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Exploring the substantial contributions and the global interactions of the microbiome in an ancient symbiosis

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Abstract

The lung lichen [*Lobaria pulmonaria* (L.) Hoffm.] was utilized in the preparation of this thesis as a novel model organism in order to explore the beneficial contributions of the associated bacterial microbiome to a symbiotic consortium. Lichen symbioses are found in a wide variety of terrestrial habitats, which also include niches that are continuously exposed to harsh environmental conditions especially drought in combination with limited nutrients. Other habitats with rich lichen populations are characterized by drastic fluctuations in environmental conditions and therefore require rapid as well as reversible adaptations of all species present. Bacterial lichen colonizers have only recently been shown to be stable and integral elements of the symbiosis. They occur in biofilm-like structures on lichen surfaces as well as endophytically in inter-hyphal spaces and provide hotspots for distinct bacteria-host interactions. While the presence and structure have been demonstrated in previous studies, their specific roles still remain uncertain and require further exploration. The hypothesis of this thesis was that the associated microbiota are involved in essential functions of the holobiont including i) defense mechanisms against lichenicolous biota, ii) the bioconversion of toxic organic and in-organic substances, iii) the provision of scarce nutrients, and iv) the biosynthesis of substantial secondary metabolites.

To expand the knowledge base in this field, an inter-disciplinary combination of microbiological (cultivation, microcopy, meta-omics), chemical (*in vitro* assays, mass spectrometry), and supporting bioinformatics methods was employed. Specific techniques were utilized to describe bacterial diversity, identify key players, and ultimately assign specific functions. Data obtained with next-generation sequencing of meta-community DNA was integrated into results obtained with metaproteomics and high-resolution mass spectrometric metabolite detection to identify beneficial contributions made by the lichen microbiome. Complementary experiments with fluorescence *in situ* hybridization and confocal laser scanning microscopy (FISH-CLSM) facilitated a detailed localization of specific taxa on lichen thalli.

This multi-phasic approach to exploring the *L. pulmonaria* microbiome has contributed to the identification of a variety of beneficial contributions, and moreover endorsed the hypothesis that specific bacterial taxa are integral components of the symbiosis. Cultivation-based experiments together with meta-omic studies have provided strong evidence that the microbiome fulfills several host-protecting roles. This includes enhanced resistance against both biotic as well as abiotic stress factors, and the detoxification of metabolites. Furthermore,

the microbiome was shown to be involved in nutrient supply, especially the provision of nitrogen and phosphate and the recycling of older thallus parts. In addition, different members of *Alphaproteobacteria* were shown to be producers of vitamins, co-factors and growth promoting hormones. More detailed approaches to exploring the potential of lichen-associated isolates have shown that specific antagonistic bacteria were also producers of metabolites that are known to promote plant growth under drought and high salinity conditions. Supplementing results were obtained with a novel FISH probe which allowed visualization of lichen-specific *Rhizobiales* communities in inter-hyphal spaces.

In contrast to the classic lichen model, which only considers the mycobiont (fungal partner) and photobiont (algal and/or cyanobacterial partner) as integral parts of the symbiosis, recent results provide strong evidence for the importance and central role of the bacterial microbiome. A more holistic model of this unique symbiosis was introduced by the integration of all recent findings to facilitate a better understanding of this miniature ecosystem.

Kurzfassung

Im Rahmen dieser Arbeit wurde die Lungenflechte [*Lobaria pulmonaria* (L.) Hoffm.] als unkonventioneller Modellorganismus eingesetzt, um nützliche Funktionen des assoziierten bakteriellen Mikrobioms in einer Symbiose näher zu beschreiben. Flechten stellen eine weit verbreitete Lebensform dar und sind mitunter in Habitaten vorzufinden, die über lange Zeiträume widrigen Umweltbedingungen wie Trockenheit und Nährstoffmangel ausgesetzt sind. Andere Habitate, die vor allem von Flechten besiedelt werden, weisen extreme Schwankungen lokaler Umweltparameter auf und erfordern deswegen sowohl schnelle als auch reversible Anpassungen aller dort vorkommenden Lebewesen. In den letzten Jahren wurden bakterielle Populationen auf Flechten als stabiler und integraler Bestandteil der Symbiose erkannt. Sie kommen als Biofilm-ähnliche Strukturen auf Oberflächen von Flechten und auch endophytisch vor und stellen einen Hotspot für ausgeprägte Wechselbeziehungen dar. Während das Vorkommen und die Struktur solcher Bakterienpopulationen schon mehrfach gezeigt wurden, ist die Rolle der einzelnen Mikroorganismen immer noch nicht ganzheitlich geklärt. Die in dieser Arbeit aufgestellte Hypothese war, dass sie im Holobiont in i) Abwehrmechanismen, ii) der Umwandlung toxischer organischer und anorganischer Verbindungen, iii) der Bereitstellung knapper Nährstoffe und iv) der Biosynthese erforderlicher Sekundärmetaboliten beteiligt sind.

