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Age, sun and substrate: triggers of bacterial communities in lichens

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Summary

Bacterial communities colonize the surfaces of lichens in a biofilm-like manner. The overall structure of the bacterial communities harboured by the lichens shows similarities, in particular the dominance of not yet cultured Alphaproteobacteria. Parameters causing variation in abundance, composition and spatial organization of the lichen-associated bacterial communities are so far poorly understood. As a first step, we used a microscopic approach to test the significance of both lichen-intrinsic and extrinsic environmental factors on the bacterial communities associated with 11 lichen samples, belonging to six species. Some of these species have thalli with a distinct age gradient. A statistically significant effect can be attributed to the age of the thallus parts, which is an intrinsic factor: growing parts of the lichens host bacterial communities that significantly differ from those of the ageing portions of the thalli. The substrate type (rock, tree, understory) and (at a lower extent) the exposition to the sun also affected the bacterial communities. Interestingly, the abundance of bacterial cells in the lichens was also influenced by the same structure-triggering factors. No effect on the composition with main bacterial groups was attributed to different lichen species, differentiated thallus parts or thallus growth type. Our results are important for the experimental designs in lichen-bacterial ecology.

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Introduction

The lichen association represents one of the most 'eye-catching' fungal symbioses. Lichens are often dominating aspects of vegetation in otherwise hostile habitats such as high mountains or subpolar regions. Lichenized fungi form long-living and compact thallus structures to position their phototrophic partners in a controlled manner towards light (the name of a lichen holobiont applies in fact to the shape-determining fungal partner). Recent studies suggest that lichens are more complex symbiotic systems than previously assumed. Diverse bacteria were characterized in lichens by rRNA gene sequencing (Cardinale *et al*., 2006; 2008; Liba *et al*., 2006; Hodkinson and Lutzoni, 2009) and we consider the concept of lichens be expanded by including the associated bacterial community (Grube *et al*., 2009). Fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM) revealed details about the abundance and localization of the bacterial communities (Cardinale *et al*., 2008). Bacteria preferentially colonize hydrophilic surfaces of the lichens, varying from individual colonies to biofilm-like communities. Alphaproteobacteria usually form a predominant fraction, while diverse other bacteria are present at lower abundances (Grube *et al*., 2009; Bates *et al*., 2011; Schneider *et al*., 2011). Functional studies of the culturable bacteria display a range of lytic activities, including chitinolysis, glucanolysis and proteolysis (Grube *et al*., 2009), while metaproteomic analysis suggested a multifunctional role of the bacterial partner in the lung lichen symbiosis (Schneider *et al*., 2011). Sequence data confirm presence of *nif*H genes in lichen-associated bacteria and suggest a contribution to the lichen's nitrogen budget. It appears that lichen symbioses resemble miniature ecosystems (Farrar, 1985; Grube *et al*., 2009), comprising algae as producers, fungi as consumers, as well as bacteria contributing to nutrient acquisition, recycling and antagonism.

The view of lichens as micro-ecosystems spurs new research questions about stability and variation of the associated microbial community. The long-living thalli of lichens grow in vastly different habitats and represent rather complex niches. Lichens can essentially maintain metabolic activity over the whole thallus area, but species can present a more or less distinct gradient of ageing, with proliferating young parts and slowly decaying distal parts

(Grube, 2010). Because FISH presents mostly physiologically active subpopulation, we can thus analyse in the lichen miniature ecosystem the succession of active microbial communities and the effects of habitat variation at a microscopic level.

In this work, we studied the bacterial communities associated with 11 lichen samples, representing six species, by FISH coupled with confocal laser scanning microscopy (FISH-CLSM) with the aim to analyse and compare the abundance and diversity. For this purpose, we developed a new method to measure the ratio between the volume and the weight of the lichens, defined as Delta volume method (Δvol). We also investigated the effect of lichen-intrinsic (lichen species, thallus age, differentiated thallus parts, growing type) and extrinsic environmental factors (sun exposure and substrate) on the taxonomic structure of the bacterial communities in lichens.

