Aurantimonadaceae*, *Rhizobiaceae*, *Acetobacteraceae*, *Rhodospirillaceae*, *Bradyrhizobiaceae*, *Hyphomicrobiaceae*, *Methylobacteriaceae*, *Methylocystaceae* and *Sphingomonadaceae*. The latter family represented the most dominant group, reaching up to 43–57% of all *Alphaproteobacteria*. Families only occurring on thalli from St. Oswald/Eibiswald and Tamischbachgraben are *Beijerinckiaceae*, *Phyllobacteriaceae* and *Xanthobacteraceae*, whereas *Rickettsiaceae* was only found in the two northern sampling sites (Johnsbach and Tamischbachgraben; Table S2).

Core microbiome across lung lichen populations

Network analysis of the microbial communities among the three localities revealed that 87% of all sequence reads are shared across sites (Fig. 2B). However, these represent only 16% of the total 3117 observed OTUs. Each sampling site also has unique OTUs as well as OTUs shared only among two sampling sites (see Fig. 2D; samples for each location were pooled). The dominant classes within this core microbiome were *Alphaproteobacteria* (34%), *Sphingobacteria* (12%), *Actinobacteria* (10%), *Nostocophycideae* (8%), *Spartobacteria* (8%) and *Deltaproteobacteria* (6%; Fig. 2C). On the other hand, less abundant phyla like *Chlorobia*, *Deinococcales*, *Firmicutes* and *Chlamydiales* were not present in the shared microbiome.

Discussion

Here, we present first indication that local dispersal via symbiotic propagules also includes a part of the bacterial communities associated with the lung lichen. Rather than depending on a de novo recruitment of bacteria on the developing lichen structure, a 'starter' community is packaged with the vegetative propagules of this lichen. It is likely that such mode of microbiome transfer is not exclusive in this lichen, but rather, a widespread phenomenon associated with vegetative dispersal. This example for community dispersal also raises interesting new questions about the dispersal of bacteria and microbial biogeography, e.g. to what extent do propagated communities generally influence the subsequent recruitment and succession in bacterial communities?

Symbiotic propagules

Using the lung lichen, we demonstrated – for the first time – the bacterial colonization on symbiotic propagules. An initial overview of bacterial diversity using microbial fingerprints already indicated a high number of bacterial strains associated with the lung lichen, and variation among the samples. While FISH-CLS found bacterial colonization to be localized on the surface, insight in the taxonomic composition of this bacterial niche was then achieved by amplicon library sequencing. Symbiotic propagules largely shared the general taxonomic composition of the microbiome with the lichen thalli at class level. However, the PCoA plot of thalli and symbiotic propagules suggested a shift in the community composition mostly at the species level of bacteria. The difference may result from the morphological and positional constraints of the symbiotic propagules. While the total thallus with its age structure and its upper and lower surface (i.e. fungal formed cortex) enables the formation of specific bacterial communities, symbiotic propagules are young and comprise only an upper cortex at maturity. The upper surface of the thalli could preferentially enrich desiccation-tolerant bacterial taxa originating from the rain, wind or small animals (e.g. insects, snails); the shaded lower surface offers different ecological conditions for enrichment. The symbiotic propagules are produced on the upper surface of the lichen thalli and might therefore lack bacterial taxa that might be more common on the lower surface of the thalli.

Distant lung lichen populations share a core microbiome

The overall compositional pattern of bacterial phyla across the locations in our study was fairly similar and reflected an overall ecological resemblance of the lichen habitats. A limited number of highly abundant bacterial OTUs were shared by all sampling sites (16% of all OTUs, ~ 87% of all sequences). The geographical range of the sampling sites represents a small fraction of the global lung lichen distribution. We therefore consider it as a regional core microbiome. The majority of the bacterial

OTUs (70% of all observed OTUs), however, only referred to 5% of all sequences and was specific for each sampling site. It remains unresolved (also because of the unfeasibility to evenly cover all microniches in forests) whether these unique species are adaptations to the specific environmental conditions at each sampling site or if they might be more common in these forest sites.