Um auf diese Fragen einzugehen, wurde eine interdisziplinäre Kombination von mikrobiologischen, chemischen und unterstützenden bioinformatischen Methoden angewendet. Spezielle Techniken wurden verwendet um die bakterielle Diversität zu beschreiben, wichtige Taxa zu identifizieren und letztendlich den detektierten Mikroorganismen funktionelle Rollen zuzuweisen. Daten die mit Hochdurchsatz-Sequenzierungen gewonnen wurden, sind mit Metaproteom-Experimenten und der Metaboliten-Detektion mittels hochauflösender Massenspektrometrie kombiniert worden. Ergänzende Versuche mit Fluoreszenz-in-situ-Hybridisierung (FISH) und konfokaler Laser-Scanning-Mikroskopie haben eine genaue Lokalisierung bestimmter bakterieller Taxa auf Flechtenthalli ermöglicht.

Dieser mehrphasige Vorgang zur Erforschung des *L. pulmonaria*-Mikrobioms hat dazu beigetragen eine Vielfalt an nützlichen Beteiligungen zu identifizieren und zusätzlich die Hypothese bekräftigt, dass spezifische bakterielle Taxa essentielle Bestandteile der Symbiose sind. Kultivierungsabhängige Experimente haben in Verbindung mit Meta-Omics-Analysen zum Nachweis von Schutzfunktionen des Flechten-assoziierten Mikrobioms beigetragen.

Diese Funktionen fördern die Widerstandsfähigkeit gegenüber biotischen und abiotischen Stressfaktoren. Ferner sind Bakterien auch an der Detoxifikation von speziellen Metaboliten beteiligt. Es wurde gezeigt, dass das Mikrobiom in der Zurverfügungstellung von Stickstoff und Phosphat, sowie der Wiederverwertung alter Flechtenteile, eine Rolle spielt. Zusätzlich konnte man zeigen, dass verschiedene Mitglieder der *Alphaproteobacteria* Vitamine, Cofaktoren und Wachstumshormone produzieren. Vertiefende Analysen mit antagonistischen Isolaten haben gezeigt, dass diese auch bestimmte Metaboliten produzieren die das Wachstum von Pflanzen bei Trockenheit und hohen Salzkonzentrationen fördern können. Ergänzende Ergebnisse wurden durch eine neue FISH-Sonde ermöglicht. Mit dieser konnte gezeigt werden das *Rhizobiales*-Populationen in Zwischenräumen der Pilzhyphen lokalisiert sein können.

Im Gegensatz zum klassischen Flechtenmodell, das nur den Mykobionten und einen oder mehrere Photobionten als integrale Bestandteile der Symbiose bezeichnet, liefern neue Ergebnisse aus mehrphasigen Studien eindeutige Beweise für die bedeutende und zentrale Rolle des bakteriellen Mikrobioms. Schlussendlich konnte ein überarbeitetes und vollständigeres Modell dieser einzigartigen Symbiose durch die Implementierung neuer Entdeckungen erstellt werden.

Thesis Introduction

Host-associated microorganisms and their importance for health and stability

A multitude of microorganisms and especially bacteria that are known to develop close interactions with humans, animals, plants and fungi are receiving increased attention due to ongoing discoveries of their beneficial contribution to health and general well-being. Current studies have demonstrated that healthy human individuals harbor a unique microbiome (The Human Microbiome Project Consortium, 2012). This microbiome remains stable over long periods of time, although the detailed mechanisms that shape the overall community structure are still not fully understood. Bacteria are outnumbering the total amount of eukaryotic cells in each healthy individual and their gene content is 100 times higher than that of the human genome (Kurokawa *et al.*, 2007). The human gut was identified as a hotspot for functionally and taxonomically diverse bacteria and their role in maintenance of physical health is the focus of many ongoing studies (Cho & Blaser, 2012). Conversely, dysbiosis of the gut microbiota was linked to obesity and other chronic medical conditions, *e.g*. inflammatory bowel disease (Qin *et al.*, 2010; Turnbaugh *et al.* 2009a). Moreover, the gut microbiome has a crucial role in immune programming and thus affects the absence or development of allergies at early life stages (Madan *et al.*, 2012). There are several indications that gut microbiota not only trigger physical conditions, but also influence the central nervous system through release of various neuroactive metabolites (Forsythe *et al.*, 2010). These metabolites can alter the mood, behavior, and pain perception of the host organism. The different effects of community shifts within the gut microbiome are studied with suitable model animals, *i.e*. humanized mice and rats, since similar adaptions of the host can be expected (Turnbaugh *et al.*, 2009b).