Results and discussion

Lichen samples were collected from above and near the timber line in subalpine altitudes in the Styria region (Austria), and from broad-leaved montane forests, in the years 2007–2009. At least four different healthy lichen thalli for each sample were collected from sites specifically chosen to study the effects of several lichen-intrinsic and extrinsic environmental factors on the lichen-associated bacterial communities. Cryosections of paraformaldehyde-fixed lichen samples were pretreated with lysozyme and then hybridized with different FISH probes (Table S1), following the protocol of Cardinale and colleagues (2008). Initial experiments showed no difference between the bacterial cell counts of Gram-positive bacteria in paraformaldehyde- and ethanol-fixed lichens, when pretreated with lysozyme. A negative control for systemic errors within FISH experiments consisted of a mixture of NONEUB FISH probes labelled with all the fluorochromes present in the positive probes. Typical signals from probe-stained bacteria were not detected in the negative control, whereas autofluorescence of algae and fungi showed the same intensity, which confirmed the absence of both probe- and fluorochrome-unspecific labelling. The FISH-stained samples were viewed under a confocal microscope and the stacks were recorded to perform both bacterial counts and 3D-reconstructions. Bacterial cell count was performed on 3D image stacks semi-automatically, supported by the counting options in the software Imaris 7.0 (Bitplane, Zurich, Switzerland). Specifically, the bacteria were tagged automatically and tags were then inspected by eye. Particular attention was paid to dense cell clusters to avoid underestimation of counts due to signal overlap. The stacks showing low signal/noise ratio or ambiguous signals were discarded from the analysis. At least 30 confocal stacks from at least five independent FISH experiments per lichen sample were recorded and analysed.

To determine the density of the EUB338MIX-stained bacterial cells per gram of lichen we developed a new measurement method, which we call Delta volume (Δvol). First, lichen thalli were saturated with water in a humid chamber until a constant wet weight (ww) was reached. The volume of the wet thalli was measured by dipping it into a graduated tube (Δvol). Then the thalli were dried at 50°C and the dry weight (dw) was assessed. The ratio Δvol/dw represents the volume of hydrated lichen thallus containing one gram of lichen biomass (dw). Results are shown in Table 1.

To check the detection efficiency of FISH, we used the nucleic acid stain Acridine Orange, which was shown to be superior to DAPI in lichens (Cardinale *et al*., 2008). On average, 82% of the Acridine orange-detected cells were stained by the EUB338MIX probe. The number of EUB338MIX-stained cells associated with the analysed lichens were in the range of $2.11 \pm 0.24 \times 10^9$ cells/g_{dw} (squamules of *Cladonia coccifera*) and $7.56 \pm 1.09 \times 10^9$ cells/g_{dw} (senescing part of the *Cladonia arbuscula* thallus, sun-exposed site). The means between lichen samples (Log_{10}-transformed values) were significantly different, $F_{10,518} = 11.598$, $P < 0.001$, $\eta^2 = 0.183$, 95% $CI_{\eta 2} = 0.113–0.227$, $1 - \beta_{\alpha = 0.05} = 1.000$. All analysed

Table 1. Correspondence between the volume and the weight of the lichens, assessed by the Delta volume method – Δvol.

Lichen species	Thallus part	Δvolume (mm^3)	Wet weight (g)	Dry weight (g)	Volume (mm^3) corresponding to 1 g of lichen dry weight
Cetraria islandica	Younger	4.0×10^3	3.7	0.77	5.19×10^3
Cetraria islandica	Older	6.0×10^3	4.4	0.72	8.33×10^3
Lobaria pulmonaria	Whole	6.5×10^3	5.13	1.58	4.11×10^3
Lecanora polytropa	Whole	5.5×10^3	5.18	3.02	1.82×10^3
Cladonia arbuscula	Younger	8.0×10^3	12.39	3.1	2.58×10^3
Cladonia arbuscula	Older	5.0×10^3	3.4	0.91	5.49×10^3
Umbilicaria cylindrica	Whole	2.6×10^4	22.94	7.04	3.69×10^3
Cladonia coccifera	Podetia	2.0×10^3	1.72	0.67	2.99×10^3
Cladonia coccifera	Squamules	6.5×10^2	0.46	0.19	3.42×10^3

