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Technical Focus

Microbial consortia of bacteria and fungi with focus on the lichen symbiosis

Martin GRUBE^{a,*}, Gabriele BERG^b

^aInstitute of Plant Sciences, Karl-Franzens-University, Holteigasse 6, 8010 Graz, Austria

^bInstitute of Environmental Biotechnology, Graz University of Technology, Petersgasse 12, 8010 Graz, Austria

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ABSTRACT

The investigation of fungal–bacterial interactions is an emerging field of research applying tools of modern microbial ecology. Studies have previously focused on the mycorrhizosphere, but in past decade, the role of bacteria in other fungal niches has been increasingly evaluated. This review presents recent progress in the understanding of fungal–bacterial interactions and contains a special focus on lichen symbioses. Lichens are traditionally considered as mutualisms between fungi and photoautotrophic species, but recent molecular approaches have revealed that lichens also harbour diverse microbial communities. Using modern DNA/RNA-based and microscopic techniques (e.g. FISH and confocal laser scanning microscopy) we are now able to analyse the abundance, composition, and structure of microbial communities in the lichen holobiont. Lichen-associated microbial communities consist of diverse taxonomic groups; the majority of bacteria belong to Alphaproteobacteria. Microbial communities can form biofilm-like structures on specific parts of the lichen thallus. Until now, the function and interaction within the microbial consortia is not fully understood. The functions displayed mainly by culturable strains suggest that bacteria have lytic activities, complement the nitrogen budget and produce bioactive substances, including hormones and antibiotics. Bacterial contribution to the lichen symbiosis is perhaps not restricted to one particular function in the lichen system, but supports a complex functional network which remains to be studied in greater detail.

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1. Introduction

The investigation of microorganisms from poorly explored ecological niches has become a fascinating biological endeavour in recent years. Unprecedented progress has been achieved with the application of new molecular and microscopic techniques (Smalla, 2004; Allen and Banfield, 2005; Amann and Fuchs, 2008). Furthermore, microbial habitats can be approached by ultra-deep sequencing and

metagenomic analyses (Velicer *et al.*, 2006; Handelsman *et al.*, 2007). These achievements have not only illuminated our understanding of microbial contributions to nutrient cycles in the environment (Leininger *et al.*, 2006), but they will also influence our concepts of symbiotic interactions.

The term symbiosis was coined in 1879 by Heinrich Anton de Bary, a German mycologist, who defined it as: “the living together of unequally named organisms”. In this broad sense symbiosis includes all kinds of close biological relationships

* Corresponding author. Tel.: +43 316 380 5650.

E-mail address: martin.grube@uni-graz.at (M. Grube).

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between species, hence spanning a continuum between pathogenic and mutualistic phenomena. Especially in European scientific schools, symbiosis was often restricted to the beneficial cases, where functions were thought to be partitioned among co-operating species for the sake of an improved whole, i.e. mutualism. However, in many cases it is hard to distinguish between mutualism and controlled slavery among partners. Alternatively, symbiosis can be seen as a long-term intimate association of organisms that lead to new structures and metabolic activities (Douglas, 1994), a view that overcomes the value-laden interpretations of interaction among two partners. In fact, owing to the presence of two visually conspicuous species in association, classic fungal (mutualistic) symbioses were originally described as bipartite partnerships. The most prominent examples are of course mycorrhizal associations of fungi with plants or lichen symbioses comprising fungi and algae. In lichens, however, it was since long realized that some of the species can – in addition to green algae – also harbour cyanobacteria in specialized organs called cephalodia. Cengia-Sambo (1924) coined the term polysymbioses for such cases (and regarded cephalodia as analogous to root nodules of legumes), though the term tripartite relationships has become more popular since then.

In addition to the easily recognized ‘key’ partners, modern studies reveal additional microbial partners in most symbioses, and show an previously unknown degree of intricate interconnectivity of nature (Hunter, 2006). An impressive example of complexity comes from recent research on the fungus leaf-cutter ant symbiosis. This symbiosis has traditionally been given as an example for fungal fermentation of plant material in a highly organised agriculture evolved by ants approximately 8–12 million years ago (Schultz and Brady, 2008). Meanwhile additional symbiotic partners have been recognized in leaf-cutter ant nests, which have recently been called homeostatic symbiotic fortresses (Hughes et al., 2008): the ant’s fungal cultures (*Leucoagaricus gongylophorus*) can be invaded by a fungal parasite of the genus *Escovopsis*. To prevent damage from this pathogen, the ants carry bacteria (*Pseudonocardia* sp., belonging in the order Actinomycetales) on their thoraces to distribute an antagonistic substance against *Escovopsis*. With the recent discovery of a Chaetothyrialean black fungus (related to *Phialophora*) in the nests, which may impair the antagonistic effect by its affinity to *Pseudonocardia* (Little and Currie, 2008), this symbiosis comprises five intricately associated partners. However, the functional interactions in multibiont fungal symbioses are not always so apparent or well-studied as in the leaf-cutter ant symbiosis.

As will be apparent from the following account, most fungal symbioses have more or less tight associations with bacteria, which may specifically contribute to the functioning of the symbiotic systems. One of the prevailing challenges of the near future will be the investigation of the functional involvements of fungal-associated bacteria by using various approaches. In this review we summarize new findings of interaction with bacteria in different fungi but we do not cover bacterial–fungal interactions of medical importance (the latter were recently reviewed by Wargo and Hogan, 2006). After a more general survey we concentrate on lichens as the most diversified forms of fungal life. We highlight recent progress in the study of lichen-associated bacteria, and set up

a reviewed conceptual framework including the involvement of bacteria in lichen symbioses.

2. Bacteria and fungi

Research on bacterial–fungal associations has focused on mycorrhizal systems for more than two decades. The earliest reports were about the positive effect of fluorescent pseudomonads on ectomycorrhizal establishment. These pseudomonads had been isolated from hyphae of the corresponding ectomycorrhizal fungi (EM), e.g. *Rhizopogon luteolus* and *Laccaria* spp. (Garbaye, 1994; Frey-Klett and Garbaye, 2005). Due to their positive effect on mycorrhiza formation, these bacteria were called ‘mycorrhizal helper bacteria’ (MHB) (Garbaye, 1994). It has been widely accepted that plants benefit from the bacterial involvement in the mycorrhiza. Interactions of plants, bacteria, and mycorrhizal fungi take place in that portion of soil, where microbial processes are primarily influenced by the root, i.e. the ‘rhizosphere’ (Hiltner, 1904), and differ from those in bulk soils (Andrade et al., 1997). Growth-promoting effects were reported from MHB *Streptomyces* Ach 505 (Riedlinger et al., 2006), including the spreading of hyphae due to a modification of the actin cytoskeleton of *Amanita* (Schrey et al., 2007). Recent work shows that bacterial strains induce differential gene expression in ectomycorrhizal (EM) fungi (Schrey et al., 2005) and can alter the fungal transcriptome dynamically over time (Deveau et al., 2007). Early responsive genes are involved in recognition processes and transcription regulation, while subsequently expressed genes encode proteins of primary metabolism. Reports also showed that the ‘helper effect’ is not restricted to ectomycorrhizae: comparable effects have also been observed for bacteria that are associated with arbuscular mycorrhiza (AM) (Johansson et al., 2004), where bacteria stimulate spore germination of AM fungi. A comprehensive review of interkingdom signals in bacterium–fungus interactions has recently been provided by Tarkka et al. (2009).