In contrast to these rather novel findings related to the human microbiome, the importance of microbes in plant development and growth is already known for more than one century. The basis was set when leguminous plants were identified as distinct members of mutualistic symbioses which harbor specific bacteria in root nodules (Ward, 1887; Beijerinck, 1888). Lorenz Hiltner (1904) was the first to suggest a widespread importance of bacteria in the plant rhizosphere (Hartmann *et al.*, 2008). More recently, molecular studies have shown that the rhizosphere of various plant species is colonized by a remarkably high number of bacteria (Berg *et al.*, 2005). This explains why studies that focus on these colonizers are of great relevance to applications in modern agriculture and facilitate advances in plant breeding strategies. Plants can stimulate the accumulation of beneficial bacteria in the rhizosphere by diverse root exudates (Bais *et al.*, 2006; Doornbos *et al.*, 2012). Different studies have

demonstrated species- and even cultivar-specific bacterial colonization of various plants (Smalla *et al.*, 2001; Cardinale *et al.*, 2015). Plant-associated bacteria are provided with carbohydrates from carbon dioxide fixation and in return fulfill several central functions in nutrient supply, provision of growth hormones and also in the protection against plant pathogens (Berg *et al.*, 2014). Distinct microorganisms from potentially diseased plants can be used as indicators for plant health (Erlacher *et al.*, 2014). Further research of such indicator species might foster the early detection of incipient pathogen propagation and thus allow directed counteractions. Bragina *et al.* (2013) have shown that lower plants without a characteristic rhizosphere also provide selective habitats for various beneficial bacteria.

Bacterial-fungal interactions were recently identified as key elements in many natural environments (Frey-Klett *et al.*, 2011). One salient and thoroughly studied phenomenon is trophic competition, which is often observed between microorganisms that share the same habitat and often results in antibiosis. The most prominent example of this negative interaction was observed by Fleming (1929) and led to a revolution in medicine. His findings established the basis for the development of numerous beta-lactam antibiotics in the ensuing years. In contrast to antibiosis, many cooperative interactions between bacteria and fungi have also been detected in various environmental niches (Frey-Klett *et al.*, 2011). 'Fungiphilic' *Pseudomonas* species were described by Warmink *et al.* (2009) and cytosolic endosymbionts by Lackner *et al.* (2009). The latter can be employed by the fungal host as producers of mycotoxins. However, the most prominent bacterial-fungal interactions are represented by lichens. Many distinct species have an obligate association with various cyanobacteria as photosynthetic partners. This close and long-lasting interaction is exceptional and represents a successful evolutionary lifestyle. Their globally widespread distribution is enabled by a unique mutualistic symbiosis and further research may reveal key elements which could be later implemented into various biotechnological applications.

Microbial ecology in the era of meta-omics

The advent of –omic techniques has largely contributed to a generally better understanding of microbial structures and their roles in complex ecosystems. Research in microbiology and related sub-disciplines was centered on cultivation-dependent methods before the first molecular techniques emerged. It became subsequently evident that only 1% of environmental bacteria are cultivable and thus represent only a small fraction of the entire diversity (Amann, 1990). Molecular fingerprinting has enabled descriptive analyses of complex microbial communities and was extensively applied to describe microbial diversity in ecological studies.