These results also address the recurrent question about the presence of 'core' microbiomes in host-associated bacterial communities (Shade and Handelsman, 2012). Assessing the 'global' core microbiome of lichens would require a thorough sampling across the entire geographic range. This was beyond the scope of this study and will remain challenging because of the remoteness of appropriate sites colonized by the worldwide-distributed lung lichen. However, our results suggest a differentiation of the lichen thallus microbiomes by distance. A first hint of geographic pattern in lichen-associated *Alphaproteobacteria* was indicated already in Cardinale and colleagues (2012). The only other analysis of geographic structure in a different lichen species, *Cetraria aculeata*, was presented by Printzen and colleagues (2012), which propagates asexually by fragments of the thalli (while sexual fruiting bodies are rare). In this species, alphaproteobacterial communities of high latitudes are depauperate and more closely related to each other than to those of extrapolar habitats. This pattern also agreed with findings for their fungal and algal symbionts (Fernández Mendoza *et al*., 2011).

Lichen-associated microbiomes in comparison

The predominance of *Alphaproteobacteria* agrees well not only with the results obtained from other foliose lichens with a green photobiont (e.g., *Parmelia sulcata, Rhizoplaca chrysoleuca*, *Umbilicaria* sp. and *Flavocetraria*), but also in lichens with cyanobacterial photobionts (e.g., *Leptogium*, *Peltigera* and *Sticta*), as well as lichens with other growth forms beyond foliose lichens (Cardinale *et al*., 2008; Grube *et al*., 2009; Bates *et al*., 2011; Hodkinson *et al*., 2012). However, some lichens displayed altered patterns, e.g. the intertidal *Hydropunctaria maura* was dominated by *Actinobacteria* (Bjelland *et al*., 2010), whereas the acid-rock inhabitant *Ophioparma ventosum* was dominated by *Acidobacteria* (Hodkinson *et al*., 2012). *Rhizobiales* is usually the most abundant order of *Alphaproteobacteria*, followed by *Sphingomonadales* and *Rhodospirillales* (Bates *et al*., 2011; Hodkinson *et al*., 2012). However, in the case of the lung lichen, *Sphingomonadales* appeared to be the prominent order. Within alphaproteobacterial families, *Acetobacteraceae* (*Rhodospirillales*) appeared to be fairly dominant in lichens from acid rock and soil habitats (Cardinale *et al*., 2008; Bates *et al*., 2011), whereas *Sphingomonadaceae* were found to be more prominent in lichens with cyanobacterial photobionts (Hodkinson *et al*., 2012). However, this observation is not well supported by our present findings of high amounts of *Sphingomonadaceae* on the symbiotic propagules, which are generally devoid of the cyanobacterial photobiont. Based on our current sampling, we did not detect LAR1 (order *Rhizobiales*), which was characterized as a lichen-specific lineage of *Alphaproteobacteria* in other studies (Hodkinson and Lutzoni, 2009; Bates *et al*., 2011; Hodkinson *et al*., 2012). LAR1, which has not been taxonomically described up to now, seems to occur on several phylogenetically unrelated species of lichenized fungi. LAR1 was so far detected in lichens from North America and in a sequence library derived from Antarctic lichens (de la Torre *et al*., 2003), but not in our data set. Bacterial communities of the lung lichen differ among geographically distant sites, which leads to an interesting aspect in relation to the Baas-Becking hypothesis ('everything is everywhere, but the environment selects'). The biogeography of host-associated bacteria could at least partly be structured by the dispersal-capacities and -modes of the host organisms and may contribute to a phenomenon of microbiome 'dissimilarity by distance', which may also be found in other plant-alike systems hosting microbial communities.