A continuum between pathogenic and mutualistic effects on fungi

Both positive and negative effects of bacteria on fungal performance (and vice versa) have been reported (Kobayashi and Crouch, 2009). In saprotrophic and ectomycorrhizal fungi this includes induction of (edible) fruiting body formation (Rainey et al., 1990), but also the activation of tyrosinase (Crowe and Olsson, 2001) and decay of fruitbodies (Soler-Rivas et al., 1999). Fungus-associated bacteria have now been described in other biological groups of fungi, where they can contribute to fungal biology in various ways. For example, bacteria may stimulate pathogenicity of fungi toward plants (Dewey et al., 1999). Diazotrophic Alphaproteobacteria were observed to be predominant in bacterial communities in truffles (Barbieri et al., 2007). Many plant (including root) associated bacteria secrete indole acetic acid (IAA), which acts as a signal molecule in fungi as well as plants and can also induce invasive growth in *Saccharomyces cerevisiae* (Prusty et al., 2004). Still little explored is the role of bacterial effects on secondary metabolite production of fungi. Recent research

showed that cocultivation with actinomycetes can activate expression of hitherto unknown biosynthetic pathways in *Aspergillus nidulans* (Schroeckh et al., 2009), including the archetypal compound orsellinic acid, the typical lichen compounds lecanoric acid, and the cathepsin K inhibitors F-9775A and F-9775. Negative effects of bacteria against fungi include inhibition of fungal ligno-cellulose degradation (De Boer et al., 2005) and inhibition of root infection by pathogenic fungi (Whipps, 2001). Bacterial mycophagy was recently reviewed by Leveau and Preston (2008), who recognized three basic bacterial strategies: necrotrophy, extracellular biotrophy and endocellular biotrophy. Each of these strategies requires a fine-tuned interaction and signalling system. The outcome of the symbiotic relationship might be specific for interacting strains of fungi and bacteria, with varying degrees of mycophagy when interacting partners are switched in natural habitats. Shifts in the frequency of strain combinations that leads to expression of 'mild' mycophagy may result in long-term interactions and evolution towards mutualisms (see below and Partida-Martinez et al., 2007a). On the other hand there are also dramatic shifts of symbiotic effects when interacting strains of fungi and bacteria are recombined. Lehr et al. (2007) found a generally antagonistic activity of *Streptomyces* AcH 505 against the mycelial growth of *Heterobasidium annosum*, except for one tested strain of this pathogenic fungus, which was not affected at all. Instead, suppression of plant defence promoted by the bacterial strain enhanced plant root colonization of the *H. annosum* strain. Such strain-specific differences have a high impact on risk management of pathogens and use of bacteria in biological control of plant diseases, where antagonistic fungal-bacterial interactions are exploited. The aspect of biological control of pathogenic fungi by bacterial antagonists has been featured in a recent review (Kobayashi and Crouch, 2009).

Fungi as determinants of microbial community composition

The presence of fungi specifically alters the composition of bacterial communities. The mycorrhizal association of *Glomus fasciculatum* (Glomeromycota) with *Zea mays* or *Trifolium subterraneum* reduced the viable counts of fluorescent pseudomonads but increased total bacterial numbers compared to plants growing without the AM fungus (Meyer and Linderman, 1986). Similarly, *G. fasciculatum* increased total viable counts of bacteria in the rhizoplane of *Panicum maximum*, but *Acaulospora laevis* (Glomeromycota) reduced this number (Secilia and Bagyaraj, 1987). Effects of the ectomycorrhizosphere on the *Pseudomonas fluorescens* populations select strains potentially beneficial to the symbiosis and to the plant (Frey-Klett et al., 2005). Such strains might be ubiquitous and widespread

or highly specific for species or soil conditions (pH, humidity). A recent study of the mycosphere – the zone below the mushroom fruitbodies – distinguished between universal and species-specific bacterial fungophiles (Warmink et al., 2008). BIOLOG-based substrate utilization assays showed a clear functional difference between bulk soil and mycosphere pseudomonads.

How can bacteria colonize fungal mycelia? Guided motility could be responsible for rather dynamic spreading of bacteria in the range of mycelia. Kohlmeier et al. (2006) showed that bacteria can move along the surface of fungal hyphae (the "fungal highways") by flagellar motility (intrinsic motility migration hypothesis). Bacterial motility apparently requires a continuous liquid film on the hyphae, because movements do not occur on hydrophobic hyphae. Migration via fungal hyphae in soil in microcosm experiments containing *Lyophyllum* sp. only occurred when bacteria were introduced at the fungal growth front and proceeded only in the direction of hyphal growth (Warmink and van Elsas, 2009). All single-strain migrators were equipped with type III secretion systems (TTSSs). Their colonization on the hyphae may also involve biofilm formation as a second mechanism for migration at the fungal tip beside motility, as was revealed by more detailed analyses of a *Burkholderia terrae* strain. An increase of TTSS-harboring bacteria has also been observed in the mycosphere of the ectomycorrhizal fungus *Laccaria proxima* by Warmink and van Elsas (2009) who suggested that TTSSs are involved in the interactions with fungi.

Bacteria as endohyphal associates

In the above-mentioned associations, the bacteria live externally of the fungal cells. However, endosymbiotic associations seem to be more common than previously thought. Intracellular bacteria are widespread in the Basidiomycota, Glomeromycota, and Zygomycota (e.g. Bertaux et al., 2003; Bianciotto et al., 2000; Lim et al., 2003; Partida-Martinez and Hertweck, 2005), but are so far rarely found in the Ascomycota. Intrahyphal endosymbionts have been reported only once from the truffle *Tuber borchii* in Ascomycota (Barbieri et al., 2000). A cautious distinction should be made between endobiosis and the intracellular occurrence of bacteria in ageing fungal cells.

