Graz University of Technology

Doctoral Thesis

Bacteria and Lichens
Elucidation of Microbial Communities Associated to Lichens based on Molecular and Proteomics Analysis

Dissertation zur Erlangung des akademischen Grades
Doktor der Naturwissenschaften (Dr. rer. nat.) an der Fakultät für
Technische Chemie, Verfahrenstechnik und Biotechnologie
der Technischen Universität Graz

vorgelegt von
Joao Vieira de Castro Junior
geboren am 11.06.1978 in Recife

Graz, im Februar 2010.

Acknowledgments

- Finally, I thank everyone who in one way or another contributed to this work.
- To God for allowing me to move on with my journey and finish my course.
- To my parents (Neide de Lima Torres and Joao Vieira de Castro), which enabled my existence and my visit to Graz (city that received me with open heart).
- To my family that despite being so far away I'm sure that are twisting for me.
- I want to especially thank Prof. Gabriele Berg, for the great help and patience, especially at the beginning of the course and all the advice and guidance that helped me to make this work.
- A special thanks to Prof. Martin Grube, from the Department of Plant Science, for all patience and attention given to me.
- To PD Dr Kathrin Riedel and the entire department of Proteomics that helped me to understand a little more about this complex and fascinating world of proteomics.
- To all the friends I made in the laboratory of environmental biotechnology (Christin, Lony, Michi, Kathrin, Florian and all the others), especially Dr. Henry Müller for all the advice and lessons that I have learned.
- The great friendships I made during my stay in Graz, mainly Miguel, Ana, Luciana, Daniela, Christiana, Yui and Jiajia.
- All the teachers who helped in my professional development, enabling me to expand my horizons and discover the wonderful world of microbiology.
- My Federica that despite the short time we met each other was always on my side encouraging me all the time.
- To Andréa Marins across dedication and support given to me before I came to Austria.
- To those who do not participate directly in spite of the work are essential to our lives (employees of the institution).
- OEAD by the financial support.

Abstract

The investigation of microorganisms from so far unexplored ecological niches has become an exciting subject in recent years. Lichens are ecologically highly adapted and also unique because the physiological integration of organisms provides conditions for persistence under extreme environmental conditions. Lichens are generally considered as symbiosis between fungi and green algae or cyanobacteria. These partnerships give raise to light-exposed and long-living joint structures, and the unique organisation of lichens provides still unexplored environments for microbial communities. To study microbial communities associated to lichens, samples from *Cladonia arbuscula, Umbilicaria cilindrica* and *Lecanora polytropa* were analyzed by a polyphasic approach. Bacteria were successfully isolated, cultured and characterized by use of selective culture media and biochemical assays. The structural and functional diversity of bacteria in lichens were done using a combination of DNA-assays based on Single Strand Conformation Polymorphism analysis (SSCP). The presence of specific groups was investigated with universal and specific primers for *Pseudmonas*, *Burkholderia* and *Alphaproteobacteria*. Dominant and unique bands were excised from the SSCP gels and sequenced to confirm the results obtained after PCR. The strains were isolated and shared into groups according to their profile based on 16S rDNA sequences (ARDRA). The biotechnological potential of bacteria associated to lichens was analyzed based on dual culture and biochemical/genetic approaches, and the structure and composition of associated bacterial communities of the lichen species was described by a polyphasic approach, using combined microscopic [(fluorescence in situ hybridization (FISH)] and confocal laser-scanning microscopy (CLSM). Microbial fingerprints performed by PCR-SSCP using universal as well as group-specific primers show distinct patterns for each lichen species and the presence of *Pseudomonas*, *Burkholderia* and *Alphaproteobacteria* groups were detected among all the lichens tested. Characterisation of cultivable strains and presence of functional genes in the total fraction suggest the involvement of associated bacteria in nutrient cycling. Ubiquitous *nifH* genes show a high diversity and are assigned to several cyanobacteria species, e.g. *Nostoc*. Cultivable strains showed lytic (chitino-, glucano-, proteolytic) activities, hormone production (IAA), as well as phosphate-mobilization and other functions such as antagonistic activity towards other microorganisms. Fluorescence in situ hybridization revealed the predominance of Alphaproteobacteria. Furthermore, the metaproteomic composition of the microbial community was analyzed on the species *Lobaria pulmonaria*. A functional screening with special interest in lytic enzymes (proteases, chitinases) and nitrogen-fixing systems (nitrogenases) was investigated to a better understanding the lichen symbiosis. Proteins were extracted using phenol method, and the proteome analysis were performed employing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and one-dimensional gelelectrophoresis (1-DE) followed by trypsin digestion and detected by multidimensional liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Proteins were identified according to Swissprot database; among them the fungal proteins were dominant in comparison with the others groups and 99% of bacterial proteins is descendant of *Alphaproteobacteria*. This study could demonstrate that the thalli space of lichens contains diverse, rich and very interesting populations of microrganisms. In order to study biogeography of microbial communities associated to the genus *Lobaria*, approaches have aimed to describe global diversity with phylogenetic analyses of 16S rDNA gene and SSCP analysis. Alphaprotebacteria were found as dominant groups in communities of bacteria-associated lichens and the SSCP fingerprint showed a very complex correlation with eco-regions. The traditional concept of lichens has to be expanded to consider multiple bacterial partners but more studies should be conducted to understand much better the interaction and function of these bacteria in lichens.

Zusammenfassung

Die Untersuchung von Mikroorganismen aus bislang unerforschten ökologischen Nischen hat sich in den letzten Jahren zu einem spannenden Thema entwickelt. Flechten sind ökologisch sehr angepasst und einzigartig, weil die physiologische Integration von Organismen Bedingungen für die Persistenz unter extremen Umgebungsbedingungen schafft. Flechten sind in der Regel als Symbiose zwischen Pilzen und Grünalgen oder Cyanobakterien bekannt. Diese Partnerschaften ermöglichen langlebige und an extreme Umweltbedigungen angepasste Strukturen, die wiederum bislang unerforschte mikrobielle Gemeinschaften beherbergen. Zur Untersuchung Flechten-assoziierter mikrobieller Gemeinschaften wurden Proben von ausgewählten Flechtenarten analysiert und mittels eines polyphasen Ansatzes charakterisiert. Bakterien wurden durch die Verwendung von selektiven Nährmedien isoliert, kultiviert und biochemisch und molekular charakterisiert. Die strukturelle und funktionelle Diversität von Bakterien in Flechten wurden mit einer Kombination von DNA-Tests auf der Grundlage von Single Strand Polymorphismus Analyse (SSCP) untersucht. Das Vorhandensein von spezifischen Gruppen wurde mit den universellen und spezifischen Primern für *Pseudmonas*, *Burkholderia* und *Alphaproteobacteria* studiert. Dominante und auch einzigartige Banden wurden aus den SSCP Gelen ausgeschnitten und sequenziert, um die Ergebnisse der PCR zu bestätigen. Bakterien wurden isoliert und gemeinsam in Gruppen, je nach ihrem Profil auf der Grundlage von 16S rDNA-Sequenzen (ARDRA), aufgeteilt. Die biotechnologische Potenzial der Bakterien Flechten assoziiert wurde basierend auf Dual-Kultur und biochemische / gentechnische Ansätze, und die Struktur und Zusammensetzung der assoziierten bakteriellen Gemeinschaften der Flechtenarten wurde von einem mehrphasigen beschriebenen Ansatz, kombiniert mit mikroskopischen [Fluoreszenz in situ Hybridisierung (FISH analysiert)] und der konfokalen Laser-Scanning-Mikroskopie (CLSM). Mikrobielle Fingerabdrücke von PCR-SSCP mit universellen sowie Gruppen-spezifische Primern zeigen verschiedene Muster für die einzelnen Flechtenarten, was für *Pseudomonas*, *Burkholdeira* und *Alphaproteobacteria* gezeigt wurde. Charakterisierung von kultivierbaren Stämmen und die Präsenz der funktionellen Gene in der gesamten Fraktion zeigen die Beteiligung der assoziierten Bakterien im Nährstoffkreislauf. Allgegenwärtige *nifH* Gene zeigen eine hohe Vielfalt und konnten mehrere Arten von Cyanobakterien zugeordnet werden, wie z. B. *Nostoc*. Kultivierbare Stämme zeigten lytische (chitino-, glucano-, proteolytischen) Fähigkeiten, Hormonproduktion (IAA), sowie Phosphat-Mobilisierung und andere Funktionen wie antagonistische Aktivität gegenüber anderen Mikroorganismen. Fluoreszenz in situ Hybridisierung zeigte die Vorherrschaft der Alphaproteobacteria. Darüber hinaus wurde das Metaproteom der Flechten analysiert; hierfür wurde die Art *Lobaria pulmonaria* ausgewählt. Weiterhin wurden lytische Enzyme (Proteasen, Chitinasen) und Stickstoff-Fixierungssysteme (Nitrogenasen) wurde zu einem besseren Verständnis der Flechten Symbiose untersucht.. Die Proteine wurden mit Hilfe der Phenol-Methode extrahiert. Die Proteom-Analyse wurden mittels Natrium Dodecyl Sulfat-Polyacrylamid-Gel-Elektrophorese (SDS-PAGE) und ein-dimensionale Gelelektrophorese (1-DE), gefolgt von Trypsin Verdauung und mehrdimensionaler Flüssigkeits-Chromatographie in Verbindung mit Tandem-Massenspektrometrie (LC-MS/MS) durchgeführt. Die Proteine wurden nach Swissprot-Datenbank identifiziert. Unter ihnen waren die pilzlichen Proteine dominant; 99% der bakteriellen Proteine wurden von *alphaproteobacteria* gefunden. Diese Studie konnte zeigen, dass Flechtenthalli ein vielfältige, reichhaltige und sehr interessante Gemeinschaft von Mikroorganismen enthält. Um Biogeographie der mikrobiellen Gemeinschaften der Gattung Lobaria verbundenen Studie haben Ansätze dazu dient, globale Vielfalt mit phylogenetischen Analysen der 16S-rDNA-Gens und SSCP-Analyse zu beschreiben. Alphaprotebacteria wurden herrschenden Gruppen in den Gemeinden von Bakterien-assoziierten Flechten und der SSCP Fingerabdruck zeigte ein sehr komplexer Zusammenhang mit Öko-Regionen. Das traditionelle Konzept der Flechten kann nun um den bakteriellen Partner erweitert werden, allerdings sollten weiterführende Untersuchungen durchgeführt werden, um die Interaktion und die Funktion dieser Bakterien in der Symbiose zu verstehen.

Table of Contents

1. Introduction

Lichens are important components of biodiversity on earth. Their species richness may be equal to or greater than that of vascular plants at given sites (Brown et al. 1994; Jarman & Kantvilas 1994). Lichens are mutualistic symbiotic organisms and consist of two unrelated components: a fungus (the mycobiont) and one or more algae or cyanobacteria (the photobionts). Lichens are the dominant organisms of *ca*. 8% terrestrial ecosystems (Larson, 1987) and are typically found in environments subject to extremes of temperature, desiccation and nutrient status. Lichens fill an important niche in areas, where extreme climatic conditions lead to an absence or seasonal inaccessibility of higher plants. According to these environmental conditions and aspects, lichens can be considered as an interesting reservoir for diverse group of microorganisms.

Although the symbiotic components of lichens have been extensively described little is known about the diversity of the microbial community inhabiting these complex systems. Detailed scientific studies on bacteria colonizing lichens have been not so far well understood, and based on the idea that lichen represents a unique ecosystem due to its physiological and morphological characteristics; it is expected that such complex system would contain novel strains with unknown metabolic capability (Cardinale et al. 2006).

The extent of microbial diversity in nature is still largely unknown, suggesting that there might be many more useful information yet to be identified from microorganisms in natural habitat. Significant diversity in genotype appears to exist in among and within lichens species and molecular methods are taking place to analyze this pool of diverse biosynthetic gene. Molecular approaches provide an invaluable assessment of biotechnological potential of unexplored groups of microorganisms associated to lichens (Tkacz & Lange, 2004), especially regarding bacterial species

The presence of nonphotosynthetic bacteria in lichens has been already described since some time ago, however these early narratives were all only based on non molecular approaches, mainly of them were found only by cultivation-dependent approaches. The use of molecular fingerprints and DNA sequences has only recently been used to characterize culturable bacteria in

lichens and no study has so far focused on the localization of microbial population in the lichen thalli (Cardinale et al. 2008; Grube et al. 2009).

2. Review of Literature

Lichens

Lichen is a symbiotic associative life form among a wide range of fungi, photosynthetic algae, or cyanobacteria, or possibly all of three together (Hawksworth, 1988). They are considered one of the best known groups of symbiotic organisms (Hill, 2009). The lichens symbiosis has been evolved probably around 400 to 600 million years ago and they can be considered as ecosystems where the interaction of partners results in specific behaviour and life forms (Yuan et al. 2005, Oksanen, 2006). Typically, these filamentous and unicellular organisms are involved in association to form undifferentiated plant-like structure referred to as thallus. The lichen is an ecologically obligate, stable mutualism between a fungal partner and a unicellular or filamentous algae or cyanobacteria cells (Oksanen, 2006). The thallus shows an interesting variety of morphological structures, colours and size, and present themselves completely different from either symbiotic partner grown in non-symbiotic culture, this differentiation is formed through complex interactions between mycobiont and photobiont cells growing together to form a body unlike either of the two symbionts grown alone (Honegger, 1993).

In general, three major life forms of lichens thalli are recognized: crustose (crust-like biofilm), foliose (leaf-like) and fruticose (branched tree-like). Gelatinous thallus is also described, although it is restricted only to some cyanobacterial lichens. Even without roots, lichens can efficiently extract nutrients from recalcitrant surfaces and very often grow in habitats with extreme light, dryness, or temperature, which are less favourable or not suitable to higher plants. The symbiosis increases the tolerance to undesirable conditions than in its partner alone (Vrablikova et al. 2006; Oksanen, 2006; Gauslaa, 2005).

According to Joneson & Lutzoni (2009), the sequence of events leading to a stratified thallus can be summarized in four basic stages: (i) pre-contact, which is prior to physical contact of symbionts but close enough that extracellular interactions are possible; (ii) early-contact, where fungal cells contact algal cells

forming an envelop through an increase in short lateral branches; (iii) incorporation of growing fungal and algal units into an undifferentiated mass; (iv) and differentiation of layered tissues, however the molecular mechanisms involved in such event are still unknown.

Galun (1988) studying the development of thallus observed that if fungi and phototrophs do not interact to form any of the initial stages of lichens development, they are considered non-compatible, indeed mycobionts and photobionts are considered compatible when they enter into stage two of lichen development as shown by the envelopment of the alga through increased lateral branching. The formation of lichens associations represents one of the most successful lifestyles among the fungi; approximately 20% of the 65.000 described species are involved in this association. Almost all of the 13.500 lichen-forming species are ascomycetes; only ~ 50 belong to the basidiomycetes group (Depriest, 2004).

As described by many authors, fungi are important for the symbiotic process and the main selective fungi advantage in becoming symbiotic in lichens is the supply of carbohydrates, as its source of energy produced by photosynthesis in the photobiont. Lutzoni *et al*. (2001) along of their studies found that about one-fifth of all known extant fungal species form obligate symbiotic associations with green algae, cyanobacteria or with both photobionts, as a way to meet their requirements for carbohydrates. This feature requires the photobiont to release the carbohydrate without fatal detriment to itself (Hill, 2009). There are several groups of organisms responsible to produce carbohydrates (polyol or glucose); these include the major groups of the algae represented not only by green algae (Chlorophyceae), but also red algae and the brown algae. All these groups capable of multicellular organisation have physiological characteristics in common which allow them to transfer carbohydrates between cells of their tissues to the fungi (Van Sandt et al. 2007). At present, an estimated 100 species in 40 genera are reported to form lichen symbiosis. They are placed in at least five phylogenetically classes: Cyanophyceae, Xanthophyceae, Phaeophyceae, Chlorophyceae and Trebouxiophyceae (Depriest, 2004).

An estimated 10% of the lichen-forming fungi form associations with cyanobionts, which provide fixed nitrogen along with photosynthetically fixed carbon (Depriest, 2004). In cyanobionts lichens, the fungus absorbs glucose from the cyanobacterial cells, through the movement of hexose produced by

photosynthesis from the vegetative cells to the heterocysts; in non-heterocystous cyanobacteria the mechanisms of nitrogen fixation is still not yet clearly understood and most of the cyanobionts appear to be heterocystous (Rikkinen, 2002).

The reproduction is carried either with fungal spores that have to find a suitable photobiont or by vegetative propagules including both partners; usually lichens grow very slow in a rate of mm up to cm per year (Büdel & Scheideger, 1996), in nature it is possible to find exemplars with more than one meter heights growing over rocks and native vegetation.

The lichen community can be found in different substrates and environments, usually in places where no other organisms would be able to survive, they are able to colonize inhospitable niches, with extreme variations on temperature and humidity, and hence they are considered as pioneers in colonization of new spaces (Käffer et al. 2009).

Microorganisms associated to lichens

Microrganisms in natural environment face conditions that are different from those experienced during assays carried in laboratory, especially growth at variable temperatures (low and high), long periods of frozen and dryness state, soil with high water content causing sometimes anaerobic conditions, low pH, and high content of organic matter on soil. These conditions may result in selectivity and evolution of different taxonomic groups of microorganisms to adapt to specifics niches (Zhou et al. 1997). It is well known, that isolated microorganisms do not reflect the true composition and structure of natural environments, and actually the analyze of small subunits of rDNA from natural ecosystems has become a powerful tool to characterize microbial communities directly *in situ*, because it avoids limitation of culturability and provide precise information regarding phylogenetic fingerprints (Zhou et al. 1997).

The ecological assessment of microbes from unexplored environmental habitats has become an interesting subject in earlier years. Niches as diverse as soil, water and air are being studied by various techniques, ranging from culture techniques to metagenomic approaches (Cardinale et al. 2006). According to these new molecular assessments, several new information about microbial communities are now being clarified and this is a promising perspective for the

investigation of further, biologically rich habitats. Among these new highlights, diverse groups of cryptogams including lower plants, fungi and lichens could be particularly suitable as hosts for bacterial communities. Lichens are considered as pioneers of terrestrial habitats colonization, they are found from arctic to tropical regions in a large diversity of environments developing among others on stones, arid soils, or as epiphytes on plants (González et al. 2005). Although the symbiotic components of lichens have been extensively described, little is known about the diversity of the microbial community inhabiting these complex systems. Petrini et al. (1990) described the presence of several microbial biofilms in the interface of saxicolous lichens; in this report they observed the existence of micro habitats constituted by a very rich diversity of microorganisms. Similar evidences were also found on the Antarctic cold desert, the place has been colonized by a considerable number of lichen community, and provide one of the most extensively locus for studying the association formed by endolithic microorganisms. The molecular examination carried by de la Torre et al. (2003) revealed the presence of vast microbial diversity inhabiting this ecosystem, where it is possible to find distinct phylotypes of bacteria belonging mainly to the Actinomycetes group.

Cyanobacteria can be found in association with lichens, and they are responsible to provide nitrogenated compounds via nitrogen fixation (Honegger, 1998); approximately 150 species in 58 genera form association with lichens. Rai (1988) proved that almost all nitrogen fixed by cyanobacteria is transferred to the fungi. Cyanobionts occurs in two type of lichen association: the first one called bipartite, where a continuous layer of photosynthetic and nitrogen-fixing cyanobionts is established, and the second one there is the integration of a second eukaryotic photobionts form with the nitrogen-fixing cyanobiont (Paulsrud et al. 2000). Usually, modifications on morphology and life cycles occur on cyanobacteria after the aggregation with lichens, including increase of 10% to 35% in hetorocysts and enhancing the fixation rates compared with non-associated cells. However, relatively little information is known regarding the nitrogen cycling in lichens (Kardish et al. 1995).

Of all fungal symbiotic relationships, however, the lichen association is a rather particular case: it contributes to a substantial primary evolutionary radiation of ascomycetous fungi (Lutzoni et al. 2001). Species that form lichens associations are equal or outnumber those that form parasitic associations (20%)

or mycorrhizal associations (8%), and they are exceeded only by saprophytic decomposers (50%). Almost 98% of all lichen-forming species belong to the ascomycetes group; only 1% is quoted as basidiomycetes. Although some authors have been also reported Actincomycota, Mastigomycota and Myomycota, these groups are no longer considered as true fungi (Depriest, 2004). On the other hand, fungus-associated bacteria have now been described for several functional groups of fungi and both positive and negative effects on fungal performance have been reported (Johansson et al. 2004).

The study on bacterial diversity in unexplored niches provides potential benefits by storing new gene pool as well as by finding new bacterial taxa. The understanding bacterial diversity also gives new insights into the biological mechanisms of adaptation and tolerance to inhospitable environments (Lee et al. 2007). Bacteria have the capacity to adhere to natural or artificial surfaces, and they are able to form sessile multicellular communities very stable and resistant to almost any kind of adverse conditions, these complex microbial structural interactions include diverse species with various functions responsible to give stability to the microbial community (Dalton & March, 1998).

Lichens-association microorganisms and their biotechnological potential

Lichens and their natural products have a long tradition of being used for decorations, brewing and distilling, perfume and dying industry, food, and natural remedies. A great number of species have proved to be source of important metabolites and with modern technology, the potential of discovering and utilizing these products has increased every day (Oksanen, 2006). Lichenologists have studied lichen chemistry for the past hundred years and have found over 800 compounds considered as secondary metabolites, especially polyketides-derived aromatic compounds (Elix, 1996; Müller, 2001).

The chemical substances produced by lichens can be grouped according to their location on the thallus, these products may be considered intra- or extracellular. The thallus has a complex structure, and some metabolites are synthesized by both the fungal and/or the photobiont partners (Hale, 1983). The intracellular compounds (carbohydrates, carothenoids, vitamins, aminoacids and proteins) are connected to cell wall and protoplast; those compounds are formed not only in lichens, but also in fungi, algae and high plants (Hale, 1983; Honda &

Vilegas, 1998). Extracellular products usually are so called secondary metabolites and are mainly found at the medulla or cortex, but very rare in both layers (Honda & Vilegas, 1993). Although, these compounds are found in fungi and high plants, the biggest part is considered exclusive of lichens; the concentration of metabolites comprehend 0.1 to 10% of lichen dry biomass, in some cases even higher (Hale, 1983).

Although many natural and cultured lichens have been screened for their biological activities and several novel compounds have been isolated and identified, lichens have been essentially ignored by the modern industry. There are two contributing reasons for this: its slow growth in nature and they are difficult to propagate and resynthesize in culture (Miao et al. 2001; Behera et al. 2005).

It is generally believed that most of secondary metabolites in lichens are provide by the mycobionts, this is evident due to the fact fungal compounds are well known in medicine. It is possible, that the photobionts also contribute to the repertoire of lichens metabolites (Miao et al. 2001).

A significant part of all lichens are colonized by cyanobacteria, and research suggests that the prokaryotic partner contributes significantly to the biosynthetically diversity of lichens. Marine and freshwater cyanobacteria produce a wide range of peptides and other bioactive compounds and are rich source of mixed peptides-polyketides. Among these metabolites, many can be considered with biotechnologically promising bioactivity; anticancer, antifungal and antiviral are also reported. Several reports has described many microcystin compounds as the most commonly isolated bioactive compound produced by the *Nostoc* strains, one of the most common genus in terrestrial cyanobacteria symbosis (Burja et al. 2001).

The extent of microbial diversity in nature is still largely unknown, suggesting that there might be many more useful products yet to be identified from microorganisms in natural habitat. Significant diversity in genotype appears to exist in among and within lichens species and molecular methods are taking place to analyze this pool of diverse biosynthetic gene. Molecular approaches provide an invaluable assessment of biotechnological potential of unexplored groups of microorganisms associated to lichens (Tkacz & Lange, 2004), especially regarding bacterial species.

The investigation of bacteria in association with symbiotic organisms has attracted considerable interest because of its great unexplored biotechnological potential for different industrial processes and in sustainable agriculture. Wang (2006) reported the rich source of bioactive secondary metabolites with antiviral, antitumor and antimicrobial activity from bacteria isolated from sponge; they are hosts of a large community of microorganisms, such as bacteria and fungi, and some of them are host-specifics.

Among these groups, the lichens have attracted special attention in concern to the complex structure presented by the interaction of different microrganisms in symbiosis and the huge amount of compounds produced as primary and secondary metabolites (Grube et al. 2009).

Detailed scientific studies on bacteria colonizing lichens have been not so far well understood, and based on the idea that lichen represents a unique ecosystem due to its physiological and morphological characteristics; it is expected that such complex system would contain novel strains with unknown metabolic capability.

Metaproteomics and lichens

During the begging of the geological earth formation, microorganisms were responsible for the primary roles in providing the environmental conditions that we can find actually. They are the major drives of the biogeochemical and nutrients cycles, as well as degraders of natural and anthropogenic wastes on the planet (Rodriguez-Velera, 2004). Many of these important reactions are catalysed by microbial enzymes that are made up by individual proteins and are regarded as environmental catalysts.

According to the functions described above, it makes sense to study the vast range of microbial proteins in different ecosystems and unexplored niches to understand better to role of these enzymes in complex systems (Wilmes & Bond, 2006). Actually, there is an increasing interest to understand microbial community composition and functions directly from their respective environments, and molecular analysis of environmental samples has greatly improved our knowledge about microbial diversity. Metagenomic approaches obtained directly from environmental samples provide large amount of data concerning genetic diversity and metabolic potential of microorganisms within selected environments (Wilmes

& Bond, 2008). Indeed, it is necessary to improve knowledge of microbial diversity with functional details of these microbial ecosystems.

In former time, the application of postgenomic techniques has been limited mainly to study pure culture in laboratory, and regarding studies on microbial communities only cultivation-independent experiments were done. However, these studies do not provide enough information about gene and protein expression in complex mixtures as found in the natural environment, guiding to a distorted understanding of microbial ecology.

Various strategies were used to study the relationship between the ecosystem functioning and the structure of microbial communities. Major goals of these efforts are to attribute key functions to specific community members and, in view of the ecosystem stability, to reveal cooperation between community members and functional redundancies (Benndorf et al. 2007). Besides measuring enzyme activities, respiration rates, metabolites concentration and nucleic acids are often used as markers for microbial identity. However, these structural data are specific only for certain group of microorganisms and there are no specific markers for all known bacteria (Kanegawa, 2003).

Although the identification of structural and functional gene can be used as a good indicator for the presence of microorganisms and their metabolic potential in the environment, it is not possible to understand completely the entire role of those organisms *in situ*. In comparison to molecular approaches, the use of proteins are a promising alternative since they reflect the actual functionality with respect to metabolic reactions, and give direct information about microbial activity more than functional genes. In other aspect, the use of proteins can also reveal the identity of microorganisms living in mixture systems via database analysis using homology with other described species (Kanegawa, 2003; Wilmes & Bond, 2006).

Proteomics is one of the fastest developing research areas and contributes substantially to our understanding of organisms at the cellular level. It is considered a new approach that enables the direct observation of proteins expressed by mixed microbial assemblages (Sowell et al. 2009). Recently, the large-scale characterization of the entire protein complement of environmental microbiota (often referred to as metaproteomics) has been proven useful to investigate the most abundant proteins in environmental samples (Wilmes & Bond, 2004; Schulze et al. 2005; Ram et al. 2005). Environmental proteomics, due to its

opportunity to study many protein functions and responses simultaneously, offers excellent possibilities to improve our understanding of microbial community architecture and composition, and ecosystem functioning. Metaproteomics can be used to study protein expression from complex systems and provide direct evidence of metabolic and physiological activities. Recently, proteomic approaches have been used to quantify and detect proteins from organisms in natural habitats and the proteomic approach is now more feasible than metagenomics sequences providing opportunity to identify proteins from microorganisms in complex mixtures (Wilmes & Bond, 2004). Until now, several authors presented metaproteome data from different environmental systems, such as soil particles, activate sludge, biofilms, and seawater, even about stress response of mixed cultures. However, the use of metaproteomic approaches to elucidate the microbial diversity in symbiotic living being is still completely unexplored, especially in lichen research area (Rustichelli et al. 2008).

3. Objectives and Aims of the Work

The aims of the study were to provide an assessment of the bacterial community in selected lichens, to clarify their structural and functional diversity, as well their proteomic composition and biotechnological potential.

1. Bacterial communities of three selected lichen species *Cladonia arbuscula, Umbilicaria cylindrica* and *Lecanora polytropa* were studied in the first part of the work. A molecular fingerprinting method was applied to give an overview about the composition of the microbial community. Fingerprinting performed by Single Strand Conformation Polymorphism analysis (SSCP) was carried out during the experiments based on 16S rDNA fragments with universal primer (UNIBAC), along with specific primers for *Burkholderia*, *Pseudomonas*, *Alphaproteobacteria*, and functional primers for the detection of *nifH* gene. Dominant and/or unique bands were excised from SSCP gels and the product re-amplified to find out their identity and confirm the results provided after PCR. Representatives of individual cultured strains were characterized by ARDRA and identified phylogenetically according to their 16S rDNA sequencing.

2. In order to verify the biotechnological and antagonistic abilities of the isolates, biochemical approaches and dual culture were accomplished as demonstrated in this work. Bacterial isolates were tested for their anti-prokaryotic and eukaryotic activity against a set of target organisms including plant pathogens as well as human pathogens; Single strains were characterized in their metabolically potential by biochemical and molecular assays. The bacteria were screened based on their ability to produce bioactive compounds, such as biopolymers, enzymes, plant growth promoters important also for clarifying the lichens symbiosis process.
3. A metaproteome study was accomplished to assess the structure and function of the lichen *Lobaria pulmonaria* based on the protein expression profiles with special interest in functional data. The experiment was carried out to provide a functional screening of the lichen associated bacterial with emphasis on the identification of key functions which might be important for symbiotic and antagonistic interactions. Expression of hydrolytic enzymes (proteases, chitinases) and nitrogen-fixing systems (nitrogenases) were verified focusing on comprehensive understanding of the lichen symbiosis.
4. Considering that the environmental conditions have a great impact on microbial community, the population composition of bacteria associated to genus *Lobaria* might differ from those in other regions. We performed the present study in order to investigate the community composition, geographic distribution, and phylogenetic relationships of the bacterial community associated to lichen thalli.

4. Final Discussion

The structure of natural communities of terrestrial habitats is often highly complex (Torsvik et al. 1990) and therefore difficult to characterize. It is possible by the use of PCR-SSCP analysis to evaluate, at the community level, a wide number of environmental samples and describe the microbial community structure in complex interaction, such as lichen symbiosis (Muyzer and Ramsing 1996). Despite the acknowledged importance of soil and root associated microorganisms, little is known about the interaction of bacteria associated with lichens.

The aim of this study was to gain knowledge about diversity and characterization of bacterial community associated to lichen thalli. We have

characterized taxonomically the bacterial population in three lichen species *C. arbuscula, U. cylindrica* and *L. polytropa.* It provided evidence to show that a subset of these bacteria is intrinsically associated with the thalli space. In the present study, a high degree of specificity of lichen-associated bacteria was found. Here we combined cultivation dependent techniques with cultivation-independent methods to analyze the lichen-associated bacterial populations. The investigation was based on the extraction of bacterial DNA and amplification of the 16S rDNA fragments, which were then separated by SSCP. The method showed us clearly the lichen specificity of the associated bacterial communities.

According to the previous results described by other authors, the presence of bacteria was already detected in lichen symbioses, however very little information about their diversity and specific location within the lichen thalli is known so far.
We found diverse bacterial representatives among the single isolates from different bacterial branches: Gammaproteobacteria, Actinobacteria and Firmicutes were the most common species among the all identified sequences. Representatives of actinomycetes have already previously been found in assoacition with lichens by Gonzales et al. 2005. Sequences obtained from SSCP bands revealed a very rich diversity of Alphaproteobacteria community involved with all three tested lichens.

Based on SSCP fingerprint and FISH analysis, the phylum Proteobacteria (in special Alphaproteobacteria) are the predominant bacterial group among the tested lichen species.

