# 9 Lichen–Bacterial Interactions

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## I. Introduction

Lichen symbioses are composed of a dominating fungal partner, which hosts green algal and/or cyanobacterial photosynthetic partners. The algal partners provide fixed carbon as the main energy source to build up the holobiont structure. The formation of a distinct symbiotic phenotype, also known as "lichen thallus," is so unique in the fungal kingdom that it was long considered a separate organismal group. The light-exposed lichen thallus represents a self-sustaining structure, which generally grows as slow rate, and usually without a predetermined lifetime. Unless the preferred ecological settings change, it can therefore persist for many years and occasionally even reach ages of several 1000 years. Periodic desiccation in the natural habitat is survived by cryptobiosis. Lichens can therefore dominate the landscape in hostile habitats characterized by drought, high and low temperatures, and excessive light intensities, where higher plants are outcompeted.

With their long-persisting fungal structures, lichens provide a habitat for other microorganisms. About 1800 host-specific lichenicolous fungi are known by their phenotypes (Lawrey and Diederich 2003). Most of these are commensals or weak parasites, while the biological relations of other, inconspicuous eukaryotic associates are less known (Bates et al. 2012). In this chapter, however, we spend the focus on the associations of lichens with bacteria, which raised more scientific interest recently. We will not particularly discuss cyanobacteria, as these are well known as photosynthetic and nitrogen-fixing partners ("blue green algae") in estimated 10 % of lichenized fungi. In addition to green algal partners, some lichens ("tripartite" lichens) also host cyanobacteria in specialized organs ("cephalodia") for nitrogen fixation (Millbank and Kershaw 1969; Hyvärinen et al. 2002). Others, primarily crust-like lichens, grow preferentially in the vicinity of cyanobacterial mats ("cyanotrophy," Poelt and Mayrhofer 1988).

Lenova and Blum (1983) already suggested bacteria as a "third component" in lichens, but it was only research of the past 10 years which contributed substantial new information about their diversity and abundance of lichen-associated bacteria (Bates et al. 2011; Bjelland et al. 2011; Cardinale et al. 2006, 2008, 2012a, b; Grube et al. 2009, 2015; Grube and Berg 2009; Hodkinson and Lutzoni 2009; Hodkinson et al. 2012; Selbmann et al. 2010; Mushegian et al. 2011). According to these results, lichen thalli

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provide unique niches for bacteria, suggesting that bacteria are an integral component of lichen symbioses. The bacterial communities are host specific and reach densities of up to $10^{10}$ bacteria per gram of lichen dry weight. Moreover, they are able to form biofilm-like communities on the thallus surfaces (Grube et al. 2009; Cardinale et al. 2012b). The associations of lichens with bacteria seem to be very old, as they were demonstrated in fossilized lichens already from the lower Devonian period (Honegger et al. 2013).

The present data, which mainly come from lichens with green algal photobionts, reveal a predominance *of Alphaproteobacteria* as well as presence of other bacterial phyla, such as *Acidobacteria* or *Actinobacteria*. Many of these likely represent novel species (Selbmann et al. 2010; Lee et al. 2014; Sigurbjörnsdóttir et al. 2014), but only few of the lichen-associated strains were so far described as new taxa (Li et al. 2007; Lang et al. 2007; An et al. 2008, 2009; Hamada et al. 2012; Yamamura et al. 2011a, b; Cardinale et al. 2011). Since only a minor fraction of lichen-associated bacteria is culturable and because fungal symbionts grow too slow for efficient experimental resynthesis of bacterial associations, the analysis of interactions is a challenging topic. In the first section, we will therefore present an outline of the different methods that have so far been used to study these hardly culturable symbioses.