Such methods were also employed to compare the bacterial colonization on various lichen samples (Cardinale *et al.*, 2006). Explorative studies to describe the diversity of uncultivable bacteria from various environmental habitats became more feasible with next-generation sequencing techniques. These high-throughput methods allowed the sequencing of high numbers of specific genetic markers (*e.g.* 16S rDNA fragments) and taxonomic assignments through suitable databases and bioinformatics tools (Rybakova *et al.*, 2015). The utilization of amplicon-based sequencing has enabled a profound description of many aquatic, terrestrial, human-made, and host-associated microbiomes (Bragina *et al.*, 2013; Oberauner *et al.*, 2013; Ding *et al.*, 2014; Hardoim *et al.*, 2014). Moreover, it was also utilized to demonstrate that an explicit part of the lichen microbiome is vertically transmitted with vegetative propagules (Aschenbrenner *et al.*, 2015). Together with amplicon-based studies, the utilization of metagenomics, metatranscriptomics, and metaproteomics is widespread in modern microbial ecology. Such meta-omic techniques allow the analysis of functional aspects in addition to taxonomic assignments and thus facilitate a more detailed picture in species-rich habitats (Grube *et al.*; 2015). In the context of lichens, the first metaproteomic study to include all organisms present in the *Lobaria* symbiome has reinforced the important role of bacteria therein (Schneider *et al.*, 2011). A combination of these emerging techniques was employed to thoroughly study lichen-associated microbiomes from three distinct sampling sites during the preparation of the present thesis (Fig. 1). This has facilitated the development of robust strategies to explain multiple ecological questions and to gain deeper insight into this unique cross-kingdom network (Cernava *et al.*, 2015a; Erlacher *et al.*, 2015; Grube *et al.*, 2015).

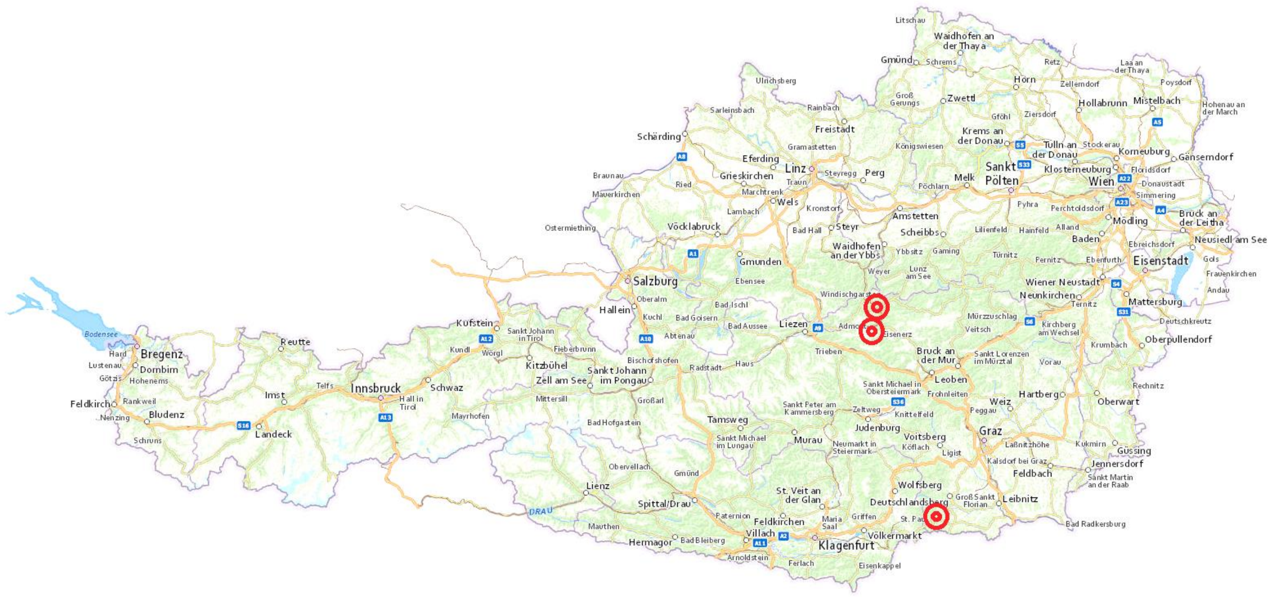

Figure 1 Location of the *L. pulmonaria* sampling sites in Styria (Austria). The samples were collected from rich lichen populations in Tamischbachgraben (N47°32'40'', E14°37'35''), Johnsbach (N 47°38'07'', E 14°44'45'') and St. Oswald ob Eibiswald (N 46°44' 50'', E 15° 04' 26''). Samples from Johnsbach were utilized for metagenomic and metaproteomic studies. Amplicon-based analyses were conducted with Lobaria samples from all indicated sampling sites.

