RESEARCH LETTER

Bacterial taxa associated with the lung lichen *Lobaria pulmonaria* are differentially shaped by geography and habitat

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Abstract

The correlation between the taxonomic composition of *Alphaproteobacteria*, *Burkholderia* and nitrogen fixers associated with the lichen *Lobaria pulmonaria* and the geographical distribution of the host was studied across four sites in Europe. Results proved that the diversity of *Alphaproteobacteria* is affected by geography, while those of *Burkholderia* and nitrogen fixers were mostly driven by local habitat. This difference indicates a higher stability of the association between *Alphaproteobacteria* and the lichen host.

Introduction

More than 18 500 species of fungi diversified in the lichen symbiotic stage (Nash, 2008). Their unique symbiotic structure, the lichen thallus, is maintained for decades and in some cases for thousands of years. While lichens are still presented in text books as a partnership of fungi and algae (and/or *Cyanobacteria*), recent research revealed a high diversity and abundance of bacteria in lichen thalli (Cardinale *et al.*, 2006, 2008, 2011; Grube *et al.*, 2009; Hodkinson & Lutzoni, 2009; Bjelland *et al.*, 2010; Selbmann *et al.*, 2010; Bates *et al.*, 2011; Mushegian *et al.*, 2011). Lichens have generally a wide distribution, which has been suggested to be the result of long-distance dispersal (Galloway, 2008). There is a fairly good knowledge about lichen biogeography (Galloway, 2008), whereas less is known about the geographical patterns of their associated bacteria. In a study analysing different lichen species, Hodkinson *et al.* (2012) found the trend that the major bacterial community was correlated with differences in large-scale geography. Despite an increasingly better understanding of microbial biogeography (Hughes Martiny *et al.*, 2006), the effects of habitat and geography on symbiotic microbial communities are still scarce. Lichens are of particular interest for such studies because of both their cosmopolitan distribution and their strict requirements for particular environmental conditions. We selected the 'lung lichen' *Lobaria pulmonaria* (Fig. 1a) widely found in the Northern hemisphere, tropical mountains and in South America. It includes a green-algal (*Dictyochloropsis reticulata*) and further cyanobacterial (*Nostoc*) photobiont. Our previous works on *Lobaria*-associated bacteria revealed yet-uncultivable *Alphaproteobacteria* as structurally dominant and metabolically active taxon (Cardinale *et al.*, 2011; Schneider *et al.*, 2011) (Fig. 1b–d).

Our hypothesis for the present study was that the association of the bacteria to the host, measured as correlation with its distribution range, will reflect their stability in the lichen symbiosis. Therefore, the differences among key bacterial taxa in lichen samples collected from different sites can be the effect of historical contingencies, that is,

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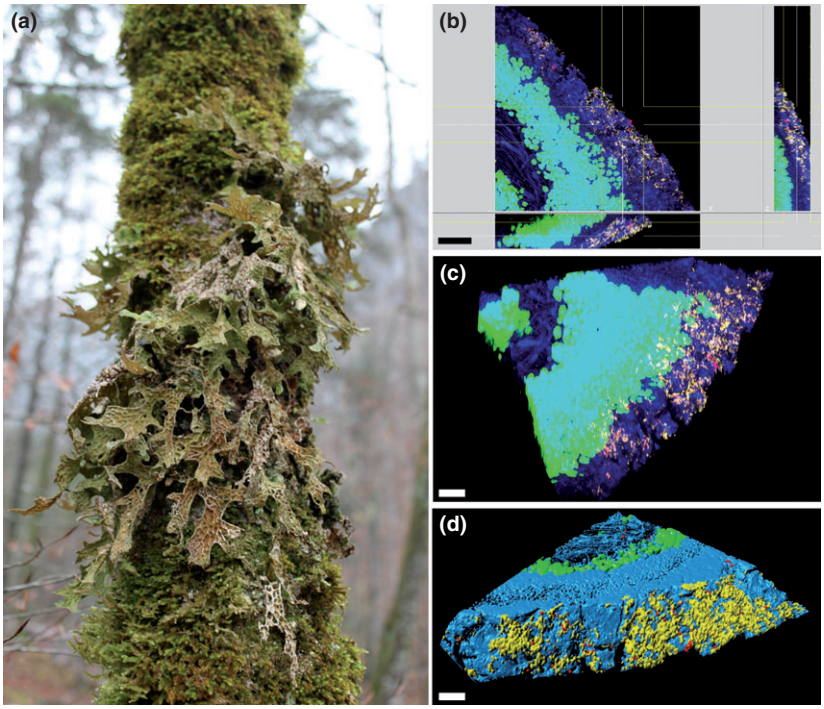