## II. Methodological Approaches

Lichen-associated bacteria have been studied using a range of techniques. Culture-dependent studies date back to the first half of the last century and were used to verify the presence of nitrogen-fixing bacteria (e.g., Henckel and Yuzhakova 1936; Iskina 1938). While Cardinale et al. (2006) confirmed the growth of isolated lichen-associated bacteria on N-free media, N-free enrichment media were used to selectively sample nitrogen-fixing bacteria from tropical green algal lichens (Liba et al. 2006). Molecular data in these studies already revealed that phylogenetically diverse bacteria colonize lichens and that the isolated bacteria also may contribute auxiliary functions to the symbiosis. Although the cultivable fraction in most of the cases represents a very low proportion of the whole microbiome, antagonistic bacteria are now well presented in the culture collections (Grube et al. 2009; Cernava et al. 2015). Altogether, 24.5 % of all isolates were shown to display antagonistic properties against plant and lichen pathogens in vitro. Cultured isolates are amenable to further experimental approaches, which may show their interaction with fungal hyphae in greater detail (Senevirratne and Indrasena 2006). Cultivation-independent methods, originally accomplished by DNA community fingerprints and clone libraries, were used to gain more inside in the diversity of the total bacterial community or of specific groups, such as *Actinobacteria*. For example, Gonzáles et al. (2005) showed differences of actinobacterial associations with lichens between tropical and cold climatic regions using fingerprinting methods.

Grube et al. (2009) provided first evidence for host specificity of bacterial communities. They compared DNA fingerprints of bacterial communities from three lichen species occurring in the same subalpine habitat. Host specificity was confirmed by subsequent studies, either using gene clone library sequencing or amplicon sequencing (Bjelland et al. 2011; Bates et al. 2012). Amplicon sequencing meanwhile became the gold standard to assess microbial community structure, and this technique also helped in exploration of ecological influence on bacterial community structure (Cardinale et al. 2012a, b; Grube et al. 2012).

In parallel with sequencing approaches, lichen-associated bacteria were also studied by advanced microscopic approaches. While previous analyses with light or electron microscopy revealed the presence of bacteria, it is only possible with fluorescence in situ hybridization (FISH) to study the structure of bacterial colonization in more detail. For this purpose, Cardinale et al. (2008) introduced a method to directly make all steps of FISH using fragments of lichens instead of fixed sections. It was then

possible to visualize the total abundance of bacteria on lichen surfaces and also to evaluate the relative abundance of certain bacterial lineages, by the use of specific DNA probes. Visualization usually requires a confocal laser scanning microscopy (CLSM), by which the blur of fluorescence signals is avoided (in comparison with epifluorescence wide field microscopy). Using sophisticated image analysis tools, it is also possible to create three-dimensional representations of the bacterial colonization (Fig. 9.1). Cardinale (2014) discussed technical challenges of FISH-CLSM and gives valuable comments on possible misinterpretation of results. Confocal laser scanning microscopy proved useful to assess the effect of thallus age and variation of habitat conditions on bacterial composition (Cardinale et al. 2012a) or to demonstrate the endohyphal occurrence of bacteria (Cardinale et al. 2008; Erlacher et al. 2015). FISH-CLSM can therefore be used to complement and validate results from sequencing approaches and to precisely localize interactions with the lichen host.

In the meantime, meta-omics methods have become popular to further explore lichen–