A more detailed statistical analysis was used to survey to which extent the differences in community composition can be explained – by geographic factors and by the tissue origin (non-parametric analysis of variance and a redundancy analysis (RDA)-based variation partitioning; see Supporting information). Both pyrosequencing-based OTUs and community fingerprinting patterns suggested that approximately 60–70 % of variation is still unexplained by statistical analyses. This high number prevents a conclusive answer to the question whether the microbiome structure is reinforced exclusively by vertical transmission. Nonetheless, microscopic evidence clearly supports that a bacterial fraction is vertically inherited by lichen vegetative propagules. It might also be possible that bacterial communities undergo successional change by the development of thalli starting from germinating propagules (similar to Shade *et al*., 2013). Even the comparison of mature thalli did not reveal a strict dominance of particular bacterial species. Burke and colleagues (2011) argue that the colonization of ecological niches is based on a random selection of species with similar functions suitable for certain habitats and not following bacterial taxonomy. It will be a further informative step to analyse the correlation between the lichen host and its microbiome by large-scale metagenomic and metatranscriptomic approaches in the future.

Vertical transmission in other systems

Increasing evidence suggests a universal role of vertical transmission for structural stability of host-associated microbiomes. Vertical transmission is common in animals, ranging from invertebrates to humans (Funkhouser and Bordenstein, 2013), as well as in plants. A microbiome core fraction is transmitted from a moss sporophyte to the gametophyte (Bragina *et al*., 2013), an invasive plant shares endobacteria in grass rhizomes across generations (Rout *et al*., 2013) and the transmission of endophytic bacteria via seeds seems to be widespread (e.g. Ferreira *et al*., 2008, López-López *et al*., 2010). Comparing bacterial communities, vertical transmission was suggested also in invasive macroalgae (Aires *et al*., 2013). In fungi, vertical transmission of symbiotic bacteria has so far been found in mycorrhizal fungi (Bianciotto *et al*., 2004; Sharma *et al*., 2008), as well as in plant pathogens (Lackner *et al*., 2009). In the fungal kingdom, however, vertical transmission of entire communities is so far unique, because it requires particular morphological structures of propagules, here resulting from pre-established symbiotic interactions, to create the habitat for its own bacterial cargo.

Experimental procedures

Sampling strategy

Lichen thalli were collected at three sampling sites in Austria: Tamischbachgraben (Styria, N 47°38′22″ E 14°41′45″; 8 June 2012), Johnsbach (Styria, N 47°32′35″, E 14°37′38″; 5 October 2012) and St. Oswald/Eibiswald (Styria, N 46°44′50″, E 15°04′26″; 9 July 2012). The sampling sites in upper Styria (Johnsbach, Tamischbachgraben) have a linear distance of about 15 km (separated by a mountain range up to 2500 m of altitude), and both of them are in about 100 km of linear distance from St. Oswald/Eibiswald. The sites differed in bedrock type – siliceous at St. Oswald and calcareous at Tamischbachgraben and Johnsbach. The substrate was uniformly bark of maple trees (*Acer pseudoplatanus*), where the lichen thalli develop either on or adjacent to pleurocarpous mosses at heights of 1–2 m above the ground. At each sampling site, five lichen samples were collected from different *Acer pseudoplatanus* trees by using sterile tweezers. Each sample consisted of about five thallus replicates with symbiotic propagules (Fig. S1). Samples were stored separately in sterile polyethylene bags and cooled on dry ice until further processing, within 4 h after sampling.

Sample preparation and DNA extraction

Prior to DNA extraction, isidioid soredia were separated from lichen thalli under a binocular microscope by using a sterile razor blade and stored separately at −20°C until processing. About 1.5 g of thalli of each sample was homogenized with sterile pestle and mortar, resuspended in 0.85% NaCl-peptone solution and centrifuged in 2 ml of fractions at 13 000 r.p.m. for 20 min at 4°C. The pellets were stored at −20°C until processing. DNA of thalli and vegetative propagules was isolated with PowerSoil® DNA Isolation Kit (MoBio, Germany) and DNeasy Plant Mini Kit (Qiagen, Austria). DNA was purified using E.Z.N.A.® Cycle-Pure Kits, Omega Bio-tek (VWR). Extraction and purification was done according to the manufacturer's protocol.