Fig. 1. Bacterial colonization of the lichen *Lobaria pulmonaria* and dominance of *Alphaproteobacteria*. Lichen samples were collected from healthy thalli (a) and cut with a cryotome. The cryosections were stained by FISH with the universal bacterial probe EUB338MIX (red) and the *Alphaproteobacteria*-specific probe ALF968 (yellow). Autofluorescence of fungi (blue) and algae (green) was also detected. A confocal laser scanning microscopy stack is displayed as XY-XZ-YZ projections (b), volume rendering (c) and three-dimensional model (d). The bacteria form a biofilm over the fungal surfaces and the *Alphaproteobacteria* dominate the bacterial community. Scale bars: B = 40μm, C and D = 20μm. Image of (a), referring to the sampling site 'northern Styria', courtesy of Armin Erlacher (Graz). Images of (b–d) refer to *Lobaria* samples collected from the site 'southern Styria' (see Materials and methods).

the diversity has evolved across time only as a consequence of the isolation of the original bacterial population (s). On the other hand, bacterial species occurring on lichens, but not critical to their survival/growth, will be less abundant and also more variable. We compared lichen samples from different parts of their geographical range and evaluated whether geography is a primary determinant shaping the taxonomical structure of different lichen-associated bacterial taxa. For this study, we selected *Alphaproteobacteria* and *Burkholderia* for a fingerprinting analysis of their geographically correlated structure. *Alphaproteobacteria* are the dominant taxon in all tested lichen species (Cardinale *et al.*, 2008, 2011) and were shown to form a biofilm-like layer over the fungal surfaces of the *L. pulmonaria*, intermingled with other bacteria (Fig. 1). *Burkholderia* is present in the culturable fraction but hardly detected by *in situ* hybridization (Cardinale *et al.*, 2006, 2008). Isolates of *Burkholderia* were retrieved from the same lichen samples used in this work (data not shown). Although evidences of either symbiotic relationship or pathogenicity were not yet shown in the lichen hosts, strains of *Burkholderia* are already known for their stable associations and symbiosis with fungi, such as mycorhiza (Partida-Martinez *et al.*, 2007). Considering the protective and self-sustaining nature of the lichen symbiosis, it can be hypothesized that some of the lichen-associated *Burkholderia* strains play functional roles, as already proved in other fungal-*Burkholderia* associations, such as enabling the vegetative reproduction (Partida-Martinez *et al.*, 2007) or supporting the nutrient uptake (Ruiz-Lozano & Bonfante, 1999) and pathogen defence (Opelt *et al.*, 2007). We also analysed the diversity of *nifH* genes, which is related to the functional group of nitrogen fixers. They include the *Nostoc* symbionts and further potential N-fixing species. The ability to grow on N-free substrate was already shown for bacterial strains belonging to different classes, isolated from different species of lichens (Cardinale *et al.*, 2006; Grube *et al.*, 2009). Grube & Berg (2009) suggested that, in the case of N-limiting conditions, bacterial N-fixation could be of considerable importance for the vitality of lichens.

To test our hypothesis, we considered the theoretical pattern of distribution proposed by Hughes Martiny *et al.* (2006) as a consequence of prevailing historical or environmental influences. *Lobaria pulmonaria* has very strict requirements for growing, so that the environmental

parameters cannot differ very much across sites where it grows. Its associated bacteria live in their habitat (the thallus) where the environmental parameters are even more stable, because of the homeostatic effect generated by the hosting organism. The assumption of our study was that the lichen *Lobaria* offers a similar habitat, even across very distant regions. The lichen should thus represent one single 'microbial habitat' and the only differences between structures of bacterial taxa associated with lichen samples from different regions would result from historical contingencies as a biogeographical effect.