The bacteria, which are involved in intracellular association with Basidiomycota are diverse and belong to different major groups (Table 1). Endosymbiotic bacteria also appear to be common in Sebaciales (Sharma et al., 2008), a phylogenetically basal order of Hymenomycetes (Basidiomycota). Sebaciales are widespread as mycorrhizal partners with the Ericaceae, Orchidaceae, as well as with mosses (Weiss

Table 1 – Bacteria as endocellular associates in hyphae of Basidiomycota

Bacterial group	Hosting fungus	Reference
<i>Paenibacillus</i> sp.	<i>Laccaria bicolor</i>	Bertaux et al., 2003, 2005
<i>Burkholderia cepacia</i> coll.	<i>Pleurotus ostreatus</i>	Yara et al., 2006
<i>Rhizobium radiobacter</i>	<i>Pyriiformospora indica</i>	Sharma et al., 2008
<i>Paenibacillus</i> sp., <i>Acinetobacter</i> sp., <i>Rhodococcus</i> sp.	<i>Sebacia vermiformis</i>	Sharma et al., 2008

et al., 2004). While single fungal isolates harbours only one particular bacterial strain, different isolated strains of the *Sebacina* species contain diverse and unrelated bacteria (Table 1). This raises further questions about the co-evolution and specificity of these endosymbioses, which need to be addressed in the future. *Rhizobium radiobacter* is also a producer of indole acetic acid, which could explain the growth-promoting effect for a wide range of plants by this fungus (Sirrenberg et al., 2007). Curiously, all attempts to cure *Piriformospora indica* from its *R. radiobacter* bacterial endobiont have failed as reported by Sharma et al. (2008). According to this work, antibiotics effective against cultures of the bacteria alone, failed to eliminate bacteria from cultures of *P. indica*, indicating that growth in the fungus shelters the bacteria from the effects of antibiotics.

The functional roles of the intracellular bacteria are still little understood. The presence of a *Burkholderia* strain in hyphae of a plant-pathogenic *Rhizopus microsporus* (Partida-Martinez and Hertweck, 2005) is an exception. This bacterium produces rhizoxin, an antimetabolic toxin for defending the habitat and required for accessing nutrients from the decaying plant host. Self-resistance of *Rhizopus* is mediated by specific mutations in the beta-tubulin gene (Schmitt et al., 2008a). Fungi without the bacterium were incapable of sporulation, indicating that the bacterial endosymbiont produces factors that are essential for the fungal life cycle (Partida-Martinez et al., 2007a). This result shows that endosymbiotic associations can result in a complex ecological integration of fungi and bacteria. Rhizoxin is not the only bacterial product in Zygomycota. Partida-Martinez et al. (2007b) discovered that rhizonin, a hepatotoxic cyclopeptide and the first mycotoxin isolated from this fungal group, is also produced by an endotrophic *Burkholderia* strain. Lackner et al. (2009) found strains of *Burkholderia rhizoxinica* (producing rhizoxin) and *Burkholderia endofungorum* (producing rhizonin) to occur in highly diverse ecological niches from different geographic origins. Interestingly, that study provided also preliminary evidence for a mutational bias toward high AT contents in the analysed genes of these strains, as found in other endosymbiotic bacteria.

The endosymbiotic associations with Basidiomycota and Zygomycota are characterized by low abundances of bacteria in fungal hyphae. In contrast, Glomeromycota can harbour a large number of bacterial endobionts in their spores (up to 20 000 individuals per fungal spore). Originally characterized as *Burkholderia* strains, present in *Gigaspora* and *Scutellospora* (Bianciotto et al., 2000), were subsequently assigned to 'Candidate Glomeribacter gigasporarum' (Bianciotto et al., 2003). With 1.35–2.35 Mb of small genome size in Betaproteobacteria (Jergeat et al., 2004), this endobiont was apparently prone to massive erosion of genetic material during the evolution of an obligately endobiontic life-style. Gene regulation and endobacterial division is in fact under influence of the fungus in mycorrhizal stage (Anca et al., 2009). Furthermore, ongoing genome-sequencing of this symbiont reveals large number of mobile elements and among other genes, those involved in type III secretion systems; interestingly, these are apparently up-regulated when the fungus is in the symbiotic stage (Bonfante and Anca, 2009).

Geosiphon pyriforme is a unique member of Glomeromycota, which forms a phototropic association characterized by

club-like bladders exposed on the soil surface. The huge vesiculate cells of *Geosiphon*, which can become 1–2 mm long, contain massive numbers of Nostoc-chains (Schüßler and Kluge, 2001), in contrast to lichens with extracellularly arranged photoautotrophs. Amidst the internal Nostoc community, Proteobacteria are also hosted in the *Geosiphon* coenocytes. They were originally termed bacteria-like organisms (BLOs; Schüßler et al., 1994), and later assigned to the *Oxalobacter* group of Betaproteobacteria (Volz, 2004).

Bacteria on complex fungal structures

The most complex and diverse structures developed by free-living fungi are their fruitbodies. The exposed structures, more or less long-living and rich in carbohydrates represent a specific habitat for bacteria. Isolation of bacteria from fruitbodies has been reported early from Lycoperdaceae (Swartz, 1929), and blotch diseases were since long recognized as bacterial infections of cultured mushrooms. On *Agaricus* and *Pleurotus*, blotch diseases are caused by *Pseudomonas tolaasii* (Soler-Rivas 1999). This species produces the toxic lipodepsipeptide tolaasin in the fruitbody infections, which causes membrane disruption by pore-formation or biosurfactant activity. The bacterial interaction with *Pleurotus eryngii* was visualized with a *gfp*-tagged *Pseudomonas tolaasii* strain (Russo et al., 2003). This highly specific strain (which does not infect the related *P. ostreatus*) forms an almost continuous layer of tightly aggregated cells around the hyphae. Toxic effects of the bacterial strain were evident by the swollen and vacuolated hyphal tips of the host, and the brown blotch symptoms appeared only when bacterial abundance exceeded 10^4 cfu g⁻¹ d.w. Non-pseudomonads which detoxify tolaasin were identified by isolation techniques from fruitbodies of various wild mushrooms (Tsukamoto et al., 2002). The detoxifying bacteria are also attached to the surface of mycelia. They might be of potential use as biological control agents as they can suppress blotch development in cultured fungi.