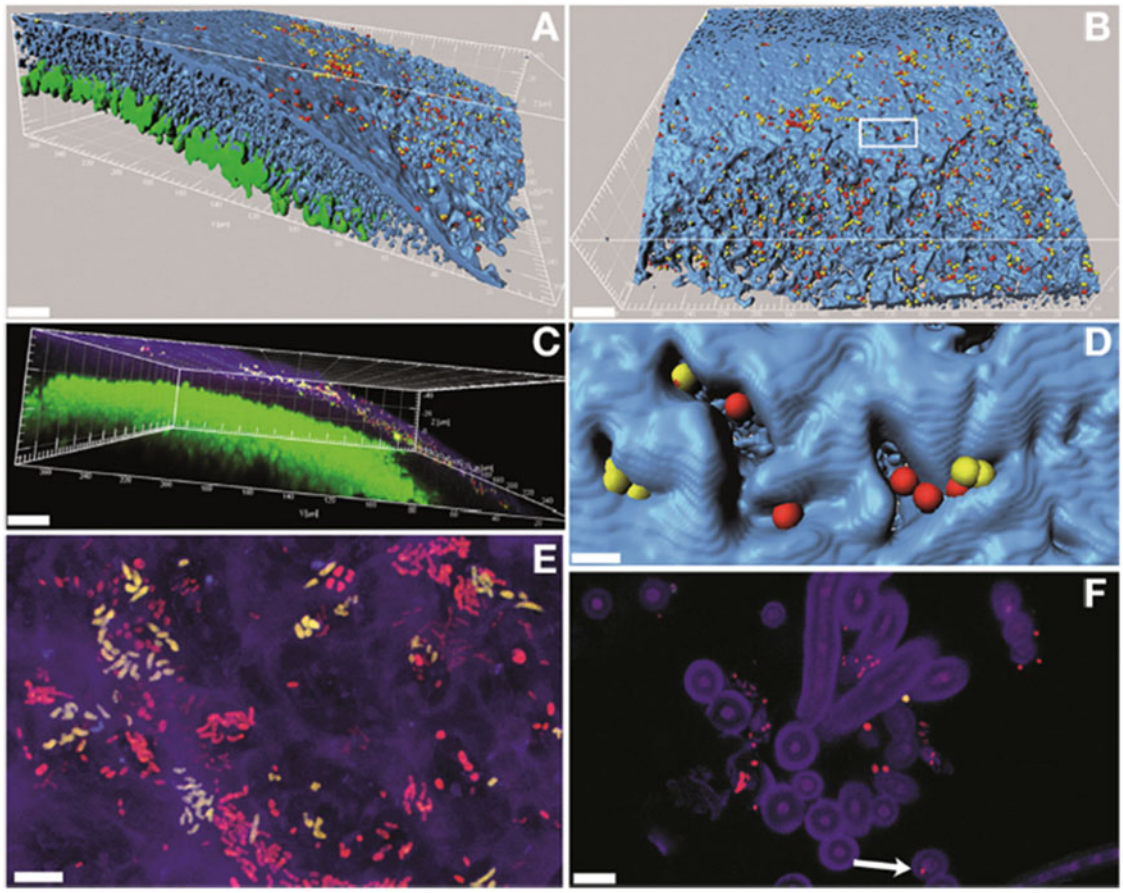


**Fig. 9.1** Confocal laser scanning microscopy (CLSM) of bacteria associated with *L. pulmonaria*. *Bacteria* are stained by fluorescence in situ hybridization (FISH). *Green*, algae; *blue/purple*, fungi; *yellow*, *Rhizobiales*; *red*, other bacteria. (**a**, **b**, **d**) Three-dimensional models made of isosurfaces and spheres; (**c**) volume rendering; (**e**) maximum projection; (**f**) single optical slice. In (**b**) *white box* indicates the region shown in (**d**). *Arrow* in (**f**) indicates an endophytic bacterial cell. Scale bars, (**a**–**c**) 30 μm, (**d**–**f**) 5 μm. (Reproduced from Erlacher et al. 2015; ©Frontiers Media S.A.)

bacterial interactions. By including the bacterial fraction, Schneider et al. (2011) provided a first environmental proteomics insight into the functional complexity of lichen holobionts. This work, conducted with the lung lichen *Lobaria pulmonaria*, was recently complemented by a metagenomic dataset (Grube et al. 2015). The comparison of metagenomic and metaproteomic data revealed that bacteria take part in several functions, which potentially contribute to the stability of the lichen symbioses. We will discuss the results of these studies in more detail further below.