Lichens: obligate symbioses and miniature ecosystems

Lichens are not only characterized by their versatility and their presence in a variety of terrestrial habitats, they are also known to be long-lived organisms, which are in some cases adapted to extreme environmental conditions. Such conditions include prolonged periods of drought, intense exposure to UV radiation, nutrient deficiency, and other abiotic factors. Even when exposed to various unfavorable conditions, established lichen symbioses can reach indeterminate ages (Øvstedal & Lewis-Smith, 2001). They can be the predominant lifeform in suitable environments and are estimated to cover up to 8% of the total land surface area (Ahmadjian, 1995). Moreover, the first ancestors of lichen symbioses can be tracked back to the Devonian 400 million years ago (Remy *et al.*, 1994; Honegger *et al.*, 2013). Since that time, more than 18,000 lichen species have emerged (Honegger *et al.*, 2013). They all include a distinctive sun-exposed fungal morphotype that is subjected to atmospheric influences such as variation in humidity. Moreover, the fungal symbiont determines the particular morphology and provides compartments for green algae and/or Cyanobacteria. These so-called photobionts fix carbon dioxide in a micro-environment similar to a greenhouse and exchange those products with the fungal partner which in return provides them with other essential nutrients. In contrast to terrestrial plants, lichens do not have roots and are therefore completely reliant on nutrients that are passively transported to their outer thallus layers (lichen cortex). Alternating layers of loose and densely packed fungal hyphae ensure efficient nutrient uptake and are perfectly adapted to the poikilohydric lifestyle that defines lichens and other cryptogams. This assembly allows all participating partners to endure in adverse habitats that would otherwise be uninhabitable for isolated organisms.

The lung lichen [*Lobaria pulmonaria* (L.) Hoffm.] harbors two photosynthetic partners which is also observed for approximately 4% of all described lichens (Honegger, 1991). However, only the green alga *Dictyochloropsis reticulata* is evenly distributed within inter-hyphal spaces, while different cyanobacterial *Nostoc* species can be stored in specific differentiated compartments (cephalodia). *L. pulmonaria* is characterized by a leaf-like structure (foliose lichen) and mainly found in old-growth forests with unpolluted air. Its sensitivity to air pollution can be employed for indirect evaluations of air quality and ecosystem integrity

(Scheidegger and Werth, 2009). *L. pulmonaria* was chosen as the model for this study due to its relatively fast growth and other facilitative characteristics, *e.g.* epiphytic growth on tree bark and a low number of secondary metabolites which could interfere with the conducted analyses.

In previous years attention was increasingly drawn to lichen-associated bacteria that are not recognized as being an integral part of the symbiosis. The textured surfaces and inter-hyphal spaces of lichen thalli provide specific niches in distinctive miniature ecosystems for various microorganisms (Erlacher *et al.*, 2015). Biofilm-like communities that mainly consist of *Alphaproteobacteria* were detected on the surface of various lichen species (Cardinale *et al.*, 2008, 2012a, b; Grube *et al.*, 2015). Furthermore, it was shown that such communities display a certain degree of host specificity when different species were compared (Grube *et al.*, 2009; Hodkinson *et al.*, 2011; Bates *et al.*, 2011). Related to these findings, experiments based on cultivable bacteria from lichens have provided additional evidence for their beneficial contributions to the overall symbiosis (Liba *et al.*, 2006; Grube *et al.*, 2009; Cernava *et al.*, 2015a, b). The detailed roles of these bacteria were previously unknown and therefore required further exploration to facilitate a more holistic lichen model.

A holistic lichen model to explain complex interactions and a unique co-existence

Although the first notable studies on lichen-associated bacteria that do not belong to cyanobacterial taxa were conducted several decades ago (Uphof, 1925; Henkel & Yuzhakova, 1936; Iskina, 1938), the thorough role of these colonizers remained to be elucidated. Early experiments based on cultivation-dependent methods have provided evidence for nitrogen fixation among isolated non-photosynthetic bacteria in the lichen symbiosis. Studies on isolates were expanded by Cardinale *et al.* (2006) with additional approaches to identify common representatives of ectophytic bacteria. In addition, the results were complemented with cultivation-independent molecular fingerprinting of bacterial communities from several lichen species. Breakthroughs towards holistic lichen models were achieved with the incorporation of specifically adapted visualizing techniques (Cardinale *et al.*, 2008). FISH-CLSM experiments have confirmed a dense colonization of the lichen cortex by diverse bacterial taxa. Furthermore, it was demonstrated that these colonizers are highly diverse, but have certain species specificity (Grube *et al.*, 2009; Bates *et al.*; 2011). In recent years, evidence has accumulated illustrating that various bacterial taxa that have been ignored so far might be an integral part of the symbiosis. This was reinforced by a metaproteomic study that demonstrated the involvement of the prokaryotic fraction in diverse metabolic pathways

(Schneider *et al.*, 2011). All aforementioned studies have cumulatively provided a solid basis for deepening the elucidation of a holistic lichen model which was also the central part of the presented thesis. The construction of this model was facilitated by a comprehensive combination of classic microbiological techniques with advanced visualizing methods and state-of-the-art meta-omic completion (Fig. 2).