In contrast to pathogenic roles, fruitbody-associated bacteria also show beneficial effects. Acetylene-reduction assays suggested nitrogen-fixing activities in fruitbodies (Larsen et al., 1978), and nitrogen-fixing *Azospirillum* was isolated from basidiocarps (Li and Castellano, 1987). Some fluorescent pseudomonads isolated from fruitbodies stimulated mycorrhiza formation (Garbaye et al., 1990; see also above). Pseudomonads were also repeatedly detected during attempts to culture *Cantharellus cibarius* (Danell et al., 1993). More recently, culture-independent and culture-based methods were used to study bacterial diversity in truffles (Barbieri et al., 2007). Clone library sequencing revealed dominance of Alphaproteobacteria (c. 82 % of screened clones), which represented *Sinorhizobium*, *Rhizobium* and *Bradyrhizobium* spp., whereas the culturable fraction comprised mostly Gammaproteobacteria (c. 55 % of cultured strains, most of which are fluorescent pseudomonads). Predominance of the Alphaproteobacteria was confirmed by fluorescence *in situ* hybridization. The difference between the culturable culture-independent fractions is striking and demonstrates that culture conditions are inadequate to mimic the specific conditions required by naturally dominating strains in fruitbody structures.

Apart from mushroom fruitbodies, the most complex and long-living morphologies of fungal structures are found in lichen symbioses. These will be specifically focused on in the following sections.

3. Bacteria and lichens

Following [Hawksworth and Honegger \(1994\)](#), a lichen organism can be characterized as an ecologically obligate, stable mutualism between an exhabitant fungal partner (the mycobiont) and an inhabitant population of extracellularly located unicellular or filamentous algal or cyanobacterial cells (the photobiont). This definition excludes the special endosymbiosis of *G. pyriforme* and also fungi which parasitize macroalgae. Some biologically unclear cases of the latter are termed borderline lichens.

Of all fungal symbiotic relationships, the lichen association is a rather particular case. While most fungal symbioses hide in the substrate (with sometimes rather extensive mycelial networks), lichens usually expose themselves on the surface where they form compact vegetative structures, the lichen thalli ([Grube and Hawksworth, 2007](#)). The emergence of the lichen habit coincides with a substantial primary evolutionary radiation of ascomycetous fungi ([Lutzoni et al., 2001](#)). As “joint venture” both partners develop complex and exposed structures under environmental situations that would usually not be favourable for them in biological solitude. Even under rather hostile circumstances, the composite organisms can reach ages of thousands of years ([Denton and Karlén, 1973](#)).

Lichen symbioses as bacterial hosts

Many lichens are extremely desiccation tolerant and can survive in cryptobiosis for months to many years. Some species can endure extreme stress conditions in a dry state, including exposure to outer space or rinsing with acetone. Others survive extremely high temperatures (90 °C) or freezing in liquid nitrogen (−196 °C) in laboratory experiments. Yet, lichens can quickly resume metabolic activity after rewetting. High UV-exposition is effectively tolerated by lichens, primarily due to their diverse secondary metabolites, which act as UV filters. Beside the externally visible crystallized and non-crystallized pigments that are deposited in the upper surface layers of the lichen thallus, colourless substances are common in parts of the thalli that are not light-exposed. Concomitant production of several related and unrelated compounds is commonly observed in morphologically and functionally different strata of the thallus structure. Differences in chemistry and structure in lichens create a great diversity of ecologically different and long-living ecological niches for additional microorganisms. The antibiotic potential of lichen compounds has been known for many years (e.g., [Burkholder et al., 1944](#); [Francolini et al., 2004](#); [Boustie and Grube, 2005](#)). It has been suggested that secondary metabolites prevent degradation of lichen thalli by fungal, bacterial or other organisms. Nevertheless, lichens host diverse other fungi, both phenotypically conspicuous as well as poorly classified epi- and endobionts (e.g., [Lawrey and Diederich, 2003](#); [Petrini et al., 1990](#); [Prillinger et al., 1997](#)).

They comprise highly specific commensals to more pathogenic species. Yet, despite their slow growth, lichens are rarely eradicated by other fungi. Growth of the parasites may require tolerance to lichen compounds or prior breakdown of the lichen’s chemical defence ([Lawrey, 2000](#)). Similar to most lichenicolous fungi, there seem to be also no massive bacterial infections. Numerous reports on *in vitro* antibacterial effects of lichen compounds or extracts suggest that bacterial growth is under control in lichens (e.g. [Ingolfssdottir et al., 1998](#); [Francolini et al., 2004](#); [Boustie and Grube, 2005](#)). However, molecular baseline information about bacterial colonization and abundance in lichen symbioses emerged only rather recently.

The earliest reports about non-cyanobacterial prokaryotes in lichens were contradictory, and suspected to be a misinterpretation of crystallized secondary compounds ([Uphof, 1925](#); [Suessenguth, 1926](#)). Clear evidence for the presence of bacteria in lichens was then provided by a series of papers that appeared long before the emergence of molecular methods. These papers reported on various bacterial genera found in lichens (e.g., *Azotobacter*: [Henkel and Yuzhakova, 1936](#); [Iskina, 1938](#). *Pseudomonas*: [Panosyan and Nikogosyan, 1966](#). *Beijerinckia*: [Henkel and Plotnikova, 1973](#). *Clostridium*: [Iskina, 1938](#) or *Bacillus*: [Panosyan and Nikogosyan, 1966](#)). The isolation of strains classified as *Azotobacter* led to speculation of a nitrogen-fixing role for lichens, and the subsequent detection of Actinobacteria suggested a possible defensive role in certain lichens ([Zook, 1983](#)). All these early investigations of bacteria relied on both cultivation-dependent and phenotypic determination methods. Thus a precise affiliation of the isolated strains was not available, nor was there any indication about the abundance in natural lichen.

Bacterial diversity and location in lichens

Recently, molecular methods have been applied to study lichen-associated bacteria. [Cardinale et al. \(2006\)](#) analysed the bacterial communities in eight lichen species from montaneous habitats of temperate latitudes. Bacterial isolates were purified and pooled into 25 phylotypes after analysis of the ribosomal internal transcribed spacer (RISA) polymorphism. Sequence analysis of 16S rDNA genes revealed presence of Firmicutes (3 genera), Actinobacteria (4 genera), and Proteobacteria (3 genera). Two phylotypes of Actinobacteria and Proteobacteria could not be identified to genus level. Differences in the structure of the culturable bacterial communities were observed between the lichen samples, though some bacterial taxa were retrieved from different lichen species sampled in the same or different sites, possibly representing general lichenophiles. Strains of *Paenibacillus* (related to the species *P. pabuli* and *P. amyloliticus*) and *Burkholderia* (related to *B. sordidicola*) were especially common constituents of the culturable fraction. These genera were also involved in bacterial associations of non-lichenized fungi ([Bertaux et al., 2003](#); [Bianciotto et al., 2000](#); [Lim et al., 2003](#); [Partida-Martinez and Hertweck, 2005](#); [Yara et al., 2006](#)). *Burkholderia* was so far not present in the culturable fraction of Antarctic lichens, which revealed a number of psychrotolerant strains, including a possibly new species of *Deinococcus* ([Selbmann et al., in press](#)). Also several other strains could not be clearly assigned

to known species or genera within main bacterial lineages, indicating a number of potentially new taxa yet to be described.