One of the greater challenges is to understand the exchange and signaling of compounds between bacteria with their host thallus in the lichen symbiosis, which requires a refined and localized chemical analysis of metabolite patterns. As the primary metabolism dominates the overall happening, analyses need to be targeted for secondary metabolites. So far, most studies have focused on the fungal secondary metabolites with a few exceptions (Boustie et al. 2011), including recent spatial analyses of compound patterns (Le Pogam et al. 2015). A stable symbiotic association is most likely maintained by a set of regulatory compounds, which may represent a complex chemical signature of symbiotic relations, for which we here introduce the term symbiotic molecular patterns (SYMPs). So far SYMPs are rather a hypothetical concept than a revealed known set of compounds. SYMPs may comprise exudates from the lichen holobiont, small molecules, which might include volatiles and other secondary metabolites (the parvome according to Davies and Ryan 2012). We also suppose that the compositions of SYMPs differ among species of lichens.

We argue that symbioses in general are valuable objects for finding new regulatory compounds and mechanisms, which can be exploited for other purposes. Lichens, with their high phylogenetic age, might offer particularly rich biological resources in this respect. It is already well known that lichens host a tremendous richness of secondary metabolites produced by the fungal partner (Elix 2014), but recent results suggest that bacteria associated with lichens may also diverse with respect to their biosynthetic potential.

## III. Bioactive Secondary Metabolites from Lichen-Associated Bacteria

One of the most productive bacterial lineages regarding the biosynthesis of complex secondary metabolites is represented by *Actinobacteria*. Apparently, many *Actinobacteria* occurring in lichens are also culturable, and preliminary data also suggest that closely related strains may occur in the same host lichens (Cardinale, unpublished data). However, only very few lichen-associated *Actinobacteria* have been characterized in more detail so far, but already these data are very promising for more future studies (Suzuki et al. 2016). *Streptomyces uncialis*, isolated from reindeer lichen *Cladonia uncialis*, was shown to be a first potent species for finding new products. *Streptomyces uncialis* produces the enediyne uncialamycin (Davies et al. 2005), which shows strong antibacterial activity against human pathogens *Burkholderia cepacia* (MIC, 0.001 μg/ml) and *Staphylococcus aureus* (MIC, 0.0064 μg/ml), as well as strong cytotoxic activities against various cancer cell lines. Further investigation of that isolate revealed new alkaloids called cladoniamides A–G. Cladoniamide G showed significant in vitro cytotoxicity against human breast cancer MCF-7 cells (Williams et al. 2008). Two other *Streptomyces* species have been isolated from lichen species and produced novel cytotoxic compounds: chlorinated anthraquinonic angucyclines (Motohashi et al. 2010) and aminocoumarins structurally closed to novobiocin (Cheenpracha et al. 2010).

*Streptomyces cyanofuscatus* was isolated by Parrot et al. (2015) from coastal lichens and is also found in marine organisms. Surprisingly, the lichen-derived strain (MOLA1488) of this species is able to produce usnic acid (Parrot 2014), a dibenzofuran compound that was until now known to be produced only by lichen-forming fungi (but not by the lichen from which the strain was isolated). Other isolates of *S. cyanofuscatus* may have identical 16S rRNA genes but differ substantially in their biosynthetic properties. Considering the generally high conservation of 16S rRNA genes in *Actinobacteria*, these strains may either repre-

sent different species or their chemical variation is driven by other factors. In addition, one new compound, cyaneodimycin, an acrylate derivative, was isolated from the lichen-inhabiting *S. cyanofuscatus*. Six other known compounds were also characterized (diketopiperazines, actinomycin derivatives, and indole derivatives). Total EtOAc extract of *S. cyanofuscatus* showed antibacterial activities against *Staphylococcus epidermidis* and antiproliferative properties against B16 and HaCaT cell lines ($IC_{50}$ 0.33 μg/ml and 0.25 μg/ml, respectively). Moreover, cynomycin exhibited antiproliferative properties against Jurkat cell lines after 72 h of incubation with $IC_{50}$ value of 18.5 μM.