The nifH sequences showed a dominance of Cyanobacteria among all studied species. All identified nifH genes belong to species that are well known for symbiotic nitrogen fixation belonging specially to the genus *Nostoc*. According to Miadlikowska and Lutzoni (2004), cyanobacteria (mainly the genus *Nostoc*) are the photobionts most frequently found in lichen thalli.

In addition, strains of the genus *Burkholderia* and *Pseudomonas* were also found among all the tested lichens according to 16S rDNA profile obtained by SSCP fingerprint. Their presence is not surprising, as these genera are known also from associations with diverse other fungi. For example, Burkholderia was isolated from
Basidiomycota, from Glomeromycota, and from Zygomycota (Lim et al. 2003; Partida-Martinez and Hertweck, 2005), and Pseudomonas was found in

Basidiomycota and in Glomeromycota (Poole et al. 2001). Several genetically distinct strains within these genera are associated with lichens.

Furthermore, ARDRA analysis was used successfully to characterize genotipically the diversity of bacterial population inhabiting lichen thalli. All ARDRA fingerprints grouped the strains into major clusters represented manly by Firmicutes, g-Proteobacteria and Actinobacteria.

Using ARDRA we found a high genotypic diversity and lichen specificity for isolates belonging to the group of the Firmicutes, g-Proteobacteria and Actinobacteria.

Aislabie et al. 2006 and Smith et al. 2006 analyzed the richness of bacterial community composition on Dry Valley soils using ARDRA screening of clone libraries, clone libraries were dominated by sequences belonging to the Actinobacteria and Firmicutes representatives.

To examine the effects of sample age and location of microbial population associated to Algae species, Brambilla et al. (2001) found that a bacterial 16S rRNA gene clone library from a Lake Fryxell algal was dominated by members of the Firmicutes and Proteobacteria group. Gammaproteobacteria has been reported to be the major bacterial group in many associations with soft coral using culturing technique (Rohwer et al. 2001). By comparing the composition of culturable isolates from two distinct coral species, Lampert et al. 2006 found that isolates were dominated by an almost equal distribution of Alpha and Gammaproteobacteria.

According to the previous results described by other authors, the presence of bacteria was already detected in lichen symbioses, however very little information about their biotechnological potential is known so far

In the present study, we have analyzed the use of lichens as an innovative source for the isolation of microorganisms with biotechnological potential, specifically regarding the bacterial group.

The investigation of bacteria in association with symbiotic organisms has attracted considerable interest because of its great unexplored biotechnological potential for different industrial processes and in sustainable agriculture. In this study we started to explore metabolic potential of a particular biotic niche, the lichen symbioses.

According to our results, we showed that lichen-thalli space presents an interesting reservoir for polyhydroxyalkanoate-producing bacteria. A relative number of potential PHA-producers were observed among the tested samples. The bacterial community in the presence of rich nutrients tends to accumulate certain storage compounds, such as volutin granules, lipids and polyhydroxyalkanoates (Du et al. 2004).

In general biotic niches (plant roots, sponges and other) are interesting targets for finding biotechnologically interesting microbes, especially when the hosting organisms have unique ecological properties and adaptations. Such adaptations may include extremotolerance, accumulation of compounds, oligotrophy etc. Search in these niches can also reveal activities that seem surprising at first glance. Wang (2006) reported the rich source of bioactive secondary metabolites with antiviral, antitumor and antimicrobial activity from bacteria isolated from sponge; they are hosts of a large community of microorganisms, such as bacteria and fungi, and some of them are host-specifics.

Lichen associated bacteria were isolated from the thalli space with the aim of selecting efficient antagonists against pathogens*. In vitro* dual culture assay on culture media was taken as preliminary screening criterion for antagonism to characterize bacteria with antagonistic potential towards, however a low antagonistic activity was found among the tested isolates.

A high proportion of moss-associated antagonistic bacteria were studied by Opelt et al. (2007), their results suggested that *Sphagnum*-associated bacteria represent an interesting source of strains with high antagonistic activity against plant and human pathogens. Similar results were also observed by Berg et al. 2005 regarding the antagonistic potential of bacteria isolated from rhizosphere.

Lichens and their natural products have a long tradition of being used for industrial proposes, a great number of species have proved to be source of important metabolites and with modern technology, the potential of discovering and utilizing these products has increased every day (Oksanen, 2006). Although many natural and cultured lichens have been screened for their biological activities and several novel compounds have been isolated and identified (Miao et al. 2001; Behera et al. 2005), still very few information regarding the metabolic potential of bacteria associated lichen is known.

Our data presented in this study showed that bacteria-inhabiting lichens have a very rich secondary metabolic system, which suggest that bacterial strains could have significant role in the lichen ecosystem and probably those bacteria are involved in nutrient cycling in the lichen tahlli, although it is still not clear.

Detailed scientific studies on bacteria colonizing lichens have been not so far well understood, and based on the idea that lichen represents a unique ecosystem due to its physiological and morphological characteristics; it is expected that such complex system would contain novel strains with unknown metabolic capability.

The microbial metaproteomic profile in our study was obtained from the lichen specie *Lobaria pulmonaria*, the wild population was extracted from the thallic part and grouped into four distinct groups: fungi, bacteria, cyanobacteria and green algae.

Proteomic analysis examines proteins, the final product of all levels of gene expression, and thus provides information that is often different from the information provided by the measurements of gene expression (Gygi et al. 1999). Genomic, transcriptomic and proteomic analyses are complementary, each contributing to our understanding of how microbial communities respond to their environment.

A detailed examination performed by Scaffold software revealed that the most abundant group of proteins were of fungal origin, followed by bacterial proteins, green algae proteins and cyanobacteria.

Interestingly, the results demonstrated by the protein fingerprint analysis were significantly different among the different taxonomic groups; a significantly higher quantity of protein was recorded by the fungal group in lichen thalli, which may suggest that this group is dominant among the living microbial community.

The functional structure of the bacterial community was characterized by protein fingerprinting method. Considering the results presented by scaffold analysis, profiles were visually complex among the bacterial group. In addition, protein fingerprints confirmed that green algae and cyanobacteria are also one of the groups colonizing the thallic space.

Studying microbial community from unexplored niches is challenging because most groups either have never been cultivated (Giovannoni et al. 1990; Amann et al. 1995) or grow to very low density in the laboratory (Rappe et al. 2002). Culture-independent molecular approaches have indicated that

environmental bacterial communities are more complex and diverse than previously thought (Ward et al. 1990).

Most of all analyzed microbial proteins could be assigned to functional categories according to Clusters of Orthologous Groups of proteins and are involved in various metabolic processes.

Metagenomics has been applied to several environments, such as water, soils and extreme environments (Beja et al. 2000; Rondon et al. 2000), however, genomic data accumulates from pure cultures and environmental communities becomes unuseful without a critical understanding of gene expression and protein function. While metagenome sequences provide valuable information on potential functions, accurately predicting ecological function from sequence is nearly impossible without information on what proteins are synthesized under specific conditions (Lopez, 1999; Petersohn et al. 2001; Eymann et al. 2002). To address this question, post-genomic molecular approaches such as microarrays to monitor mRNA abundance (Conway & Schoolnik 2003) have been developed. In addition, as proteins/proteomes are the ultimate functional products of genes/genomes, proteomic studies of microbial communities (metaproteomics) are an obvious approach to advance our understanding of microbial community function. Metaproteomics can provide a direct measurement of functional gene expression in terms of the presence, relative abundance and modification state of proteins (Blackstock & Weir, 1999; Wilmes & Bond, 2004).

In the context of our experiment, this may illustrate the importance of microbial functional diversity and metabolic versatility for maintenance of lichens species. According to literature, still little concrete information on the sequences or identities of induced proteins from microorganisms associated to symbiotic organisms is known, and most of studies reported on literature are dealing with low-complexity microbial communities. So far, no studies have yet applied proteomic approaches to lichen-associated microbial communities.

Three geographic distinct areas were analyzed in an attempt to understand community structures within the lichen thalli.

Biogeography as applied to microbial distribution in the natural environment is still an undeveloped area. Staley and Gosink (1999) suggested a logical model for biogeographical analysis which involves isolation of several strains from different sites with confirmation of species similarity by using molecular approaches such as

DNA-DNA hybridization. For many environments the difficulties involved in cultivation make it possible to make comparisons only at higher taxonomic levels for most prokaryotes.

Although the microbial community structure in soil ecosystems varies at the microscale, owing to variable nutrient distribution (Azam and Long, 2001), the same soil prokaryotic species and genera inhabit ecologically similar niches separated over wide geographical scales, with populations shifting in space and time depending on environmental fluctuations. Even though various physiological characteristics are not necessarily conserved within the phylogenetic groups found in this study, the environmental conditions and energy sources available would tend not only to dictate microbial community composition and species richness but also to constrain the physiological characteristics of the community members. In the case of the bacterial population associated to the studied lichen species, most community members must be adapted to unfavorable conditions and generally able to grow in such extreme environmental conditions.

Distributional comparisons of prokaryotes (Andrews and Harry, 2000) have only recently been possible as the 16S rDNA database has expanded sufficiently to cover most global prokaryote diversity. In this study, for example, representative of Burkholderia and Pseudomonas group were found among all sampled areas; as well as cyanobacteria (especially the genus *Nostoc*) represented the majority taxonomically nitrogenase reductase (nifH gene) group of microorganisms associated to lichens of bacteria. A very rich diversity of Alphaproteobacteria were found according to sequences obtained from SSCP gels, suggesting that this group should be dominant and well conserved among the studied *Lobaria* species.

Certain phylogenetic groups did not show any similarity between different sampling sites ediment samples, this may indicate that the distribution of certain microorganisms is potentially endemic, with localized populations specifically adapted to the in situ properties of the given environment. Finlay (2002) suggested that the high abundance, short generation time, small size, and high dispersal rates of prokaryotes makes them unlikely to be endemic, as they are able to overcome large geographical barriers

The findings presented here show that the bacterial strains inhabiting the thallic structure could play a more important role on lichen adaptation to extreme environment. .Further studies could help to clarify with more detail the role of

bacterial community on lichen symbiosis. In conclusion, a high microbial diversity was detected for the thallic part of all lichen species described here. Lichen species present a unique environment for microorganisms and according to our results they might be colonized by specific bacterial population which are adapted to these special conditions. Therefore, they may provide important functions for their hosts, which likely are essential for the lichen growth on adverse conditions.

5. REFERENCES

Aislabie JM, Chhour K, Saul DJ, Miyauchi S, Ayton J, Paetzold RF, Balks MR (2006) Dominant bacteria in soils of Marble Point and Wright Valley, Victoria Land, Antarctica. S Biol Biochem 38(10):3041-3056.

Andrews JH, Harris RF (2000) The ecology and biogeography of microorganisms of plant surfaces. Annu Rev Phytopathol. 38:145-180.

Azam F, Long RA (2001) Oceanography—sea snow microcosms. Nature. 414:495-497.

Behera BC, Adawadkar B, Makhija U (2006) Tissue-culture of selected species of the *Graphis* lichen and their biological activities. Fitoterapia. 77(3):208–215.

Benndorf D, Balcke GU, Harms H, Bergen M (1997) Functional metaproteome analysis of proteins extracts from contaminated soil and groundwater. ISME Journal. 1:224-234.

Brambilla E, Hippe H, Hagelstein A, Tindall BJ, Stackebrandt E (2001) 16S rDNA diversity of cultured and uncultured prokaryotes of a mat sample from Lake Fryxell, McMurdo Dry Valleys, Antarctica. Extremophiles 5(1):23-33.

Brown DH, Avalos A, Miller JE, Bargagli R (1994) Interactions of lichens with their mineral environment. Crypt. Bot. 4:135–142.

Büdel B, Scheidegger C (1996) Thallus morphology and anatomy. In: Nash TH III (ed) Lichen biology. Cambridge University Press, Cambridge, pp 37–64.

Burja AM, Banaigs B, Abou-Mansour E, Burgess JG, Wright PC (2001) Marine cyanobacteria—a prolific source of natural products. Tetrahedron. 57:9347–9377.

Cardinale M, Puglia AM, Grube M (2006) Molecular analysis of lichen-associated bacterial communities. FEMS Microbiol. Ecol. 57(3): 484-495.

Cardinale M, Castro Jr, Mueller H, Berg G, Grube M (2008) In situ analysis of the bacterial community associated with the reindeer lichen Cladonia arbuscula reveals predominance of Alphaproteobacteria. FEMS Microbiol. Ecol. 66:63-1.

Dalton HM, March PE (1998) Molecular genetics of bacteria attachment and biofouling. Curr. Opin. Biotechnol. 9:252-255.

De la Torre JR, Goebel BM, Friedmann EI, Pace NR (2003) Microbial diversity of cryptoendolithic communities from the McMurdo dry valleys, Antarctica. Appl. Environ. Microbiol. 69:3858-3867.

Depriest PT (2004) Early molecular investigations of lichen-forming symbionts: 1986-2001. Annu Rev Microbiol. 58:273-301.

Du G, Chen LXL, Yu J (2004) High-efficiency production of bioplastics from biodegradable organic solids. J Polym and the Environ. 12(2):89–94.

Elix JA (1996) Biochemistry and secondary metabolites. In: Nash TH III (ed) Lichen biology. Cambridge University Press, Cambridge, pp 154–180.

Finlay BJ (2002) Global dispersal of free-living microbial eukaryote species. Science. 296:1061-1063.

Halle ME (1983) The biology of lichens. Baltimore, Edwards Arnold.

Hawksworth DL (1988) The variety of fungal-algal symbiosis, their evolutionary significance, and the nature of lichens. Bot J Linn Soc 96:3-20.

Hill DJ (2009) Asymetric Co-evolution in the lichen symbiosis caused by a limited capacity for adaptation in the photobiont. Bot Rev 75:326-338.

Honda NK and Vilegas W (1998) The chemistry of lichens. Quimica Nova. 21(6):110-125.

Honegger R (1993) Developmental biology of lichens. New Phytol. 125:659-677

Honegger R (1998) The lichen symbiosis: what is so spectacular about it? Lichenologist. 30:193-212.

Galun M (1998) Lichenization, In: CRC Handbook of Lichenology, Volume II. Galun, M., ed. CRC Press, Boca Raton, pp. 153-169.

Gauslaa Y (2005) Lichen palatability depends on investments in herbivore defence. Oecologia. 143:94–105.

Gonzales I, Ayuso AS, Anderson A, Genilloud O (2005) Actinomycetes isolated from lichens: evaluation of their diversity and detection of biosynthetic gene sequences. FEMS Microbiol. Ecol. 54:401-415.

Grube M, Cardinale M, Castro Jr JV, Mueller H, Berg G (2009) Species-specific structure and functional diversity of bacterial communities in lichen symbioses. ISME Journal. 1-11.

Jarman S J, Kantvilas G (1994) Lichens and bryophytes of the Tasmanian world heritage area II–Three forest sites at Pelion Plains. Tasforests 6:102–120.

Johansson JF, Paul LR, Finlay RD (2004) Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. FEMS Microbiol. Ecol. 481–13.

Joneson S, Lutzoni F (2009) Compatibility and thigmotropism in the lichen symbiosis: A reappraisal. Symbiosis 47:109-115.

Kanagawa T (2003) Bias and artifacts in multitemplate polymerase chain reactions (PCR). J. Biosci. Bioneng. 96:317-323.

Kaeffer MI, Ganade G, Marcelli MP (2009) Lichen diversity and composition in araucaria forest and tree monocultures in southern brazil. Biodvers. Conser. 18:3543-3561.

Kardish N, Rotem-Abarbanell D, Zilberstein A, Galun M (1990) Comparison between the symbiotic Nostoc of the lichen *Nephroma laevigatum* Ach. and its cultured, isolated *Nostoc* by recombinant DNA. Symbiosis. 8:135–45.

Lampert Y, Kelman D, Dubinsk Z, Nitzan Y, Hill RT (2006) Diversity of culturable bacteria in the mucus of the red sea coral Fungria scutaria. FEMS Microbiol Ecol 58:99-108.

Larson DW (1987) The absorption and release of water by lichens. Bibl. Lichenol. 25:351–336

Lee HK, Lee YK, Hong S (1997) Diversity of polar and terrestrial lichens. In. The International Symposium, Asian Collaboration in IPY 2007-2008 (http://polaris.nipr.ac.jp/~ipy/usr/sympo/proc-files/12-Hongkum_Lee.pdf).

Lim YW, Baik KS, Han SK, Kim SB & Bae KS (2003) Burkholderia sordidicola sp. nov., isolated from the white-rot fungus Phanerochaete sordida. Int J Syst Evol Microbiol 53: 1631–1636.

Lutzoni F, Pagel M, Reeb V (2001) Major Fungal lineages are derived from lichens ancestors. Nature. 411:937-940.

Miao V, Coeffet-LeGal MF, Brown D (2001) Genetic approaches to harvesting lichen products. Trends Biotechnol. 19:349–355.

Müller K (2001) Pharmaceutically relevant metabolites from lichens. Appl. Microbiol. Biotechnol. 56:9–16.

Muyzer G, Ramsing NB (1996) Molecular methods to study the organization of microbial communities. Water Sci Technol 32:1-9

Miadlikowska J, Lutzoni F (2004) Phylogenetic classification of Peltigeralean fungi (Peltigerales, Ascomycota) based on ribosomal RNA small and large subunits. Amer J Bot 91(3): 449–464. 2004.

Oksanen I (2006) Ecological and biotechnological aspects of lichens. Appl Microb Biotech. 73:723-734.

Partida-Martinez LP & Hertweck C (2005) Pathogenic fungus harbours endosymbiotic bacteria for toxin production. Nature 437: 884–888.

Petrini O, Hake U, Dreyfuss MM (1990) Lichens. Mycologia. 82:444-451.

Paulsrud P, Rikkinen J, Lindblad P (2000) Spatial patterns of photobiont diversity in some *Nostoc*-containing lichens. New Phytol. 146:291–99.

Poole EJ, Bendino GD, Whipps JM & Read DJ (2001) Bacteria associated with Pinus sylvestris-Lactarius rufus ectomycorrhizas and their effects on mycorrhiza formation in vitro. New Phytol 151: 743–751.

Rai AN (1988) Nitrogen metabolism. In Handbook of lichenology I ed. Galun, M. pp. 201-237. Boca Raton, FL: CRC Press.

Ram RJ, Verberkmoes NC, Thelen MP, Tyson GW, et al (2005) Community proteomics of a natural microbial biofilm. Science. 308:1915-1920.

Rikkinen J (2002) Cyanobionts evolutionary overview. Pp. 31-72. In: Rae, A. N. (ed.),

Rodriguez-Valera F (2004) Environmental genomics, the big picture? FEMS Microbiol. Lett. 231:153-158.

Rohwer FM, Breitbart JJ, Azam F, Knowlton N (2001) Diversity of bacteria associated with the Caribean coral Montastraea franksi. Coral Reefs. 20:85-95.

Rustichelli C, Visioli G, Kostecka D, Vurro E, Toppi LS, Marmiroli N (2008) Proteomics analysis in the lichen Physcia adscendens exposed to cadmium stress. Environm. Pollut. 156:1121-1127.

Schulze WX, gleixner G, Kaiser K, Guggenberger G, Mann M, Schulze ED (2005) A proteomic fingerprint of dissolved organic carbon and of soil particles. Oecologia. 142:335-343.

Smith JJ, Tow LA, Stafford W, Cary C, Cowan DA (2006) Bacterial Diversity in Three Different Antarctic Cold Desert Mineral Soils. Microb Ecol 51(4):413 – 422.

Sowell SM, Wilhelm LJ, Norbeck AD, Lipton MS et al (2009) Transport functions dominate the SAR11 metaproteome at low-nutrient extremes in the Sargasso sea. ISME Journal. 3:93-105.

Staley JT, Gosink JJ (1999) Poles apart: biodiversity and biogeography of sea ice bacteria. Annu. Rev. Microbiol. 53:189-215.

Tkacz JS, Lange L (2004) Advances in fungal biotechnology for industry, agriculture and medicine. Hardcover. Pp. 468. Cyanobacteria in Symbiosis. Dordrecht: Kluwer.

Torsvik V, Goksoyr J, Daae FL (1990) High diversity in DNA of soil bacteria. Appl Environ Microbiol 56:782-787

Van Sandt VST, Stieperaere H, Guisez Y, Verbelen JP, Vissenberg K (2007) XET activity is found near sites of growth and cell elongation in bryophytes and some green algae; new insights into the evolution of primary cells wall elongation. Ann. Bot. 99:39-51.

Vrablikova H, McEvoy M, Solhaug KA, Bartak M, Gauslaa Y (2006) Annual variation in photoacclimation and photoprotection of the photobiont in the foliose lichen *Xanthoria parietina* 83(2):151–162

Wang G (2006) Diversity and biotechnological potential of the sponge-associated microbial consortia. J. Ind. Microbiol. Biotechnol. 33:545-551.

Wilmes P, Bond PL (2004) The application of two-dimensional polyacrilamide gel electrophoresis and downstream analyses to a mixed community of prokaryotic microorganisms. Environm. Microbiol. 6:911-920.

Wilmes P, Bond PL (2006) Metaproteomics: studying functional gene expression in microbial ecosystems. Trends Microbiol. 14(2):92-97.

Wilmes P, Wexler M, Bond PL (2008) Metaproteomics provides functional insight into activated sludge wastewater treatment. Plos ONE. 3(3):1778-1789.

Yuan X, Xiao A, Taylor TN (2005) Lichen-like symbiosis 600 million years ago. Science. 308:1017.

Zhou J, Davey ME, Figueras JB, Rivkina E, Gilichinsky D, Tiedje JM (1997) Phylogenetic iversity of bacterial community determined from siberian tundra soil DNA. Microbiology. 143:3913-3919.

FEMS Microbiology Ecology 66 (2008) 63-71

Publication I

In situ analysis of the bacterial community associated with the reindeer lichen Cladonia arbuscula reveals predominance of Alphaproteobacteria

Massimiliano Cardinale, Joao Vieira de Castro Jr, Henry Müller, Gabriele Berg and Martin Grube

Institute of Plant Sciences, Karl-Franzens-Universität Graz, Graz, Austria; and Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria

RESEARCH ARTICLE

In situ analysis of the bacterial community associated with the reindeer lichen *Cladonia arbuscula* reveals predominance of *Alphaproteobacteria*

Massimiliano Cardinale[1,2], João Vieira de Castro Jr[2], Henry Müller[2], Gabriele Berg[2] & Martin Grube[1]

[1]Institute of Plant Sciences, Karl-Franzens-Universität Graz, Graz, Austria; and [2]Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria

Correspondence: Martin Grube, Institute of Plant Sciences, Karl-Franzens-Universität Graz, Holteigasse 6, A-8010 Graz, Austria. Tel.: +43 316 380 5650; fax: +43 316 380 9883; e-mail: martin.grube@uni-graz.at

Received 30 October 2007; revised 19 May 2008; accepted 19 May 2008.
First published online 8 July 2008.

DOI:10.1111/j.1574-6941.2008.00546.x

Editor: Jan Dirk van Elsas

Keywords
fluorescent *in situ* hybridization; confocal laser scanning microscopy; *Cladonia arbuscula*; bacterial community; *Alphaproteobacteria*; *Acetobacteraceae*.

Abstract

The diversity and spatial pattern of the bacterial community hosted by the shrub-like reindeer lichen *Cladonia arbuscula* were investigated by general DNA staining and FISH, coupled with confocal laser scanning microscopy (CLSM). Using an optimized protocol for FISH using cryosections of small lichen fragments, we found about 6×10^7 bacteria g^{-1} of *C. arbuscula*. 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%). *Firmicutes* were rarely detected, and no *Gammaproteobacteria* were present. Bacterial cells of different taxonomic groups are embedded in a biofilm-like, continuous layer on the internal surface of the *C. arbuscula* podetia, mainly occurring in small colonies of a few to a few hundred cells. The other parts of the lichen showed a lower bacterial colonization. α-proteobacterial 16S rRNA genes were amplified using total DNA extracts from *C. arbuscula* and separated by single-strand conformation polymorphism (SSCP). Sequencing of excised bands revealed the dominance of *Acetobacteraceae*.

Introduction

Lichens are highly integrated associations of fungi and photoautotrophs (algae and/or cyanobacteria). Suitable photoautotrophs are required by the fungus to develop the characteristic symbiotic phenotypes. The resulting thalli have diverse macroscopic shapes and are in most cases highly structured, with several functionally and anatomically different layers. Some of these fungal-determined morphologies represent what might perhaps be the most complex structures that can be formed by fungi. Lichens are also unique because the physiological integration of organisms provides conditions for persistance under extreme environmental situations that would not favour accumulation of the partners in biological solitude. In contrast to many fungi, which reside inside their substrates, lichens expose their vegetative parts at the surface of the substrates to harvest sunlight. Lichens grow very slowly, in the range of a few millimetres per year, but they can also survive for thousands of years, and represent a reasonably stable ecological niche for further microorganisms.

The presence of nonphotosynthetic bacteria in lichens has been detected some time ago (see references in Cardinale *et al.*, 2006). These early reports were all based on isolated cultures and nonmolecular approaches for further characterization. DNA sequence data or fingerprinting approaches have only recently been used to characterize culturable bacteria in lichens (Cardinale *et al.*, 2006; Liba *et al.*, 2006), and fingerprinting approaches of the unculturable fraction suggested that diverse bacteria are present in lichens (Gonzáles *et al.*, 2005; Cardinale *et al.*, 2006). No study has so far focused on the localization of bacterial populations in the lichen thalli. Few studies included electron microscopic images of bacteria in lichen thalli (e.g. Meier & Chapman, 1983; Souza-Egipsy *et al.*, 2002; De los Ríos *et al.*, 2005), which gave the impression that bacteria are sporadic in the investigated lichens.

In this study, we propose a general DNA-staining technique using acridine orange, for the rapid assessment of bacterial abundances, and apply FISH for more detailed investigation. Using this approach, we illustrate the spatial pattern of bacterial diversity in the lichen *Cladonia arbuscula*. This lichen produces erect and branched thalli (named podetia) that are several centimetres high. We chose this species because it is a common representative of the shrub-like reindeer lichens, which are of ecological significance in the ground layer of subalpine–alpine, boreal and subarctic habitats of the Northern Hemisphere.

In situ hybridization experiments using fluorescence-labelled nucleic acid probes that are complementary to target signature regions of rRNA have become a golden standard for localization of bacteria in many contexts (DeLong *et al.*, 1989; Amann *et al.*, 1995; Wagner *et al.*, 2003). The complexity of the lichen symbiosis, however, requires special considerations. Autofluorescence of lichen metabolites, of fungal hyphae, or algal chlorophylls can be a major nuisance in FISH experiments. In addition, the strongly conglutinated extracellular matrix of hyphae in lichen thalli interferes with the adherence of cryosectioned samples to the precoated slides. We developed and applied a modified protocol and propose optimized parameters for *in situ* hybridization experiments for lichens and other complex fungal structures.

Materials and methods

Sampling and fixation conditions

Lichens were collected at the Koralpe mountain ridge (Handalm) in the South of the province Styria, Austria, at an elevation of *c.* 1700 m. Samples were carefully taken using sterile forceps and immediately placed in sterile bags, at environmental temperature. Several individual thalli were always taken in order to have statistically representative samples. Lichen samples were quickly washed in phosphate-buffered saline (PBS) and fixed within 6 h after collection in 3 : 1 4% paraformaldehyde solution/PBS for 12 h at 4 °C, and then washed three times in ice-cold PBS before storage at − 20 °C in 1 : 1 ice-cold PBS/ice-cold 96% ethanol. A subset of the samples was quickly washed in PBS, placed into ice and then directly fixed in 1 : 1 ice-cold PBS/ice-cold 96% ethanol and immediately stored at − 20 °C. Nonfixed (fresh) subsamples were quickly washed in PBS and immediately stained with a general dye for fluorescent detection of nucleic acids (see 'Nucleic acid staining'), in order to compare the bacterial colonization with that of fixed samples. For each of the staining experiments, we analysed at least 20 different lichen sections. Fresh lichen subsamples were also used for the extraction of metagenomic DNA [see 'Single-strand conformation polymorphism (SSCP) analysis'].

Nucleic acid staining

Cryosections of both fresh and fixed lichen samples were stained with acridine orange, 4,6-diamino-2-phenyl indole (DAPI) and BacLight green, to compare the suitability of the stains. Acridine orange (Molecular Probes Inc., Eugene, OR) was used at 1 mg mL^{-1} in 1 : 3 permeabilizing solution PS (0.1% Triton X-100, 80 mM HCl, 150 mM NaCl)/staining solution STS (37 mM citric acid, 126 mM Na_2HPO4, 150 mM NaCl, 1 mM Na_2EDTA) or 1 : 3 : 1 PS/STS/5 M NaCl, as described by Bertaux *et al.* (2005). DAPI was used at 0.7 μg mL^{-1} in PBS. BacLight green (Invitrogen, Carlsbad, CA) was used at 0.2 μM, following the manufacturer's instructions. All incubations were performed at room temperature in the dark for 30 min (acridine orange and DAPI) to overnight (BacLight green). The samples were observed with the confocal laser scanning microscope (CLSM).

FISH probes and fluorochromes

Detailed information about the probes used for FISH is provided in Table 1. The stringency conditions for optimal staining are also reported therein. FITC-, Cy3- and Cy5-labelled probes or 6FAM-, Cy3- and Cy5-labelled probes were applied sequentially or simultaneously depending on the required formamide concentrations. Samples stained with multiple probes were simultaneously observed, taking advantage of the nonoverlapping emission wavelengths of the fluorochromes (max. excitation/emission in nm: 6FAM 492/518, FITC 490/525, Cy3 548/562 and Cy5 650/670).

In tube FISH

Small fragments (10–15 mm length) of fixed thalli were embedded in water, rapidly frozen at − 25 °C and cut into 30-μm-thick sections with a cryotome. Sections from different thalli were collected separately. Some fragments were embedded in O.C.T.™ Tissue-Tek® (Sakura, Finetech Europe BV, Zoeterwoude, The Netherlands), which prevents tissue damage. Comparison with the latter was used to exclude the possibility of artefacts with water-embedded samples. The cryosections were directly placed in a 1.5-mL microcentrifuge tube (without mounting on slides) and incubated with 1 mg mL^{-1} lysozyme (Sigma-Aldrich, Steinheim, Germany) for 10 min at room temperature, to increase the bacterial cell wall permeability for the FISH probes. The samples were then rinsed twice with ice-cold PBS. An ethanolic series (50–70–96% ethanol solutions, 3 min each) was carried out in the same tube. The samples were quickly rinsed with ice-cold PBS and washed in ice-cold PBS for 3 min at room temperature. All hybridizations

Table 1. Characteristics of the probes used for FISH

Name	Sequence (5′–3′)	Target	Formamide concentration*	Reference
EUB338†	gctgcctcccgtaggagt	Most bacteria	10–20	Amann *et al*. (1990)
EUB338II†	gcagccacccgtaggtgt	*Planctomycetales*	10–20	Daims *et al*. (1999)
EUB338III†	gctgccacccgtaggtgt	*Verrucomicrobiales*	10–20	Daims *et al*. (1999)
ALF968	ggtaaggttctgcgcgtt	*Alphaproteobacteria*, except *Rickettsiales*	45	Neef (1997)
BET42a†	gccttcccacttcgttt	*Betaproteobateria*	45	Manz *et al*. (1992)
GAM42a†	gccttcccacatcgttt	*Gammaproteobacteria*	45	Manz *et al*. (1992)
LGC354A†	tggaagattccctactgc	*Firmicutes* (low G+C Gram-positive bacteria)	35	Meier *et al*. (1999)
LGC354B†	cggaagattccctactgc	*Firmicutes* (low G+C Gram-positive bacteria)	35	Meier *et al*. (1999)
LGC354C†	ccgaagattccctactgc	*Firmicutes* (low G+C Gram-positive bacteria)	35	Meier *et al*. (1999)
HGC236	aacaagctgataggccgc	*Actinobacteria* (high G+C Gram-positive bacteria)	20	Erhart *et al*. (1997)
Burkho	accctctgttccgaccat	*Burkholderia* spp.	40	Hogardt *et al*. (2000)
ARCH915	gtgctcccccgccaattcct	Archaea	> 60	Stahl & Amann (1991)
Univ-1390	gacgggcggtgtgtacaa	All organisms	5	Zheng *et al*. (1996)
NONEUB	actcctacgggaggcagc	–	‡	Wallner *et al*. (1993)

*The indicated concentrations of formamide are intended for hybridizations at 41 °C.