Materials and methods

Sampling of lichens

Lichen samples were collected from northern Styria (47°37′35″ N, 14°41′35″ E), southern Styria (46°44′35″ N, 15°04′30″ E), Montenegro (42°53′55″ N, 19°35′51″ E) and Madeira (32°44′09″ N, 16°53′17″ W). These locations lie within a range of relative distances (102.4–3367 km) that allows the occurrence of both historical contingencies and contemporary environmental factors (Hughes Martiny *et al.*, 2006). Four to seven independent replicates (composite samples of four lichen thalli) per sampling site were collected.

PCR-SSCP

Single strand conformation polymorphism (SSCP) analysis of *nifH* genes and 16SrRNA genes of *Alphaproteobacteria* and *Burkholderia* carried out as described in Grube *et al.* (2009). The fingerprinting method was selected because it allows an appropriate statistical analysis, which based on the dominant members of the bacterial community. The SSCP profiles were normalized with GelCompar II (Applied Maths, Kortrijk, Belgium). Single DNA bands, characterized by the relative position and abundance on the gel, were defined as response variables and used for detrended correspondence analysis (DCA), as implemented in the software CANOCO for Windows (ter Braak & Šmilauer, 2002). Both parametric (Pearson) and nonparametric (Sperman's rho) correlations between the relative geographical distances of sampling sites and their relative position in the DCA plots were calculated with PASW Statistic 18 (SPSS Inc., Chicago, IL).

FISH-CLSM

Fluorescence *in situ* hybridization (FISH) was performed as described in Cardinale *et al.* (2008) with the FISH-probes Cy3-EUB338MIX (universal for bacteria) and Cy5-ALF968 (specific for *Alphaproteobacteria*). Samples were pretreated with lysozyme (Sigma-Aldrich, Steinheim, Germany) to ensure permeability to the FISH-probes, and negative controls were performed using a mixture of both Cy3- and Cy5-labelled NONEUB probes. FISH-stained samples were observed with the confocal laser scanning microscope Leica TCS SPE (Leica microsystems GmbH, Mannheim, Germany) and three-dimensional models were created with the software IMARIS 7.0 (Bitplane, Zurich, Switzerland).

Results and discussion

FISH images showed that the bacterial colonization is similar in all samples, irrespective of the sampling site (Fig. 1b-d). Relative proportion of *Alphaproteobacteria* ranged between 47.3% and 93.9%; they were detected in all confocal stacks. *Betaproteobacteria* were detected in some confocal stacks and their relative proportion ranged between 0.2% and 0.6%.

All microbial fingerprints showed a high diversity but the functional patterns were more heterogeneous than those using group-specific primers. Pearson correlation based on converted fingerprints demonstrated that the distribution of the bacterial assemblage in the DCA plots was significantly correlated with the relative distances between the sampling sites only in the case of *Alphaproteobacteria* ($r = 0.722$, $P = 0.05$) but not for *Burkholderia* ($r = 0.162$, $P = 0.38$) and *nifH* genes ($r = -0.251$, $P = 0.32$) (Fig. 2). Nonparametric test showed also a higher (although not statistically significant) correlation between DCA plot assemblages of *Alphaproteobacteria* and the relative distances between the sampling sites ($P = 0.11$), in comparison with *Burkholderia* ($P = 0.31$) and *nifH* genes ($P = 0.31$). In both *Burkholderia* and *nifH* DCA, a clustering of the samples from the same site is clearly evident. This implies that additional site-specific environmental factors affected the taxonomic structure of the investigated taxa, and moreover, it indicates that our assumption of lichen as unique microbial habitat across sampling sites was not met. These environmental factors were the only triggers in the case of *Burkholderia* and *nifH* genes while, in the case of *Alphaproteobacteria*, their influence was generally overcome by the biogeographical effect, and this also explains why samples of *Burkholderia* and *nifH* cluster more tightly than *Alphaproteobacteria* based on sampling location.