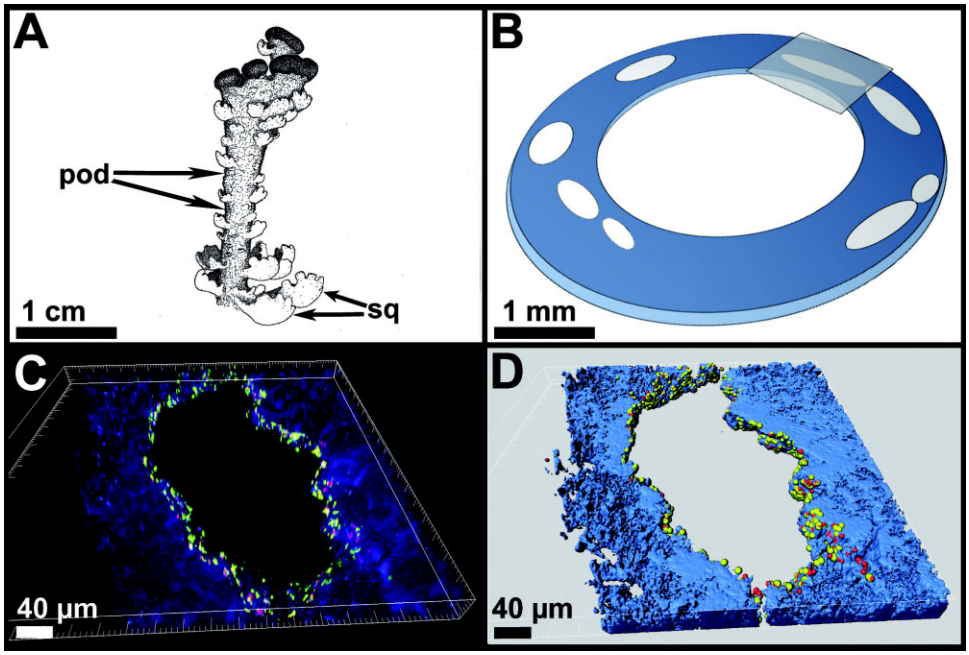

Fig. 1. A. The lichen *Cladonia coccifera* with the differentiated plectenchyma forming squamules (sq) and podetia (pod).
B. A transversal section of a *C. coccifera* podetium, showing the characteristic cylindric shape, and with cavities within the fungal thallus structure.
C. Volume rendering of a confocal stack showing the bacterial colonization of the lichen *C. coccifera*; the bacteria densely colonize the surface of the internal cavities in the podetium [see rectangle in (B); blue: lichen-fungal plectenchyma; yellow: Alphaproteobacteria; red: other bacteria (algae not present in this section)].
D. Three-dimensional model of the image in (C); blue surface: lichen-fungal plectenchyma; yellow spheres: Alphaproteobacteria; red spheres: other bacteria. Bacteria were stained by FISH as described by Cardinale and colleagues (2008) using the universal bacterial probe EUB338MIX (Cy3-labelled) and the Alphaproteobacteria-specific probe ALF968 (Cy5-labelled). The confocal microscope Leica TCS SPE (Leica microsystems GmbH, Mannheim, Germany) was used for the image acquisition; the Z-step size, laser intensity and detector settings (gain and offset) were optimized to get the optimal resolution and the best signal/noise ratio; the software Imaris 7.0 (Bitplane, Zurich, Switzerland) was used to create the 3D models.

lichens hosted a very high number of bacteria, comparable with the abundance in the rhizosphere (Berg and Smalla, 2009), and similarly represents bacterial hot spot.

According to our data *Lobaria pulmonaria* and the old thalli of *C. arbuscula* harboured the highest number of active bacteria, at a confidence level of 0.05 (Tukey test). Lichens grown under shaded conditions harboured a higher total number of bacteria than the sun-exposed ones, $t_{527} = 3.242$, $P = 0.001$, $d = 0.28$. Interestingly, the old senescing parts of lichen thalli host a significantly higher number of bacteria, than the young growing parts, $t_{527} = 7.890$, $P < 0.001$, $d = 0.70$. This difference suggests a succession or gradient of the lichen-associated bacterial community. The ageing structures allow growth of diverse bacterial taxa, whereas young parts with prolifering fungal and algal partners constrain the spectrum of bacteria to highly adapted ones, mostly Alphaproteobacteria. ANOVA followed by Tukey test also showed that the lichens growing on rock (*C. coccifera, Lecanora polytropa* and *Umbilicaria cylindrica*) harbour fewer bacteria than lichens from other substrate types (e.g. soil and bark), $F_{3,525} = 22.762$, $P < 0.001$, $\eta^2 = 0.115$, 95% $CI_{\eta 2} = 0.066–0.164$, $1 - \beta_{\alpha = 0.05} = 1.000$. Alphaproteobacteria dominated the vital structures in all lichens (Fig. 1), ranging from 37.6 ± 10.5% in *U. cylindrica* to 82.1 ± 3.8% in the squamules of *C. coccifera* (Fig. 2A). Only in the older basal part of *C. arbuscula* collected from a shaded site the dominant group was Betaproteobacteria (53.5 ± 5.1%) and the Alphaproteobacteria represented 15.4 ± 2.6% (Fig. 2A). Other bacterial groups were relatively rare in the lichens (Fig. 2A).