†Used together in equimolar concentration.

‡Used as negative control at the same formamide concentration as that used for the positive FISH probe.

were performed at 41 °C for 2–3 h in a buffer containing 0.9 M NaCl, 0.02 M Tris-HCl, pH 8, 0.01% vol sodium dodecyl sulphate (SDS) and 1.5–2.5 ng μL^{-1} of each used probe. The concentration of ultrapure formamide™ (Invitrogen) was adjusted individually for the respective FISH probe. Double-distilled sterile water (Fresenius Kabi, Graz, Austria) was added up to an appropriate volume until the lichen sections were completely immersed (usually 100–200 µL). Different concentrations of formamide and incubation temperatures were tested to assess the optimal parameters. The hybridization buffer was immediately removed and the samples were rinsed twice with prewarmed (42 °C) washing buffer, containing 0.02 M Tris-HCl, pH 8, and 30–450 mM NaCl (depending on the concentration of formamide in the hybridization buffer). The washing buffer was adjusted to 5 mM EDTA when formamide concentration exceeded 20%. The samples were incubated in the washing buffer in a water bath for 10–15 min at a temperature 1 °C above the hybridization temperature. The washing buffer was removed and the samples were rinsed with ice-cold water. The sections were incubated with a 5 µM PBS solution of the general nucleic acid stain Sytox Blue (Molecular Probes Inc.; maximum excitation/emission 444/480 nm; no overlap with any of the emission wavelengths of the FISH probe fluorochromes) for 1 h at room temperature in the dark. The samples were then rinsed with ice-cold water. The sections were finally placed on a regular microscopic glass-slide and quickly dried with compressed air. The sections were immediately mounted with ProLong Gold antifadent (Molecular Probes), incubated for 24 h at room temperature as recommended by the manufacturer and finally observed under the CLSM.

CLSM

Stained lichens were observed with a Leica TCS SP confocal laser-scanning microscope (Leica Microsystems, Heidelberg, Germany) equipped with argon and helium/neon lasers. Three confocal light channels were observed simultaneously along with further bright-field channel. Filter settings were adjusted to achieve the maximum signal from each fluorochrome (the detection of some sample autofluorescence was often impossible to avoid). Up to three FISH probe signals were observed simultaneously (with or without the bright-field image) and compared with the image from Sytox Blue staining only. Maximum projections of an appropriate number of 0.8–1-µm-depth optical slices were applied to visualize the lichen sections (confocal stacks). Up to 15 scans per optical slice were averaged to improve the image resolution and to reduce noise. The digital images were further observed and analysed with the NIH IMAGEJ software implemented with suitable plugins (http://rsbweb.nih.gov/ij/docs/index.html). Bacterial cells were manually counted using the 'POINT PICKER' plugin (http://bigwww.epfl.ch/thevenaz/pointpicker/). Images showing a high number of ambiguous objects or a high noise-to-signal ratio were excluded from the analysis. The software IMARIS 6.0 (Bitplane, Zurich, Switzerland) was used for three-dimensional (3D) rendering of confocal stacks.

Single-strand conformation polymorphism (SSCP) analysis

The SSCP analysis was carried out according to Schwieger & Tebbe (1998). Metagenomic DNA extracted from fresh lichen samples (see 'Sampling strategy' above) was used as

a template for the PCR using *Alphaproteobacteria*-specific primers ADF 681F (5′-AGTGTAGAGGTGAAATT-3′) (Blackwood *et al.*, 2005). A semi-nested PCR was performed using the primer pair ADF 681F/1492r (5′-TACGGYT ACCTTGTTACGACTT-3′) for the first PCR and the primer pair ADF 681F/927r (5′-CCCGTCAATTYMTTTGAGTT-3′) for the second PCR. Dominant bands were excised from SSCP gels as described by Schwieger & Tebbe (1998). Gel-extracted DNA was reamplified and cloned as described by Opelt & Berg (2004). For phylogenetic analysis and identification of related sequences, the BLAST algorithm according to Altschul *et al.* (1997) was used. The sequences obtained from SSCP bands are available in GenBank/EMBL/DDBJ database under accession numbers AM944536–AM944545.

Results

Nucleic acid staining and assessment of bacterial density

Aspecific staining of nucleic acids in sectioned *C. arbuscula* using acridine orange indicates that the abundance of bacterial colonization of lichens varied among areas of lichen thallus. The quality of the images was better as compared with both DAPI and BacLight green staining. The high cytoplasmic content of bacterial RNA allowed a well-distinguishable staining of the bacterial cells. The lichen medulla mainly showed a green colour, resulting from cytoplasmic autofluorescence, some aspecific DNA staining and weak staining of hyphal walls (Fig. 1). Furthermore, the chlorophyll autofluorescence allowed us to detect algal cells easily (Fig. 1). Dense bacterial colonization is particularly seen on the internal surface of the cylindric thalli of *C. arbuscula* (Fig. 1). The external surfaces were less abundantly colonized, either by single bacteria or by small clusters of cells attached to fungal hyphae (Fig. 2). In some cases, we detected bacteria inside hyphae, between the outer cell wall and the cytoplasm (Fig. 2b). Different morphotypes were clearly detected (Fig. 2b). We calculated an average density of $6.27 \pm 4.37 \times 10^7$ bacteria g^{-1} *C. arbuscula* thallus. The highest density of bacterial colonization ($8.9 \pm 1.6 \times 10^4$ bacteria mm^{-2}, corresponding to $2.1 \pm 0.38 \times 10^8$ bacteria g^{-1} thallus) was found on the internal layer on *C. arbuscula* podetia, where bacterial populations colocalized in a biofilm-like continuous layer (Fig. 1). Other

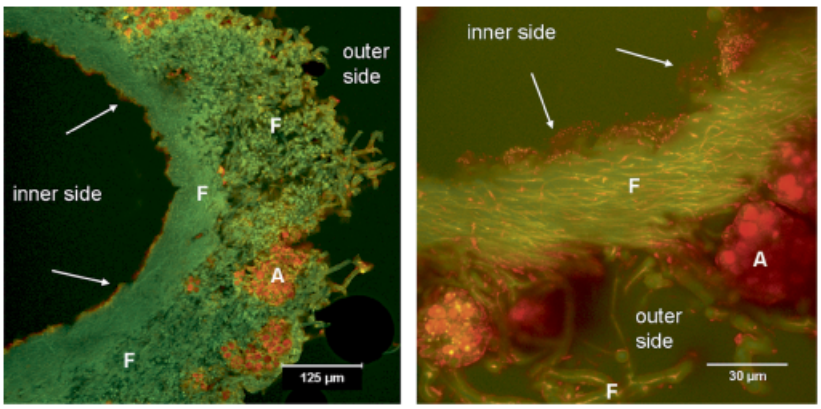


Fig. 1. Acridine orange-stained transversal sections of *Cladonia arbuscula* showing bacterial colonization on the internal surface and also to a lower extent on the outside (CLSM image). Left panel, overview; right panel, detail. A, algal cells; F, fungal hyphae; arrows, bacteria.

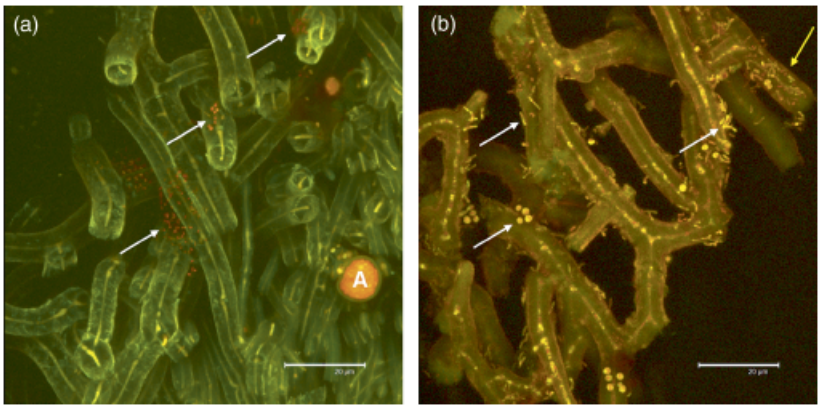


Fig. 2. Acridine orange-stained sections of *Cladonia arbuscula* showing bacteria on hyphae of the external lichen surface (CLSM image). Thin yellow central lines in hyphae represent the cytoplasm of the fungus. (a) Clusters of bacterial colonies. (b) Colonization by different bacterial morphotypes. A, algal cells; white arrows, bacteria adhering to the hyphae; yellow arrow, bacteria inside hyphae.

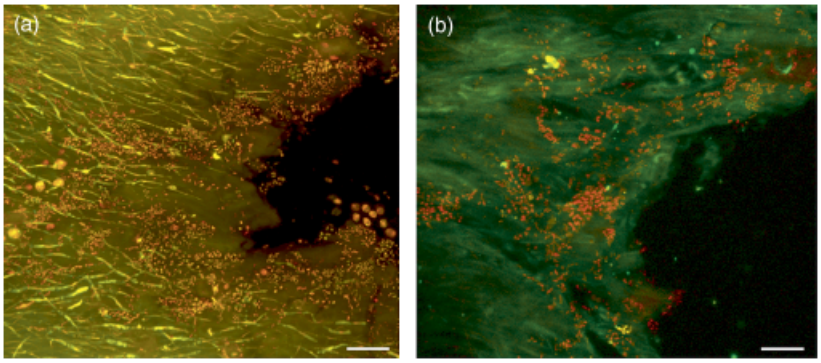


Fig. 3. Detection of bacteria by acridine orange and FISH in *Cladonia arbuscula* (diagonal sections of inner surface, CLSM images). (a) Acridine orange: bacterial cells are yellow-orange particles. (b) EUB338MIX-Cy3 and HGC236-Cy5 bacterial FISH probes: *Actinobacteria* are yellow objects, and other bacterial cells are red. Scale bars are 15 μm.

nonprokaryotic microorganisms were also detected on the surfaces, including fungal spores not belonging to the lichen mycobiont and protists (data not shown).

In-tube FISH and assessment of bacterial diversity

In-tube FISH was used to overcome the detachment problem of the lichen sections from the precoated slides. All FISH steps were performed in a single 1.5-mL tube by exchanging the solutions with a 20–200-μL micropipette and filter tips. The hybridization temperature of 41 °C proved to be optimal. Higher hybridization temperatures led to the production of autofluorescent aggregates of unknown nature and origin in the gelatinous matrix of the conglutinated hyphal network. The optimal concentration of formamide for each probe is reported in Table 1. The conglutinate nature of the lichen thallus preserved the integrity of the sample during the sectioning and the hybridization/washing steps. In fact, no differences in terms of number of cells and distribution pattern were detected between the fresh samples and fixed samples and between the samples embedded in O.C.T.™ Tissue-Tek® compound before the cryosectioning and the samples embedded in water. With in-tube FISH using universal bacterial probes EUB338MIX and CLSM, we could detect 85.8 ± 16.8% of the acridine orange-stained cells and confirm the biofilm-like structure of the bacterial community (Fig. 3). Negative controls with a nonsense FISH probe (NONEUB, Table 1) were always without positive signals. Using FISH probes targeting different bacteria, we detected the occurrence of distinguishable bacterial cells belonging to different phylogenetic groups (Fig. 4) that can form neighbouring or mixed colonies. The dominant group was *Alphaproteobacteria*, with a percentage of 75.0 ± 12.0% of the EUB338MIX-stained cells (Fig. 5); other bacterial groups were *Actinobacteria* (5.7 ± 2.9%, Fig. 3) and *Betaproteobacteria* (4.4 ± 1.2%). Only in a few cases was stratification of different populations visible (Fig. 6). *Firmicutes* were only present in small numbers, in a number of images too low to allow a statistically supported count. No *Gammaproteobacteria* or *Archaea* were detected in any lichen sample. 13.3 ± 1.4% of EUB338MIX-stained cells remained unidentified.

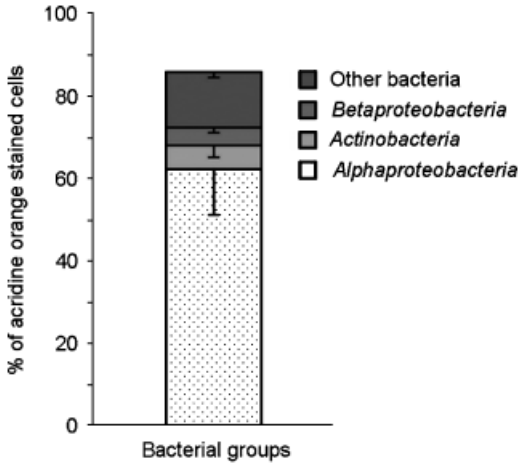


Fig. 4. Abundance of bacterial groups in *Cladonia arbuscula* revealed by FISH and CLSM image analysis of FISH experiments.

SSCP analysis

16S-SSCP profiles containing between five and seven bands of different intensity were obtained using specific primers for *Alphaproteobacteria* (data not shown). Representative samples of the four major bands were excised, reamplified and sequenced. BLAST searches with three distinct sequences revealed a relationship with members of the family *Acetobacteraceae*, close to the genera *Gluconacetobacter, Acidisphaera*, and also to the recently proposed new species *Rhodovastum atsumiense* (GenBank accession no.: AB381935, unpublished). The remaining distinct sequence was not identified at the genus level, and it matched only with unculturable *Alphaproteobacteria* of the SAR11 cluster (Stingl *et al.*, 2007). Sequences obtained from dominant SSCP bands of *Acetobacteraceae* from two different sampling times were identical.

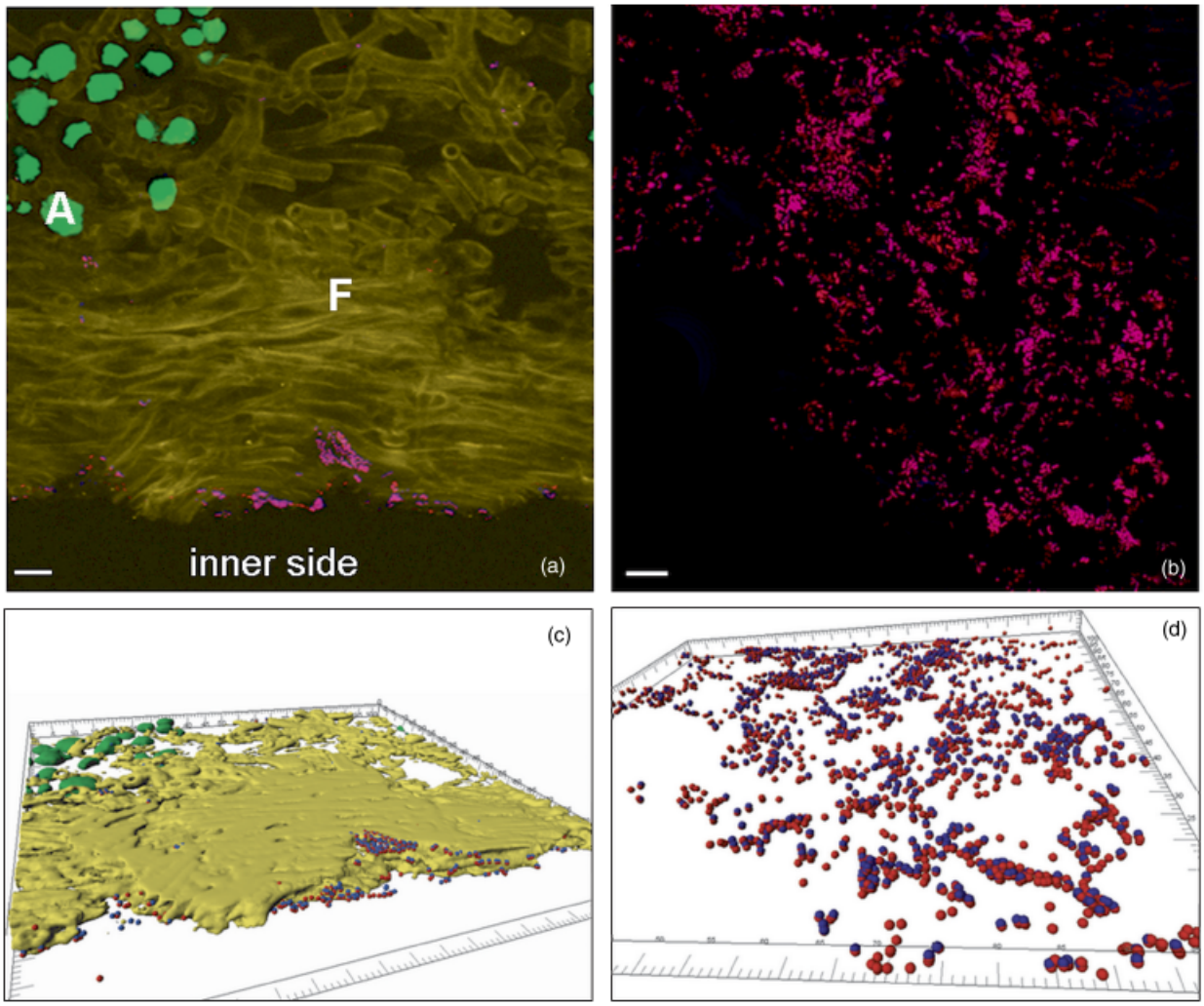


Fig. 5. Predominance of *Alphaproteobacteria* in *Cladonia arbuscula* as detected by simultaneous use of EUB338MIX-Cy3 and ALF968-Cy5 FISH probes (CLSM images). (a) Transversal and (b) tangential sections. Pink, *Alphaproteobacteria* (resulting from a positive signal with both probes); red, other bacteria (stained by EUB338MIX-Cy3 only); green, chlorophyll autofluorescence; yellow/brown, fungal autofluorescence; A, algal cells; F, fungal hyphae. Scale bars are 10 μm. (c, d) 3D reconstructions (formed by spots and isosurfaces) of the images in (a) and (b), respectively. Red/blue spots, *Alphaproteobacteria*; red spot, other bacteria; yellowish isosurface, fungi; green isosurface, algal cells.

Discussion

Although the presence of bacteria in lichen symbioses has been detected previously, still little is known so far about their diversity and specific localization within the lichen structure. Here, we show that a microscopic approach is suitable to properly interpret data obtained from culture-based approaches or from analyses of unculturable fractions. Cardinale *et al*. (2006) studied the diversity of culturable and unculturable bacteria associated with several *Cladonia* spp., including the reindeer lichen *Cladonia rangiferina*, by sequencing the 16S rRNA gene of pure cultures isolated from both internal and external parts of the lichen thalli. Common phylotypes represented genera of *Firmicutes* and *Betaproteobacteria*, whereas *Actinobacteria* were only found in *Cladonia pyxidata*. Differences in the structure of the culturable bacterial communities were observed among the lichen species, but some bacterial taxa were retrieved from different lichens sampled at the same or different sites. Strains of *Paenibacillus* and *Burkholderia* were particularly common and these bacterial genera are also involved in other fungal–bacterial associations of nonlichenized fungi (Bianciotto *et al*., 2000; Bertaux *et al*., 2003; Partida-Martinez & Hertweck, 2005). Further strains were found by sequencing the dominant bands obtained by ribosomal intergenic spacer analysis (RISA) using the DNA extracted from the same lichens (Cardinale *et al*., 2006). In other recent studies, a number of *Gammaproteobacteria* were

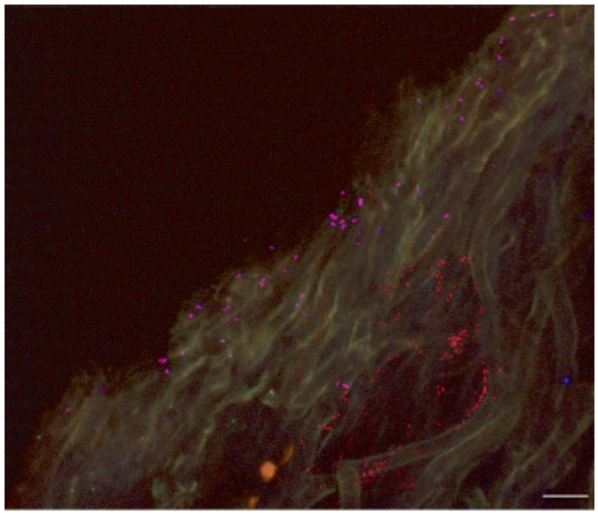


Fig. 6. Stratified bacterial colonies in the inner surface of *Cladonia arbuscula* as detected by simultaneous use of EUB338MIX-Cy3 and ALF968-Cy5 FISH probes (CLSM image of transversal section). Pink, *Alphaproteobacteria* (stained by the universal bacterial probe EUB338MIX-Cy3 and the specific probe ALF968-Cy5); red, other bacteria (stained by EUB338MIX-Cy3 only). Scale bar is 10 μm.

found in lichens (Liba *et al*., 2006), while fingerprinting techniques also revealed a considerable diversity of *Actinobacteria*, especially in lichens from tropical habitats (Gonzáles *et al*., 2005). Lichens certainly represent a promising source of new bacterial species (e.g. Li *et al*., 2007). However, a thorough assessment of specificity and variation of the associations by considering the diversity of the hosts will provide new clues about the biology of lichens. To achieve this, the relative abundances of bacterial groups and their preferred location need to be studied directly in the host lichens (*in situ*). Here, a two-step approach elucidated the spatial distribution of bacteria in the reindeer lichen *C. arbuscula*.

First, we tested which of the aspecific DNA-staining methods is suitable to visualize bacteria and their general abundance in lichens. We found that acridine orange has the optimal properties for rapid general assessment of bacterial colonization. Second, for a more detailed investigation we optimized FISH with lichens. So far, FISH has only rarely been applied in lichens, e.g. when Grube & de los Rios (2001) visualized the presence of parasitic fungi in lichens. In that study, so-called microslides (small rectangular pieces cut out from cover-slips) were used to carry out the *in situ* hybridization with small volumes of liquids in PCR tubes. Only rather thin sections adhered properly to the microslides, while washing steps were still carried out with greatest care to avoid section detachment. Once transversal sections of more than 10 μm thickness are required, e.g. to obtain a better 3D representation of structures, detachment becomes a serious problem even with precoated slides. We suppose that the adherence is decreased due to the hygroscopic movements of the strongly conglutinated hyphal structures in lichens. The problem cannot be solved by stronger fixation of the material because this apparently decreases the permeability for the probes and makes targets inaccessible. Lichen sections resided on the slide after they were covered by a layer of 1% agarose (dried with compressed air), but this treatment led to the deposition of autofluorescent agarose residues, which cause artefacts in the CLSM analysis (data not shown). We therefore skipped the use of supporting slides during the FISH procedure. Taking advantage of the strong coherence of the lichen structures, we used thick sections (between 15 and 30 μm) in an approach designated here as in-tube FISH. We found that the integrity of the lichen structures and the distribution of the bacterial communities are more easily preserved than in material placed on slides. Furthermore, the possibility of adapting the reaction volume to the sample size allowed us to minimize the amount of chemicals and toxic wastes. This optimized FISH protocol was successfully tested with other lichen species, including those with different macroscopic organizations (M. Cardinale, unpublished data).

Our experiments revealed an abundant bacterial colonization in lichens, which range from single cells attached to fungal hyphae to dense biofilm-like aggregations. Group-specific probes showed that *Alphaproteobacteria* form a common component in the bacterial community associated with *C. arbuscula*. Interestingly, *Alphaproteobacteria* were hardly found in previous culture-dependent approaches (only *Inquilinus*, Cardinale *et al*., 2006, and *Methylobacterium*, unpublished data). This discrepancy could indicate that certain, not yet cultured *Alphaproteobacteria* require the lichen symbiosis for growth, whereas culturable bacteria could belong to a minor and perhaps less specific fraction in vital parts of the lichen. Moreover, sequences obtained after PCR amplification of excised major bands of SSCP gels using specific primers revealed that the dominant strains belong to *Acetobacteraceae*. The SSCP-derived sequences are not long enough for a thorough phylogenetic analysis, but their alignment with reference sequences indicated low sequence diversity. Lichen-associated strains could form a monophyletic cluster inside the *Acetobacteraceae* family, which suggests a possibly specific association between bacteria and lichen partners. Several examples of highly specific associations between *Eukaryota* and *Alphaproteobacteria* strains (e.g. Beier *et al*., 2002; Bertaux *et al*., 2005; Andam & Parker, 2007) and even members of *Acetobacteraceae* (Favia *et al*., 2007) are known. Further investigations are under way to assess the diversity of bacterial groups in other lichens collected from both the same or different sampling sites. These future works will use additional methods, including 16S rRNA gene clone library construction, which will more precisely represent the composition of the whole bacterial community, including the faction that was not identified here.

Our data and published images of bacteria in lichen thalli always show their presence in intercellular spaces and on surfaces (see e.g. Meier & Chapman, 1983; Souza-Egipsy *et al.*, 2002; De los Ríos *et al.*, 2005), but not inside the vital cells of the lichen symbionts. Bacteria inside hyphal cells have been described already in *Ascomycota* [*Cytophaga—Flexibacter–Bacterioides* phylogroup (CFB) bacterium in *Tuber borchii*, Barbieri *et al.*, 2000], *Basidiomycota* (*Paenibacillus* in *Laccaria bicolor*, Bertaux *et al.*, 2003), *Glomeromycota* (*Burkholderia* in *Gigaspora* and *Scutellospora*, Bianciotto *et al.*, 2000) and *Zygomycota* (*Burkholderia* in *Rhizopus*, Partida-Martinez & Hertweck, 2005). We do not exclude the possibility of endocytoplasmatic bacteria in vital hyphae, but so far, we only have evidence for bacterial colonization within hyphal walls, external of the cytoplasm.

In this work, we present results for a shrub-like species that does not form an exploiting mycelium in the soil substrate. However, initial acridine orange staining experiments with lichen species that are tightly attached to the underlying substrates show a likewise considerable bacterial colonization (preliminary data). It will be interesting to explore differences of bacterial communities in a variety of lichens and their substrates, and to study how the abundance of bacterial colonization is controlled in lichens. One of several possible factors will be secondary lichen compounds with antibiotic effects. Peripheric parts of *C. arbuscula* thalli, with extracellular, crystallized usnic acid deposits on the surface of hyphae, harbour significantly fewer bacteria. Because usnic acid is well known for its antibiotic potential (Boustie & Grube, 2005), the lower abundance of bacteria is not surprising. Highly structured lichen thalli provide a variety of chemically distinct microniches for bacteria, and varying hydrophobicity of mycelial surfaces in lichens (Büdel & Scheidegger, 1996) may additionally influence the composition of associated bacterial communities.

We hypothesize that associated bacteria contribute significantly to the integrity and robustness of lichen biology. This contribution is perhaps not restricted to one particular function in the lichen system. We hypothesize that bacteria in lichens are parts of a complex functional network, which remains to be studied in the future.

Acknowledgements

We are grateful to Walter Obermayer for an introduction to confocal microscopy and technical support. The F.W.F. is acknowledged for financial support of G.B. and M.G.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R & Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919–1925.

Amann RI, Ludwig W & Schleifer KH (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* **59**: 143–169.

Andam CP & Parker MA (2007) Novel alphaproteobacterial root nodule symbiont associated with *Lupinus texensis*. *Appl Environ Microbiol* **73**: 5687–5691.

Barbieri E, Potenza L, Rossi I, Sisti D, Giomaro G, Rossetti S, Beimfohr C & Stocchi V (2000) Phylogenetic characterisation and *in situ* detection of a *Cytophaga-Flexibacter-Bacteroides* phylogroup bacterium in *Tuber borchii* Vittad. ectomycorrhizal mycelium. *Appl Environ Microbiol* **66**: 5035–5042.

Beier CL, Horn M, Michel R, Schweikert M, Görtz HD & Wagner M (2002) The genus *Caedibacter* comprises endosymbionts of *Paramecium* spp. related to the *Rickettsiales* (*Alphaproteobacteria*) and to *Francisella tularensis* (*Gammaproteobacteria*). *Appl Environ Microbiol* **68**: 6043–6050.

Bertaux J, Schmid M, Chemidlin Prevost-Boure N, Churin JL, Hartmann A, Garbaye J & Frey-Klett P (2003) *In situ* identification of intracellular bacteria related to *Paenibacillus* sp. in the mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N. *Appl Environ Microbiol* **69**: 4243–4248.

Bertaux J, Schmid M, Hutzler P, Hartmann A, Garbaye J & Frey-Klett P (2005) Occurrence and distribution of endobacteria in the plant-associated mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N. *Environ Microbiol* **7**: 1786–1795.

Bianciotto V, Lumini E, Lanfranco L, Minerdi D, Bonfante P & Perotto S (2000) Detection and identification of bacterial endosymbionts in arbuscular mycorrhizal fungi belonging to Gigasporaceae. *Appl Environ Microbiol* **66**: 4503–4509.

Blackwood CB, Adam O & Buyer JS (2005) Phylum- and class-specific PCR primers for general microbial community analysis. *Appl Environ Microbiol* **71**: 6193–6198.

Boustie J & Grube M (2005) Lichens-a promising source of bioactive secondary metabolites. *Pl Gen Res* **3**: 273–287.

Büdel B & Scheidegger C (1996) Thallus morphology and anatomy. *Lichen Biology* (Nash TH III, ed), pp. 37–51. Cambridge University, Press, Cambridge, UK.

Cardinale M, Puglia AM & Grube M (2006) Molecular analysis of lichen-associated bacterial communities. *FEMS Microbiol Ecol* **57**: 484–495.

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

DeLong EF, Wickham GS & Stahl DA (1989) Phylogenetic strains: ribosomal RNA-based probes for the identification of single microbial cells. *Science* **243**: 1360–1363.

De los Ríos A, Wierzchos J, Sancho LG, Green A & Ascaso C (2005) Ecology of endolithic lichens colonizing granite in continental Antarctica. *Lichenologist* **37**: 383–395.

Erhart RD, Bradford RJ, Seviour R, Amann RI & Blackall LL (1997) Development and use of fluorescent *in situ* hybridization probes for the detection and identification of *Microthrix parvicella* in activated sludge. *Syst Appl Microbiol* **20**: 310–318.

Favia G, Ricci I, Damiani C *et al*. (2007) Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. *Proc Natl Acad Sci USA* **104**: 9047–9051.

Gonzáles I, Ayuso-Sacido A, Anderson A & Genilloud O (2005) Actinomycetes isolated from lichens: evaluation of their diversity and detection of biosynthetic gene sequences. *FEMS Microbiol Ecol* **54**: 401–415.

Grube M & de los Rios A (2001) Observations in *Biatoropsis usnearum* and other gall forming lichenicolous fungi using different microscopic techniques. *Mycol Res* **105**: 1116–1122.

Hogardt M, Trebesius K, Geiger AM, Hornef M, Rosenecker J & Heesemann J (2000) Specific and rapid detection by fluorescent *in situ* hybridization of bacteria in clinical samples obtained from cystic fibrosis patients. *J Clin Microbiol* **38**: 818–825.

Li B, Xie CH & Yokota A (2007) *Nocardioides exalbidus* sp. nov., a novel actinomycete isolated from lichen in Izu-Oshima Island, Japan. *Actinomycetologica* **1**: 22–26.

Liba CM, Ferrara FIS, Manfio GP, Fantinatti-Garboggini F, Albuquerque RC, Pavan C, Ramos PL, Moreira-Filho CA & Barbosa HR (2006) Nitrogen-fixing chemo-organotrophic bacteria isolated from cyanobacteria-deprived lichens and their ability to solubilize phosphate and to release amino acids and phytohormones. *J Appl Microbiol* **101**: 1076–1086.