Liba *et al.* (2006) used enrichment selection with nitrogen-free minimal medium to find acetylene-reduction positive strains of bacteria. These were isolated from 5 foliose species of lichens from Atlantic rain forest in Brazil. Dot-blot detection of *nifH* genes in strains confirmed the nitrogen-fixation capacity of these strains. All 17 strains belonged to different genera of Gammaproteobacteria: *Acinetobacter*, *Pantoea*, *Pseudomonas*, *Serratia*, and *Stenotrophomonas*. Fourteen strains were further analysed for other functions: all of them excreted amino acids and indole acetic acid (IAA), eight solubilized phosphate and four released ethylene. All *Stenotrophomonas* strains released both ethylene and IAA. *Stenotrophomonas* contains typically plant-associated species and also was isolated from extraordinary plant-associated habitats such as root nodules of herbaceous legumes (Kan *et al.*, 2007). Similar to the other detected bacteria from lichens, *Stenotrophomonas* seems to have a low substrate specificity, and is also present in associations with other, ecologically different fungi, including the plant-pathogenic *Fusarium oxysporum* (Minerdi *et al.*, 2008), and spores of arbuscular mycorrhizal fungi (Bharadwaj *et al.*, 2008).

Gonzales *et al.* (2005) used a selective culture medium to isolate actinomycetous populations in lichens collected in tropical and cold areas (Hawaii and Réunion vs Alaska). Unfortunately, it was not stated which lichen species were investigated. The number of bacterial isolates ranged from 1 to 45 strains per sample. Generally a lower number of strains (in total 22) were isolated from cold habitats than from tropical ones (in total 315). The most abundant genera were *Micromonospora* and *Streptomyces*. The diversity of the microbial population was further evaluated using fatty acid analysis and tRNA fingerprinting. A PCR approach to screen the isolates for genes associated with secondary metabolite production (targeting non-ribosomal peptide synthetases, type I and II polyketide synthetases, aminoglycoside phosphotransferases, and 3-hydroxy-3-methylglutaryl coenzyme A reductases) was applied to evaluate the biosynthetic potential of culturable Actinobacteria. The isolate-specific profiles were compared to the antimicrobial activity exhibited by these isolates in agar diffusion tests against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. The results indicate considerable potential of metabolite biosynthesis: 62.6 % of the tested 337 strains were positive for PKS-I, 64.7 % for PKS-II and 58.5 % for NRPS, while the other targeted genes were present at lower frequencies. The highest detected rates were found with the Pseudonocardiaceae. Half of the isolated strains showed antimicrobial activities, with activity against gram-positive *S. aureus* occurring at highest frequency (23 %). In agreement with the high biosynthetic potential, Pseudonocardiaceae was the group with highest antimicrobial potential.

Isolation of strains from lichens will be interesting for the description of new bacterial species or for discovery of bioactive products (Li *et al.*, 2007; Davies *et al.*, 2005; Williams *et al.*, 2008), but the culturable fraction generally represents only a minor component of the total bacterial diversity in a sample. We assume that specific organismic habitats such as lichens also host a large number of strains which require the natural

host organism for growth. One of several methods to uncover the unculturable bacteria is microbial fingerprinting, by which PCR products with distinct sequence composition are separated to yield characteristic banding patterns (fingerprints) (Smalla, 2004). The advantages of fingerprint methods are the comparatively low costs, little time consumption and the possibility to directly compare patterns from the gel images. The choice of primers (universal or group-specific) defines a taxonomic window to the bacterial community (Fig. 1). Individual bands of interest – either dominant, ubiquitous, or unique bands – can then be cut out and sequence-characterized. Because the bands are rather short, in the range of a few hundred base pairs, a thorough phylogenetic inference is not possible with fingerprint fragments. For this purpose, the analysis of 16S rDNA clone libraries is the common alternative. High throughput ultra-deep sequencing, now in the reach, has the advantage of producing a very high number of sequences in one run, which can also reflect a statistical representation of strain frequency. In a recent study we amplified bacterial 16S rRNA genes from lichens with group-specific primers. Using total DNA extracts we amplified the Alphaproteobacterial fraction from the reindeer lichen *Cladonia arbuscula*, sequences in the product were separated by a single-strand conformation polymorphism (SSCP) approach. Sequencing of excised bands revealed that Acetobacteraceae are the dominant Alphaproteobacteria in this species, which occurs on nutrient-poor acidic soils (Cardinale *et al.*, 2008).

The abundance and specific location of bacteria in the symbiotic structures of the host has been established by fluorescent *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM, Fig. 2). Using an optimized protocol for cryosections of small lichen fragments, Cardinale *et al.* (2008) found about 6×10^7 bacteria per gram of *C. arbuscula* thallus (Fig. 2A,B). Approximately 86 % of acridine orange-stained cells were also stained by the universal FISH probe EUB338. Using group-specific FISH probes, we detected a dominance of Alphaproteobacteria (more than 60 % of all bacteria), while the abundance of Actinobacteria and Betaproteobacteria was much lower (<10 %, Fig. 2D,E). Members of these groups form small colonies that grow adjacent or are mixed with larger colonies of Alphaproteobacteria. Firmicutes were rarely detected, and no Gammaproteobacteria were present. Phenotypically different bacterial colonies are concentrated in a biofilm-like, continuous layer on the surface lining the central cavity in *C. arbuscula* podetia, mainly occurring in small colonies of a few to a few hundred cells. Similar patterns with the dominance of Alphaproteobacteria are also observed in other lichen species, including *Cetraria islandica* (Fig. 2F).