Our results suggest that these bacterial groups are differentially shaped by geography and habitat and that the *Alphaproteobacteria* in *Lobaria* are maintained across space and evolve across time. As stated above, *Alphaproteobacteria* are the dominant lichen-associated bacterial group, whereas other taxa, including *Burkholderia*, are present at lower abundances. Our results demonstrate a

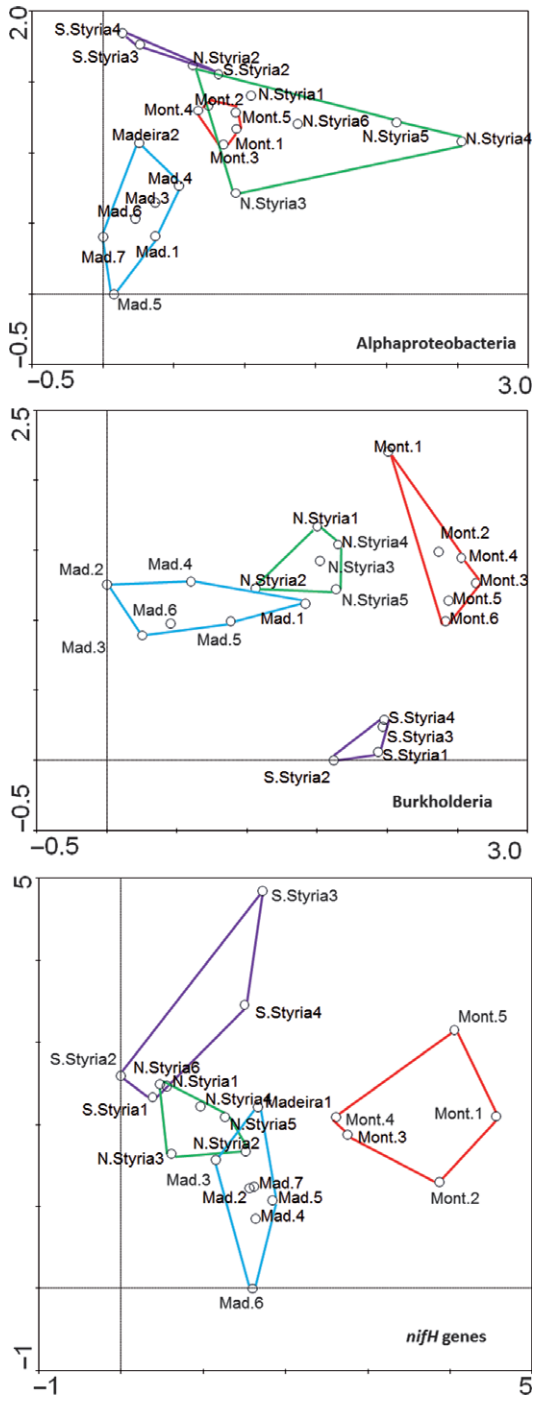

Fig. 2. DCA of both *Alphaproteobacteria* and *Burkholderia* population structures and of the *nifH* gene diversity in *Lobaria pulmonaria* samples collected from different sites. Population from the same site cluster together but only the distribution of *Alphaproteobacteria* is significantly correlated with the geographical distances between sampling sites, so indicating a biogeographical effect (see text for details). S. Styria, southern Styria; N. Styria, northern Styria; Mad., Madeira; Mont., Montenegro.

differential effect of habitat and geography on the composition of these groups of the lichen-associated bacteria. The structure of *Alphaproteobacteria* correlated well with geography, whereas this effect could not be observed in *Burkholderia* and, surprisingly, also in *nifH* genes. Our results shed light on the ecological significance of different bacterial groups of the lichen microbiome, indicating which taxa are maintained across space, thus suggesting a necessary involvement in the lichen symbiosis. Fierer (2008) suggested that both dispersal and colonization success depend on the original density of the population. We suppose that when vegetative lichen propagules are dispersed, the high-abundant *Alphaproteobacteria* are maintained for successful colonization of the new site; on the contrary, the original species of both *Burkholderia* and nitrogen fixers will be lost, and local, better adapted competitors will be uploaded from the new environment.

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