Manz W, Amann R, Ludwig W, Wagner M & Schleifer KH (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst Appl Microbiol* **15**: 593–600.

Meier H, Amann R, Ludwig W & Schleifer KH (1999) Specific oligonucleotide probes for *in situ* detection of a major group of Gram-positive bacteria with low DNA G+C content. *Syst Appl Microbiol* **22**: 186–196.

Meier JL & Chapman RL (1983) Ultrastructure of the lichen *Coenogonium interplexum* Nyl. *Am J Bot* **70**: 400–407.

Neef A (1997) Anwendung der *In Situ* Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. PhD thesis, Technische Universität, München.

Opelt K & Berg G (2004) Diversity and antagonistic potential of bacteria associated with bryophytes from nutrient-poor habitats of the Baltic Sea Coast. *Appl Environ Microbiol* **70**: 6569–6579.

Partida-Martinez L & Hertweck C (2005) Pathogenic fungus harbours endosymbiotic bacteria for toxin production. *Nature* **437**: 884–888.

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

Souza-Egipsy V, Wierzchos J, Garcia-Ramos JV & Ascaso C (2002) Chemical and ultrastructural features of the lichen-volcanic/sedimentary rock interface in a semiarid region (Almeria, Spain). *Lichenologist* **34**: 155–167.

Stahl DA & Amann R (1991) Development and application of nucleic acid probes. *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt E & Goodfellow M, eds), pp. 205–248. John Wiley & Sons Ltd, Chichester.

Stingl U, Harry J, Tripp HJ, Stephen J & Giovannoni SJ (2007) Improvements of high-throughput culturing yielded novel SAR11 strains and other abundant marine bacteria from the Oregon coast and the Bermuda Atlantic Time Series study site. *ISME J* **1**: 361–371.

Wagner M, Horn M & Daims H (2003) Fluorescence *in situ* hybridisation for the identification and characterisation of prokaryotes. *Curr Opin Microbiol* **6**: 302–309.

Wallner G, Amann R & Beisker W (1993) Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14**: 136–143.

Zheng D, Alm EW, Stahl DA & Raskin L (1996) Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies. *Appl Environ Microbiol* **62**: 4504–4513.

Publication II

Species-specific structural and functional diversity of bacterial communities in lichen symbioses

Martin Grube, Massimiliano Cardinale, Joao Vieira de Castro Jr, Henry Müller and Gabriele Berg

Institute of Plant Sciences, Karl-Franzens-Universität Graz, Graz, Austria; and Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria

The ISME Journal (2009), 1–11

www.nature.com/ismej

ORIGINAL ARTICLE

Species-specific structural and functional diversity of bacterial communities in lichen symbioses

Martin Grube[1], Massimiliano Cardinale[2], João Vieira de Castro Jr[2], Henry Müller[2] and Gabriele Berg[2]

[1]*Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria and*
[2]*Institute of Plant Sciences, Karl-Franzens-University of Graz, Graz, Austria*

Lichens are generally considered as mutualisms between fungi and green algae or cyanobacteria. These partnerships allow light-exposed and long-living joint structures. The unique organization of lichens provides still unexplored environments for microbial communities. To study lichen-associated bacterial communities, we analyze samples, by a polyphasic approach, from three lichen species (*Cladonia arbuscula*, *Lecanora polytropa* and *Umbilicaria cylindrica*) from alpine environments. Our results indicate that bacteria can form highly structured, biofilm-like assemblages on fungal surfaces and reach considerable abundances of up to 10^8 cells per gram fresh weight. Fluorescence *in situ* hybridization reveals the predominance of *Alphaproteobacteria*. Microbial fingerprints performed by PCR-single-strand conformation polymorphism analysis using universal and group-specific primers show distinct patterns for each lichen species. Characterization of cultivable strains and presence of functional genes in the total fraction suggest the involvement of associated bacteria in nutrient cycling. Ubiquitous *nifH* genes, which encode the nitrogenase reductase, show a high diversity and are assigned to *Alphaproteobacteria* and *Firmicutes*, for example, *Paenibacillus*. Cultivable strains mainly belonging to the genera *Acinetobacter*, *Bacillus*, *Burkholderia*, *Methylobacterium* and *Paenibacillus* show lytic (chitinolytic, glucanolytic, and proteolytic) activities, hormone production (indole-3-acetic acid) as well as phosphate mobilization and antagonistic activity toward other microorganisms. The traditional concept of lichens has to be expanded to consider multiple bacterial partners.
The ISME Journal advance online publication, 25 June 2009; doi:10.1038/ismej.2009.63
Subject Category: microbial ecology and functional diversity of natural habitats
Keywords: lichen symbiosis; FISH; SSCP; bacteria; nitrogen fixation

Introduction

Fossil and molecular phylogenetic evidence show that the lichen lifestyle was established at least 600 million years ago (Yuan *et al.*, 2005) and that it is basal in filamentous ascomycete fungi (Lutzoni *et al.*, 2001). Lichens are ecologically diverse and many, often pioneering species, can grow with exceptionally low supply of nutrients. Many lichen symbioses tolerate extreme environmental conditions that are unfavorable for long-term survival of individual partners. Even under rather hostile circumstances, the composite organisms or thalli can live up to thousand years (Denton and Karlén, 1973; Grube and Hawksworth, 2007). Lichens are traditionally considered as symbioses between a fungal species and one or more algae or cyanobacteria; yet, there is a considerable range of further associations and numbers of partners (Hawksworth, 1988). The diversity of co-inhabiting fungi is better known because of morphologically distinct structures: more than a thousand fungal species grow specifically on lichens, ranging from commensals to pathogens (Lawrey and Diederich, 2003). However, abundance and diversity of the externally inconspicuous bacterial communities are still poorly understood.

Lichen-associated bacteria were found only by previous studies applying cultivation-dependent approaches: the detected strains were assigned to *Azotobacter*, *Bacillus*, *Beijerinckia*, *Clostridium*, and *Pseudomonas* (Henkel and Yuzhakova, 1936; Iskina, 1938; Scott 1956; Panosyan and Nikogosyan, 1966; Henkel and Plotnikova, 1973). A few recent molecular approaches, still based on cultivation, indicated that the diversity of bacteria might be much higher (Lenova and Blum, 1983; Gonzáles *et al.*, 2005; Cardinale *et al.*, 2006; Liba *et al.*, 2006) and

Correspondence: G Berg, Institute of Plant Sciences, Karl-Franzens-University of Graz, Holteigasse 6, Graz, Austria.
E-mail: gabriele.berg@TUGraz.at
Received 9 February 2009; revised 15 April 2009; accepted 21 April 2009

suggested that up to millions of bacterial cells could be present per gram of a lichen thallus but abundance and specific location in the symbiotic structures in different species has only recently been established directly in one lichen species. Cardinale *et al*. (2008) showed that *Alphaproteobacteria* form a dominant part of a community in a reindeer lichen.

The objective of this work was to analyze and compare the structure and composition of associated bacterial communities of the lichen species *Cladonia arbuscula*, *Lecanora polytropa* and *Umbilicaria cylindrica* by a polyphasic approach, using combined microscopic (fluorescence *in situ* hybridization (FISH) and confocal laser-scanning microscopy (CLSM)) and molecular techniques (microbial fingerprints by PCR-single-strand conformation polymorphism analysis (SSCP) using different primer systems). The lichens were selected because they represent the predominant growth types evolved by lichens: brush-like, crust-forming and leaf-like, respectively (Figure 1). The studied species further grow on different substrates (rocks and soil). In addition, we studied variation in *nifH* genes for evaluation of nitrogen-fixing populations in the lichen symbiosis, as well as functions of the culturable bacterial fraction. The ability to fix atmospheric nitrogen by the nitrogenase enzyme complex is restricted to some bacteria and is a key element in symbiotic interactions of a variety of host organisms, including plants and fungi (Kneip *et al.*, 2007).

Material and methods

Sampling procedure

Specimens of the lichens *C. arbuscula*, *L. polytropa* and *U. cylindrica* (Figure 1) were collected from a wind-swept heath above the tree line in November 2006 and in May 2007 (Austria, Styria, Koralpe, Handalm, approximately 1800 m altitude, 46°50′ N, 15°01′ O). Lichen samples were collected at three locations within an area of 2 Ha. At each location, composite samples of five individuals per species were prepared. Using sterile tweezers, fragments of *Cladonia* and *Umbilicaria* were transferred into sterile polyethylene bags. Samples of *Lecanora* were collected together with underlying rock substrate (to avoid disintegration of the lichen) by chiselling using a hammer. The samples were stored in sterile polyethylene bags until processing. Samples were processed within 3 h after sampling.

FISH, CLSM and image analysis

Lichen samples were fixed within 3 h after collection in 3:1 4% paraformaldehyde/phosphate-buffered saline (PBS) for 12 h at 4 °C, washed three times in ice-cold PBS and then stored at −20 °C in 1:1 PBS/96% ethanol. Small pieces (5–10 mm length) of fixed thalli were cut in 30 μm cryosections and placed into 1.5 ml microcentrifuge tubes. FISH was performed as described by Cardinale *et al.* (2008). An equimolar mixture of Cy3-labeled EUB338, EUB338II and EUB338III probes (Amann *et al.*, 1990) was used for the detection of all bacteria and 6FAM-labeled Bet42a probe for the detection of *Burkholderia* and other *Betaproteobacteria* (Daims *et al.*, 1999; Manz *et al.*, 1992). Bet42a probe was used together with an unlabeled competitor probe to avoid unspecific hybridization of closely related bacteria, or together with the Cy5-labeled Gam42a probe (Manz *et al.*, 1992) for detection of *Gammaproteobacteria*. For detection of *Alphaproteobacteria*, *Actinobacteria* and *Firmicutes* we used respectively Cy5-labeled ALF968 (Loy *et al.*, 2007), Cy5-labeled HGC 236 (Erhart *et al.*, 1997) and an equimolar mixture of fluorescein-isothiocyanate-labeled LGC354A, LGC354B and LGC354C (Meier *et al.*, 1999). Negative controls were performed with a non-sense FISH probe (NONEUB; Wallner *et al.*, 1993). Different non-sense probes labeled with all the fluorochromes used in the experiments were

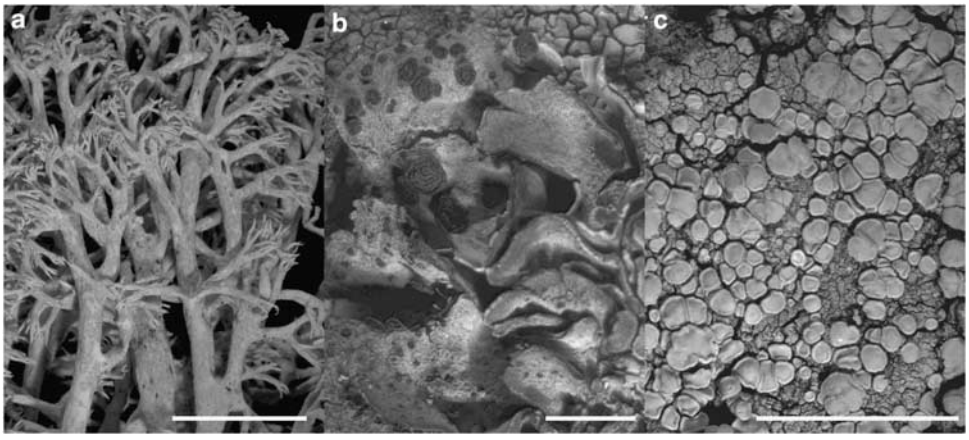


Figure 1 The lichen species studied. (**a**) The shrub-forming reindeer lichen *C. arbuscula* on soil. (**b**) The leaf-like *U. cylindrica* on rock. (**c**) The crust-forming *L. polytropa* on rock. Scale bar = 1 cm.

Table 1 Properties of the probes used for FISH

Name	*Sequence (5′–3′)*	*Target*	*Formamide concentration (%)*[a]	*Reference*
EUB338[b]	gctgcctcccgtaggagt	Most bacteria	10	Amann *et al.* (1990)
EUB338II[b]	gcagccacccgtaggtgt	*Planctomycetales*	10	Daims *et al.* (1999)
EUB338III[b]	gctgccacccgtaggtgt	*Verrucomicrobiales*	10	Daims *et al.* (1999)
ALF968	ggtaaggttctgcgcgtt	*Alphaproteobacteria*	40	Loy *et al.* (2007)
BET42a[b]	gccttcccacttcgttt	*Betaproteobacteria*	40	Manz *et al.* (1992)
GAM42a[b]	gccttcccacatcgttt	*Gammaproteobacteria*	40	Manz *et al.* (1992)
LGC354A[b]	tggaagattccctactgc	*Firmicutes*	35	Meier *et al.* (1999)
LGC354B[b]	cggaagattccctactgc	*Firmicutes*	35	Meier *et al.* (1999)
LGC354C[b]	ccgaagattccctactgc	*Firmicutes*	35	Meier *et al.* (1999)
HGC236	aacaagctgataggccgc	*Actinobacteria*	20	Erhart *et al.* (1997)
NONEUB	actcctacgggaggcagc	—	[c]	Wallner *et al.* (1993)

[a]The indicated percentage of formamide are intended for hybridizations at 41 °C.
[b]Used together in equimolar concentration.
[c]Used as negative control at same formamide concentration as used for positive FISH probe.

applied, as they can show different adhesion properties. All hybridizations were performed at 41 °C for 1.5–2.5 h. Formamide concentrations and other properties of the FISH probes are described in Table 1. Washing steps with appropriate washing buffer matching the formamide concentration were carried out at 42 °C for 10 min. Stained samples were immediately dried with compressed air, mounted with ProLong antifadent (Molecular Probes Inc., Eugene, OR, USA) and viewed under a CLSM within 24 h.

Stained lichens were examined using a Leica TCS SP confocal laser-scanning microscope equipped with argon and helium/neon lasers. SYTOX Blue (Ex/Em maximum 444/480 nm) for general visualization of bacteria was excited with the 458 nm argon laser line. To optimize the resolution of the CLSM, we set the size of the pinhole to 50–60% of its optimal size to match the Airy disk diameter of the lens used. Sampling of raster points was according to the Nyquist's sampling theorem (for example, 1024×1024 for a $\times 2$ zoom, 512×512 for a $\times 4$ zoom). The voltage of the photomultiplier tube was set to 460–560 V. Maximum intensity projections of an appropriate number of optical slices (0.5–1 μm deep) were applied to visualize the lichen sections. Up to 20 scans per optical slice were averaged to improve the image quality and to reduce noise.

Confocal stacks containing signals from three different fluorescent simultaneously were analyzed by z-projection and 3D rendering with the software Imaris 6.0.0 (Bitplane, Zurich, Switzerland). Images containing large amount of unidentifiable autofluorescent objects were discarded. Three-dimensional rendering was carried out by both analyses of extended volumes of the original fluorescent signals and reconstruction of artificial objects (isosurfaces and points).

For every FISH probe staining, 15–30 pictures obtained at least two different thalli and two different FISH experiments per lichen species were used to calculate the number of cells per volume of dry lichen. Every picture is the maximum z-projection of a confocal stack formed by 5–60 confocal planes showing the whole section of an undamaged part of the thallus. The software ImageJ (http://rsbweb.nih.gov/ij/docs/index.html) implemented with suitable plug-ins was used to count the fluorescent cells and to calculate the lichen volume in every picture.

Molecular fingerprints of lichen-associated bacterial communities

Fingerprinting of the ecto- and endolichenic bacterial communities by SSCP was carried out as described by Schwieger and Tebbe (1998). Microorganisms of the lichen communities were extracted by mechanical disruption and homogenization of 400 μg material in a FastPrep Instrument (BIO101 Systems; Qbiogene, Carlsbad, CA, USA) for 30 s at speed 5.0. DNA was purified by the GENECLEAN Turbo kit (Qbiogene) containing the special binding buffer guanidine thiocyanate for removal of humic acids. Bacterial 16S rDNA fragments (positions 515–927 according to *Escherichia coli* rDNA sequence numbering) were amplified by PCR with the primers Unibac-II-515f and Unibac-II-927rP (Berg *et al.*, 2005). *Alphaproteobacteria* were selectively amplified with primers ADF 681F/1492r (5′-AGTGTAGAG GTGAAATT-3′/5′-TACGGYTACCTTGTTACGACTT-3′) followed by a second PCR with the primers ADF 681F/927r (5′-CCCGCTAATTYMTTTGACTT-3′) (Blackwood *et al.*, 2005). For specific patterns of *Burkholderia*, a double-nested PCR was applied using the following primer pair Eub1/Eub2 (5′-GAGTTTGATCCTGGCTCAG-3′/5′-AGAAAGGAGGT GATCCAGCC-3′), BKH143Fw/BKH1434Rw (5′-TGGG GGATAGCYCGGGG-3′/5′-TGCGGTTAGRCTASCYAC T-3′) and the eubacterial primer pair Unibac-II-515f and Unibac-II-927rP. *Pseudomonas* was investigated by the primer pairs F311Ps/1459rPs-P (5′-CTGGTC TGAGAGGATGATCAGT-3′/5′-AATCACTCCGTGGTA AACGT-3′) and Unibac-II-515f and Unibac-II-927rP

(Opelt and Berg, 2004), respectively. *nifH* fragments were obtained with nifH3/19F (5′-ATAGTTAGTTA GCTGCAGCTGCAGTA-3′/5′-GCXATTCTTACTGGXA AAG-3′) and nifH11/nifH22 (5′-GACTCCAGCTAA AGGCAGCTGACTC-3′/5′-AAGTATGCCATCATCTT CAGCC-3′) (Yeager *et al.*, 2004).

The amplicons were separated using the TGGE Maxi System (Biometra, Göttingen, Germany) and visualized by silver staining. Computer-assisted evaluation of bacterial community profiles obtained by SSCP was performed by using the GelCompar program version 4.1 (Applied Maths, Kortrijk, Belgium). Sequence-confirmed bands of plastid origin were excluded from further analysis. The pair-wise similarities of lanes were calculated for each gel by Pearson's correlation. To validate the significance of differences between separated groups, we performed the permutation test according to Kropf *et al.* (2004).

Bacterial isolation and culturing

For the isolation of ecto- and endolichenic bacterial communities, 2–4 g of *C. arbuscula* and *U. cylindrica* were transferred to a sterile stomacher bag. Extraction of the lichen-associated bacteria was carried out as described by Opelt and Berg (2004). Bacteria from *L. polytropa* were isolated by scraping about 0.2 g lichen from the rock before suspending in NaCl solution (0.85%) and vortexing for 5 min. For the isolation of the endolichenic bacterial communities of *C. arbuscula* and *U. cylindrica*, the lichens were surface-sterilized in 20% hydrogen peroxide (H_2O_2) for 5 min followed by three washes for 5 min in sterile 0.85% NaCl solution. The surface-sterilized material was imprinted on nutrient agar as a sterility check. To each sample, 2 ml of sterile NaCl solution (0.85%) was added and all samples were homogenized with mortar and pestle. Suspensions containing material from *L. polytropa* as well as suspensions obtained by extracting the ecto- and endolichenic bacterial fraction of *C. arbuscula* and *U. cylindrica* were serially diluted and plated onto nutrient-poor R2A agar and incubated at 20 °C for 5 days. Between 100 and 150 colonies of for each lichen were randomly selected, purified and stored in broth containing 15% glycerol at −70 °C.

Screening for functions of cultured strains

Macromolecular hydrolytic activity. Chitinase activity (β-1,4-glucosamine polymer degradation) was tested on chitin minimal medium by the method described by Chernin *et al.* (1995). Clearing zones were detected 5 days after incubation at 20 °C. β-Glucanase activity was tested by using chromogenic (azurine-dyed, cross-linked) substrates (Megazyme, Bray, Ireland). Formation of blue halos was recorded until 5 days after incubation. Protease activity (casein degradation) was determined from clearing zones on skim milk agar (50 ml of sterilized skim milk mixed at 55 °C with 50 ml of one-fifth tryptic soy agar and 4% agar) 5 days after incubation at 20 °C.

Nitrogen fixation. The ability to fix atmospheric nitrogen under *in vitro* conditions was tested similar to Cardinale *et al.* (2006), but here using a semisolid Brown's N-free medium: 5 g glucose, 5 g mannitol, 0.8 g K_2PO_4, 0.2 g $MgSO_4 \times 7H_2O$, 0.15 g $CaCl_2$, 0.04 g $FeSO_4 \times 7H_2O$, 0.005 g $Na_2MO_4 \times 2H_2O$, 0.3 g NEEO Agarose (Carl Roth, Karlsruhe, Germany), distilled water to 1 liter. Bacterial growth on the N-free medium was compared with the growth on Brown's medium amended with an organic nitrogen source ($2\,g\,l^{-1}$ casamino acids) after 1–2 days at 20 °C. Isolates growing in absence of organic nitrogen as fast as on the control medium were denoted as nitrogen-fixing bacteria.

Phosphate solubilization. Antagonistic bacteria were screened for their ability to solubilize phosphate by an *in vitro* plate assay using NBRIP media supplemented with 1.5% agar as described by Nautiyal (1999).

Detection of indole-3-acetic acid. Indole-3-acetic acid (IAA) excretion by bacterial strains was determined by means of a modified colorimetric analysis developed by Sawar and Kremer (1995). Growth medium consisting of glucose (5.0 g), yeast extract (25.0 mg), L-tryptophane (0.2 g) and distilled water (to 1.0 liter) was inoculated with cell material from the preculture (0.5 × TSA, 30 °C, 24 h) to an OD_{600} of 0.5. After cultivation at 20 °C and 150 r.p.m. for 72 h in absence of light, cell-free supernatant was mixed with Salkowski reagent (50.0 mM $FeCl_3$, 35.0% (v/v) perchloric acid) at the ratio of 3:2 and incubated for 30 min in absence of light. IAA concentration was measured photospectrometrically using the microplate reader Spectramax 250 (Molecular Devices, Union City, CA, USA) at 530 nm and quantified using standard curve.

Antagonistic activity. Bacterial isolates were screened for their activity toward fungi and bacteria *Verticillium dahliae* KLEB, *Rhizoctonia solani*, *Botrytis cinerea*, *Phytopthora infestans*, *Candida albicans* as well as against *Erwinia carotovora* and *Staphylococcus aureus* by a dual-culture *in vitro* assay on the current media: Waksman agar containing 5 g of proteose-peptone (Merck, Darmstadt, Germany), 10 g of glucose (Merck), 3 g of meat extract (Chemex, Munich, Germany), 5 g of NaCl (Merck), 20 g of agar (Difco, Detroit, MI, USA) and distilled water (to 1 liter) (pH 6.8); potato-extract glucose agar (PGA) containing 26.5 g of potato extract glucose (Roth), 20 g of agar (Difco) in distilled water (1 liter); pea agar with extract of pea (150 g) boiled for 20 min, 20 g of agar (Difco) and distilled water (1 liter) and LB agar containing 25 g of LB broth (Roth), 20 g of agar and distilled water

(1 liter). Zones of inhibition were measured after 3 and 7 days of incubation at 20 °C according to the method described by Berg *et al*. (2002).

Identification of cultured strains and phylogenetic analysis

Bacterial genomic DNAs were extracted by the lysozyme–proteinase K–sodium dodecyl sulfate method (modified by increasing the reagent concentration to 2.4 mg ml^{-1} of lysozyme, 0.5 mg ml^{-1} of proteinase K and of 0.8% of sodium dodecyl sulfate). Amplified ribosomal DNA restriction analysis (ARDRA) was performed with the endonuclease *Hha*I. Representatives of individual cultured strains as assessed by ARDRA were identified by PCR amplification of the 16S rDNA fragment between positions 14 and 907 (according to *E. coli* 16S rDNA sequence numbering) and sequencing using the primers Eub1 (5′-AGATTTGATCMTGGCT CAG-3′) and 907r (5′-CCGTCAATTCMTTTGAGTTT-3′). PCR products were purified with the GENECLEAN Turbo kit (BIO101 Systems; Qbiogene). Complementary strands of DNA were sequenced using the BigDye Terminator Ready Reaction kit (Applied Biosystems, Norwalk, CT, USA) and sequencing reactions were separated on an ABI 310 automated sequencer (Applied Biosystems). Relationships were assessed by BLAST searches. Sequences were submitted to GenBank (accession nos. FN298879–FN298916).

The partial sequences of the 16S rRNA genes from our isolates, the best BLAST-matching sequences and some reference sequences were aligned by ClustalX (Thompson *et al.*, 1994). A 550 nt multialignment was obtained and the PHYLIP package (Felsenstein, 2004; http://evolution.genetics.washington.edu/phylip.html) was used for producing the bootstrap replicates (program SeqBoot), for inferring the maximum likelihood tree (program DNAml), the neighbor-joining (programs dnadist and neighbor) and for calculating the consensus trees (program consense). The phylogenetic tree was constructed with the Neighbor software of the PHYLIP package. TreeView (Page, 1996; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) was used for the visualization of the trees.

Results

Fluorescence in situ *hybridization*

Functionally distinct layers of lichens are differentially colonized by bacteria. We investigated the location and abundance of bacteria directly in the stratified lichen thalli using CLSM. Reindeer lichens of the genus *Cladonia* form mycelial cylinders with the photosynthetic algal partners exposed on their external surface. In contrast, bacteria form morphologically diverse, biofilm-like communities on the internal surface of these cylinders (Figure 2a), and much fewer bacteria colonize the external surface. Semi-automated quantification of CLSM images uncovers an abundance that ranged from 10^4 to 10^6 bacteria per cubic millimeter of lichen volume (*C. arbuscula*: $1.2 \times 10^6 \pm 0.2$, *L. polytropa*: $7.4 \times 10^5 \pm 1.2$ and *U. cylindrica*: $9.0 \times 10^5 \pm 2.0$). We found similar abundances in primordial tips of the thalli as well as in their senescing bases. FISH with group-specific probes reveal a prevalence of *Alphaproteobacteria* (45–75%) in these communities, whereas other groups of bacteria were present at lower abundances. Bacteria colonized not only the surface of the extracellular polysaccharides, but also immersed into the intercellular gelatinous matrix (Figure 2b). In *U. cylindrica* bacteria were highly abundant in shallow pits on the lower surface, well separated from the algal partners (Figure 2c,d), and in the crustose *L. polytropa*, these form dense clusters between hyphal strands and algae (Figure 2e, f).

Microbial fingerprints of lichen-associated bacterial communities

PCR-SSCP fingerprints of bacterial communities from the three lichen species were compared for both universal bacterial and group-specific fingerprints (*Alphaproteobacteria*, *Pseudomonas*, *Burkholderia*). Specific primers for *Alphaproteobacteria*, as well as the genus-specific primers for either *Burkholderia* or *Pseudomonas* revealed complex SSCP patterns: *Burkholderia* and *Pseudomonas* communities are highly diverse in all lichens, whereas more similarity throughout was found for *Alphaproteobacteria*. The qualitative analysis of microbial fingerprints showed specific patterns for each of the investigated lichens. However, specificity was different and depended on the primer system used. The application of universal primer for bacteria resulted in statistically significant differences at both sampling times (*C. arbuscula—U. cylindrica* $P \leqslant 0.0029$, 0.0021; *C. arbuscula—L. polytropa* $P \leqslant 0.0115$, 0.0360; *U. cylindrica—L. polytropa* $P \leqslant 0.0108$, 0.0118). This is shown in Figure 3, which represents the cluster analysis for the first sampling time. These species-specific patterns were confirmed by using primers targeting *Alphaproteobacteria* (*C. arbuscula—U. cylindrica* $P \leqslant 0.0046$, 0.0021; *C. arbuscula—L. polytropa* $P \leqslant 0.0286$, 0.0118; *U. cylindrica—L. polytropa* $P \leqslant 0.0356$, 0.5957) as well as *Burkholderia* (*C. arbuscula—U. cylindrica* $P \leqslant 0.0043$, 0.0021; *C. arbuscula—L. polytropa* $P \leqslant 0.0116$, 0.0372; *U. cylindrica—L. polytropa* $P \leqslant 0.0108$, 0.2506). In contrast, the applications of *Pseudomonas*-specific primers showed no distinct patterns. Two fractions of the lichen thallus (endo- and ectolichenic) were investigated (with exception of *Lecanora*). The endolichenic microbial community, which was obtained after surface sterilization of lichens, was generally more specific than those which contain also bacterial communities from the lichen surface. Furthermore, the bacterial

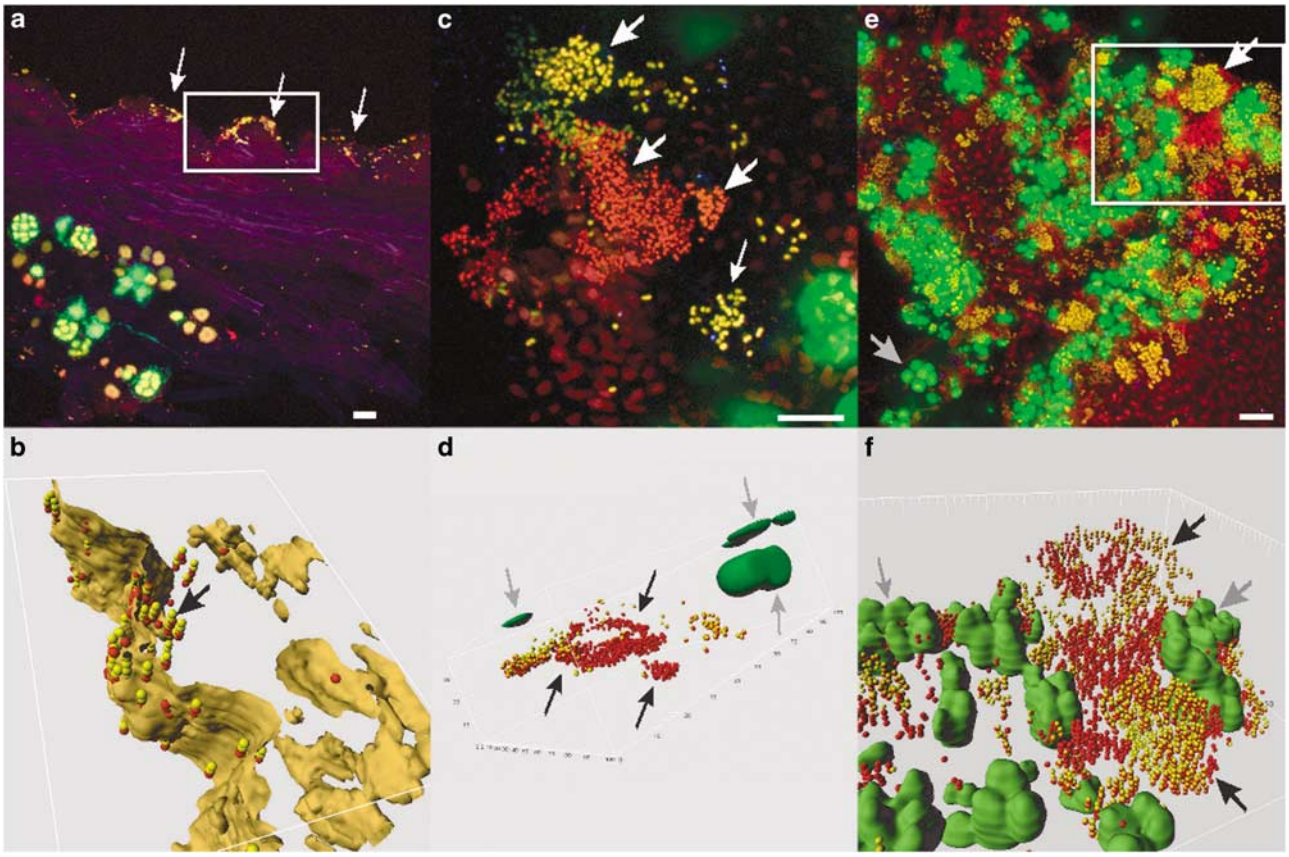


Figure 2 Localization of bacteria in lichens by confocal laser-scanning microscopy (CLSM) and fluorescence *in situ* hybridization (FISH). Yellow: *Alphaproteobacteria*, red: other bacteria, green: eukaryotic algal cells, blue/pink: fungal hyphae. (**a**) Colonization of inner surfaces in *C. arbuscula* (arrow). (**b**) 3D reconstruction from the framed region in (**a**) shows bacteria below the surface of extracellular polysaccharides (arrow). (**c**) Lower surface of *U. cylindrica* with dense clusters of bacteria (arrow). (**d**) 3D reconstruction of (**c**) reveals bacterial community (black arrow) growing well separated from the algal cells (gray arrow). (**e**) Basal parts of *L. polytropa* with mixture of algal cells (gray arrow) and bacterial colonies (white arrow). (**f**) 3D reconstructions of the framed region in (**e**) with bacteria growing (black arrow) abundantly among the algal colonies (gray arrow). Scale bar = 15 μm.