Community fingerprinting using SSCP of 16S rRNA genes

SSCP of the lichen-associated microbiome was carried out as described by Schwieger and Tebbe (1998). For bacterial 16S rRNA amplification universal bacterial primers Com1/Com2^{P} (Schwieger and Tebbe, 1998) were used. Thermocycling was conducted in a G-STORM™ GS482 instrument (AlphaMetrix Biotech), starting with an initial denaturation for 3 min at 95°C. Each of the 35 cycles included 45 s at 95°C, 45 s at 55°C and 60 s at 72°C. The final extension step was done for 4 min at 72°C. The amplicons were separated using TGGE Maxi System (Biometra). Electrophoresis was carried out in an 8% Polyacrylamide gel at 26°C and 400 V. Silver-staining visualized band patterns.

FISH-CLSM

Symbiotic propagules (also called isidioid soredia) were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) (v/v, 3:1) at 4°C for at least 4 h, followed by three washing steps with ice-cold 1x PBS. The samples were stored at −20°C in ethanol absolute/PBS (v/v, 1:1). The isidioid soredia were used for hybridization without cryosectioning in 1.5 ml tubes (following Cardinale *et al*., 2008). For detection of *Alphaproteobacteria*, the Cy5-labelled probe ALF968 (46°C, 45% formamide) and for eubacteria an equimolar mixture of the Cy3-labelled probes EUB338, EUB338II, EUB338III (46°C, 10% formamide) were used (Amann *et al*., 1990; Neef, 1997; Daims *et al*., 1999). Details on oligonucleotide probes are available at probeBase (Loy *et al*., 2007). Confocal laser scanning microscopy was carried out with a Leica TCS (Leica Microsystems). IMARIS 7.0 (Bitplane) was used for volume rendering and 3D reconstructions of the confocal stacks.

454-Pyrosequencing of 16S rRNA genes

The microbiome of *L. pulmonaria* was studied using a 454-pyrosequencing approach. From each sample (15 thallus and 5 isidiod soredia samples) 50 ng of bacterial template DNA was amplified with a universal bacterial primer set, 515F/806R (Caporaso *et al*., 2011), and the Taq&Go Ready-to-use PCR Mix (MP Biomedicals). From each template, a triplet of PCR amplicons was pooled and purified with E.Z.N.A.® Cycle-Pure Kits (Omega Bio-tek, VWR). High-throughput DNA sequencing was carried out by Macrogen Korea using 454 GS FLX Titanium platform. 16S rRNA gene sequences from the lichen microbiome were analysed using the open-source software package QIIME 1.6.0 (Quantitative Insights Into Microbial Ecology; Caporaso *et al*., 2010a,b). Raw 16S rRNA 454 reads were filtered by quality (minimal quality

score: 25) and length (≥ 250 nt; ≤ 550 nt), followed by a denoising step (denoise wrapper and denoiser script; Reeder and Knight, 2010) and trimming of the reverse primer. OTUs were clustered with UCLUST (Edgar, 2010) at a similarity of 97%, 95% and 90%, which correlates with the taxonomic levels of species, genus and family. Sequence alignment was done with PYNAST using the Greengenes core set as template sequences (DeSantis *et al*., 2006; Caporaso *et al*., 2010a,b). For chimera identification the method Chimera Slayer was used, and taxonomy was assigned with the Ribosomal Database Project (RDP) classifier (Wang *et al*., 2007; Haas *et al*., 2011). Mitochondrial and Chloroplast sequences were excluded as well as sequences assigned to the genus *Nostoc*, because it represents the second photobiont of the lung lichen, and therefore, these sequences were not evaluated as part of the microbiome. Singletons were not removed. Alpha rarefaction was analysed at 97%, 95% and 90% sequence similarity. Normalization of the data set to the same sequence count per sample was carried out before calculation of diversity indices and richness estimation. Based on jackknifed, unweighted UNIFRAC distance matrix the beta diversity was calculated and shown as PCoA plots created with QIIME (Lozupone and Knight, 2005). Determination of richness was done at family level (genetic distance: 10%). Alpha diversity was resolved using Shannon diversity index and Simpson diversity calculated at 3% genetic distance. Network analysis was performed with QIIME and visualized as Venn diagrams. Therefore, the five thallus/propagules samples of each sampling location were pooled. Pie charts were created with KRONA (Ondov *et al*., 2011). Statistical analysis was performed with the R packages VEGAN and APE (http://www.r-project.org/; see also Supporting information).