## IV. Omics Technologies as Indicators of Interactions

Grube et al. (2015) explored the metabolic potentials of the bacterial microbiome of the lung lichen *Lobaria pulmonaria*. Metagenomic and proteomic data were compared and visualized by Voronoi treemaps. The study was further complemented by molecular, microscopic, and physiological assays. It was found that more than 800 bacterial species grow on the lung lichen. This diverse collective may contribute multiple aspects to the symbiotic system, including essential functions such as (1) nutrient supply, especially nitrogen, phosphorous, and sulfur, (2) resistance against biotic stress factors (i.e., pathogen defense), (3) resistance against abiotic factors, (4) support of photosynthesis by provision of vitamin B12, (5) fungal and algal growth support by provision of hormones, (6) detoxification of metabolites, and (7) degradation of older parts of the lichen thallus. These findings showed the considerable potential of lichen-associated bacteria to interact with the fungal as well as algal partner to support health, growth, and fitness of their hosts.

Contributing to one third (32.2 %) of the overall bacterial community, *Rhizobiales* (*Alphaproteobacteria*) are the most common partners in the symbiosis of *Lobaria pulmonaria*, and most of the *Rhizobiales* belonged to the families Methylobacteriaceae, *Bradyrhizobiaceae*, and *Rhizobiaceae*. Erlacher et al. (2015) studied this order in more detail using the available metagenomic dataset. About 20 % of our metagenomic assignments could not be placed in any of the *Rhizobiales* lineages, which indicates the incomplete knowledge of this order. Focused on *Rhizobiales*, the SEED-based functional analysis revealed again functions supporting the symbiosis, including auxin and vitamin production, nitrogen fixation, and stress protection.

Similar results were found meanwhile also in other lichens, e.g., in an analysis of metagenomically derived 454 sequences from *Peltigera membranacea* after subtraction of sequences attributed to the primary fungal and cyanobacterial symbionts (Sigurbjörnsdóttir et al. 2015). The dominant groups in *P. membranacea* are *Proteobacteria* (*Alphaproteobacteria* 59 %, *Betaproteobacteria* 29 %), while *Actinobacteria* and *Bacteroidetes* represent minor fractions. This metagenomic data agrees largely with microscopic and amplicon sequencing studies (e.g., Cardinale et al. 2008; Mushegian et al. 2011; Hodkinson et al. 2012). Also *P. membranacea* hosts bacteria capable of synthesizing indole acetic acid, albeit in a small number according to BLASTX hits to indoleacetimide hydrolase (most similar to those from *Actinobacteria* and *Betaproteobacteria*). The few hits for chitinase A were nearly exclusively actinobacterial. This is in accordance with observations that *Actinobacteria* are particularly associated with senescing thalli (Cardinale et al. 2012a). Several further glycosyl hydrolases were representative for other bacterial classes, including *Alphaproteobacteria*. The use of AppA phytase and AcpA acid phosphatase genes as query sequences for *Peltigera* metagenome data yielded diverse hits, with alphaproteobacterial *appA* and betaproteobacterial *acpA* homologs being particularly prominent. This supports the hypothesis that inorganic phosphate solubilization may be among the roles of these abundant members (Sigurbjörnsdóttir et al. 2015; Grube and Berg 2009; Grube et al. 2015).

## V. Localization of Bacteria

Microscopic analyses complement the results from amplicon sequencing and other metaomics approaches by visualizing the bacterial colonization. Cardinale et al. (2008) originally demonstrated that bacteria colonized lichens in a biofilm-like manner but also provided further insights. Because the hyphae of lichenized fungi conglutinate and are embedded in a common matrix of polysaccharides, they also provide colonizable surfaces for bacteria. Generally, hydrophilic surfaces of lichens are the preferred environment for dense bacterial colonization. In contrast, hydrophobic hyphal surfaces (due to a self-assembled layer of hydrophobins or crystallized lichen metabolites) are often less abundantly colonized or colonized only by small colonies of bacteria.