In previous TEM studies, lichen-associated bacteria were only sporadically found in lichens (e.g., de los Rios *et al.*, 2005; Meier and Chapman, 1983; Souza-Egipsy *et al.*, 2002). With the FISH approach we could show that bacteria are actually much more common in lichens than previously thought. We also observed that bacteria can potentially penetrate the fungal cell wall (Cardinale *et al.*, 2008), but observation of endocellular biotrophy has not yet been achieved. The bacterial fingerprints obtained from the three lichen species from the same habitat were found to be species-specific by Grube *et al.* (2009). In this work, functional characterization of the culturable fraction revealed chitinolytic, glucanolytic, and

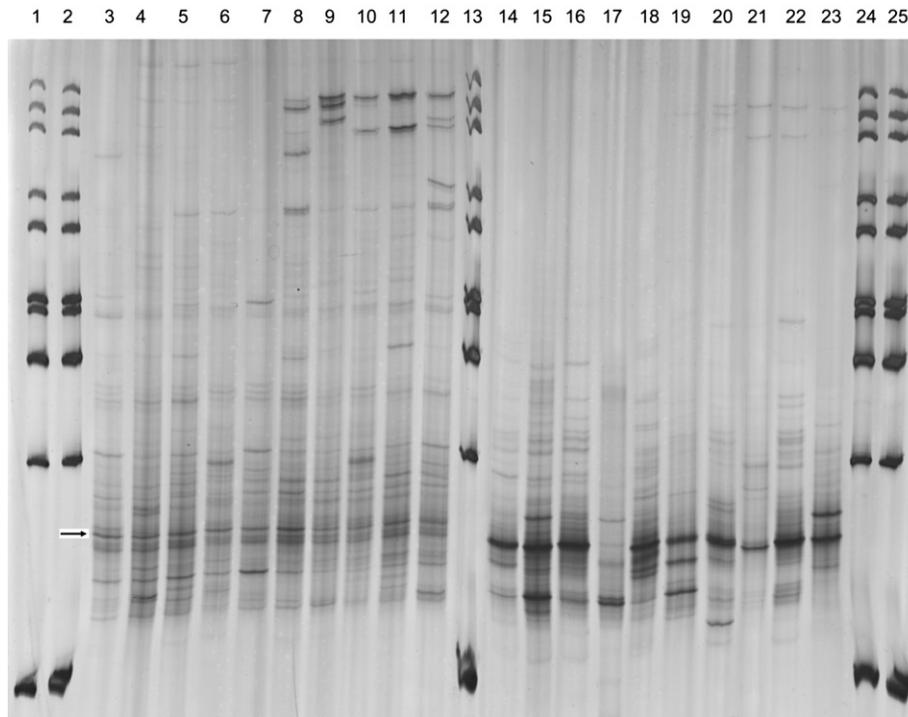


Fig. 1 – SSCP analysis of bacterial communities in the lichens *Lobaria pulmonaria* (Biogradska Gora, Montenegro, 2009, coll. P. Bilovitz). Lanes 1, 2, 13, 24, 25: marker. Lanes 3–12: SSCP bands produced with universal bacterial primers (with an arrow pointing to the band of *Nostoc* sequence of cephalodia). Lanes 14–23: bands produced with a *Pseudomonas* specific primer pair.

proteolytic activities, as well as hormone production, phosphate mobilization, and antagonistic activity towards other microorganisms. Besides these results from functional assays, *nifH* genes were detected in the culture-independent fractions. These data suggest several functional roles of associated bacteria in the lichen symbiosis

Functional roles of bacteria in lichen symbioses

In addition to the well established knowledge about the fungal–algal interaction in the lichen symbiosis, we think that bacteria contribute to a complex symbiotic network with multiple functions (Fig. 2G): The lichen photobiont provides carbohydrates to the mycobiont, which develops the morphological scaffold of the entire symbiotic system and offers niches for establishment of bacteriobiont communities. In the case of cyanobacterial photobionts in lichen thalli, which can also occur in tripartite associations (i.e. together with green-algal photobionts), there is an obvious sharing of bacteriobiont (N-fixation) and photobiont (C-fixation) functions. In the following, however, we want to concentrate on possible functions of other, non-cyanobacterial prokaryotes.

Nitrogen-fixing strains play a role in delivering nitrogen to the symbiotic partners. This could involve both bacteria living inside the thalli, or those in parts of the substrate influenced or accessed by mycobiont hyphae (which we call the “hypothallosphere”). Release of nitrogenated compounds such as amino acids, could improve the nitrogen budget of lichens.

This may be particularly important in lichens without contacts to nitrogen-delivering cyanobacteria, which otherwise need to rely on nitrogenated compounds from the substrate or from external sources (air, water, bird manure, pollution). Ellis *et al.* (2004) found no support for efficient uptake of soil organic N by mat-forming lichens. Thus, in the case of N-limiting conditions, bacterial N-fixation could be of considerable importance for the vitality of lichens. Recent experiments showed increased solubilisation of mineral substrate in co-cultures of an isolated lichen fungus with N-fixing *Bradyrhizobium elkanii* (Seneviratne and Indrasena, 2006). Nitrogenases are ubiquitous in Alphaproteobacteria, but nitrogen fixation could also be accomplished by fungal-associated bacteria of other groups, notably Betaproteobacteria (Leveau and Preston, 2008). Nitrogen-fixing systems are also known from other bacterial groups involved in symbiotic relations (Kneip *et al.*, 2007), and can also include alternative nitrogenase systems (Zhao *et al.*, 2006). Growth of isolated strains on N-free media (Cardinale *et al.*, 2006; Grube *et al.*, 2009) and the results from acetylene-reduction assays by Liba *et al.* (2006) provided support for nitrogen fixation being undergone by lichen-associated bacteria. It is likely that the highly regulated nitrogenase systems require special microclimatic conditions or severely depleted nitrogen levels for their function. This could be a reason for contrasting earlier reports about the N-fixing role of bacteria (Caldwell *et al.*, 1979).

Bacteria can be involved in defence against lichen pathogens and feeders. Antimicrobial properties and biosynthetic potential of Actinobacteria, which seem to be common as