Acknowledgements

We are grateful to Tomislav Cernava (TU Graz) and Christian Westendorf (KFU Graz) for critical remarks on the manuscript, to Stefanie Maier (KFU Graz) for the valuable discussions about bioinformatic analyses and to Fernando Fernandez Mendoza (KFU Graz) for the statistical analysis. The authors declare that they have no conflict of interest. This research was supported by a grant of FWF to GB and MG (FWF-DACH I882).

References

Aires, T., Serrão, E.A., Kendrick, G., Duarte, C.M., and Arnaud-Haond, S. (2013) Invasion is a community affair: clandestine followers in the bacterial community associated to green algae, *Caulerpa racemosa*, track the invasion source. *PLoS ONE* **8:** e68429.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56:** 1919–1925.

Bates, S.T., Cropsey, G.W., Caporaso, J.G., Knight, R., and Fierer, N. (2011) Bacterial communities associated with the lichen symbiosis. *Appl Environ Microbiol* **77:** 1309–1314.

Bianciotto, V., Genre, A., Jargeat, P., Lumini, E., Bécard, G., and Bonfante, P. (2004) Vertical transmission of endobacteria in the arbuscular mycorrhizal fungus *Gigaspora margarita* through generation of vegetative spores. *Appl Environ Microbiol* **70:** 3600–3608.

Bidussi, M., Gauslaa, Y., and Solhaug, K.A. (2013) Prolonging the hydration and active metabolism from light periods into nights substantially enhances lichen growth. *Planta* **237:** 1359–1366.

Bjelland, T., Grube, M., Hoem, S., Jorgensen, S.L., Daae, F.L., Thorseth, I.H., and Øvreas, L. (2010) Microbial metacommunities in the lichen–rock habitat. *Environ Microbiol Rep* **3:** 434–443.

Bragina, A., Cardinale, M., Berg, C., and Berg, G. (2013) Vertical transmission explains the specific *Burkholderia* pattern in *Sphagnum* mosses at multi-geographic scale. *Front Microbiol* **4:** 394.

Burke, C., Steinberg, P., Rusch, D., Kjelleberg, S., and Thomas, T. (2011) Bacterial community assembly based on functional genes rather than species. *Proc Natl Acad Sci* **108:** 14288–14293.

Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., and Knight, R. (2010a) PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26:** 266–267.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., *et al*. (2010b) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7:** 335–336.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., *et al*. (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci* **108:** 4516–4522.

Cardinale, M., De Castro, J.V., Müller, H., Berg, G., and Grube, M. (2008) In situ analysis of the bacterial community associated with the reindeer lichen *Cladonia arbuscula* reveals predominance of *Alphaproteobacteria*. *FEMS Microbiol Ecol* **66:** 63–71.

Cardinale, M., Grube, M., de Castro, J.V., Jr, Müller, H., and Berg, G. (2012) Bacterial taxa associated with the lung lichen *Lobaria pulmonaria* are differentially shaped by geography and habitat. *FEMS Microbiol Lett* **329:** 111–115.

Cornejo, C., and Scheidegger, C. (2013) New morphological aspects of cephalodium formation in the lichen *Lobaria pulmonaria* (Lecanorales, Ascomycota). *The Lichenologist* **45:** 77–78.

Daims, H., Brühl, A., Amann, R., Schleifer, K.H., and Wagner, M. (1999) The domain-specific probe EUB338 is insufficient fort the detection of all Bacteria: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22:** 434–444.