Canonical correspondence analysis showed that morphologically different lichens harbour communities, which are similar with respect to the principal bacterial groups according to FISH/CLSM analyses (Fig. 2B). Testing the environmental variables for significance demonstrated that the lichen species does not exert an exclusive effect on the overall community structure ($P = 0\ 640$) (Fig. 2B). This observation does not exclude variation between the bacterial communities at the species/strain level in different lichen species; specificity was already shown in different lichens for the composition of microbial fingerprints

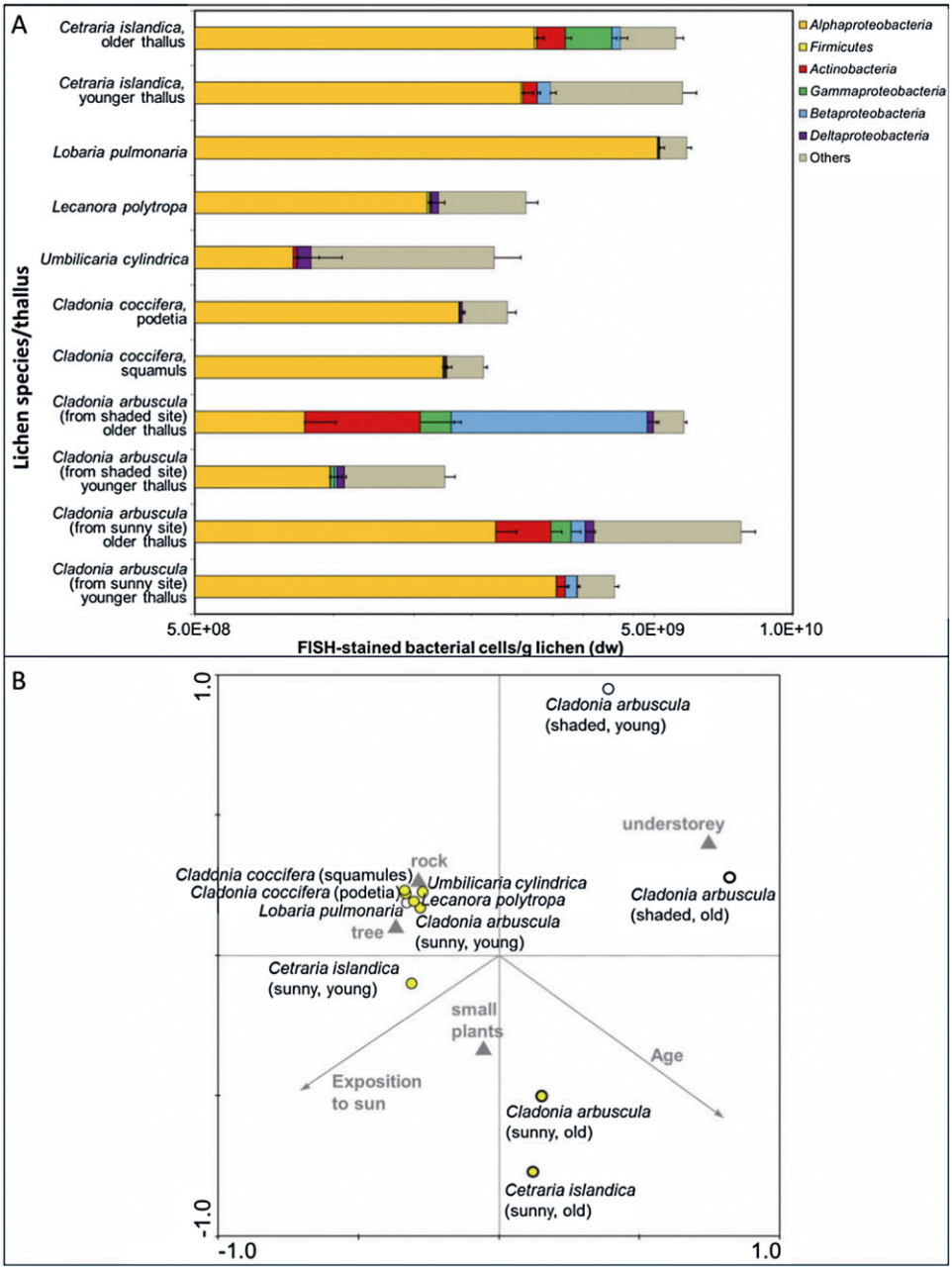

Fig. 2. A. Density of principal bacterial groups associated with different lichen species/thalli assessed by fluorescence *in situ* hybridization-confocal laser scanning microscopy (FISH-CLSM). FISH was performed as described by Cardinale and colleagues (2008) on 30–50 μm thick cryosections of PFA-fixed lichens; for details on the used FISH probes, see Table S1.
B. Canonical correspondence analysis (CCA) performed with CANOCO for Windows (ter Braak and Šmilauer, 2002), showing the similarity between the bacterial community structures, assessed by FISH-CLSM; yellow-filled circles indicate bacterial communities of lichens samples collected from sun-exposed sites; circles with bold lines indicate bacterial communities of the older parts of the thalli. Statistically significant environmental factors are shown (grey labels); for the nominal variables 'substrate type' the centroid (triangle) is shown. Significance of environmental factors was evaluated by Monte Carlo permutation test, as implemented in CANOCO.

(Grube *et al.*, 2009) and corroborated recently by 16S rRNA gene deep-sequencing (Bates *et al.*, 2011).

A statistically significant effect on the bacterial community structure was found for the age of the thallus ($P = 0.002$): in the older thallus parts the bacterial community displayed a drastic change due both to the reduction of the otherwise dominant Alphaproteobacteria and to the increased abundance of other groups including Actinobacteria, Gamma- and Betaproteobacteria. On the other hand, Deltaproteobacteria and Firmicutes were not

significantly affected (Fig. 2A). It is worth noting that no Alphaproteobacteria were isolated in culture experiments from the same lichens as used for FISH-CLSM (data not shown). This suggests that the dominant Alphaproteobacteria have special requirements for growth, possibly expressed by the physiologically active lichen. The exposition to sun was also found to be of significant effect on the bacterial communities ($P = 0.010$), as well as the substrate type ($P = 0.004$) (Fig. 2B). Other apparently important factors such as differentiated thallus forms and growth types also did not affect the main taxonomic structure of the bacterial communities ($P = 0.244$ and 0.490, respectively).