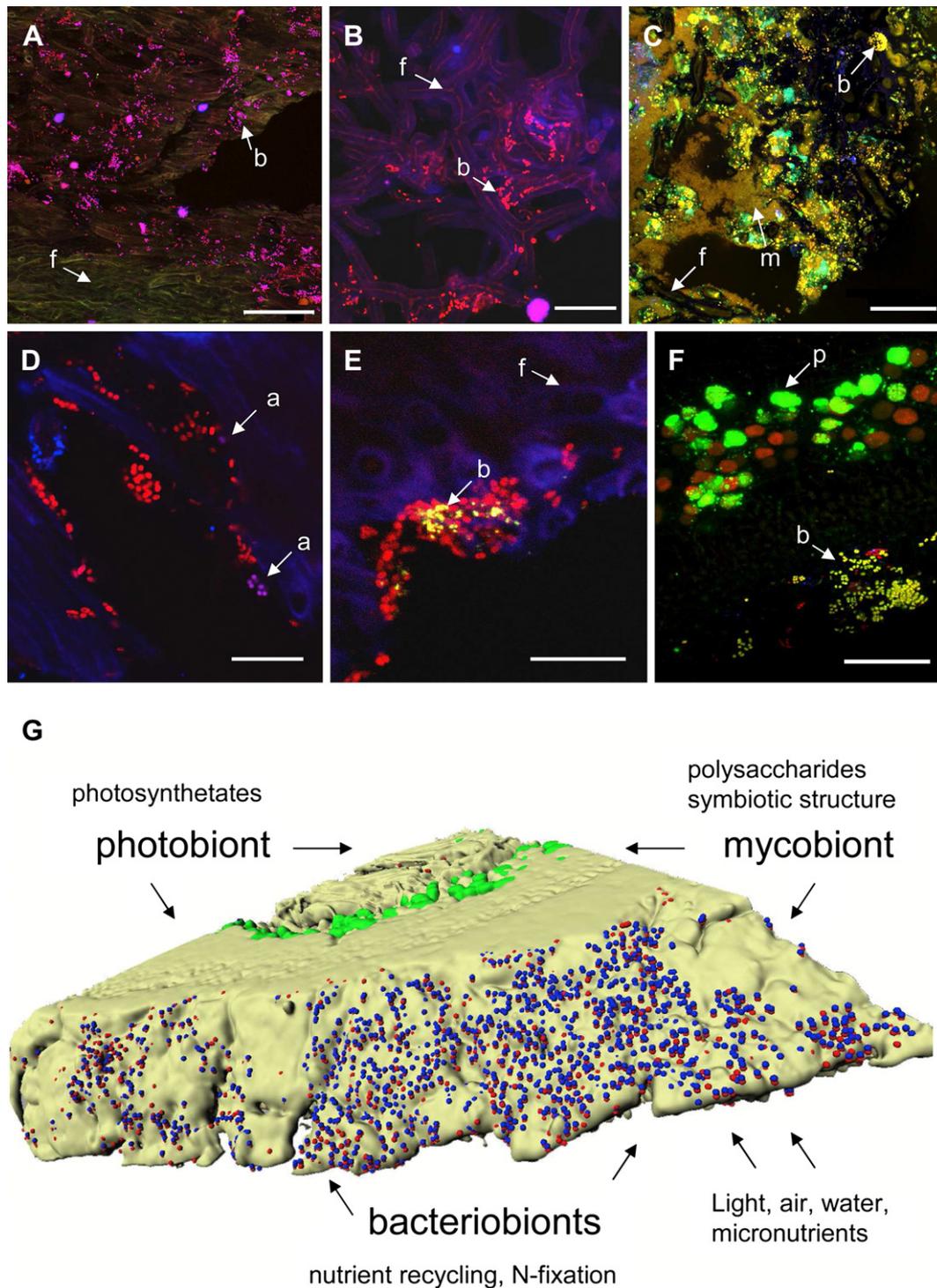


Fig. 2 – Bacterial colonization of lichens. (A–F), CLSM images. (G), 3D-reconstruction of CLSM image. (A), FISH-stained bacteria on internal podetia surface of *Cladonia arbuscula* (f, fungal plectenchyma; b, bacterial colonies); Bar = 50 μm . (B), FISH-stained bacteria (b) on hyphae (f) of the external surface of *Cladonia arbuscula*; Bar = 30 μm . (C), abundance of bacteria (yellow spots) in the hypothallosphere below the soil crust lichen *Lecidoma demissum* (fungal hyphae, f; unidentified soil matrix, m; bacterial colonies, b); Acridine orange staining; Bar = 30 μm . (D), section showing lilac actinobacterial colonies (a) neighbouring other eubacteria in *Cladonia arbuscula*; Bar = 10 μm . (E), section with yellow betaproteobacterial colony (b) amidst other eubacteria; Bar = 10 μm . (F), *Cetraria islandica*, cross section of thallus showing dominance of Alphaproteobacteria (b) on the thallus surface, while the algal layer (p) is bacteria-free; Bar = 30 μm . (G), Colonization of bacteria on a thallus fragment of *Lobaria pulmonaria*, with general schematic outline of functional contributions in lichen symbioses. More explanation in the text.

lichen-associates, could be of particular importance in this respect. Products of some lichen-associated bacteria were shown to be potent antibiotics at very low concentrations (Davies et al., 2005), which also suggests that low-abundance strains could play significant functional roles in the lichen micro-ecosystem.

Bacteria can degrade parts of lichen thalli to facilitate biomass mobilisation. Assays for lytic properties of isolated strains show a considerable potential for glucanolytic, cellulolytic, and proteolytic activity (unpublished results). As fungal cell walls are complex mixtures of polysaccharides and proteins, these strains can be involved in the degradation of senescing lichen parts, where antibacterial lichen compounds are no longer produced. Degraded lichen material can be remobilized in the thallus structures to promote growth in other, vital parts of the lichens. Experimental studies demonstrated the translocation of fixed nitrogen compounds from the base to the tip in mat-forming reindeer lichens, which lack a substrate-exploiting mycelial system (Ellis et al., 2005). Nutrient mobilization may also be promoted by phosphate-solubilizing activity of bacteria.

Bacteria can influence the growth by producing hormones. The production of indole acetic acid (IAA) (Liba et al., 2006; Grube et al., 2009), ethylene, and possibly other compounds potentially acting as signalling molecules could alter morphogenetic processes in the holobiont symbiotic community. *In vitro* re-synthesis of lichens using separately cultured fungal and algal symbionts is notoriously difficult to achieve, and often does not complete the original morphology (e.g. Ahmadian, 1993). As bacteria have been noticed on the native lichens used for such experiments, we are now proceeding with re-syntheses including lichen-associated bacteria (Grube and Stocker in prep.) to test morphogenetic modulation by bacteria. IAA could influence both the fungal and algal partner: varied effects were found previously on fungal growth (e.g. Řeřábek, 1970; Nakamura et al., 1985), and induction of cell division was found in unicellular algae, although signalling pathways known from plants are not present in microalgae (Lau et al., 2009).

Further functions could be envisioned: A study of phenolic compound production using lichen mycobionts and associated bacteria (co-immobilized in polyhydroxyurethane spheres) suggested the possibility that lichen cells might use bacterial cofactors for compound production (Blanch et al., 2001). It is also possible that development of lichen primordia is supported by bacterially produced extracellular polymeric matrices, and bacterial communities are thought to play a role in conditioning the development of saxicolous lichens (de los Ríos et al., 2002).