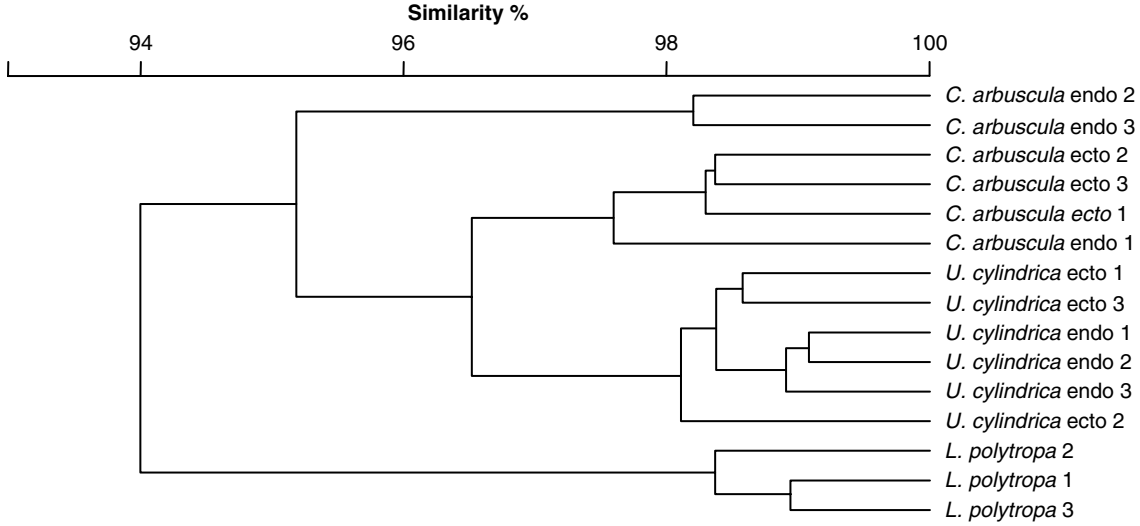


Figure 3 Similarities of single-strand conformation polymorphism (SSCP) profiles from total bacterial communities in lichens. Ecto- and endolichenic bacterial communities designated endo and ecto, respectively, were analyzed from *C. arbuscula* and *U. cylindrica.*

community of the soil-inhabiting reindeer lichen *C. arbuscula* was more distinct from the more similar communities of the rock-inhabiting *U. cylindrica* and *L. polytropa*. In addition, SSCPs using *nifH*-specific primers indicate the presence of nitrogenase genes in the total bacterial fraction. Our sequence

Table 2 Identification and taxonomic classification of DNA bands separated by SSCP from different lichens

No.	*Origin: lichen species*	*Primer in SSCP*	*Closest NCBI database match and accession number*	*Similarity (%)*
N1	*C. arbuscula*	*NifH gene*	*Beijerinckia derxii* subsp. *derxii* AJ563940	86
N3	*C. arbuscula*	*NifH gene*	*Paenibacillus durus* AJ515294	87
A3	*C. arbuscula*	*Alphaproteobacteria*	*Acidisphaera rubrifaciens* D86512	95
N4	*U. cylindrica*	*NifH gene*	*Stenotrophomonas maltophilia* DQ431165	94
N7	*U. cylindrica*	*NifH gene*	*Methylocystis echinoides* AM110703	88
B6	*U. cylindrica*	*Alphaproteobacteria*	*Uncultured Gluconacetobacter* sp. DQ499979	95
B8	*U. cylindrica*	*Alphaproteobacteria*	*Acidisoma tundrae* AM947652	96
B9	*U. cylindrica*	*Alphaproteobacteria*	*Uncultured Sphingomonas* sp. AM944544	100
B11	*U. cylindrica*	*Alphaproteobacteria*	*Acidisoma sibirica* AM947654	97

data show that *nifH* genes present in all lichens are from *Alphaproteobacteria*, *Gammaproteobacteria* and *Firmicutes* (Table 2). Sequencing of excised SSCP bands showed that most *Alphaproteobacteria* belong to strains of *Acetobacteraceae* (for example, *Gluconacetobacter* and *Acidisoma*), but others were also present (*Sphingomonas*) (Table 2).

Bacterial abundances in lichens

The counts of colony-forming units (CFU) in the two sampling were in agreement with high abundances of bacteria found by microscopy in the soil-inhabiting *C. arbuscula* ($3.4–4.7 \times 10^7$ CFU per gram) and in the rock-inhabiting *U. cylindrica* ($1.3–2.1 \times 10^7$ CFU per gram). Lower counts were found in *L. polytropa* ($1.6–4.1 \times 10^4$ CFU per gram).

Identification of cultured strains

To identify the bacterial strains, partial 16S rRNA gene sequences were used to reconstruct a phylogenetic tree (Figure 4). In contrast to the microscopic observations that showed a dominance of *Alphaproteobacteria*, a broad phylogenetic spectrum of the culturable fraction was found. Altogether, 12 bacterial genera were identified: *Acinetobacter*, *Bacillus*, *Burkholderia*, *Frondicola*, *Leifsonia*, *Luteibacter*, *Methylobacterium*, *Microbacterium*, *Micrococcus*, *Paenibacillus*, *Pseudomonas* and *Sphingomonas*. Data obtained from cultured strains assign the ubiquity strains to *Acinetobacter*, *Bacillus*, *Burkholderia* and *Paenibacillus*, whereas other bacteria were less abundant (for example, strains assigned to *Frondicola*, *Luteibacter*, *Methylobacterium* as well as unnamed and potentially new lineages) or retrieved from one sampling time only (for example, *Pseudomonas* and *Leifsonia*). The group including Actinobacteria-related isolates seem to be the most complex and less stable but it was not possible in this work to obtain a consistent phylogenetic inference, due to the fact that most of our actinobacterial isolates showed the same similarity with different known species and the best BLAST-matching sequences refer to unidentified environmental strains (data not shown).

About one-tenth of the culturable colonies retrieved from the lichen species ($N = 261$) were able to grow both on semisolid N-free and on nutrient-rich medium. Many of our culturable strains displayed lytic activities (proteolytic: 50%, chitinolytic: 14%, glucanolytic: 29%), whereas 23% of the strains showed phosphate-solubilizing activity. In addition, 21% of our cultured strains produced the growth-promoting hormone IAA. The strains were also tested regarding their *in vitro* antagonism toward other microorganisms. In general, a low antagonistic activity was found for the lichen-associated bacteria. The proportion of antagonistic isolates was 4.2% against *B. cinerea* and 2.3% against *V. dahliae* (both plant pathogenic ascomycetes), 1.5% against *R. solani* (plant pathogenic basidiomycetes), 3.4% against *P. infestans* (plant pathogenic oomycetes), 2% against *S. aureus* (Gram-positive, human pathogenic bacterium) and 1.5% against *E. carotovora* (Gram-negative, plant pathogenic bacterium).

Discussion

Our data present lichens as more complex symbiotic systems than thought previously. According to the traditional view of functions, lichen photobionts provide carbohydrates to their fungal partners, which develop the structural framework rendering

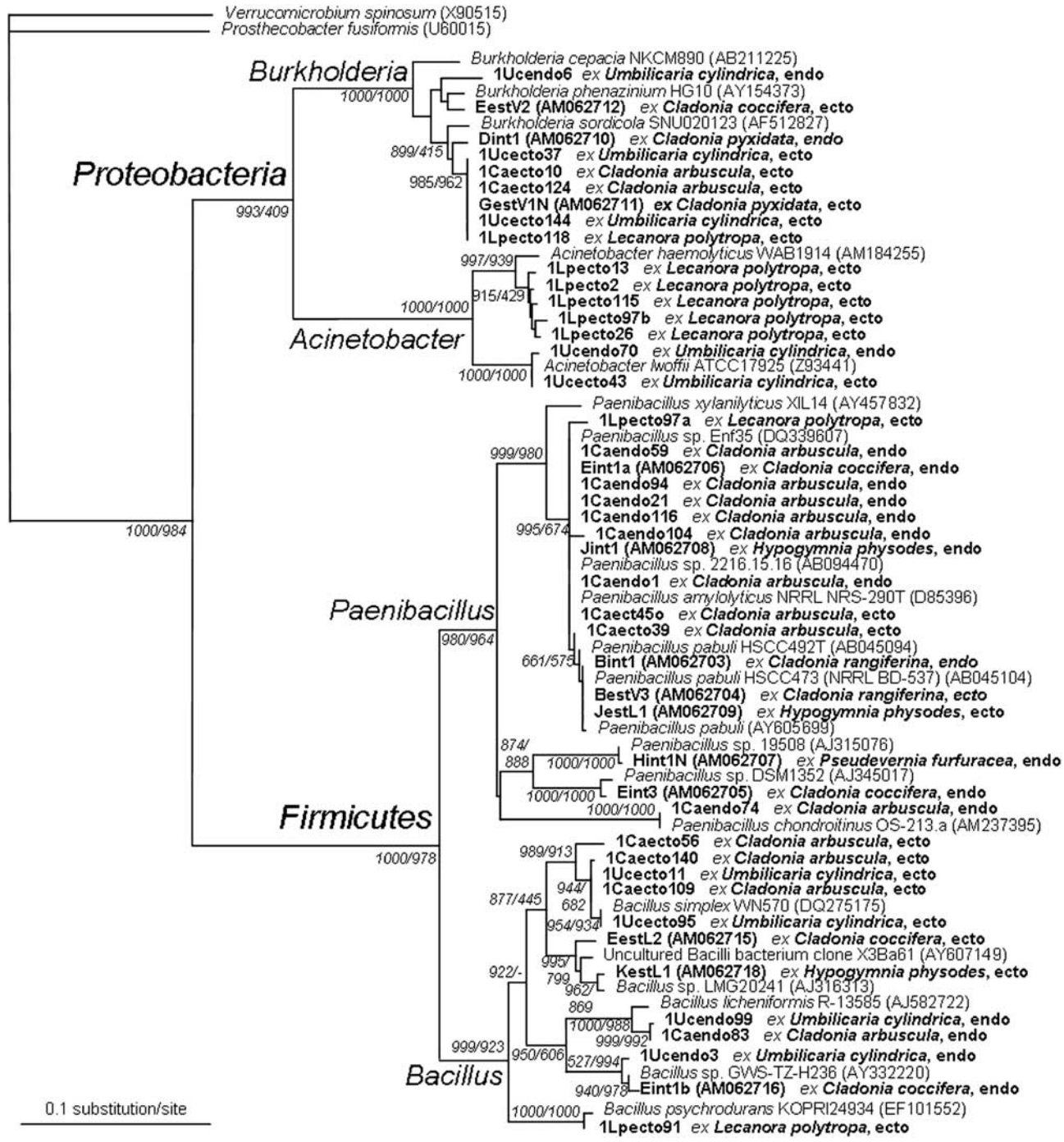


Figure 4 Maximum likelihood tree based on 16S rRNA gene multialignment (550 nt) showing the phylogenetic relationships between sequences of lichen-associated bacteria, the correspondent best BLAST-matching sequences and some reference sequences. Sequences from lichen-associated bacteria are highlighted in bold; lichen host and location (ecto- or endophytic) are indicated after the isolate name. The sequences from lichen-associated bacteria including an accession number were obtained from Cardinale *et al.* (2006). The numbers at node positions indicate the bootstrap values (calculated by maximum likelihood/neighbor-joining methods) out of 1000 resampling of the data. Roots of genus clades are labeled at the correspondent nodes.

optimal performance of the entire symbiotic system. In addition to the two keystone partners, lichens can benefit from complementary functions of associated bacterial communities as microsymbionts. For example, diazotrophic bacteria potentially complement the nitrogen budget in lichens with eukaryotic algae. Diazotrophic bacteria in lichens are not restricted to a particular group. We found diverse *nifH* sequences representative for different bacterial branches: *Gammaproteobacteria*, and *Firmicutes* but with a dominance of *Alphaproteobacteria*. All identified *nifH* genes belong to species that are well known for symbiotic nitrogen fixation and their beneficial and endophytic interaction with

plants resulting in plant growth promotion (Ryan *et al*., 2008). For tropical lichens, dot-blot detection of *nifH* genes in strains from N-free enrichment cultures as well as positive acetylene reduction (Liba *et al*., 2006) suggests widespread potential and importance of bacterial nitrogen fixation in green-algal lichens. The proportion of bacteria with lytic activities was higher than found with plant-associated bacteria (Berg *et al*., 2002) whereas antifungal activity was lower (less than 5% of the strains). We hypothesize that lytic bacteria are involved in nutrient cycling in the lichen thalli, although metabolite fluxes in the natural lichens are not yet quantified. The occurrence of a substantial proportion of auxin-producing bacteria in the lichen symbiosis supports the intimate interaction between the bacterial community and the lichen fungus because the phytohormone auxin has not only a function in plant–microbe interaction but also on fungal growth (Grube and Berg, 2009).

Extracellular polysaccharides and cell wall components of lichen fungi are lasting nutrient sources, which are also exploitable when the fungal and algal partners are metabolically inactive. Differences in the composition and abundance of bacteria in lichens are apparently regulated by still unknown mechanisms that also help to protect against invasions by pathogenic bacteria. Diverse secondary metabolites with antibacterial activities known from lichens potentially contribute to this function (Boustie and Grube, 2005). The dibenzofurane usnic acid is active against Gram-positive bacteria (Ingólfsdóttir, 2002) and strongly influences bacterial biofilm formation (Francolini *et al*., 2004). This agrees with the low abundance of bacteria on usnic acid-containing external surfaces in growing upper parts of *C. arbuscula*, whereas the degrading basal parts were richly colonized also on the external side (Cardinale and Steinova, personal communication).

The growth of most lichens is indeterminate and not limited by age-specific death rates under undisturbed environmental conditions (for example, absence of grazing animals or air pollution). Parts of a lichen thallus can then locally senesce, whereas biomass increases in actively growing parts. Recycling and translocation of components are of key importance for robustness and longevity of lichens in nutrient-poor habitats. Such parsimonious management of resources will be of particular importance for surface-detached lichens with little contact to the underlying substrate. *Umbilicaria* species connect to the substrate only with a narrow central holdfast, whereas shrub-like thalli of reindeer lichens have senescing basal parts and completely lack a soil-exploiting mycelium. Experimental studies demonstrated the translocation of fixed nitrogen compounds from the base to the tip in mat-forming reindeer lichens (Ellis *et al*., 2005). We argue that lytic, N-fixing and phosphate-solubilizing bacteria may significantly enhance the mobilization of nutrients. The mobilized nutrients may originate either from the lichen itself or from the supporting substrate. Recent experiments showed increased solubilization of mineral substrate in co-cultures of an isolated lichen fungus with N-fixing *Bradyrhizobium elkanii* (Seneviratne and Indrasena, 2006). Efficient rock weathering by cooperating bacteria and fungi could explain the abundance of bacterial communities in basal parts of *L. polytropa*.

The analogy proposed by Farrar (1985), that lichens be viewed as self-contained ecosystems, is here extended to include associated, nonphotosynthetic bacterial communities as multifunctional partners in the symbiotic assembly. This adds to recent amendments in other symbiotic systems, for example, sponges and corals, but also invertebrates, which now turn out to be complex multisymbioses (Hunter, 2006). The abundance of bacteria and their potential function in these long-living holobionts will initiate more detailed studies on further aspects of these unique associations, including their evolution and biogeographic patterns, and also the function of particular bacteria for the entire lichen holobiont. Products of some lichen-associated bacteria were shown to be potent antibiotics at very low concentrations (Davies *et al*., 2005), which suggests that also less abundant strains could have significant functional role in the lichen microecosystem. The evolutionary diversification of lichens is characterized by adaptation to an extremely wide range of environments in which bacterial communities are involved. We suppose that bacterial strains that maintain the lichen symbiosis in different ecological situations might in a long run assist in the ecological adaptation of lichens. Thus, lichen evolution could to some extent be pushed by bacterial powers. On the other hand, lichens as unique habitats could promote bacterial diversification, especially in dense biofilm-like structures (Hansen *et al*., 2007). Lichens can also tolerate extreme abiotic stressors (including extreme climates, salt, radionuclides and so on) and accumulate toxic compounds (heavy metals and so on). We are therefore convinced that they may turn out as hot bed of bacterial diversity and as sources of biotechnologically interesting strains, compounds and enzymes (Davies *et al*., 2005; Gonzáles *et al*., 2005).

Acknowledgements

We thank David Hawksworth, Ilse Kranner and Martin Hagemann for comments. This research was funded by a grant from the Austrian Science Foundation (FWF) to MG and GB.

References

Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry

for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919–1925.

Berg G, Krechel A, Ditz M, Faupel A, Ulrich A, Hallmann J. (2005). Comparison of endophytic and ectophytic potato-associated bacterial communities and their antagonistic activity against plant pathogenic fungi. *FEMS Microbiol Ecol* **51**: 215–229.

Berg G, Roskot N, Steidle A, Eberl L, Zock A, Smalla K. (2002). Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. *Appl Environ Microbiol* **68**: 3328–3338.

Blackwood CB, Oaks A, Byuer JS. (2005). Phylum- and class-specific PCR primers for general microbial community analysis. *Appl Environm Microbiol* **71**: 6193–6198.

Boustie J, Grube M. (2005). Lichens—a promising source of bioactive secondary metabolites. *Pl Gen Res* **3**: 273–287.

Cardinale M, Puglia AM, Grube M. (2006). Molecular analysis of lichen-associated bacterial communities. *FEMS Microbiol Ecol* **57**: 484–495.

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

Chernin L, Ismailov Z, Haran S, Chet I. (1995). Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Appl Environ Microbiol* **61**: 1720–1726.

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

Davies J, Wang H, Taylor T, Warabi K, Huang X-H, Andersen RJ. (2005). Uncialamycin, a new enediyne antibiotic. *Org Lett* **7**: 5233–5236.

Denton GH, Karlén W. (1973). Lichenometry: its application to Holocene moraine studies in Southern Alaska and Swedish Lapland. *Arctic Alpine Res* **5**: 347–372.

Ellis CJ, Crittenden PD, Scrimgeour CM, Ashcroft CJ. (2005). Translocation of ^{15}N indicates nitrogen recycling in the mat-forming lichen *Cladonia portentosa*. *New Phytol* **168**: 423–434.

Erhart RD, Bradford RJ, Seviour R, Amann RI, Blackall LL. (1997). Development and use of fluorescent *in situ* hybridization probes for the detection and identification of *Microthrix parvicella* in activated sludge. *Syst Appl Microbiol* **20**: 310–318.

Farrar JF. (1985). The lichen as an ecosystem: observation and experiment. In: Brown DH, Hawksworth DL, Bailey RH (eds). *Lichenology: Progress and Problems*. Academic Press: London, pp 385–406.

Felsenstein J. (2004). PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author Department of Genome Sciences, University of Washington, Seattle.

Francolini I, Norris P, Piozzi A, Donelli G, Stoodley P. (2004). Usnic acid, a natural antimicrobial agent able to inhibit bacterial biofilm formation on polymer surfaces. *Antimicrob Agents Chemother* **48**: 4360–4365.

Gonzáles I, Ayuso-Sacido A, Anderson A, Genilloud O. (2005). Actinomycetes isolated from lichens: evaluation of their diversity and detection of biosynthetic gene sequences. *FEMS Microbiol Ecol* **54**: 401–415.

Grube M, Hawksworth DL. (2007). Trouble with lichen: the re-evaluation and re-interpretation of thallus form and fruit body types in the molecular era. *Mycol Res* **111**: 1116–1132.

Grube M, Berg G. (2009). Bacterial associations with fungi, focusing on the lichen symbiosis. *Fungal Biol Rev* (in press).

Hansen SK, Rainey RB, Haagensen JAJ, Molin S. (2007). Evolution of species interactions in a biofilm community. *Nature* **445**: 533–536.

Hawksworth DL. (1988). The variety of fungal–algal symbioses, their evolutionary significance, and the nature of lichens. *Bot J Linn Soc* **96**: 3–20.

Henkel PA, Plotnikova TT. (1973). Nitrogen-fixing bacteria in lichens. *Izv Akad Nauk Ser Biol* **1973**: 807–813.

Henkel PA, Yuzhakova LA. (1936). Nitrogen-fixing bacteria in lichens. *Izv Biol Inst Permsk Gos Univ* **10**: 9–10.

Hunter P. (2006). Entente cordiale: multiple symbiosis illustrates the intricate interconnectivity of nature. *EMBO Rep* **7**: 861–864.

Ingólfsdóttir K. (2002). Usnic acid. *Phytochem* **61**: 729–736.

Iskina RY. (1938). On nitrogen fixing bacteria in lichens. *Isv Biol Inst Permsk* **11**: 133–139.

Kneip C, Lockhart P, Voss C, Maier UG. (2007). Nitrogen fixation in eukaryotes—new models for symbiosis. *BMC Evol Biol* **7**: 55.

Kropf S, Heuer H, Grüning, Smalla K. (2004). Significance test for comparing complex microbial community fingerprints using pairwise similarity measures. *J Microbiol Methods* **57**: 187–195.

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

Lenova LI, Blum O. (1983). To the question on the third component of lichens. *Bot J* **68**: 21–28.

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

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

Lutzoni F, Pagel M, Reeb V. (2001). Major fungal lineages are derived from lichen symbiotic ancestors. *Nature* **411**: 937–940.

Manz W, Amann R, Ludwig W, Wagner M, Schleifer KH. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst Appl Microbiol* **15**: 593–600.

Meier H, Amann R, Ludwig W, Schleifer KH. (1999). Specific oligonucleotide probes for *in situ* detection of a major group of Gram-positive bacteria with low DNA G1C content. *Syst Appl Microbiol* **22**: 186–196.

Nautiyal CS. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol Ecol* **170**: 265–270.

Opelt K, Berg G. (2004). Diversity and antagonistic potential of bacteria associated with bryophytes from

nutrient-poor habitats of the Baltic Sea Coast. *Appl Environ Microbiol* **70**: 6569–6579.
Page RD. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**: 357–358.
Panosyan AK, Nikogosyan VG. (1966). The presence of *Azotobacter* in lichens. *Akad Nauk Armian SSR Biol Zhurn Armen* **19**: 3–11.
Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN. (2008). Bacterial endophytes: recent developments and applications. *FEMS Microbiol Lett* **278**: 1–9.
Sawar M, Kremer RJ. (1995). Determination of bacterially derived auxins using a microplate method. *Lett Appl Microbiol* **20**: 282–285.
Schwieger F, Tebbe CC. (1998). A new approach to utilize PCR-single-strand-conformation polymorphism for 16S rRNA gene-based microbial community analysis. *Appl Environ Microbiol* **64**: 4870–4876.
Scott GD. (1956). Further investigations of some lichens for fixation of nitrogen. *New Phytol* **55**: 111–116.
Seneviratne G, Indrasena IK. (2006). Nitrogen fixation in lichens is important for improved rock weathering. *J Biosci* **31**: 639–643.
Thompson JD, Higgins DG, Gibson TJ. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–4680.
Wallner G, Amann R, Beisker W. (1993). Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14**: 136–143.
Yeager C, Kornosky JL, Housman DC, Grote EE, Belnap J, Kuske CR. (2004). Diazotrophic community structure and function in two successional stages of biological soil crusts from the Colorado plateau and Chihuahuan desert. *Appl Environ Microbiol* **70**: 973–983.
Yuan X, Xiao S, Taylor TN. (2005). Lichen-like symbiosis 600 million years ago. *Science* **308**: 1017–1020.

Manuscript I

Lichens represent a rich reservoir for bacteria with biotechnological potential

Joao Vieira de Castro Junior[1], Ilona Gasser[1], Henry Müller[1], Martin Grube[2] and Gabriele Berg[1*]

[1]Graz University of Technology, Institute of Environmental Biotechnology, Petersgasse 12, A-8010 Graz, Austria

[2]Karl-Franzens University of Graz, Institute of Plant Sciences, Holteigasse 6, A-8010 Graz, Austria

***Corresponding author:** Gabriele Berg

Tel. ++43 316 873 8310

Fax: ++43 316 873 8199

Email: gabriele.berg@tugraz.at

Submitted to: Applied Microbiology and Biotechnology

Abstract Recent studies showed that lichens harbour high abundances and diversity of bacteria but their potential for applications in biotechnology is still unexplored. Bacteria associated to the lichens *Cladonia arbuscula, Umbilicaria cylindrica, Lecanora polytropa* and *Lobaria pulmonaria* were characterised regarding their biotechnological potential by biochemical and molecular assays. A set of 263 selected strains were tested for i) their antagonistic activity towards plant and human pathogenic microorganisms ii) their lytic abilities, iii) solubilization of phosphate, iv) mobilization of iron, and v) production of auxin. Additionally, 87 strains were tested for accumulation of biopolymers (Polyhydroxyalkanoates, PHAs). Furthermore, the production of *N*-acyl-homoserine lactones (AHLs) was also tested. Results showed a remarkable low antagonistic activity against pathogens; a proportion of 3.4% expressed antibacterial activity, while 7.8% of the isolates were able to suppress fungal growth. The highest proportion of antagonists was found against the plant pathogenic fungus *Botrytis cinerea* while no antagonism was shown towards *Candida albicans*. In contrast, the analysed strains showed a high hydrolytic activity: proteolytic (47%), chitinolytic (29.2%), glucanolytic (29.3%), and lipolytic (20%). In addition, a high proportion of bacteria produced metabolites involved in growth promotion such as phosphate-solubilizing activity (16.7%); the ability to excrete siderophores (48%), and produce auxin (21%). Surprisingly only few bacterial strains produced the AHL signaling molecules. One third of the lichen-associated bacteria have the potential to produce PHAs. According to these first results, bacteria from lichens have a considerable biotechnological potential which can be explained by putative functions in the lichen mini-ecosystem.

Key words: lichens, bacteria, biotechnological potential, PHA, antagonism

Introduction

The microbial world is the largest unexplored reservoir of biodiversity which exists in diverse ecological niches, including extreme environments. Eukaryotic multicellular organisms, although more remarkable, are limited in their biochemical capacities (Haygood et al. 1999). Exploration of microbial diversity holds great promise because of the role of microbes in nutrient cycling, environmental detoxification and novel metabolic abilities and products in pharmaceutical and industrial area (Lal and Tabacchioni 2009). There has been a growing interest in the occurrence and the biotechnological potential of bacteria in so far unexplored biotopes, including extreme environment, or microbes involved in symbiotic associations of multicellular organisms. Detailed scientific information about the microbiota of these habitats is not well understood so far. As these comprise also unique ecosystems, they likely contain novel microorganisms with interesting properties (Hallmann et al. 1997). Currently, the primary focus on this kind of research is to detect biologically non-harmful products, such as nutrients for plants, new drugs and enzymes, as well as food additives (Assumpção et al. 2009).

Lichens represent symbiotic life-styles of fungi with a photosynthetic partner (algae and/or cyanobacteria). Recent studies based on cultivation (Gonzáles et al. 2005; Cardinale et al. 2006; Liba et al. 2006) and molecular analysis (Cardinale et al. 2008; Grube et al. 2009) showed that a third group of organisms is involved in the lichen symbiosis. All investigated lichens contain a highly diverse bacterial community, suggesting the expansion the traditional concept of lichens. The lichen-associated bacterial community forms stable biofilm-like layers. Although Alphaproteobacteria are the dominant component in all investigated lichens, the composition of bacteria was specific for the analysed species of lichenised fungi (Grube et al. 2009). Characterisation of cultivable strains and presence of functional genes in the total fraction suggest the involvement of associated bacteria in nutrient cycling.

Lichens represent unique and specific habitats for bacteria. In the course of evolution the association of fungi with algae has created a new morphological organisation with unique properties, the lichen thallus. Even under rather hostile circumstances, the composite thallus can live up to thousands years (Denton and

Karlén 1973; Grube and Hawksworth 2007). Further, more than 1000 secondary metabolites are produced in the lichenised stage and the number of lichen compounds is still increasing (Oksanen 2006; Jack Elix, pers. comm.). Most of them detected till now are produced by the fungal partners. Interesting bioactive properties, including antimicrobial effects, have also been reported for a number of lichen compounds (Boustie and Grube 2005; Boustie et al. in rev.). On the other side, lichens are often pioneering species and can grow with exceptionally low supply of nutrients under extreme environmental conditions (Nash 2008) which are unfavourable for long-term survival of individual partners. Altogether, diverse growth forms, surface types, environmental adaptations and the substantial accumulation of extracellular secondary compounds make lichens a highly diverse and selective habitat for bacteria. However, the biotechnological potential of the bacterial communities integrated in the lichen ecosystem is still unknown.

The objective of this work was to isolate, identify and analyse the biotechnological potential of bacteria associated with lichens, regarding their antagonistic potential against plant and human pathogenic microorganisms based on a dual culture method toward a set of target microorganisms; assessment of exo-enzymatic activity by biochemical assays onto agar plate and production of storage substances as polyhydroxyalkanoates (PHA) under extreme conditions.

Material and Methods

Experimental design; isolation and cultivation of bacteria

The bacteria used in this study were isolated from the lichens species *Cladonia arbuscula, Umbilicaria cylindrica, Lecanora polytropa* and *Lobaria pulmonaria*. The lichens specimens were sampled in Austria (Styria), Portugal (Madeira), Switzerland (St. Gallen) and Norway (Bergen). Fresh material (2-4g) was washed with sterile sodium chloride solution (0.85%) and transferred into a sterile stomacher bag. The samples were suspended in NaCl (0.85%) and homogenised for 3 min by agitation. The samples were serially diluted and plated onto R2A agar medium (Difco, Detroit, USA) for 5 days at 20 ^{0}C (Opelt and Berg 2004). After incubation in R2A medium, the colonies were counted for calculation to determine the mean number of colonies (CFU) based on fresh weight. The bacteria were selected according to their morphological characteristics and purified by cultivation

in Nutrient Agar medium (NA, Difco) for 48 hours at 20 ^{0}C and maintained at -80 ^{0}C in sterile glycerol (50%) solution for subsequently experiments.

Identification of bacteria

A single colony on NA-medium was picked with a sterile toothpick into 50 µl de-mineralised water in a 1.5 ml Eppendorf tube. The cell suspension was heated to 96°C for 10 min. The 30 µl reaction mixture contained 6 µl 5xTaq&Go (MP Biomedicals, Carlsbad, USA), 1.5 µl of primer pair mix EubI-forward (5'-GAG TTT GAT CCT GGC TCA G-3') and 1492r-reverse (5'- TAC GGY TAC CTT GTT ACG ACT T-3') both in a concentration of 10 pmol µl^{-1} and 20-30 ng template. The PCR products were purified with GeneClean Turbo Kit (MP Biomedicals) as recommended by the manufacturer. The fragments were sequenced using the reverse primer 1492r. The microbial community DNA of the lichens were extracted by mechanical disruption and homogenization of 300 mg material in a FastPrep Instrument (BIO 101 Systems: Qbiogene, Carlsbad, CA, USA) for 30 s at speed 5.0. The identification of related strains was done according to sequences-database alignment using BLAST algorithm.