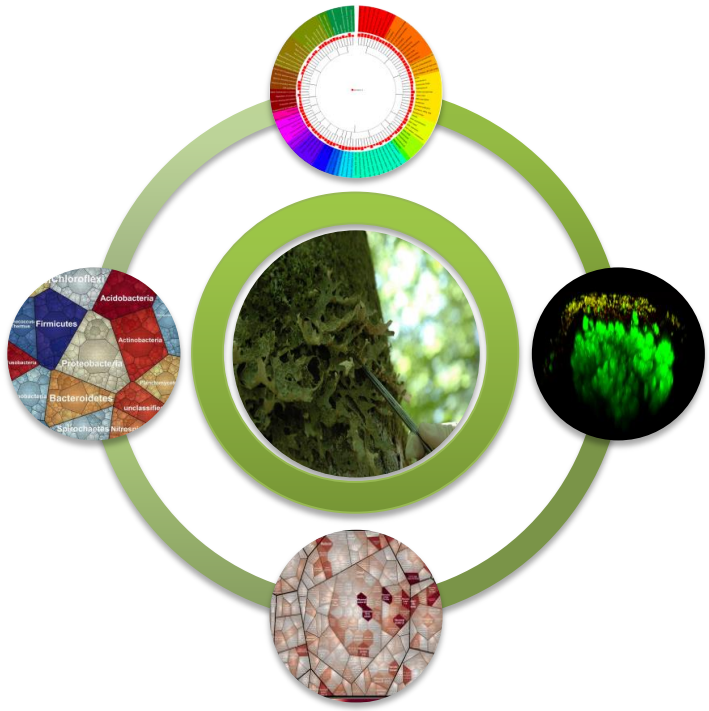

Figure 2 An integrative approach to explore the *L. pulmonaria* microbiome. Processed lichen samples were subjected to a multi-phasic approach which included cultivation-based analyses, FISH-CLSM visualization, metagenomics, metaproteomics, as well as amplicon sequencing. The integrated results were implemented in a holistic model of the symbiome.

Objectives and Summary of the Thesis

The focus of the thesis was a thorough taxonomic and functional characterization of bacteria that are associated with an unconventional model organism. Moreover, the aim was to identify particular beneficial contributions of microbial key players in the overall symbiome. This was facilitated by a multi-phasic and integrative approach which combined classic microbiological techniques with novel high-throughput analyses.

The lichen-associated microbiome plays a central role in the holobiont (Publication I)

Although the biofilm-like colonization of various lichen species by highly diversified bacterial populations has been described in previous studies, their functions remained largely

unknown. Meta-omic techniques were identified as suitable tools to globally identify potentially beneficial contributions of the bacterial population. It is of particular significance that a combination of metagenomics and metaproteomics was shown to harbor the potential to screen for present genetic information and to verify the expression at the protein level. In order to obtain a broader insight into the host associated microbiome, lichen samples were collected from a montane lichen population in Johnsbach/Austria. They were mechanically enriched for the bacterial fraction prior to molecular extractions. The prepared extracts were later subjected to meta-omic and complementary analyses. A representative number of bacterial isolates was used for various physiological assays which were previously identified as potentially relevant. Moreover, quantitative PCR (qPCR) was utilized to determine the nifH gene copy number in total genomic DNA extracts.

The results provided strong evidence that the bacterial microbiome is involved in nutrient provision and degradation of older lichen thallus parts, biosynthesis of vitamins and hormones, detoxification processes, and the protection against biotic as well as abiotic stress. Furthermore, a predominance of *Alphaproteobacteria* within a total of more than 800 taxa was confirmed, while other *Proteobacteria* were less abundant. The functional roles of the most abundant taxa were elucidated and it was shown that they contribute substantially to the lichen symbiome. These findings were verified at the protein level in addition to metagenomics analyses. Consequently, it was demonstrated that most functional classes from the analyzed metagenome can also be retrieved from the correlated metaproteome. The high prevalence of bacterial nitrogen fixation was confirmed with –omic data and qPCR. A comparison of the whole *Lobaria*-associated metagenome with a representative set of publicly available metagenomes highlighted its uniqueness. The closest related metagenomes were found to be those obtained from plant-associated habitats. All collected data were integrated into a holistic lichen model including the previously unknown bacterial contributions, making a substantial contribution to an improved understanding of this unique symbiosis.