Recently, Erlacher et al. (2015) developed a specific oligonucleotide probe to localize *Rhizobiales* by confocal laser scanning microscopy and fluorescence in situ hybridization (FISH-CLSM). The bulk of *Rhizobiales* again preferred the thallus surfaces, but there was also clear evidence that members of the *Rhizobiales* are also able to intrude at varying depths into the gelatinous matrix of the upper lichen cortical layer (Fig. 9.1). At least occasionally, some bacteria also are capable to colonize the interior of fungal hyphae. The penetration of fungal polysaccharide matrix agrees well with the presence of lytic function of bacteria (Grube et al. 2009).

So far, the microscopic data could not directly support the potential metabolic functions of the lichen-associated bacteria. Also, experimental data from coculture experiments of lichen symbionts with bacteria are still rare. Seneviratne and Indrasena (2006) presented results from coculture of lichen fungi with *Rhizobiales*. They could demonstrate that growth of rock-inhabiting lichenized fungi is enhanced in the presence of *Bradyrhizobium*. While evidence from cocultivation studies is valuable to reveal effects of associated bacteria on fungi, we are aware that the bacteria of lichens act on the whole symbiotic system of the lichen thallus. Future experiments need thus be carried out with the fully differentiated thalli of lichens, comparable to mesocosm experiments in microbial ecology.

## VI. Functional Model of the Lichen Symbiosis

On the basis of the recent evidence, we outline a revised model of the lichen symbiosis depicting the functional multiplayer network of the participants (Fig. 9.2; Grube et al. 2015). In addition to the newly gained knowledge about bacteria, the metaproteomic approach by Schneider et al. (2011) provided a holistic view of the lichen symbiosis together with the fungal and algal partner. The vast majority of proteins retrieved from the algal partner belong to the functional complex of energy production and conversion as well as carbohydrate transport and metabolism, which agrees well with their function as primary producers in the system. Fungi on the other hand have a much richer spectrum of functions (including secretion and vesicular transport), which can be attributed to their function in guiding the entire system by its architectural shape. A similarly rich spectrum of functions can be attributed to the bacterial fraction, with a significant number of hits also involved in stress responses (posttranslational modifications, protein turnover, chaperones). This is not very surprising, since the surfaces of lichens are stressful habitats. Bacterial degradative functions may help to mobilize carbon in lichens, which may be reincorporated in growing parts (Ellis et al. 2005). We suppose that the functional diversification of lichen-associated bacteria support the longevity and persistence of lichens under extreme and fluctuating ecological conditions. Internalization in the lichen structure of the algae might be correlated with functional specialization (either carbohydrate fixation by algae or cyanobacterial N-fixation in specialized organs, cephalodia, present in c. 2 % of lichens), whereas the functionally more diverse bacteria are external colonizers that may vary in composition with the age state of the lichen structure (Cardinale et al. 2012a; Mushegian et al. 2011).