Analysis of antagonistic activity

A dual culture method was used to determine the ability of the isolated lichens strains to inhibit the growth of various species of fungal and bacterial pathogens. Representative isolates of all lichens were tested for their anti-pro/eukaryotic activity against a set of target organisms. These organisms included plant pathogenic (*Rhizoctonia solani, Botrytis cinerea, Verticillium dahliae, Phytophora infestans, Erwinia carotovora*) as well as human pathogenic species (*Candida albicans, Staphylococcus aureus*). These assays were carried out in agar plate containing specific medium for each pathogen, Waksman agar for fungi, and LB medium for bacteria (Berg et al. 2002).

Screening for extracellular compounds

The chitin-degrading ability was tested on chitin minimal medium, and after 5 days the clearing zones were analysed; to investigate proteolytic activity an enzymatic assay with skim milk agar was conducted for 5 days at 20^{o}C. For detection of extracellular glucanase activity a medium with Azuridine dyed cross-linked AZCL

(Megazyme Bay Ireland) was used as substrate. Bacteria were screened for their ability to solubilize phosphate by an *in vitro* plate assay using NBRIP media supplemented with 1.5% agar. A dual culture was performed with the single strains to observe the production of N-Acyl-Homoserine Lactone (AHLs) using *Chromobacterium violaceum* as indicator. Detection of lipase was performed using a universal assay with Tween 80 (polysorbate surfactant) (Opelt and Berg 2004).

Screening of PHA-producing bacteria

Bacterial strains were screened for their genetic potential for PHA (polyhydroxyalkanoate) production and their *in vitro* ability to produce PHAs. The screening was based upon both molecular and microscopic approaches. Colony PCR techniques were employed for screening of bacterial PHA producers isolated from lichens by using a *phaC* specific primer set (Sheu et al. 2000) in a PCR reaction mixture with a total volume of 30 µl consisting of 5x Taq&Go PCR Mastermix (MP Biomedicals), 0.5µM each primer (phaCF1: 5'-ATCAACAA(GGG/A)T(TT/A) CTAC(AA/G)TC(CC/T)T(CC/G)GACCT-3' and phaCR4:5'-GGTAGTTGT (TT/C)GAC(CCC/GG)(AAA/CC)(AAA/CC)(GGG/A)TAG (TTT/G)TCCA-3'), 3% dimethyl sulfoxide (DMSO) and 1 M betaine (Sigma, Vienna). The thermal cycle programme consisted of one cycle of 94 °C for 10 min, 51 °C for 2 min, 72 °C for 2 min, and 35 cycles of 94 °C for 20 s, 57 °C for 45 s, 72 °C for 1 min, a final incubation step at 72 °C for 5 min. As template served 1.0 µl of a DNA preparation obtained by suspending cell material from an over-night culture in 50 µl de-mineralized water and incubating at 96 °C for 10 min. PCR-amplified DNA fragments were observed by gel electrophoresis using 1.0 % agarose gels. Five microliters of each amplification mixture were subjected to agarose gel electrophoresis and ethidium bromid staining. The amplified DNA fragments were visualised by UV illumination. To detect formation of PHA-containing granules microscopically, bacteria were plated onto a solid minimal medium containing high amount of glucose (10 $g.l^{-1}$) and low amount of nitrogen (0.5 $g.l^{-1}$ NH_4Cl) which is optimised for PHB-production. Plates were incubated for at least five days at 30 °C prior cells were stained by sudan black B (Steinbüchel and Oppermann-Sanio 2003).

Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in the EMBL Data Library under accession numbers FN555439 to FN555467.

Results

Bacterial sampling and identification

The microbial community of four lichen species - *Cladonia arbuscula, Umbilicaria cylindrica, Lecanora polytropa* and *Lobaria pulmonaria* - were screened to determine the type and number of viable colonies. The counts of colony-forming units (CFU) observed on the selected lichens showed high abundance of bacteria found in the soil-inhabiting *Cladonia arbuscula* (3.4-4.7 x 10^7 CFU g^{-1}), in the rock-inhabiting *Umbillicaria cylindrica* (1.3-2.1 x 10^7 CFU g^{-1}) and tree-inhabiting *Lobaria pulmonaria* (3.8-5.2 x 10^7 CFU g^{-1}). The number of colonies was rather similar for these three species different species, whereas a lower numbers of bacteria was found in *Lecanora polytropa* (1.6-4.1 x 10^4 CFU g^{-1}).

To identify the bacterial isolates, 263 colonies were chosen for functional screening according to their morphological characteristics and all of them were grouped according to their ARDRA profiles (data not shown). Representatives of each cluster were selected and identified according to partial 16S rDNA sequencing. Altogether, 13 bacterial genera were found: *Pseudomonas*, *Burkholderia*, *Paenibacillus*, *Bacillus*, *Acinetobacter*, *Leifsonia*, *Luteibacter*, *Subtercola*, *Micrococcus*, *Microbacterium*, *Frondihabitans, Kytococcus* and *Methylobacterium* (Table 1).

Screening of the isolates for antagonism

All isolates from different lichens were screened for their potential antagonistic activity against in an *in vitro* dual culture assay. All of the isolates were tested for their ability to produce inhibitory activity against seven target microorganisms. They included four fungi, one Gram-positive bacterium, one Gram-negative bacterium and one yeast species. Among all the different bacterial isolates tested for dual culture plate assay, only a few of the isolates tested were able to suppress the growth of the pathogenic strains (Fig. 1). Antifungal activity was exhibited by 7.8% among all tested isolates towards all the target fungi species. Lower activity was exhibited against the bacterial pathogens strains (3.4%). According to results

observed in solid culture medium, none of the isolates were able to active against the species *Candida albicans*. As demonstrated on antifungal activity, the antibacterial activity of the lichens isolates strains against the bacterial pathogens *S. aureus* and *E. carotovora* was also low, results showed approximately 1.3% of inhibition for both pathogens. Antifungal activity against *B. cinerea* showed best results among all the fungal pathogens tested, antagonistic inhibition was detected by 4.2% of all isolates, followed respectively by *P. infestans* (3.4%), *V. dahliae* (2.3%) and species who showed more resistant to the suppressor substances compared to others fungi was *R. solani* (1.5%).

Production of extracellular metabolites

All isolates in this study were also screened for their *in vitro* metabolic potential (Fig. 2). Lipase activity was observed in 53 strains (20%) by their growth on nutrient agar (NA) medium containing Tween 80. In addition, some strains produced cell wall-degrading enzymes such as proteases (47%), chitinases (29.2%), and -glucanases (29.3%). Siderophore production was exhibited by half of the bacterial isolates (48.3%). Since the direct measurement of phosphate solubilization in medium assays is likely to give more direct and reliable results, the 263 strains were further tested for their ability to solubilize phosphate in NBRIP medium; 44 strains (16.7%) showed phosphatase activity by inducing clear zones around the cells on agar medium. Despite the low number, the ability to produce signalling molecules (AHL) was detected in 1.1% of all the strains tested. Among all tested isolates, 56 strains (21.3%) proved positive for the production of plant growth-promoting hormone, IAA.

Screening of PHA-producing bacteria

Bacterial isolates from lichens species were screened for their potential to produce biopolymers. In this study, we focused on the isolation of microorganisms able to accumulate polyhydroxyalkanoates (PHAs). We used a nucleic acid based method to identify the capability of polyhydroxyalkanoate (PHA) production of lichen-associated bacteria sampled from different geographical regions, based on the amplification of *phaC* gene. The use of total community DNA extracted from the selected lichens in this study (*C. arbuscula*, *U. cilindrica*, *L. polytropa* and *L. pulmonria*) was used as first-line screening for the confirmation of PHA-producing

bacteria inhabiting the thalli (Fig. 3). According to the results obtained by this initial analysis, all the tested lichen species showed the presence of the *phaC* gene.

We then randomly selected 87 cultured strains for analysis of *phaC* genes. Genomic DNA from the isolates was used as templates for PCR reaction. 23 (27%) strains were considered positive (Fig. 4). To confirm the PCR results, bacterial isolates were grown in minimal medium with high amount of glucose ($10g.l^{-1}$) and low amount of nitrogen ($0.5g.l^{-1}$) to observe the formation of intracellular PHA granules by microscopy. Accumulation of polyhydroxyalkanoate granules was found in 28 (32.5%) strains, i.e. in more strains than revealed by PCR. Twelve (14%) strains were considered PHA positives for both approaches (Fig. 4).

Discussion

In this study, we have explored lichens as a source for the isolation of biotechnologically interesting bacteria. First, in the present study, lichen-associated bacteria were isolated from the lichen thalli with the aim of selecting efficient antagonists against pathogens. *In vitro* dual culture assay on culture media was taken as an initial step for isolation of efficient bacterial antagonists for human and plant disease management (Anith et al. 2003). The proportion of antagonistic isolates against the tested pathogens was relatively low. With respect to effects against fungi this situation is plausible, since broad antagonistic effects towards other fungi may also interfere with the fungal partner of the lichen symbiosis. This result clearly contrasts the situation with moss-associated or plant-associated bacteria where a significant number of strains showed antifungal effects (Opelt and Berg 2004, Berg et al. 2002, 2006). For antagonists against plant pathogens, there is general agreement that suppression *in vitro* does not necessarily relate to the same in field conditions (Viswanathan et al. 2003; Berg 2009). The next step will be to test the properties of promising strains in *ad planta* systems.

Furthermore, the number of bacteria with enzymatic activities in lichens is higher than found by Berg et al. (2002) for plant-associated bacteria. It is now well known that fungi can re-allocate fungal material from senescing parts towards growing tips in durable mycelial systems. The lytic activities of bacteria might

assist this phenomenon in the long-living lichen systems. The lichens thallus harbours a great siderophore-producing bacterial diversity. Due to the fact that many lichens are pioneering species and live under low-iron conditions, these iron-catching inhabitants can also contribute to the successful lichen life-style. Kloepper et al. (1980) showed that the excretion of siderophores by bacteria may stimulate plant growth by improving Fe nutrition of the plants. Also Tian et al. (2009) detected a large number of siderophore-producing bacteria under low-iron conditions in the rhizosphere. However, the exchange and flux of iron in the lichen symbiosis has to be detected.

Quorum sensing enables bacteria to regulate expression of certain genes according to population density. In the present study, an approach using dual culture for detecting AHL (*N*-acyl homoserine lactone) production revealed that only a very low number of isolates were able to produce signaling molecules. In contrast, in the rhizosphere, a high amount of AHL-producing isolates was found (Berg et al. 2002).

According to our results lichen thalli represent an interesting reservoir for polyhydroxyalkanoate (PHA)-producing bacteria. Polyhydroxyalkanoates (PHAs) are polymers of hydroxy fatty acids that are naturally produced by many different bacteria as an intracellular carbon and energy reserve material. Accumulation of these polymers under aerobic conditions often occurs when the carbon source is in excess but one or several other nutrients are limited. Under anaerobic conditions, a few facultative aerobic bacteria or anaerobic bacteria have been reported to accumulate PHA (Lillo and Rodriguez-Valera 1990). Gasser et al. (2009) used cultivation-independent methods including microscopy and molecular techniques to detect PHA-producers in bacterial fractions isolated from rhizosphere, which proves to be an interesting and yet unexploited reservoir of these bacteria. In this study, we could show that also lichens present a habitat, which harbours a high proportion of PHA-producers.

Here we started to explore biotechnological potential of a particular biotic niche, the lichen symbioses. In general biotic niches (plant roots, mosses and others) are interesting targets for finding biotechnologically interesting microbes, especially when the hosting organisms have unique ecological properties and adaptations. Furthermore, it is interesting to correlate ecological functions of bacteria with their biotechnological potential. In this report, we could show that

lichen thalli harbour a diverse functional bacterial community, which can be exploited for biotechnology. However, here we only studied the biotechnological potential of culturable strains – metagenomic approaches should be used to assess the whole potential of lichen-associated bacterial communities. .

References

Anith KN, Radhakrishnan NV, Manomohandas TP (2003) Screening of antagonistic bacteria for biological control of nursery wilt of black pepper *(Piper nigrum)*. Microb Res 158:91-97

Assumpcao LC, Lacava PT, Dias AF, Azevedo JL, Menten JOM (2009) Diversity and biotechnological potential of endophytic bacterial community of soybean seeds. Pesq Agrop Bras 44: 503-510

Berg G, Eberl L, Hartmann A (2005) The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. Envir Microb 7:1673-1685

Berg G, Roskot N, Steidle, A, Eberl L, Zock A, Smalla K (2002) Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. Appl Environ Microb 68: 3328-38

Boustie J, Grube M. (2005) Lichens-a promising source of bioactive secondary metabolites. Plant Gen Resourc 3: 273–278

Boustie J Tomasi S, Grube M. Bioactive lichen metabolites: a focus on some alpine species. Phytochem Rev (under review)

Cardinale M, Puglia AM, Grube M (2006) Molecular analysis of lichen-associated bacterial communities. FEMS Microb Ecol 57(3): 484-495

Cardinale M, Castro Jr JV, Müller H, Berg G, Grube M (2008) *In situ* analysis of the bacterial community associated with the reindeer lichen *Cladonia arbuscula* reveals predominance of *Alphaproteobacteria*. FEMS Microb Ecol 66: 63-71

Denton GH, Karlén W (1973) Lichenometry: ist application to Holocene moraine studies in Southern Alaska and Swedish Lapland. Artic Alpine Res 5:347-372

Du G, Chen LXL, Yu J (2004) High-efficiency production of bioplastics from biodegradable organic solids. J Polym Envir 12(2):89–94

Gasser I, Müller H, Berg G (2009) Ecology and characterization of polyhydroxyalkanoate-producing microorganisms on and in plants. FEMS Microb Ecol 70:142-150

Gonzalez I, Ayuso-Sacido A, Anderson A, Genilloud O (2005) Actinomycetes isolated from lichens: Evaluation of their diversity and detection of biosynthetic gene sequences. FEMS Microb Ecol 54:401–415

Grube M, Cardinale M, Castro Jr JV, Müller H, Berg G (2009) Species-specific structural and functional diversity of bacterial communities in lichen symbiosis The ISME Journal 3(9): 1005-1015

Grube M, Hawksworth DL (2007) Trouble with lichen: the re-evaluation and re-interpretation of thallus form and fruit body types in the molecular era. Mycol Res 111:1116-1132

Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997) Bacterial endophytes in agricultural crops. Can J Microb 43: 895-914

Haygood MG, Schmidt EW, Davidson SK, Faulkner J (1999) Microbial symbionts of marine invertebrates: opportunities for microbial biotechnology. J Mol Microb Biotec 1: 33-43.

Kloepper JW, John L, Teintze M (1980). Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286:885-886

Lal S, Tabacchioni S (2009) Ecology and biotechnological potential of *Paenibacillus polymyxa*: a minireview. Indian J Microb 49: 2-10

Liba CM, Ferrara FIS., Mangio GP, Fantinatti-Garboggini F, Albuquerque RC, Pavan C, Ramos PL, Moreira-Filho CA, Barbosa CR (2006) Nitrogen-fixing chemo-organotrophic bacteria isolated from cyanobacteria-deprived lichens and their ability to solubilize phosphate and to release amino acids and phytohormones. J Appl Microb 101: 1076–1086

Lillo JG, Rodriguez-Valera F (1990) Effects of culture conditions on poly (b-hydroxybutyric acid) production by Haloferax mediterranei. Appl Envir Microb 56:2517–2521

Nash T (2008) Lichen biology. Cambridge Univ. Press

Oksanen K (2006) Ecological and biotechnological aspects of lichens. Appl Microb Biotech 73:723–734

Opelt K, Berg G (2004) Diversity and antagonistic potential of bacteria associated with bryophytes from nutrient poor habitats of the Baltic Sea Coast. Appl Envir Microb 70 6569-6579

Sheu DS, Wang, YT, Lee CY (2000) Rapid detection of polyhyroxyalkanoate-accumulating bacteria isolated from the environment by colony PCR. Microb 146: 2019-2025

Steinbüchel A, Oppermann-Sanio FB (2003) Mikrobiologisches Praktikum. Springer-Verlag, Berlin

Tian F, Ding Y, Zhu H, Yao L, Du B (2009) Genetic diversity of siderophore-producing bacteria of tobacco rhizosphere. Braz J Microb 40(2):276-284

Viswanathan R, Rajitha R, RameshSundar A, Ramamoorthy V (2003) Isolation and identification of endophytic bacterial strains from sugarcane stalks and in vitro antagonism against the red rot pathogen. Sugar Technol 5:25–29

Table 1. Examples of bacterial isolates from lichens: identification based on partial sequencing of the 16S rDNA gene and biotechnological potential

Internal no.	**Origen: Lichens specie**	**Closest NCBI database match and accession number**	**Similarity (%)**	**Secondary metabolites**							
				Siderophore	Protease	Glucanase	Chitinase	Auxin	Lipase	Phosphate solubilizat	AHL
C2 ecto 3	*C. arbuscula*	*Bacillus circulans* FN393823	98	-	-	-	-	-	-	-	-
C1 ecto 5	*C. arbuscula*	*Burkholderia sp.* FJ796427	98	-	-	-	+	+	-	-	-
C1 ecto 15	*C. arbuscula*	*Bacillus cereus* GQ495663	98	-	+	-	+	-	-	-	-
C1 ecto 20	*C. arbuscula*	*Burkholderia sp.* FJ796427	98	-	-	-	-	-	-	-	-
C1 ecto 2	*C. arbuscula*	*Paenibacillus xylanilyticus* EU977802	98	-	+	+	-	-	-	-	-
C1 ecto 9	*C. arbuscula*	*Microbacterium sp.* FJ966939	99	-	-	-	+	+	-	-	-
C1 ecto 1	*C. arbuscula*	*Pseudomonas graminis* FJ999944	99	**-**	**+**	**-**	**-**	**+**	**-**	**-**	**-**
C1 ecto 19	*C. arbuscula*	*Pseudomonas sp.* FJ668331	98	-	-	-	-	+	-	-	-
C2 ecto 4	*C. arbuscula*	*Pseudomonas sp.* FN547772	98	-	+	-	-	-	-	-	-

C1 ecto 10	*C. arbuscula*	*Micrococcus luteus* FJ906730	99	-	-	-	-	-	-	-	-
2 Caecto 14	*C. arbuscula*	*Luteibactor rhizovicina* AJ580498	98	+	+	-	-	-	-	-	-
U1 endo 4	*U. cylindrica*	*Bacillus pumilus* EF061516	99	+	-	+	-	-	-	-	-
U2 ecto 1	*U. cylindrica*	*Micrococcus luteus* FJ205746	98	-	+	-	-	+	-	-	-
U1 ecto 1	*U. cylindrica*	*Frondihabitans australicus* DQ525859	98	-	-	-	-	-	-	-	-
U2 ecto 3	*U. cylindrica*	*Micrococcus luteus* FJ205746	99	-	+	-	-	+	-	-	-
U3 ecto 1	*U. cylindrica*	*Burkholderia sp.* FJ796427	99	+	-	-	-	-	-	-	-
U3 ecto 10	*U. cylindrica*	*Microbacterium sp* AM421786	98	+	+	-	-	-	-	-	-
U2 ecto 7	*U. cylindrica*	*Micrococcus luteus* FJ906730	98	-	+	-	-	+	-	-	-
U1 ecto 9	*U. cylindrica*	*Bacillus cereus* DQ884352	99	-	+	-	-	+	-	-	-
U3 endo 1	*U. cylindrica*	*Bacillus sp.* GQ284382	98	+	-	-	-	-	-	-	-
U1 ecto 4	*U. cylindrica*	*Burkholderia sp.* FJ796427	98	-	-	-	-	-	-	-	-
3 Ucecto 2	*U. cylindrica*	*Leifsonia xyli*	98	-	-	-	-	-	-	-	-

		FJ189782									
3 Ucecto 18	*U. cylindrica*	*Acinetobacter sp.*	100	+	-	-	-	+	-	-	-
		FN298909									
L3 ecto 15	*L. polytropa*	*Kytococcus sedentarius*	98	-	+	+	-	-	-	-	-
		CP001686									
L2 ecto 7	*L. polytropa*	*Bacillus pumilus*	99	+	+	-	-	-	-	-	-
		EF061516									
L2 ecto 3	*L. polytropa*	*Burkholderia sp.*	99	-	-	-	-	-	-	-	-
		FJ796427									
L3 ecto 9	*L. polytropa*	*Microbacterium sp.*	99	-	+	-	-	+	-	-	-
		GQ495643									
L3 ecto 14	*L. polytropa*	*Methylobacterium*	98	+	-	-	-	-	-	-	-
		podarium									
		AB302930									
L3 ecto 5	*L. polytropa*	*Bacillus cereus*	99	-	-	+	+	-	-	-	-
		DQ884352									

Production of secondary metabolites: + detected, - not detected

Legends to the figures

Fig. 1

Proportion of *in vitro* antagonistic potential of bacterial strains isolated from lichens determined in dual culture assay towards *Botrytis cinerea, Rhizoctonia solani, Phytophora infestans, Verticillium dahliae, Staphylococcus aureus, Erwinia carotovora* and *Candida albicans.*

Fig. 2

Characterization of bacterial isolates according to their metabolic properties.

Fig. 3

Screening for PHA-Producers from total community DNA extracted from *C. arbuscula, U. cilindrica, L. polytropa* and *L. pulmonata*.

Fig. 4

Screening of bacterial strains for their genetic potential for PHA (polyhydroxyalkanoate) production and their *in vitro* ability to produce PHAs.

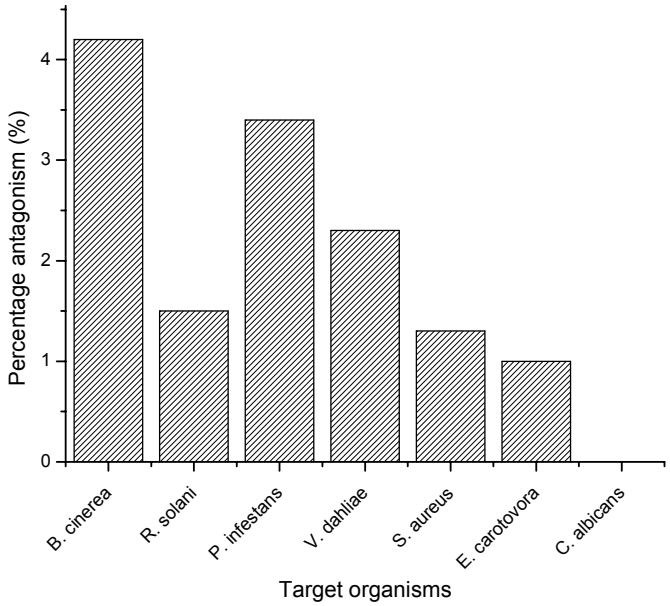


Figure 1

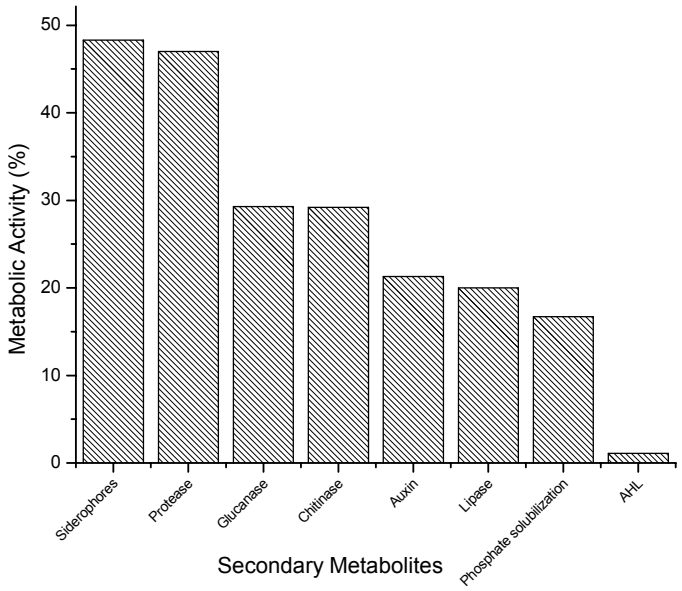


Figure 2

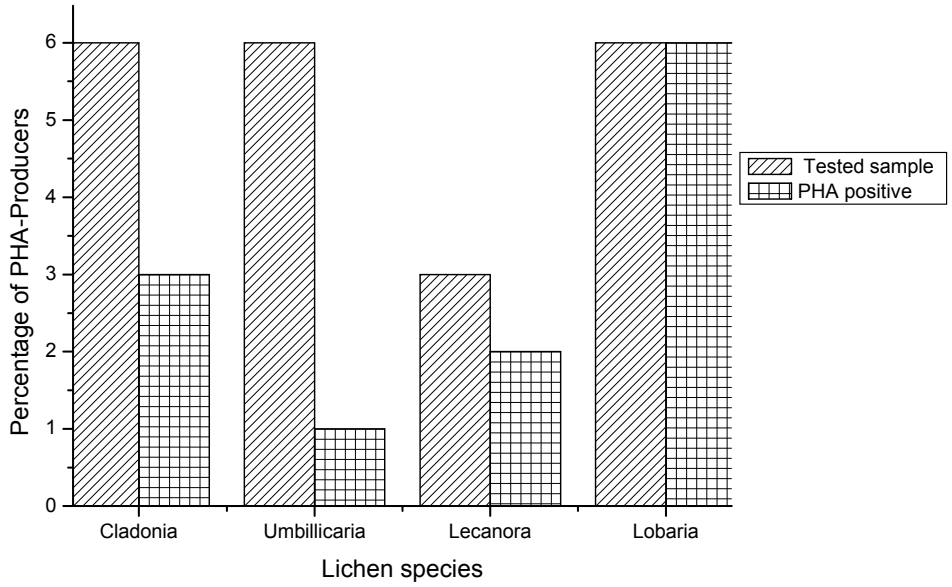


Figure 3

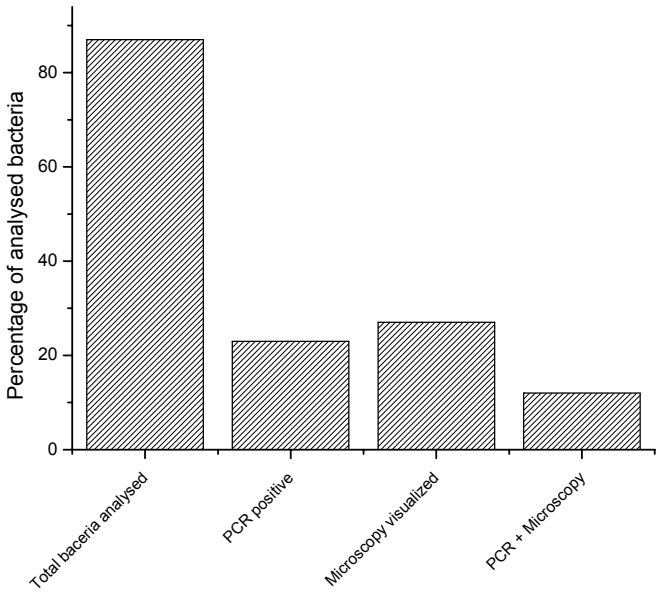


Figure 4

Manuscript II

Metaproteomics to link structure and function of symbiosis partners of the lung lichen (*Lobaria pulmonaria*)

Joao Vieira de Castro Junior[1], Kathrin Riedel[2], Thomas Schneider[2], Massimiliano Cardinale[1], Martin Grube[3] and Gabriele Berg[1*]

[1]Graz University of Technology, Institute of Environmental Biotechnology, Petersgasse 12, A-8010 Graz, Austria
[2]University of Zurich, Institute of Plant Biology, Department of Microbiology, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland
[3]Karl-Franzens University of Graz, Institute of Plant Sciences, Holteigasse 6, A-8010 Graz, Austria

***Corresponding author:** Gabriele Berg
Tel. ++43 316 873 8310
Fax: ++43 316 873 8199
Email: gabriele.berg@tugraz.at

In preparation to: The ISME Journal

Abstract

A metaproteomics approach comprising one-dimensional gel electrophoresis and liquid chromatography-coupled mass spectrometry (LC-MS) was applied to characterize the microbial community associated to the lichen *Lobaria pulmonaria* for the first time. Identification of proteins expressed by in situ population was used to understand the microbial composition and functions directly within their respective thallic environment. The microbial metaproteome profiles showed a very rich and diverse population inhabiting the inner structure of lichen, as well as demonstrating the applicability of the proteomics approach for the complex ecosystem.

Introduction

Lichens are ecologically highly adapted and robust associations, and certain species can endure hostile environmental and become up to thousands of years old, providing an almost continuous niche for other microscopic organisms. Bacterial communities in lichens are diverse and abundant (Cardinale et al. 2006, Cardinale et al. 2008), and due to their morphological and physiological characteristics, lichens can present a complex structure which might be responsible to give stability to the microbial community.

Proteins are the ultimate functional products of gene, and proteomic studies of microbial communities (metaproteomics) are an obvious approach to advance our understanding of microbial community function.

Proteomics is one of the fastest developing research areas and contributes substantially to our understanding of organisms at the cellular level. Recently, the large-scale characterization of the entire protein complement of environmental microbiota has been proven useful to investigate the most abundant proteins in environmental samples from activated sludge (Wilmes and Bond, 2004), soil (Schulze et al. 2005) and an acid-mine drainage microbial biofilm (Ram et al. 2005).

Environmental proteomics, due to its opportunity to study many protein functions and responses simultaneously, offers excellent possibilities to improve our understanding of microbial community architecture and composition, and ecosystem functioning (Schneider and Riedel, 2009). Our study aims were the assessment of structure and function of microbial community associated to the lichen *Lobaria pulmonaria* based on their protein expression profiles, with focus on the identification of key functions, which might be important for symbiotic interactions. Detailed

descriptions of the sample preparation, protein separation and identification, data validation and protein quantification are given in supplementary materials.

The microbial community associated to specie *L. pulmonaria* was analysed by fluorescence in situ hybridisation (FISH). Most of the cells that stained positively with universal bacterial probes (EUB338I, II and III) and specific probe for Alphaproteobacteria were identified as bacteria (Figure 1). The thallic space is profusely colonized by Alphaproteobacteria being similar to those results described by (Cardinale et al. 2008, Grube et al. 2009).

For proteomic analysis, 1D SDS/PAGE was carried out and the gels were sliced into consecutive blocks from top to bottom, proteins from the microbiota of the lichen specie *L. pulmonaria* were identified after tryptic digestion, and the extracted peptides were analyzed using high-accuracy LC-MS/MS. The proteins were processed as described in Materials and Methods, and MS/MS spectra were searched against a database consisting of protein sequences obtained from the public RefSeq database (Uniref 100). In total, we identified 2890 unique proteins which were considered for further interpretation, where protein abundance was roughly identified by Scaffold software. Metaproteomic approaches have been well described for laboratory scale bioprocess (Wilmes and Bond, 2004) and a low-complexity natural microbial biofilm (Ram et al. 2005). However, extending this approach to complex environmental samples is not trivial (Poretsky et al., 2005; Frias-Lopez et al., 2008).

A detailed examination performed by Scaffold software revealed that the most abundant group of proteins were of fungal origin, followed by bacterial proteins, green algae proteins and archaea (Figure 2A). In agreement with other studies reporting, the lichenic environment provides an interesting niche for microrganisms, and those may be involved with the symbiotic process (Cardinale et al. 2006, Cardinale et al. 2008; Grube et al. 2009). Interestingly, the results demonstrated by the protein fingerprint analysis were significantly different among the different taxonomic groups. Overall, from these block gels, 403 unique proteins were identified according to Database matching to the bacterial taxonomic group (Figure 2B). The LC-MS/MS analysis identified other bacterial groups, including proteins from Actinobacteria, Aquificae, Bacteroidetes, Chloroflexi, Firmicutes and Verrucomicrobia

In addition, there were cyanobacterial proteins. According to DuRand et al. (2001) cyanobacteria is the dominant phototrophic organism in the Sargasso Sea. Furthermore, there were three minor group identified as Acidobacteria, Chlorobi and

Planctomycetes with less than 1% of prevalence. Among the identified bacterial proteins the Proteobacteria group seemed to be more prevalent and the alphaproteobacteria group seems to be dominant among the bacteria living in interaction with lichen, followed in order of abundance by gammaproteobacteria, betaproteobacteria, epsilonproteobacteria and deltaproteobacteria (Figure 2C). This result highlighted the robustness of the metaproteomic method used to characterize bacterial communities in lichen thalli. Furthermore, protein fingerprints were more complex and structured by the presence of major results belonged to Alphaproteobacteria. This finding was consistent with the *in situ* analysis by FISH experiment conduced by Cardinale et al. 2008, where the bacterial community associated to the specie *Cladonia arbuscuka* revealed predominance of Alphaproteobacteria in comparison with other groups; hence alphaproteobacteria seems to be the dominant group among the bacteria associated to lichen.