Intrinsic *Rhizobiales* communities provide diverse supporting functions (Publication II)

Rhizobiales (*Alphaproteobacteria*) were previously shown to be remarkably abundant in the *L. pulmonaria* microbiome. Although they are well known for their beneficial interactions with many higher plants, less is known about their specific roles in terms of the lichens. In order to elucidate potential roles of these numerous colonizers, a combination of visualizing and bioinformatic techniques was employed. A metagenomics-based approach was utilized to globally assign functions to a taxonomically pre-filtered dataset. Identified functions with

relevant roles for the symbiosis were explored in more detail. The same metagenomic dataset was utilized for the design of a highly specific FISH probe, since other previously described probes were found to have an insufficient taxonomic coverage. Subsequently, this probe was utilized for a number of FISH-CLSM experiments to explore colonization structures and for a detailed localization of this bacterial fraction within lichen tissues.

The *Rhizobiales* community was shown to be mainly represented by the families: *Methylobacteriaceae*, *Bradyrhizobiaceae*, and *Rhizobiaceae*. Functional assignments based on hierarchical SEED classification indicated an involvement of *Rhizobiales* in various beneficial functions (*e.g.* auxin, folate, and vitamin B_{12} biosynthesis). A further breakdown demonstrated that the predominant *Methylobacteriaceae* were also the most potent producers of the examined metabolites. These findings suggest the potential for various biotechnological applications of this group. The novel metagenome-based FISH probe design has facilitated the development of a highly sample-specific visualization method. *In silico* experiments confirmed a low proportion of mismatches which would result in unspecific detection of taxa unrelated to *Rhizobiales*. FISH-CLSM visualization has shown a dense colonization of the lichen cortex by *Rhizobiales* and also demonstrated their inter-hyphal occurrence as endophytes. Moreover, bacteria that could not be assigned to a taxonomic group were detected inside lichen hyphae for the first time. These findings require further exploration since an intra-cellular lifestyle requires exceptional adaptions to the host and thus might reveal novel mechanisms of bacterial-fungal interactions.

The pathogen defense is supported by antagonistic members of the microbiome (Publication III)

The high proportion of antagonistic bacteria in the lichen-associated microbiome has raised questions as to whether the observed protective effects can be transferred to non-lichen hosts. For this purpose a culture collection obtained from multiple *L. pulmonaria* samples was screened for the most abundant and most active isolates. The approach included metagenomic screening for stress-reducing metabolites. Moreover, these results were additionally expanded with the identification and quantification of specific bacterial compounds from pure cultures. Data from amplicon sequencing was compared to sequences of distinct isolates to evaluate their representability within the microbiome. Moreover, co-occurrence networks were used to evaluate interactions of antagonistic bacteria with other present taxa. The most promising lichen-associated isolates were used for seed priming of a model plant.

The most abundant antagonists were assigned to *Stenotrophomonas*, *Pseudomonas*, *Micorcoccus* and *Burkholderia*. These genera accounted for 67% of all identified antagonistic bacteria. Metagenomic screening revealed the presence of genes involved in the biosynthesis of stress-reducing metabolites, but could not assure the contribution of any antagonistic taxa in their production. Complementary HPLC-MS analyses enabled the detection of *Stenotrophomonas*-produced spermidine which is known to reduce desiccation- and high salinity-induced stress in plants. It was shown that the intracellular spermidine concentrations differed significantly amongst different isolates. Moreover, analysis of 16S gene fragments from isolates and amplicon libraries from the same sampling sites revealed an overall high sequence similarity. In addition, co-occurrence analysis with alphaproteobacterial taxa indicated positive interactions among the three most abundant antagonists. A total of three spermidine-producing *Stenotrophomonas* isolates were selected for greenhouse experiments with primed tomato (*Solanum lycopersicum*) strains. As a result, two isolates were shown to significantly increase the root and stem lengths under water-limited conditions. The application of lichen-associated bacteria in plant protection and growth promotion may prove to be a useful alternative to conventional approaches. However, further studies are required to evaluate the host range and to elucidate the overall applicability.