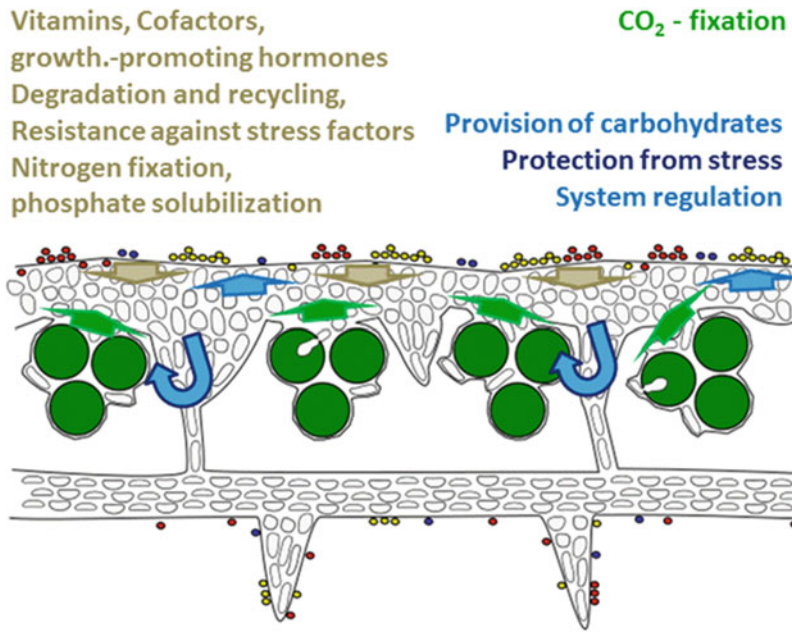


**Fig. 9.2** Functional model of the lichen symbiosis, including bacteria as third interacting partner on fungal surfaces. Bacterial functions, *light brown*; algal functions, *green*; fungal functions, *blue*. Notes: (1) nitrogen fixation of lichens with cyanobacterial partners, either in the algal layer or in cephalodia, is not considered here; (2) the *green* algal photobionts form an apoplastic continuum with the mycobiont due to a surface layer with hydrophobins (Honegger 1998)

## VII. Applications

The study of fungal–bacterial interactions in lichens could be of considerable interest for a range of biotechnological applications (Suzuki et al. 2016). Since the early work of Burkholder et al. (1944), a large number of publications only studied the effects of fungal secondary metabolites on bacteria (reviewed in Boustie and Grube 2005). For the most part, the assays in these studies demonstrated antibiotic effects of lichen compounds. Only exceptionally, the activities of these compounds were analyzed in greater detail. For example, Francolini et al. (2004) reported the inhibition of biofilm formation of bacteria using the lichen compound usnic acid. Even less known is about any positive effect of fungal lichen metabolites on bacteria, but those activities seem to exist as well. Gaikwad et al. (2014) discovered the positive effects of lichen compounds on probiotic lactobacilli.

So far, the biotechnological potential of lichen-associated bacteria has been little exploited, but certainly goes beyond the search for bioactive small metabolites. One third of the lichen-associated bacteria have the potential to produce PHA biopolymers (Gasser et al. 2012). Interestingly, the strains isolated also showed a remarkable high antagonistic potential against plant pathogens, including the common plant pathogen *Alternaria alternata* (Gasser et al. 2012). The antimicrobial properties of lichen-associated bacteria were meanwhile confirmed by other studies (Kim et al. 2013, 2014), and Cernava et al. (2015) studied the activity against several model bacteria and fungi by an integrative approach combining isolate screening, omics techniques, and high-resolution mass spectrometry. To efficiently select bacteria as stress-protecting agents for plants, a screening assay was developed which uses plants as "baits" for the lichen-associated fraction (Zachow et al. 2013).

## VIII. Conclusions and Outlook

In this chapter, we characterized lichens as a more complex system than previously known, by involving their relations with bacteria. These new insights may stimulate further research, especially to find out what regulative mechanisms help to keep the entire symbiotic systems in balance and regulate the partnering physiologies. Lichen–bacterial interactions are apparently as old as the lichen lifestyle and eventually have also contributed to the fungal genome evolution. There are already indications for horizontal gene transfer events from bacterial to fungal genomes, for example, in the diverse polyketide synthase genes (Schmitt and Lumbsch 2009). Yet, there are still many other new questions for research. For example, little is known so far about the diversity of lichen-associated *Archaea*. Lichens are a highly interesting case of interkingdom interactions (Berg 2015), and they are specifically designed for persistence and longevity under diverse ecological conditions. We think this symbiosis deserves more study in the future, since understanding and exploiting of the functional principles may be of considerable interest for biotechnological applications.

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