In concordance with the literature, there was also found proteins identified that are expressed in green algae, all the protein analyzed belonged to the group of Chlorophyta. This result was consistent with the results reported by Lewis and McCourt (2004), which showed that most of the lichenized fungi form lichen symbiosis with green alga inhering to the division Chlorophyta.

Interestingly, although there were a large number of fungal proteins identified, most of them belonged only to one group, the ascomycota group (~96%) in comparison with the basidiomycota fungi that represented only 4% of the expressed proteins (see supplementary material). According to literature, more than 98% of lichenized fungal species belong to phylum Ascomycota a few to orders of phylum Basidiomycota (Hawksworth, 1988), these reports are in concordance with the fingerprint showed by the annotated proteins for the fungi group.

Most of all analyzed microbial proteins (84%) could be assigned to functional categories according to Clusters of Orthologous Groups of proteins and are involved in various processes. Identified protein for which positive identifications were obtained are highlighted on the Figure 2. Details on the protein identification are listed in details on supplementary material.

Most of the bacterial proteins belong to the following categories: translation; RNA processing and modification; replication, recombination and repair; chromatin structure and dynamics; defence mechanisms and signal transduction mechanisms (Figure 2D).

Proteomic analyses examine proteins, the final product of all levels of gene expression, and thus provide information that is often different from the information provided by the measurements of gene expression (Gygi et al. 1999).

Overall, from these fungal sequences, most identified proteins were assigned to the seven functional groups on fungal genera (Figure 2E). This included proteins known to be involved in biosynthesis of cell wall, membrane and envelope, as well as the LC-MS/MS analysis identified multiple cytoskeleton integrative proteins, including proteins responsible for intracellular transport; production and conversion of ATP; transport and metabolism of carbohydrate and amino acids and proteins involved in stress response such as chaperones. In a course of evolution, about 13,000 extant fungal species (Hawksworth, 2001) have specialized in gaining their carbon and about 1,500 species also in gaining their nitrogen from a photosynthesizing partner (photobiont; Hawksworth et al. 1995).

In total, 201 proteins were identified on the basis of Database information that did reveal most of green algae sequence identities are related to transport and metabolism of nucleotides, coenzymes, lipids and inorganic ions; it can not be forgotten that some of these are also involved in secondary metabolites synthesis, as well as some functions were not possible to be identified (Figure 2F). According to the literature records, lichens are traditionally considered as symbioses between a fungal species and one or more algae or cyanobacteria (Hawksworth, 1988), the findings revealed by the metaproteomic approach confirmed the theory about the nature of lichen symbiosis quoted by many authors.

In the context of our experiment, this may illustrate the importance of microbial functional diversity and metabolic versatility for maintenance of lichens species. It would be interesting to perform a comparative study with other lichen species to evaluate the interaction among microrganisms to have a better comprehension about the symbiotic phenomenon and importance of microrganisms in supporting lichen structure.

To our knowledge, this study represents the first application of a metaproteomic approach to a high-complex system as lichens. The metaproteome elucidation of *L. pulmonaria* provided ample evidence of microbial interaction and adaptations to an extreme environment in which cells are subjected to damage by light stress and drought, while competing for essential nutrients that are at extremely low concentrations; as well as the understanding of microbial role on symbiosis

processes. This study found that the lichen thalli presents a very rich and active microbial fauna, and future studies should be conducted to identify a much larger number of proteins from microbial communities associated to lichens to address provide insights into microbial community dynamics, function and symbiotic role.

References

Cardinale M, Puglia AM, Grube M (2006) Molecular analysis of lichen-associated bacterial communities. FEMS Microb Ecol 57(3): 484-495

Cardinale M, Castro Jr JV, Müller H, Berg G, Grube M (2008) *In situ* analysis of the bacterial community associated with the reindeer lichen *Cladonia arbuscula* reveals predominance of *Alphaproteobacteria*. FEMS Microb Ecol 66: 63-71

DuRand MD, Olson RJ, Chisholm SW. (2001). Phytoplankton population dynamics at the Bermuda Atlantic Time-series station in the Sargasso Sea. Deep Sea Res II 48:1983–2003

Frias-Lopez J, Shi Y, Tyson GW, Coleman ML, Schuster SC, Chisholm SW et al. (2008) Microbial community gene expression in ocean surface waters. Proc Natl Acad Sci USA 105: 3805–3810

Grube M, Cardinale M, Castro Jr JV, Müller H, Berg G (2009) Species-specific structural and functional diversity of bacterial communities in lichen symbiosis The ISME Journal 3(9): 1005-1015

Gygi SP, Rochon Y, Franza BR, Aebersold R. (1999) Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19: 1720–1730

Hawksworth DL. (1988). The variety of fungal–algal symbioses, their evolutionary significance, and the nature of lichens. Bot J Linn Soc 96: 3–20

Hawksworth DL (2001) The magnitude of fungal diversity: the 1.5 million species estimate revisited. Mycol Res 105: 1422–1432

Hawksworth DL, Kirk PM, Sutton BC, Pegler DN (1995) Dictionary of the fungi. CAB, Wallingford

Lewis LA, McCourt RM (2004) Green algae and the origin of the land plants. Am J Bot 91:1535–1556

Poretsky RS, Bano N, Buchan A, LeCleir G, Kleikemper J, Pickering M (2005) Analysis of microbial gene transcripts in environmental samples. Appl Environ Microbiol 71: 4121–4126

Ram RJ, Verberkmoes NC, Thelen MP, Tyson GW, Baker BJ, Blake RC, Shah M, Hettich RL, Banfield JF (2005) Community proteomics of a natural microbial biofilm. Science 308, 1915-1920

Schneider T, Riedel K (2009) Environmental proteomics: Analysis of structure and function of microbial communities. Proteomics (in press)

Schulze W, Gleixner G, Kaiser K, Guggenberger G, Mann M, Schulze ED (2005) A proteomic fingerprint of dissolved organic carbon and soil particles. Oecol. 142, 335-343

Wilmes P, Bond PL (2004) The application of two-dimensional polyacrylamide gel electrophoresis and downstream analyses to a mixed community of prokaryotic microorganisms. Environ. Microbiol. 6, 911-920

Figures

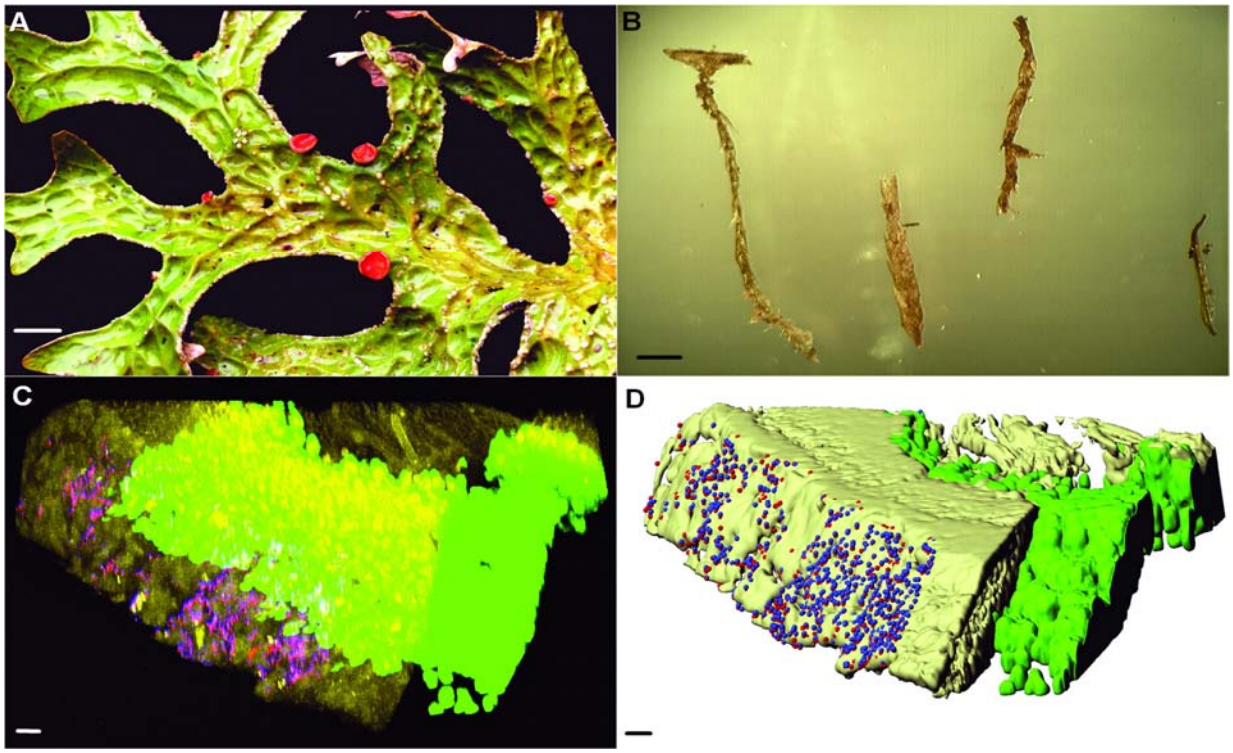


Figure 1. Bacterial colonization of *Lobaria pulmonaria*. Thalli of *Lobaria pulmonaria* (A) were sampled using sterilized forceps and then cut in 30 µm-thick cryosections (B) after fixation with 3% paraformaldehyd. The cryosections were stained by FISH using universal bacterial probes (EUB338I, II and III) and specific probe for Alphaproteobacteria (ALF968). Confocal stacks were recorded with a Leica TSC SP (Leica Microsystems GmbH, Germany) using a Z-step of 1.14 µm and assigning different color codes to the different fluorescents (C). Three-dimensional surface-spot reconstruction (D) was created with the software Imaris 6.4.0 (Bitplane, Switzerland). Panel C: green: algal chlorophyll; yellowish: fungal tissues; pink: *Alphaproteobacteria*; red: other bacteria. Panel D: green surface: algal chlorophyll; yellowish surface: fungal tissues; blue/red spots: *Alphaproteobacteria*; red spots: other bacteria. In the panels C and D the fluorescent signals from bacteria and fungal tissues were partially cropped, to evidence the stratification of layers. Scale bars: 1cm (A), 1mm (B), 10µm (C and D). For details about the FISH-CLSM procedures, see Cardinale et al. 2008.

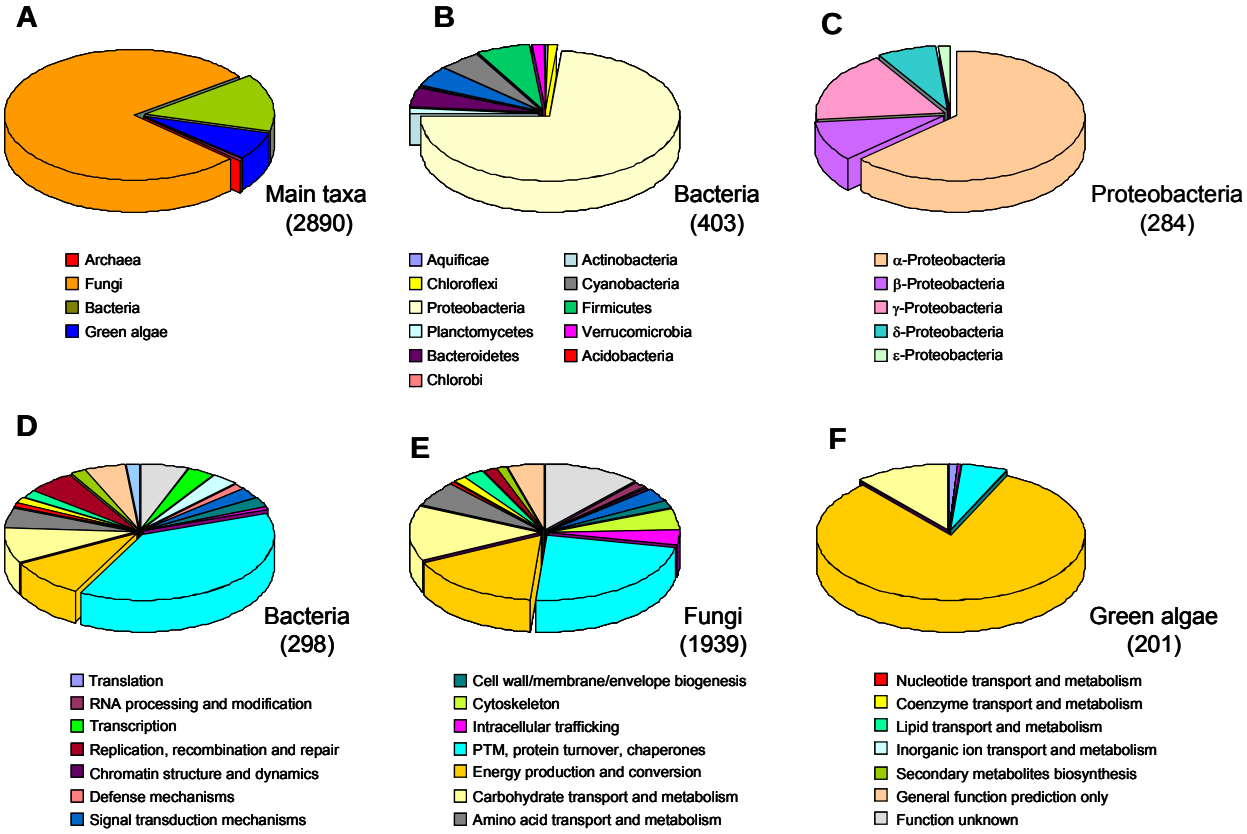


Figure 2. Identification and characterization of microbial taxonomic groups associated to the lichen thalli and organic functions of proteins revealed by 1D-PAGE gel and analysed by mass spectrometry

Manuscript III

Geographical and ecological signals from the bacterial community in selected species of *Lobaria* (lichenized Ascomycota)

Joao Vieira de Castro Junior[1], Massimiliano Cardinale[1], Lucia Muggia[2], Martin Grube[2] and Gabriele Berg[1*]

[1]Graz University of Technology, Institute of Environmental Biotechnology, Petersgasse 12, A-8010 Graz, Austria
[2]Karl-Franzens University of Graz, Institute of Plant Sciences, Holteigasse 6, A-8010 Graz, Austria

***Corresponding author:** Gabriele Berg
Tel. ++43 316 873 8310
Fax: ++43 316 873 8199
Email: gabriele.berg@tugraz.at

In preparation to: The ISME Journal

Abstract

For a long time, biologists have studied patterns of plant and animal diversity regarding the biogeographycal aspects at continental scales. However, very few information is known about the environmental factors influencing the biodiversity of bacterial communities associated to 'complex' host organisms, especially concerning complex symbiotic organisms such as lichens. Microbial biogeography has focused largely on free-living microbes, yet those microbes associated with host organisms are also of interest from a biogeographical perspective. We studied 2 lichens species (*L. pulmonaria* and *L. immixta*) from across 3 distinct geographic areas: Austria (Northern/Southern Styria), Portugal (Madeira), Montenegro (Bjelasica) and used a ribosomal DNA-fingerprinting method (SSCP) to compare bacterial community composition and diversity qualitatively across sites. SSCP banding patterns (and phylogenetic analysis of excised DGGE bands) indicated different communities in between both lichen species from all sampled regions studied. Bacterial diversity was very complex and not related to geographical region. According to our results, we speculate that the divergent communities in different geographical regions provide evidence of endemism attributed to host association, although variation in environmental factors could also explain the observed results.

Introduction

Most of the biogeography studies focused on the macroorganisms as plants or animals, whereas it was still little known about the distribution of microbial species and their communities. The understanding of microbial distribution in temporal and spatial dimensions, i.e. microbial biogeography, is still in its infancy (Fierer, 2008), but received a rapid increasing interest in the recent past. The study of biogeography offers insight in those mechanisms, which generate and maintain diversity among organisms in general, such speciation, extinction or dispersal (Brown et al. 2000).

Microbial biogeography studies have explored a wide range of habitats, taking in consideration soil bacteria communities (Fierer and Jackson, 2006), bacterial assemblages in lakes and streams (Crump et al. 2007), or extremophilous microorganisms in soda lakes (Foti et al. 2006). Genetic methodological approaches based on fingerprint profiles and large scale sequencing make easier nowadays to

discover the dominating unculturable fraction of microbial diversity. The statement of Baas Becking (1934) that “everything is everywhere but the environment selects”, meaning that species can be found where their environmental requirements are met, has been leading almost all the biogeography studies. However, not only ecological parameters determine the distribution of miroorganisms. When bacteria form intimated association with macroorganisms, as many bacteria-animal and bacteria-plant symbioses, the distribution of the first is often affected by the geographic distribution of their hosts (Martiny et al. 2006). A high degree of host specificity was found for bacterial associated with two *Sphagnum* species independent of the geographical region (Opelt et al. 2007), whereas the extent of gene deletions, duplications, and acquisitions in the genome of facultative symbiotic soil bacteria of the genus *Frankia* (Actinomycetes) was correlated to the biogeography distribution of the associated plants in the family Casuarinaceae (Normand et al 2009). However, at smaller scales the microbial biodiversity may remain hidden to us because many species occur at a density below the limit of our detection (de Wit and Bouvier, 2006).

Long-living organisms may represent ecological stable niches, which can host still undiscovered microbial communities. Textbook examples for longevity are lichens. These organisms represent an ecologically obligate and stable mutualism between a fungus, the mycobiont (the exhabitant partner) and a population of extracellularly located green algae or cynobacteria, the photobionts (Hawksworth and Honegger, 1994). Recently molecular methods confirmed the conspicuous presence of microorganisms in lichen thalli of diverse growth forms (Cardinale et al. 2006, 2008, Grube et al. 2009). The complex morphologies of the lichen thalli, from crustose to foliose and shrubby, offer more or less exposed surfaces, where microorganisms can form highly structured biofilm-like assemblages on the fungal surface and can reach considerable abundances (Grube et al. 2009). More than 17.000 lichen species are described (Hawksworth, 1988). Many lichen species are highly cosmopolitan, show a wide range of ecological tolerance, and are widespread on different substrata (Hawksworth et al. 1995). Alternatively there are lichen species, which have an extremely wide geographic distribution but are restricted to particular ecological conditions. In this context one of the major limiting factor affecting the distribution of some lichen species is their sensitivity to air pollution (Giordani et al. 2002).

Most of the lichens, especially the large shrubby and leaf-like forms are easily recognized in the nature. Data about the geographic distribution of lichens has therefore accumulated over the past 200 years. In fact, lichens are the sole group for which a vast knowledge on biogeography already exists. Many lichens have an extremely wide geographic distribution, and the same species can be found on several continents under similar ecological conditions. There has been a lot of debate how the wide distribution patterns emerged. In the past the long range dispersal hypothesis was much favoured, but recent evidence shows that the pattern could in many cases also be explained by historic changes and the separation of previously coherent distribution ranges. In recent times anthropogenic fragmentation of distribution ranges became a major threat to lichens, especially when these are particularly sensitive to other parameters such as air pollution

Lichens are well known to be reliable indicators of air quality and have been used as biomonitors in several studies (Adamo et al. 2001, Augusto et al. 2007). One of the best-known lichen genera, which is particularly sensitive to air pollution and represents excellent indicators of forest ecosystems with long ecological continuity and with a large number of rare species is *Lobaria* (Kalwij et al. 2005). In particular *Lobaria pulmonaria* is often used as a flagship species for conservation activities. *Lobaria pulmonaria* is widely distributed in temperate and circumboreal regions of the Nothern Hemisphere, and tropical mountain systems, and in South Africa. While species is still abundant in North America it suffered a decline in many parts o its European range. It is now considered endangered in many parts of Central Europe, and further industrial regions.

Considering that the environmental conditions have a great impact on microbial community, the population composition of bacteria associated to genus *Lobaria* might differ from those in other regions. We performed the present study in order to investigate the community composition, geographic distribution, and phylogenetic relationships of the bacterial community associated to lichen thalli.

Material and Methods

Bacterial isolation and cultivation - The bacteria used in this study were isolated from the lichens species *Lobaria immixta* and *Lobaria pulmonaria*. The lichens specimens used in the current study were sampled in Austria (Northern/Southern Styria), Montenegro and Portugal (Madeira) (Table 1). Fresh material (2-4 g) was washed with sterile sodium chloride solution (0.85%) and transferred into a sterile stomacher

bag. The samples were suspended in NaCl (0.85%) and homogenized for 3 min by agitation. The samples were serially diluted and plated onto poor R2A agar medium (Difco) for 5 days at 20 °C (Opelt and Berg 2004). After incubation in R2A medium, the colonies were counted for calculation to determine the mean number of colonies (CFU) based on fresh weight. The bacteria were selected according to their morphological characteristics and purified by cultivation in Nutrient Agar medium (NA, Difco^{tm}) for 48 hours at 20 °C and maintained at -80 °C in sterile glycerol (50%) solution for subsequently experiments.

Identification of cultured strains and phylogenetic analysis - Bacterial genomic DNA was extracted using the lysozyme–proteinase K–sodium dodecyl sulfate method (modified by increasing the reagent concentration to 2.4 mg/ml of lysozyme, 0.5 mg/ml of proteinase K and of 0.8% of sodium dodecyl sulfate). Amplified ribosomal DNA restriction analysis (ARDRA) was performed with the endonuclease HhaI. Representatives of individual cultured strains as assessed by ARDRA were identified by PCR amplification of the 16S rDNA fragment between positions 14 and 907 (according to E. coli 16S rDNA sequence numbering) and sequencing using the primers Eub1 (5'-AGATTTGATCMTGGCTCAG-3') and 907r (5'-CCGTCAATTCMTTTGAGTTT- 30). PCR products were purified with the GENECLEAN Turbo kit (BIO101 Systems; Qbiogene, CA). Complementary strands of DNA were sequenced using the BigDye Terminator Ready Reaction kit (Applied Biosystems, Norwalk, CT, USA) and sequencing reactions were separated on an ABI 310 automated sequencer (Applied Biosystems). The identity of the sequences was assessed by BLAST searches. Sequences were submitted to GenBank (accessions FN298879–FN298916). The partial sequences of the 16S rRNA genes from our isolates, the best BLAST-matching sequences, and reference sequences were aligned by ClustalX (Thompson et al., 1994). A 550 nt multialignment was obtained and the PHYLIP package (Felsenstein, 2004; http://evolution.genetics.washington.edu/phylip.html) was used for producing the bootstrap replicates (program SeqBoot), for inferring the maximum likelihood tree (program DNAml), the neighbor-joining (programs dnadist and neighbor) and for calculating the consensus trees (program consense). The phylogenetic tree was constructed with the Neighbor software of the PHYLIP package. The trees were visualized in TreeView (Page, 1996; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Characterization of bacterial isolates by ARDRA - ARDRA (Amplified Ribosomal DNA Restriction Analysis) was carried out as described by Opelt et al. (2007); it was used for grouping isolates at the genus level. The 16S ribosomal DNA of bacteria isolated from lichen thalli was amplified with the universal eubacterial primer EubI (5' GAG TTT GAT CCT GGC TCA G 3') and EubII (5' AGA AAG GAG GTG ATC CAGCC 3'). *Hha*I was chosen as restriction enzyme. Enzymatic reactions were digested for 3 h at 37°C in 20 µL volumes containing 15µL of the PCR product solution, 2 µL of commercially supplied incubation buffer (NEB Inc., UK) 2.55 µL of water, 0.2 µL of 100xBSA, and 0.25 µL (20 U/µL) of *Hha*I. Restriction products were run on a 2% Agarose gel (AppliChem, Darmstadt, Germany) in a 1xTris-borate-EDTA buffer for 5 h at 100 V/m. The resulting bands were made visible with ethidium bromide. Isolates showing the same band pattern were arranged to form a group. The reproducibility of the results was verified at least in two independent experiments.

The PCR products were purified using the Geneclean Spin Kit (Qbiogene, Bio 101, Carlsbad, USA) according to the manufacturer's protocol. DNA templates were sequenced and the results were aligned with sequences of the NCBI sequence databases.

BOX-PCR fingerprints - BOX-PCR was carried out as described by Rademaker and De Bruijn (1997). Using the BOXA1R primer (5´-CTA CGG CAA GGC GAC GCT GAC G-3´), PCR amplification was performed as described in Opelt and Berg (2004). A 10 µl aliquot of the amplified PCR product was separated by gel electrophoresis on 1.5% agarose gels in 0.5x Tris-borate-EDTA buffer for 5 h, stained with ethidium bromide, and then photographed under UV transillumination. The reproducibility of the results was verified in at least two independent experiments.

Identification of bacteria isolated from lichens - The isolates were identified by partial sequencing of the 16S rDNA genes as described by Urzi et al. (2001). The gene was PCR amplified using the EUB1 (5'-AGATTTGATCMTGGCTCAG-3') and 907r (5'-CCGTCAATTCMTTTGAGTTT-3') and the primer 907r was used for sequencing. The identity of the sequences was confirmed by BLAST search in GenBank.

DNA extraction of total community - DNA of bacterial-cell consortia was extracted from the lichen species *Lobaria immixta* and *Lobaria pulmonaria* by using the FastDNA Spin Kit for Soil (Bio 101, Carlsbad, California) according to the manufacturer's instruction.

SSCP analyses. Fingerprinting of the lichen bacterial communities by SSCP was carried out as described by Schwieger and Tebbe (1998). Microorganisms of the lichen thalli communities were extracted by mechanical disruption and homogenization of 400 mg material in a FastPrep Instrument (BIO101 Systems; Qbiogene, Carlsbad, CA, USA) for 30 s at speed 5.0. DNA was purified by the GENECLEAN Turbo kit (Qbiogene, CA). The special binding buffer guanidine thiocyanate integrated in the kit was used to remove humic acids. Bacterial 16S rDNA fragments were amplified by PCR with specific. Alphaproteobacteria were selectively amplified with primers ADF681F/1492r (5´-AGTGTAGAG GTGAAATT-3´, 5´-TACGGYTACCT TGTTACGAC TT-3´) followed by a second semi-nested PCR with the primers ADF681F/927r (5´-CCCGCTAATTYMTTTGACTT-3´, Blackwood et al. 2005). For specific patterns of *Burkholderia* a double-nested PCR was applied using the following primer pair Eub1/Eub2 (5´-GAGTTTGATCCTGGCTCAG-3´, 5´-AGAAAGGAGGTGATCCAGCC-3´), BKH143Fw/BKH1434Rw (5´-TGGGG GATAGCYCGGGG-3´, 5´-TGCGGTTA GRCTASC YACT-3´) and the eubacterial primer pair Unibac-II-515f and Unibac-II-927rP. *Pseudomonas* was amplified by the primer pairs F311Ps/1459rPs-P (5´-CTGGTCTGAGAGGATGATCAG T-3´, 5´-AATCACTCCGTGGTAAACGT-3´) and Unibac-II-515f and Unibac-II-927rP (Opelt and Berg, 2004), respectively. nifH fragments were obtained with nifH3/19F (5´-ATAGTTAGTTAGCTGCAGCTGCAGTA-3´, 5´-GCXATTCTTACTGGXAAAG-3´) and nifH11/nifH22 (5´-GACTCCAGCTAAAGGCAGCTGACTC-3 ´, 5´-AAGTATGCCATCAT CTTCAGCC-3´) (Yeager et al. 2004). The PCR products were purified by the GeneClean Turbo Kit (Qbiogene, BIO101®), before an exonuclease digestion and DNA single strand folding according to Lieber et al. (2003). The amplicons were separated by using the TGGE Maxi system (Biometra, Göttingen, Germany) at 400 V and 26°C. Eubacterial as well as *Burkholderia* and *Pseudomonas*-specific amplicons were separated in 8% (w/v) acryl amide gels for 26 h. DNA fragments of the Alphaproteobacteria-specific PCR were separated in 9% (w/v) acryl amide gels for 16 h. Afterwards the gels were silver-stained according to Bassam et al. (1991). Dominant and unique bands were excised from SSCP gels as described by Schwieger and Tebbe (1998). Gel-extracted DNA was reamplified with the respective primer pair for each PCR and sequenced to prove their identity. To validate the significance of differences between separated groups, we performed the permutation test according to Kropf et al. (2004).

Computer-assisted cluster analysis - GelCompar program (version 4.1: Applied Maths, Kortrijk, Belgium) was used for the evaluation of the bacterial community profiles obtained by SSCP. Cluster analysis was performed with the UPGMA (unweighted pair group method with arithmetic averages) algorithm.

Results

Isolation of bacteria from lichen thalli

We investigated the culturability and diversity of microorganisms associated to lichen thalli of two selected species *L. pulmonaria* and *L. immixta*. A combination of serial dilution method and plating onto a poor agar medium (R2A) was used to avoid the problem of fast growing microrganisms observed when organisms growing under rich nutritional conditions.

CFU (colony forming unit) determined for lichen samples were rather similar for *L. pulmonaria* among all the geographical region studied (Nothern/Southern-Styria and Montenegro), (CFU per gram of lichen, were 3.4-4.7 x 10^7, 6.6-8.1 x 10^7 and 1.3-2.1 x 10^7, respectively), lower number of bacteria was found in *L. immixta* isolated from Maderia (1.6-4.1 x 10^6 $CFU.g^{-1}$). However, the microbial abundances at the different lichen species did not show high significantly difference.

We obtained 45 isolates of bacteria from a series of thallic material collected on different locations on the same sampling period. These isolates were subcultured from randomly selected colonies that appeared on plates of a range of media. The plates had been inoculated with the most diluted aliquots of bacterial suspension that had resulted in colony formation. Approximately 868-907 bp of the nucleotide sequence were determined from the 5' region of the 16S rDNA gene for each of these 45 isolates, and compared to sequences available in the GenBank databases using BLAST. Preliminary phylogenetic placements based on the results of the BLAST analyses revealed that all isolates could be assigned to recognised phyla of the domain Bacteria.

According to their 16S rDNA sequencing (data not shown), we identified 45 bacterial strains, belonging to 11 distinctly genres: *Pseudomonas, Paenibacillus, Bacillus, Plantibacter, Frigobacterium, Variovorax, Dietzia, Erwinia, Frigoribacterium, Buttaxiella* and *Stenotrophomonas.*

Molecular fingerprint by SSCP

The SSCP fingerprints of the bacterial groups associated with the two lichens species from Montenegro, Madeira and Northern/Southern-Styria are displayed in Figure 1.

The SSCP fingerprints have good banding patterns (data not shown) and a significantly higher number of Bands was detected in the Burkholderia community (15-22 bands), nifH gene (13-16 bands) and Pseudomonas (12-18), while the least number was detected among the alphaproteobacterial community (7-10 bands); most of all geographical regions gave rise to specific community profiles as shown on Pseudomonas and Alphaproteobacteria dendograms, as well as specific primers for Burkholderia and nifH gene revealed complex SSCP patterns. The results showed Burkholderia community and functional nifH gene group are highly diverse among all studied regions, whereas more similarity throughout was found for Alphaproteobacteria and Pseudomonas. No PCR amplification was found by the *L. immixta* specie isolated from Madeira, according to result demonstrated on dendogram after the use of specific Pseudomonas primer.