Dal Grande, F., Widmer, I., Wagner, H., and Scheidegger, C. (2012) Vertical and horizontal photobiont transmission within populations of a lichen symbiosis. *Mol Ecol* **21:** 3159–3172.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., *et al*. (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72:** 5069–5072.

Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26:** 2460–2461.

Fernández Mendoza, F., Domaschke, S., García, M.A., Jordan, P., Martín, M.P., and Printzen, C. (2011) Population structure of mycobionts and photobionts of the widespread lichen *Cetraria aculeata*. *Mol Ecol* **20:** 1208–1232.

Ferreira, A., Quecine, M.C., Lacava, P.T., Oda, S., Azevedo, J.L., and Araújo, W.L. (2008) Diversity of endophytic bacteria from *Eucalyptus* species seeds and colonization of seedlings by *Pantoea agglomerans*. *FEMS Microbiol Lett* **287:** 8–14.

Funkhouser, L.J., and Bordenstein, S.R. (2013) Mom knows best: the universality of maternal microbial transmission. *PLoS Biol* **11:** e1001631.

Grube, M., and Berg, G. (2009) Microbial consortia of bacteria and fungi with focus on the lichen symbiosis. *Fung Biol Rev* **23:** 72–85.

Grube, M., Cardinale, M., Vieira de Castro, J., Müller, H., and Berg, G. (2009) Species-specific structural and functional diversity of bacterial communities in lichen symbiosis. *ISME J* **3:** 1105–1115.

Grube, M., Köberl, M., Lackner, S., Berg, C., and Berg, G. (2012) Host-parasite interaction and microbiome response: effects of fungal infections on the bacterial community of the Alpine lichen *Solorina crocea*. *FEMS Microbiol Ecol* **82:** 472–481.

Grube, M., Cernava, T., Soh, J., Fuchs, S., Aschenbrenner, I., Lassek, C., *et al*. (2014) Exploring functional contexts of symbiotic sustain within lichen-associated bacteria by comparative omics. *ISME J* doi:10.1038/ismej.2014.138.

Haas, B.J., Gevers, D., Earl, A.M., Feldgarden, M., Ward, D.V., Giannoukos, G., *et al*. (2011) Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res* **21:** 494–504.

Hodkinson, B., and Lutzoni, F. (2009) A microbiotic survey of lichen-associated bacteria reveals a new lineage from the Rhizobiales. *Symbiosis* **49:** 163–180.

Hodkinson, B.P., Gottel, N.R., Schadt, C.W., and Lutzoni, F. (2012) Photoautotrophic symbiont and geography are major factors affecting highly structured and diverse bacterial communities in the lichen microbiome. *Environ Microbiol* **14:** 147–161.

Honegger, R. (2012) The symbiotic phenotype of lichen-forming ascomycetes and their endo- and epibionts. In *Fungal Associations (The Mycota)*. Hock, B. (ed.). Berlin, Germany: Springer, pp. 287–339.

Jordan, W. (1970) The internal cephalodia of the genus *Lobaria*. *Bryologist* **73:** 669–681.

Jüriado, I., Liira, J., Csencsics, D., Widmer, I., Adolf, C., Kohv, K., and Scheidegger, C. (2011) Dispersal ecology of the endangered woodland lichen Lobaria pulmonaria in managed hemiboreal forest landscape. *Biodivers Conserv* **20:** 1803–1819.

Lackner, G., Möbius, N., Scherlach, K., Partida-Martinez, L.P., Winkler, R., Schmitt, I., and Hertweck, C. (2009) Global distribution and evolution of a toxigenic *Burkholderia-Rhizopus* symbiosis. *Appl Environ Microbiol* **75:** 2982–2986.

Lawrey, J.D., and Diederich, P. (2003) Lichenicolous fungi: interactions, evolution, and biodiversity. *Bryologist* **106:** 81–120.

Liba, C.M., Ferrara, F.I.S., Mangio, G.P., Fantinatti-Garboggini, F., Albuquerque, R.C., Pavan, C., *et al*. (2006) Nitrogen-fixing chemo-organotrophic bacteria isolated from cyanobacteria-deprived lichens and their ability to solubilize phosphate and to release amino acids and phytohormones. *J Appl Microbiol* **101:** 1076–1086.