We have so far considered the effects of bacteria on the hosting lichen, but lichens are a selective habitat for bacteria recruitment and diversification. As lichens comprise a wide variety of hyphal textures that are sometimes impregnated with crystallized bioactive compounds, bacteria are faced with unique constraints. We expect that lichens, which are producing substantial amounts of acidic secondary metabolites, have significantly different (acidotolerant) bacterial communities than species without such compounds. Bacteria are so far observed in low numbers around living algae in lichens, perhaps because algal cells are often coated with

a hydrophobic layer of self-aggregating proteins secreted by the fungal partner (Scherrer et al., 2000). In contrast, hydrophilic parts of the thallus are richly colonized by bacteria. These parts are usually characterized by conglutinated fungal hyphae, which may form massive amounts of extracellular polysaccharides. Embedding of bacteria in polymeric sugars could also promote the growth of anaerobic bacteria. *Eubacterium rangiferina*, an usnic acid-resistant bacterium isolated from reindeer rumen, is able to actively colonize lichen particles of *Stereocaulon paschale* (without usnic acid) and the usnic acid containing *Cladonia stellaris* in liquid co-culture (Sundset et al., 2008). Further investigations will reveal whether *E. rangiferina* can be detected in natural lichens and whether lichen feeders might directly recruit gut bacteria from their food source. It has earlier also been discussed whether bacteria could benefit from certain phenolic substances leaching from lichens, but with inconclusive results (Stark and Hyvärinen, 2003). These hypotheses require further investigation and also need to consider potential antibiotic properties of many compounds.

Lichens can produce layers of decaying or dead material that comprise remnants of cell walls of fungi, algae or both. Such parts, known as epinecral layers, are present as protective structures at the upper surfaces of many crustose lichens. The epinecral layers are often colonized by other fungi and bacteria, which grow more or less saprotrophically in these gelatinized matrices (unpublished observations). Colonization of these materials adds yet another strategy of bacterial mycophagy to the ones sketched by Leveau and Preston (2008). We suggest the term episaprotrophy for this case. Different to the other bacterial strategies, episaprotrophy on gelatinized fungal and/or algal cell wall material does not depend on the physiological states of the living eukaryotic partners. Bacteria can therefore also be active when the fungal-algal interaction performs suboptimally, e.g. during phases of water oversaturation. Synchronization of metabolic activities of bacteria with other lichen biotics is thus not strictly necessary. The system may also tolerate to some extent differences in humidity preferences of the bacterial symbionts.

Bacteria are also commonly observed on decaying lichen material or in the lichen-substrate interface (e.g., Asta et al., 2001), where bacteria may benefit from extracellular compounds of lichens and/or compounds available in the substrate. Of special interest are also lichen-dominated soil crusts, which seem to have an extremely abundant bacterial community below the lichen crust (Fig. 2C). This highly complex bacterial niche does not only comprise the substrate hyphae of the lichen, but certainly other organisms as well (Muggia and Grube in prep.).

4. Outlook

The discovery of potential bacterial functions in the lichen holobiont is paralleled by similar results from other symbiotic systems. Sponges and corals, but also invertebrates, now turn out to be complex symbioses of several to many partners (Hunter, 2006). Clearly, the diverse niches for bacterial growth corroborate the view of lichens as self-contained ecosystems

(Farrar, 1985). Further aspects of these unique associations now need to be studied. Many lichen species have extremely wide geographic distributions. The investigation of biogeographic patterns of the associated bacterial communities will contribute to the discussion of ubiquity of bacteria (by addressing the “everything is everywhere” hypothesis).

With a dominance of Alphaproteobacteria, the general species spectrum of lichen-associated bacteria recalls bacterial communities of plant surfaces. Typically plant-endophytic species are also known from fungal habitats, such as species of *Pseudomonas*, *Paenibacillus*, *Stenotrophomonas* and *Burkholderia* (Berg and Hallmann, 2006). A further parallel aspect is that strains of these genera from lichens are closely related to opportunistic human pathogens. Similar to the plant rhizosphere (Berg et al., 2005), lichens could represent a niche for diversification of bacteria, some of which could eventually evolve to become opportunistic human pathogens. Bacterial diversification could be accelerated in dense biofilm-like structures (Kirkelund Hansen et al., 2007), which are common in lichens. Because lichens tolerate extreme abiotic stressors (including extreme climates, salt, etc.) and accumulate toxic compounds (heavy metals, radionuclides, etc.), we are convinced that they may turn out as ‘treasure chests’ of bacterial diversity and as sources of biotechnologically interesting strains, compounds and enzymes (González et al., 2005; Davies et al., 2005; Williams et al., 2008).

We think that associations of lichens with bacterial communities are as old as the lichen symbioses. Whether or not bacteria contributed to the diversification of lichen fungi cannot be proved, but it is possible that adaptation of lichen populations to new habitats is accompanied by shifts in bacterial communities, similar to observed photobiont switches correlating with different ecological conditions (Blaha et al., 2006). Bacterial community shifts also likely lead to slight changes in interaction patterns which may further enforce segregation of mycobiont populations. The already available studies indicate that bacterial community structures in lichens differ among climatic regions. Further, as lichens have exposed morphologies, we also think that rainfall could be an important force in shaping community structures, whereby only strains that evolved to firmly attach to the fungal surfaces of their hosts have a selective advantage.

A long history of interactions with bacteria could also have left direct traces in fungal genomes. While the substantial proteobacterial contribution to the yeast genome (e.g., Esser et al., 2004; Hall et al., 2005) might derive from events that predate fungal diversification, it could be possible that horizontal gene transfer (HGT) from bacteria to fungi occurred at any time in the evolutionary history. An excreted phospholipase involved in *Tuber*-plant root recognition (Miozzi et al., 2005) is strikingly similar to homologous proteins from soil bacteria (*Streptomyces*, unpublished observation), and Garcia-Vallve et al. (2000) suggested that HGT of the glycosyl hydrolases from bacteria to rumen fungi enabled the latter to degrade cellulose and other plant polysaccharides in their ecological niche. Occasional HGT events were detected in ascomycetous fungi also by Fitzpatrick et al. (2008). Yet, HGT is rare and can be hampered by differences in gene codon usage. Further screens of fungal genomes, especially of those groups with endocellular bacterial associations, will provide challenges

to further address the role of horizontal gene uptake in fungi and its role for ecological diversification. During the suggested 600 million years of lichen evolution (Yuan et al., 2005), bacteria might well have contributed to genetic material for lichen fungi. Schmitt et al. (2008b) found that a group of PKS genes obtained from axenic cultures of lichen mycobionts is similar to bacterial 6-MSAS type genes. This suggests the possibility that typical lichen compound production, i.e. orsellinic acid derivatives, could have its origin in a horizontal transfer of bacterial polyketide synthase genes from an actinobacterial source to the mycobiont genome (Schmitt and Lumbsch, 2009; for an alternative orsellinic acid synthase see Schroeckh et al., 2009). The forthcoming genomes of lichen-forming fungi will likely reveal further cases of HGT from bacteria to fungi. Horizontal transfer of genetic material may have also affected the algal partners of lichens. In a recent study, del Campo et al. (2009) showed that group I introns at three positions contain homing endonuclease signatures and are closely similar to introns located at homologous insertion sites in bacterial rDNA genes.

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