Cluster analysis indicates a clear pattern in community structure in each of the two lichens, with well defined clusters for Burkholderia, Pseudomonas and nifH gene; however, Alphaproteobacteria dendogram showed lower correlation between the two species. The dendogram expressed on figure 1 show the phylogenetic relationship of all lichen-associated bacterial groups represented on SSCP fingerprint. The UPMGA analysis divided the lichens species into three main groups as shown on Burkholderia (Figure 1A) and Pseudomonas group (Figure 1B), more complex clusters are observed on alphaproteobacteria and nifH patterns (Figure 1C and Figure 1D, respectively). According to the data generated by 16S rDNA fragments amplified by PCR, the patterns of different lichens produced with specific primers for actinomycete showed a high degree of similarity among the four distinct groups. The permutation test revealed the application of specific primer for Burkholderia, Pseudomonas, Alphaproteobacteria and nifH gene resulted in statistically significant differences among all of the studied regions, except for the results showed by nifH gene, which no significant difference was found between Northern Styria – Southern Styria (Pp0.5552) (Table 2).

According to the cluster analysis using the UPGMA algorithm obtained from SSCP patterns of all four studied regions displayed a similarity of 69%, 43%, 8% and 73%, respectively obtained from Burkholderia, Pseudomonas, nifH gene and Alphaproteobacteria groups. The similarity among Alphaproteobacteria group was higher in comparison with the others species, demonstrating this specific group seem to be more conserved among the studied lichen species.

Identification of Lichen-associated bacteria from excised SSCP bands

Some bands of the SSCP gels performed with Alphaproteobacteria, Burkholderia, Pseudomonas and nifH gene primers were excised and sequenced to obtain further information about the dominant bacterial populations and to better understand the differences in bacterial diversity among the three studied lichen species. The resulting species list, with the strain name, nucleotide sequence similarity along with partial sequence analysis data and phylogenetic affiliations of sequenced SSCP bands, is shown in Table 3. A total of thirty four discriminable bands were individually identified as members of different eubacterial phyla, as shown in Table 3.

Ten bands (A8, A16, M_A1, M_A2, M_A3, M_A4, M_A5, M_A6, M_A7 and M_A9) were grouped with the phylum Alphaproteobacteria, and thirteen (N1, N2, N3, N4, N5, N6, N11, N12, N17, M_N1, M_N4, M_N6 and M_N7) were clustered within the group dinitrogenase reductase (nifH) gene, as well as four bands (PS2B, PS8D, PS10A, PS10B) and seven bands (1BK, 2BK, 13BK, 14BK, 15BK, 16BK and 20BK) were identified from Pseudomonas and Burkholderia groups, respectively .

The obtained DNA sequences from the Alphaproteobacteria gels showed similarities in the range of 86 to 100% to sequences from the NCBI database and most of their closest relative showed homology to *Uncultured alphaproteobacteria*; one band was identified as *Uncultured Methylobacterium* originated from the specie *L. pulmonaria* (Montenegro); one bands belonging to genus *Methylobacterium* from *L. pulmonaria* (Southern Styria).

Only one band affiliated to the lichen *L. pulmonaria* (Southern Styria) was identified as *Uncultured bacteria*. Due to the fact that the DNA sequences obtained are relatively short (~200bp), it was difficult to find a better identification of the strains with higher index of similarity.

Dominant SSCP bands were also excised and sequenced from gels generated with the nifH gene-specific primers as indicated in Table 3. All sequences could be affiliated to the group dinitrogenase reductase (nifH) gene with similarity between 76-95% to nucleotides sequences (*Nostoc sp, Scytonema sp, Nostoc commune, Cyanobacterium, uncultured Nostoc sp, Nostoc punctiform* and *uncultured nitrogen-fixing bacteria*). These results indicated the great diversity of nifH gene species associated with the tested lichens belongs to group of cyanobacteria, particularly to genus *Nostoc*.

Furthermore, two sequences obtained from Pseudomonas gel were related to *uncultured gammaproteobacteria*. *Uncultured bacteria* (band PS8D) and *Pseudomonas sp* (band PS2B) were identified as bacterial group from *L. pulmonaria* (Southern Styria). Interestingly, excised SSCP bands showed that all the sequences from Burkholderia gel belong to uncultured strains, with special emphasis to the betaproteobacteria species.

Phylogenetic analysis of the lichen-associated bacteria based on ARDRA fingerprint

The taxonomic relatedness of the bacteria associated with the two Lobaria species was deduced by the construction of phylogenetic trees inferred from comparative sequence analyses of the partial 16S rDNA sequence from each single bacterial strain. Based on this comparison, the lichen-associated bacteria fell into the firmicutes, actinobacteria or g-Proteobacteria group (Figure 2). We used the neighbour-joining program in UPMGA to help in the construction of the phylogenetic dendogram.

Four isolates from the thallic material have phylogenetic affiliation with members of the phylum Actinobacteria, greatly increasing the number of known cultured representatives of this phylum associated to thalli space; three isolates could be reproducibly assigned to of the Class g-Proteobacteria, which is currently represented by *Erwinia*, *Pseudomonas* and *Stenotrophomonas*. Furthermore, six isolates had phylogenetic affiliation with the group Firmicutes, with4 belonging to the well characterized genus *Paenibacillus* and two related to members of the widely distributed class *Bacilli*.

The results of the computer analysis are presented as ARDRA dendrogram in Figure 2. All of the strains examined were linked at the 53% similarity level, and it was possible to generated 5 distinct clusters among all ARDRA patterns. The first cluster (G1), which we examined were grouped together, contain only one isolate from the sample site Madeira, three from Northern Styria and six from Southern Styria. The cluster G2 contains 4 isolates from Madeira, 2 from Northern Styria and 3 from Southern Styria. Only representatives from Madeira (4) and Southern Styria (4) were grouped on cluster G3; in addition, Northern (4) and Southern Styria (3) were clustered on G4. The cluster G5 provided the biggest group comprehended representatives from Madeira (3), Northern Styria (2) and Southern Styria (6). Regarding the distribution of the isolates according to similarity, the group G1

presented three subgroups with 59% of similarity. Cluster group G3 and G4 were divided in two subgroups with 68% and 66% similarity respectively. Genotypic group G2 could also be divided into three subgroup, the same as group G5 at 66% and 58% similarity.

Characterization of bacteria associated to lichen thalli based on molecular fingerprint by BOX-A1

A total of 44 representatives bacterial isolates were characterized by 16S rDNA and could be assigned to 6 distinct BOX clusters. A total of 12 (sampled at Madeira), 21 (sampled at Southern Styria) and 11 (sampled at Northern Styria) isolates were genotypically characterized by their BOX fingerprints to detect lichen-specific genotypes according to sampling site (Figure 3).

The cluster analysis of BOX-fingerprint showed a very complex genotypic diversity and low sampling site specificity, except for two cluster group which contained only representatives from Northern Styria and Madeira.

The intraspecies diversity of BOX patterns analyzed in three independent replicates for each isolate was shown at 41% similarity. Analysis of BOX patterns for bacterial strain isolated from lichen thalli resulted in 6 different cluster or genotype groups, although 1 of them contained only one isolate. Only two groups contained only isolates from Northern Styria and Madeira.

References

Adamo P, Basile A, Cobianchi RC, Giordano S, Sorbo S, Vingiani S, Violante P (2001) Monitoraggio dell'inquinamento atmosferico nell'area metropolitana de Napoli mediante l'impiego di "moss" e "lichen bags". Notiziario della Società Lichenologica Italiana. 14:48-49.

Augusto S, Catarino F, Branquinho C (2007) Interpreting the dioxin and furan profiles in the lichen Ramalina canariensis Steiner for monitoring air pollution. Science of the Total Environm. 377(1):114-123.

Baas-Becking, L.G.M. (1934). Geobiologie of inleiding tot de milieukunde. The Hague, The Netherlands: W.P. van Stockum and Zoon.

Bassam BJ, Caetano-anolles G, Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. Analyt Biochem. 80:81-84.

Brown JH, West GB, Enquist BJ (2000) Scaling in biology: patterns and processes, causes and consequences. In: Scaling in Biology. Oxford University Press. Oxford pp. 1-24.

Cardinale M, Puglia AM, Grube M (2006) Molecular analysis of lichen-associated bacterial communities. FEMS microbiol ecol. 57(3): 484-495.

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

Crump BC, Adams HE, Hobbie JE, Kling GW (2007) Biogeography of bacterioplankton in lakes and streams of an arctic tundra catchment. Ecology. 88(6): 1365-1378.

de Wit R, Bouvier T (2006) 'Everything is everywhere, but, the environment selects'; what did Baas Becking and Beijerinck really say? Environm Microbiol. 8:755-758.

Fierer N (2008) Microbial biogeography: patterns in microbial diversity across space and time. In: Accessing Uncultivated Microorganisms: from the Environment to Organisms and Genomes and Back. ASM Press, Washington DC pp. 95-115

Fierer N**,** Jackson RB (2006). The diversity and biogeography of soil bacterial communities. PNAS. 103(3): 626-631

Foti M, Ma S, Yu D, Rademaker JLW, Kuenen JG, Muyze G (2006) Genetic diversity and biogeography of haloalkaliphilic sulphur-oxidizing bacteria belonging to the genus Thioalkalivibrio FEMS Microbiol. Ecol 56(1):95-101.

Giordani P, Brunialti G, Alleteo D (2002) Effects of atmospheric pollution on lichen biodiversity (LB) in a Mediterranean region (Liguria, northwest Italy). Environm Pollut 118:53-64.

Grube M, Cardinale M, Castro Jr JV, Müller H, Berg G (2009) Species-specific structural and functional diversity of bacterial communities in lichen symbiosis. The ISME Journal. 3(9): 1005-1015.

Hawksworth DL (1988) The variety of fungal-algal symbioses, their evolutionary significance, and the nature of lichens. Bot J Linn Soc 96:3–20.

Hawksworth DL, Honegger R (1994) The lichen thallus: a symbiotic phenotype of nutritionally specialized fungi and its response to gall producers. In: Plant Galls: Organisms, interactions, populations. Clarendon Press. Oxford. Pp. 77-98.

Hawksworth DL, Kirk PM, Sutton BC, Pegler DN (1995). Ainsworth & Bisby's dictionary of the fungi. 8. ed. Wallingford:CAB international.

Kalwij JM, Wagner HH, Scheidgger C (2005) Effects of stand-level disturbances on the spatial distribution of a lichen indicator. Ecol Applic. 15(6):2015-2024.

Kropf S, Heuer H, Gruening, Smalla K (2004) Significance test for comparing complex microbial community fingerprints using pairwise similarity measures. J Microbiol Methods 57: 187–195.

Lieber A, Kiesel B, Babel W (2003) Microbial diversity analysis of soil by SSCP fingerprint technique using TGGE Maxi System. In: Ökophysiologie des Wurzelraumes. Teubener Verlag, Stuttgart. Germany. Pp. 61-65.

Martiny J, Hughes B, Bohannan BJM, Brown J, Colwell R, Fuhrman J, Green J, Devine MH, Kane M, Krumins J, Kuske C, Morin P, Naeem S, Ovreas L. (2006) Microbial biogeography: putting microorganism on the map. Nature Rev Microbiol. 4:102-112.

Normand P, Lapierre P, Tisa LS (2009) Genome characteristics of facultatively symbiotic Frankia sp. Strains reflect host range and host plant biogeography. Genome Res. 17:7-15.

Opelt K, Berg G (2004) Diversity and antagonistic potential of bacteria associated with bryophytes from nutrient poor habitats of the Baltic Sea Coast. Appl Envir Microb 70:6569-6579.

Opelt K, Chobot V, Hadacek F, Schoenmann S, Berg G (2007) Investigations of the structure and function of bacterial communities associated with *Sphagnum* mosses. Environ Microbiol 9(11): 2795–2809.

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

Urzi C, Brusetti L, Salamone P, Sorlini C, Stackebrandt E, Daffonchio, D (2001) Biodiversity of Geodermatophilaceae isolated from altered stones and monuments in the Mediterranean basin. Environmental Microbiology. 3: 471-479.

Yeager C, Kornosky JL, Housman DC, Grote EE, Belnap J, Kuske CR. (2004). Diazotrophic community structure and function in two successional stages of biological soil crusts from the Colorado plateau and Chihuahuan desert. Appl Environ Microbiol 70:973–983.

Tables.

Table 1. Description of sampling sites and lichen species

Region	Site	Lichen specie	Geographic coordinates	Climate	Host tree	Annual Precipitation	Average temperature
Austria	Northern Styria	Lobaria pulmonaria	$47^0$15’0’’N $15^0$10’0’’E	Temperate		924mm	8.9^0C
	Southern Styria	Lobaria pulmonaria	$25^0$19’0’’E $34^0$43’33’’E	Temperate		924 mm	8.9^0C
Portugal	Madeira	Lobaria immixta	$32^0$44’0’’N $16^0$58’0’’W	Oceanic subtropical	*Clethra arborea*	636 mm	17^0C
Montenegro	Bjelasica	Lobaria pulmonaria	$42^0$53’55’’N $19^0$35’51’’E	Mediterranean	Fagus sylvatica	2000 mm	22^0C

Table 2. SSCP cluster analysis based on Pearson's correlation index

Bacterial group	Geographical site	P value	Significance level
Burkholderia	Madeira - Montenegro	0.0006	****
	Northern Styria – Southern Styria	0.008	***
Alphaproteobacteria	Madeira - Montenegro	0.0007	****
	Northern Styria – Southern Styria	0.0009	****
NifH gene	Madeira - Montenegro	0.0013	***
	Northern Styria – Southern Styria	0.5552	-
Pseudomonas	Northern Styria – Southern Styria	0.0159	**

Table 3. Identification and taxonomic classification of DNA bands separated by SSCP from different lichens

No.	Origin and SSCP band		Most closely related sequence(s)	% Identity	GenBank accession no.	bp
A8	*L. pulmonaria* Southern Styria	Alphaproteobacteria	*Uncultured bacteria*	86	FM873493	179
A16	*L. pulmonaria* Southern-Styria	Alphaproteobacteria	*Methylobacterium*	98	AM989021	205
M_A1	*L. pulmonaria* Montenegro	Alphaproteobacteria	*Uncultered alphaproteobacteria*	100	AY629335	188
M_A2	*L. pulmonaria* Montenegro	Alphaproteobacteria	*Uncultered alphaproteobacteria*	94	CU920714	178
M_A3	*L. pulmonaria* Montenegro	Alphaproteobacteria	*Uncultured bacteria*	93	FJ184918	178
M_A4	*L. pulmonaria* Montenegro	Alphaproteobacteria	*Uncultered alphaproteobacteria*	94	CU926049	180
M_A5	*L. pulmonaria* Montenegro	Alphaproteobacteria	*Uncultured Methylobacterium*	98	GQ249213	180
M_A6	*L. pulmonaria* Montenegro	Alphaproteobacteria	*Uncultered alphaproteobacteria*	94	AY629335	177
M_A7	*L. pulmonaria* Montenegro	Alphaproteobacteria	*Uncultered alphaproteobacteria*	93	CU926950	176
M_A9	*L. pulmonaria*	Alphaproteobacteria	*Uncultered*	93	AY250859	183

	Montenegro		*alphaproteobacteria*			
1BK	*L. pulmonaria*	Burkholderia	*Uncultured*	99	FJ569069	302
	Northern Styria		*betaproteobacteria*			
2BK	*L. pulmonaria*	Burkholderia	*Bacterium*	99	DQ156144	306
	Northern Styria					
13BK	*L. immixta*	Burkholderia	*Uncultured*	98	FJ036994	370
	Madeira		*betaproteobacteria*			
14BK	*L. pulmonaria*	Burkholderia	*Uncultured bacteria*	94	EU869563	305
	Northern Styria					
15BK	*L. pulmonaria*	Burkholderia	*Uncultured*	95	GQ306062	305
	Northern Styria		*betaproteobacteria*			
16BK	*L. pulmonaria*	Burkholderia	*Uncultured bacteria*	98	EU869563	325
	Southern Styria					
20BK	*L. pulmonaria*	Burkholderia	*Uncultured bacteria*	99	FJ592530	363
	Southern Styria					
PS2B	*L. pulmonaria*	Pseudomonas	*Pseudomonas sp*	99	FJ937929	360
	Southern Styria					
PS8D	*L. pulmonaria*	Pseudomonas	*Uncultured bacteria*	92	EU356793	281
	Montenegro					
PS10A	*L. pulmonaria*	Pseudomonas	*Uncultured*	97	GQ453019	366
	Montenegro		*gammaproteobacteria*			

PS10B	*L. pulmonaria* Montenegro	Pseudomonas	*Uncultured gammaproteobacteria*	97	GQ453019	349
N1	*L. pulmonaria* Southern Styria	NIfH gene	*Uncultured N-fixing bacterium*	86	AJ871111	237
N2	*L. pulmonaria* Southern Styria	NIfH gene	*Scytonema sp*	92	DQ531675	279
N3	*L. pulmonaria* Southern Styria	NIfH gene	*Nostoc sp*	93	EU915069	227
N4	*L. pulmonaria* Southern Styria	NIfH gene	*Uncultured bacteria*	76	EU912883	225
N5	*L. pulmonaria* Southern Styria	NIfH gene	*Nostoc commune*	92	DQ531672	234
N6	*L. pulmonaria* Southern Styria	NIfH gene	*Uncultured bacteria*	89	DQ776326	241
N11	*L. pulmonaria* Southern Styria	NIfH gene	*Nostoc punctiforme*	95	CP001037	245
N12	*L. pulmonaria* Southern Styria	NIfH gene	*Uncultured bacteria*	95	EF548043	247
N17	*L. pulmonaria* Southern Styria	NIfH gene	*Cyanobacterium*	95	U43440	241
M_N1	*L. pulmonaria*	NIfH gene	*Uncultured*	94	EF548118	142

	Montenegro		*Nostoc sp*			
M_N4	*L. pulmonaria*	NIfH gene	*Uncultured*	94	EF548118	157
	Montenegro		*Nostoc sp*			
M_N6	*L. pulmonaria*	NIfH gene	*Uncultured bacteria*	95	EF548066	153
	Montenegro					
M_N7	*L. pulmonaria*	NIfH gene	*Uncultured*	94	EF548090	153
	Montenegro		*Nostoc sp*			

Figures

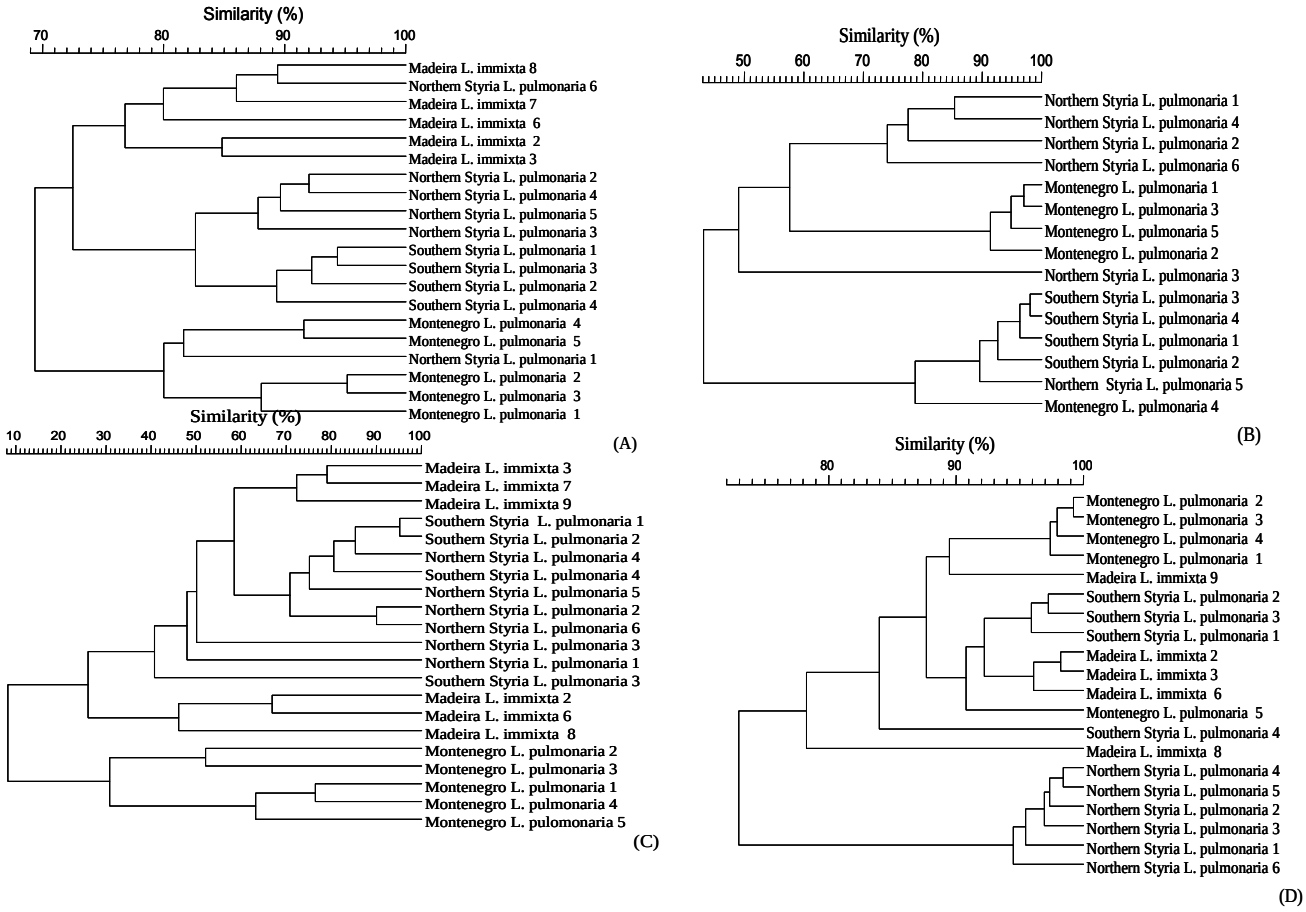


Figure1. Dendogram based on PCR-amplified 16S rDNA gene fragments of bacterial communities from *L. pulmonaria* and *L. immixta* sampled at Northern/Southern-Styria (Austria), Madeira (Portugal) and Montenegro obtained by using Burkholderia primers (A), specific primers for Pseudomonas (B), nifH gene (C), Alphaproteobacteria (D) and separated by SSCP. The patterns obtained were grouped by UPGMA.

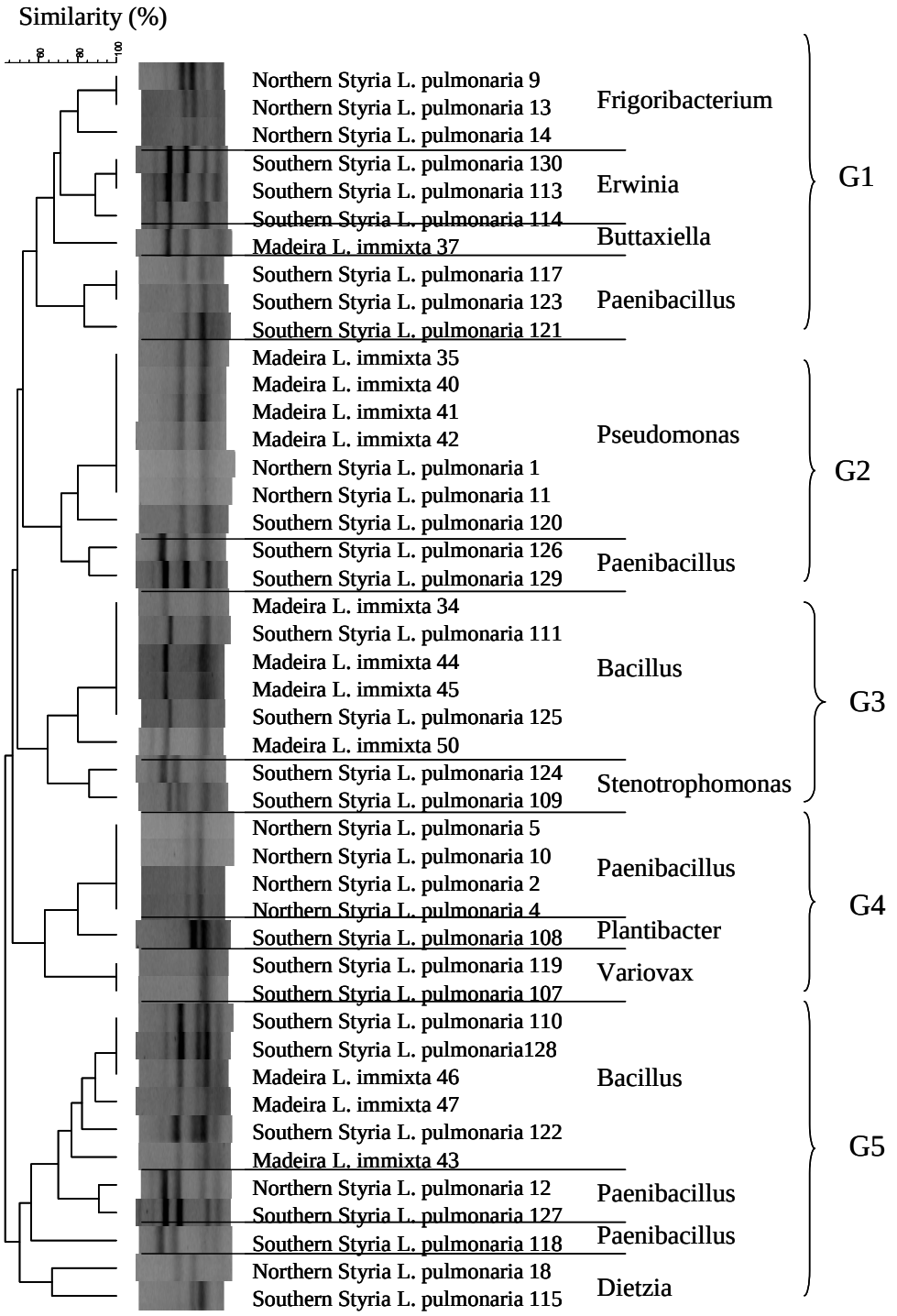


Figure 2. Dendrogram of 16S rDNA ARDRA similarities among bacteria isolated from lichen thalli (*L. immixta* – Madeira and *L. pulmonaria* – Northern/Southern Styria) calculated on the basis of similarity coefficients with the clustering algorithm of UPGMA.

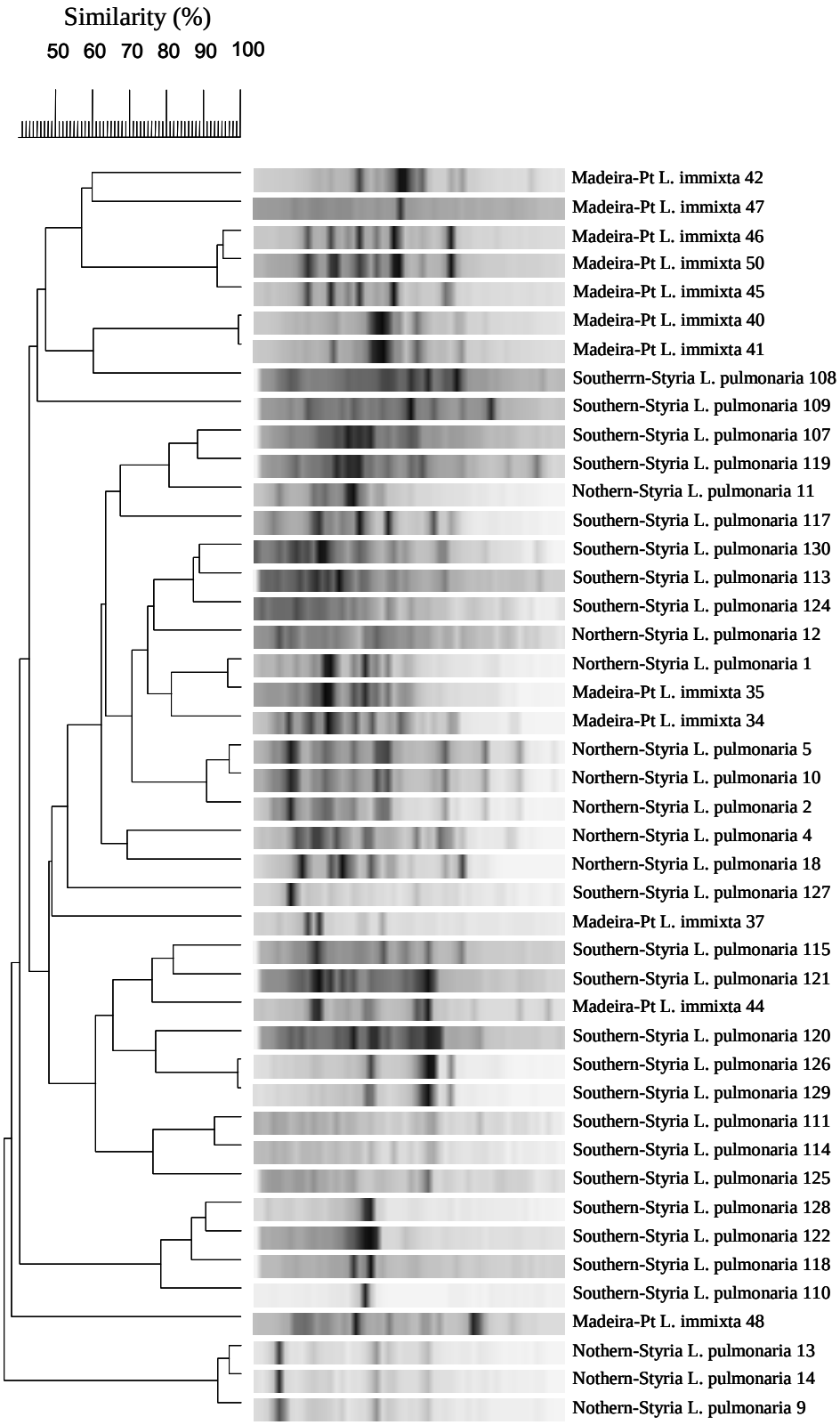
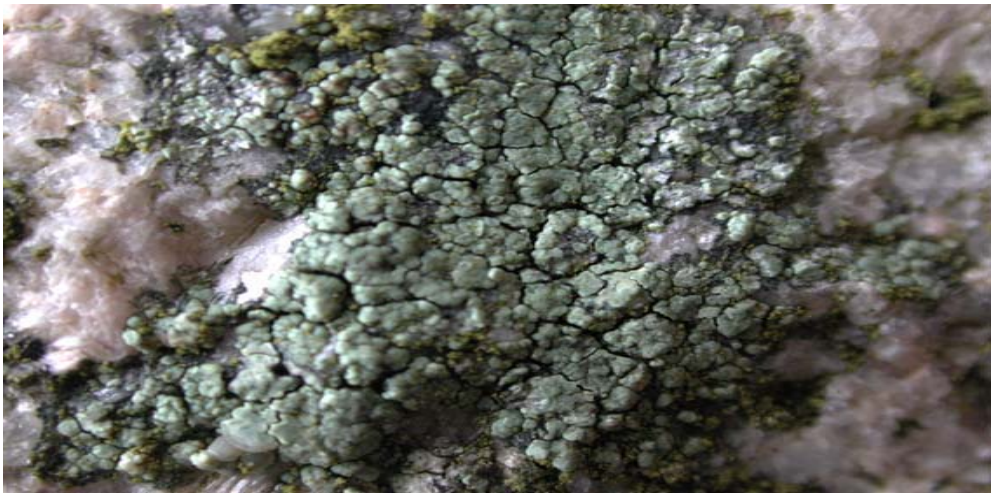


Figure 3. Dendrogram based on amplified 16S rDNA fragments of the lichen-associated bacterial communities from Madeira (L. immixta) and Northern/Southern Styria (L. pulmonaria) obtained by using BOX-A1 primer and separated by SSCP. The patterns obtained were grouped by UPGMA.

Appendix

Cladonia arbuscula

Umbilicaria cylindrica



Lecanora polytropa

Lobaria pulmonaria

Lobaria immixta