López-López, A., Rogel, M.A., Ormeno-Orillo, E., Martínez-Romero, J., and Martínez-Romero, E. (2010) *Phaseolus vulgaris* seed-borne endophytic community with novel bacterial species such as *Rhizobium endophyticum* sp. nov. *Syst Appl Microbiol* **33:** 322–327.

Loy, A., Maixner, F., Wagner, M., and Horn, M. (2007) probeBase – an online resource for rRNA-targeted oligonucleotide probes: new features 2007. *Nucleic Acids Res* **35:** D800–D804.

Lozupone, C., and Knight, R. (2005) UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* **71:** 8228–8235.

McEvoy, M., Gauslaa, Y., and Solhaug, K.A. (2007) Changes in pools of depsidones and melanins, and their function, during growth and acclimation under contrasting natural light in the lichen *Lobaria pulmonaria*. *New Phytol* **175:** 271–282.

Mushegian, A.A., Peterson, C.N., Baker, C.C.M., and Pringle, A. (2011) Bacterial diversity across individual lichens. *Appl Environ Microbiol* **77:** 4249–4252.

Myllys, L., Stenroos, S., Thell, A., and Kuusinen, M. (2007) High cyanobiont selectivity of epiphytic lichens in old growth boreal forest of Finland. *New Phytol* **173:** 621–629.

Nash, T.H.I.I.I. (2008) Introduction. In *Lichen Biology*. Nash, T.H. (ed.). Cambridge, UK: Cambridge University Press, pp. 1–8.

Neef, A. (1997) Anwendung der in situ Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. PhD Thesis. Munich, Germany: Munich Technological University.

Ondov, B.D., Bergman, N.H., and Phillippy, A.M. (2011) Interactive metagenomic visualization in a Web browser. *BMC Bioinformatics* **12:** 385.

Printzen, C., Fernández-Mendoza, F., Muggia, L., Berg, G., and Grube, M. (2012) Alphaproteobacterial communities in geographically distant populations of the lichen *Cetraria aculeata*. *FEMS Microbiol Ecol* **82:** 316–325.

Reeder, J., and Knight, R. (2010) Rapidly denoising pyrosequencing amplicon reads by exploiting rank-abundance distributions. *Nat Methods* **7:** 668–669.

Rose, F. (1992) Temperate forest management: its effect on bryophyte and lichen floras and habitats. In *Bryophytes and Lichens in a Changing Environment*. Bates, J.W., and Farmer, A.M. (eds). Oxford, UK: Oxford University Press, pp. 211–233.

Rout, M.E., Chrzanowski, T.H., Westlie, T.K., DeLuca, T.H., Callaway, R.M., and Holben, W.E. (2013) Bacterial endophytes enhance competition by invasive plants. *Am J Bot* **100:** 1726–1737.

Scheidegger, C. (1995) Early development of transplanted isidioid soredia of *Lobaria pulmonaria* in an endangered population. *Lichenologist* **27:** 361–374.

Scheidegger, C., and Werth, S. (2009) Conservation strategies for lichens: insights from population biology. *Fung Biol Rev* **23:** 55–66.

Schneider, T., Schmid, E., Vieria de Castro, J., Jr, Cardinale, M., Eberl, L., Grube, M., *et al*. (2011) Structure and function of the symbiosis partners of the lung lichen (*Lobaria pulmonaria* L. Hoffm.) analyzed by metaproteomics. *Proteomics* **11:** 2752–2756.

Schwieger, F., and Tebbe, C.C. (1998) A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. *Appl Environ Microbiol* **64:** 4870–4876.

Shade, A., and Handelsman, J. (2012) Beyond the Venn diagram: the hunt for a core microbiome. *Environ Microbiol* **14:** 4–12.

Shade, A., McManus, P.S., and Handelsman, J. (2013) Unexpected diversity during community succession in the apple flower microbiome. *mBio* **4:** e602–e612. doi:10.1128/mBio.00602-12.