The analysed lichens show an interesting duality in their associated bacteria, with a constrained and stable community in the healthy areas and more variable one in the ageing, or senescing, parts. The divergence occurs at distances of millimetres to centimetres depending on the size of the thalli. The contributions by additional bacterial diversity in ageing portions could be interpreted as transient bacterial fraction in lichens. Further research using deep amplicon sequencing data needs to take into consideration the differences within a single lichen thallus that result from the considerable age of the structures. As lichen thalli can live for decades to hundreds of years, we conclude that the lichens maintain a dynamic equilibrium. The growing apices with alphaproteobacterial dominance act as anabolic centres, whereas the senescing parts might represent catabolic sinks of the lichen system. We assume that the diverse bacteria of these parts help to convert the old biomass into simple molecules, which might be released into the substrate or be recycled by translocation to the growing parts of the lichens. Such recycling of nutrients has been demonstrated previously in lichens (Ellis *et al*., 2005).

Confocal laser scanning microscopy is a valuable tool to complement diversity studies based on fingerprinting or sequencing methods, by providing localized information on microbial diversity and circumventing biased views of diversity at small scales (Bent and Forney, 2008). The limited resolution of the probes, however, precludes comments on differences at the level of species/strains. Such level of detail was not necessary to demonstrate that thallus vitality is a principal factor for intra-individual variation of lichen-associated bacterial communities. Two additional triggers were found, namely the substrate type and the sun exposition, whereas growth type does not play a significant role in modifying the main composition of lichen-associated bacterial communities. Interestingly, the same factors affecting the structure of the bacterial community also affected the abundance of FISH-detected cells in the analysed lichens. This work represents a study of the ecology of lichen-associated bacterial communities and provides first evidence for environmental triggers of their main composition. These results will be informative and could serve as a guideline for experimental design in lichen-bacterial ecology, and more generally for exposed and long-living terrestrial symbiotic systems.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Characteristics of the FISH probes used in this work.

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