Sharma, M., Schmid, M., Rothballer, M., Hause, G., Zuccaro, A., Imani, J., *et al*. (2008) Detection and identification of bacteria intimately associated with fungi of the order Sebacinales. *Cell Microbiol* **10:** 2235–2246.

de la Torre, J.R., Goebel, B.M., Friedmann, E.I., and Pace, N.R. (2003) Microbial diversity of cryptoendolithic communities from the McMurdo Dry Valleys, Antarctica. *Appl Environ Microbiol* **69:** 3858–3867.

Walser, J.C. (2004) Molecular evidence for limited dispersal of vegetative propagules in the epiphytic lichen *Lobaria pulmonaria*. *Am J Bot* **91:** 1273–1276.

Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* **73:** 5261–5267.

Werth, S., and Scheidegger, C. (2012) Congruent genetic structure in the lichen-forming fungus *Lobaria pulmonaria* and its green-algal photobiont. *Mol Plant Microbe Interact* **25:** 220–230.

Werth, S., Millanes, A.M., Wedin, M., and Scheidegger, C. (2013) Lichenicolous fungi show population subdivision by host species but do not share population history with their hosts. *Fung Biol* **117:** 71–84.

Widmer, I., Dal Grande, F., Excoffier, L., Holderegger, R., Keller, C., Mikryukov, V.S., and Scheidegger, C. (2012) European phylogeography of the epiphytic lichen fungus *Lobaria pulmonaria* and its green algal symbiont. *Mol Ecol* **21:** 5827–5844.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Microscopic pictures of the vegetative propagules of lung lichen. A. young stage of propagules (soredia) made up of algal cell clusters wrapped by fungal hyphae (wet state). B. late developmental stage of propagules as cylindrical outgrowths stratified with fungal cortex (isidioid soredia; dry state).

Fig. S2. Single strand conformation polymorphism analysis of the lung lichen thalli and vegetative propagules of the three sampling sites (Tamischbachgraben, St. Oswald/Eibiswald, Johnsbach, white bars). M: marker; red bar: vegetative propagules; blue bar: thalli.

Fig. S3. A. Distribution of the relative abundances of either sequences or OTUs across lichen parts. B. Venn diagram of OTUs from lichen thalli and propagules (sampled at Johnsbach). Thalli and propagules shared 689 OTUs, but had also unique OTUs for each lichen part.

Fig. S4. Composition of the core microbial community of the two lichen parts at phylum, class, order and family level (starting from the inner circle). Within the *Proteobacteria*, *Deltaproteobacteria* was the predominant group (52%).

Fig. S5. Principal coordinate analysis (PCoA) of bacterial communities based on unweighted, jackknifed unifrac distance matrix. A. Beta diversity of the bacterial communities of lichen thalli and propagules sampled at Johnsbach (Data set I; 5 thallus and 5 propagule samples). Samples were represented by: blue rectangles – lichen thalli, red points – isidioid soredia; $P < 0.022$ (Anosim) B. Beta diversity of the bacterial communities of the three different sampling sites (Data set II; 5 thallus samples at each site); $P < 0.001$ (Anosim). Samples were represented by: green rectangles – St. Oswald/Eibiswald, blue points – Johnsbach and brown triangles – Tamischbachgraben.

Fig. S6. Alphaproteobacterial families on isidiod soredia. The most dominant one was *Sphingomonadaceae* comprising more than 60%. Alphaproteobacterial sequences, which could not be assigned to a certain family taxon are summarized in the category 'Other'. All further families found comprised only 8%.

Table S1. Alpha diversity* and richness indices of lichen thalli and vegetative propagules.

Table S2. Distribution of alphaproteobacterial families across all three sampling sites.

Table S3. Multivariate analysis. Statistical significance was tested with Adonis and ANOVA on the testable RDA fractions. Support values are indicated by an asterisk (*) when $P < 0.05$ and by two asterisks (**) when $P < 0.01$.