Bioactive volatiles enhance the antagonistic potential of the microbiome (Publication IV)

Bioprospecting for novel bioactive substances still plays an enduring role in modern biotechnology. In a global context, the high prevalence of microbes with antibiotic resistances to commercially available substances requires a steady development of counteracting agents. Screening for soluble or fluid substances is facilitated by a wide array of well-established tools, while methods for volatile organic compounds (VOCs) are less feasible. Therefore, a novel plate-based assay was developed to enable robust and fast screening for bioactive volatiles. The applicability of the assay was tested with a representative number of lichen-associated bacteria and a set of model pathogens. Further validation was conducted with a qPCR-based quantification of livable target bacteria after exposition in the VOCs assay. Additionally, VOC profiles of selected isolates were obtained with headspace solid phase micro extraction and GC-MS.

The usability of the new screening assay was confirmed with replicative experiments which included *E. coli* and *B. cinerea* as target organisms. Moreover, it was shown that the assay presented makes it possible to differentiate between distinct effects. Within the tested isolates, *Pseudomonas* and *Stenotrophomonas* were identified as the most active VOCs producers.

Pseudomonas isolates were also shown to emit detectable amounts of hydrogen cyanide into the headspace. Preselected isolates were subjected to a complementary qPCR approach and it was shown that the number of livable *E. coli* was significantly reduced after co-cultivation in the VOCs assay. Furthermore, VOCs profiles from three distinct isolates showed that lichen-associated bacteria are emitting a broad range of volatile substances. These molecules are most likely involved in various interactions (*e.g.* communication between microorganisms and the host) and might also increase the overall resistance against various pathogens.

The microbiome provides complementary detoxification mechanisms (Manuscript in preparation)

The lichen-associated microbiome was shown to play a central role in a variety of stability-promoting functions. Identified functions include a variety of defense mechanisms, which confer enhanced resistance against biotic as well as abiotic stress. While global studies of the microbiome provided a first evidence for the involvement in the detoxification of inorganic substances (*e.g.* As, Cu, Zn), the detailed mechanisms remained unknown. In order to obtain a deeper insight into these beneficial contributions, the effects of elevated arsenic concentration on the microbiome were analyzed. Therefore, lichen samples were obtained from sampling sites with differing arsenic concentrations and subjected to a multi-phasic approach combining metagenomics, bioinformatics, analytics, and cultivation studies. A novel bioinformatics workflow was utilized to identify the spectrum of relevant functions in the respective metagenomes. These results were complemented with a qPCR-based approach to quantify As(III) methyltransferase (arsM) genes and a screening approach with cultivable bacteria. Supplementing analytical quantification of arsenic levels in the utilized samples was used to reinforce the findings and to elucidate the rate of related bioconversions.

Metagenomic analyses have revealed that the global microbial community structures from all three samples were similar irrespective of the arsenic concentrations. Although the bacterial composition was not altered by elevated arsenic concentrations, the spectrum of arsenic-related functions was extended. These functions include bioconversion mechanisms that are involved in the methylation of inorganic arsenic and consequently generate less toxic substances. Furthermore, the abundance of numerous detoxification related genes was enhanced in arsenic-polluted samples. Supplementary qPCR approaches have shown that the arsM gene copy number is not strictly related to the determined arsenic concentrations. The analyzed samples contained up to 4.4×10^6 gene copies g^{-1} lyophilized lichen samples. Lichen species with cyanobacterial photobionts showed generally higher arsM levels than species,

which solely incorporate green algae as photosynthetic partners. Additionally, a culture collection of bacterial isolates obtained from three lichen species was screened for the arsM gene. Detected carriers of arsM were later identified as members of the genera *Leifsonia, Micrococcus, Pedobacter, Staphylococcus*, and *Streptomycetes*. These isolates were exposed to different As(III) and As(V) concentration in further experiments. It was shown that distinct isolates can tolerate more than 60 mM arsenic in fluid media. The overall results reinforced the important role of the microbiome in host protection and provided detailed insights into the taxonomic structure of involved microorganisms.

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Publication I

Exploring functional contexts of symbiotic sustain within lichen-associated bacteria by comparative omics

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ORIGINAL ARTICLE

Exploring functional contexts of symbiotic sustain within lichen-associated bacteria by comparative omics

Martin Grube[1,5], Tomislav Cernava[2,5], Jung Soh[3,5], Stephan Fuchs[4], Ines Aschenbrenner[1,2], Christian Lassek[4], Uwe Wegner[4], Dörte Becher[4], Katharina Riedel[4], Christoph W Sensen[3] and Gabriele Berg[2]

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Symbioses represent a frequent and successful lifestyle on earth and lichens are one of their classic examples. Recently, bacterial communities were identified as stable, specific and structurally integrated partners of the lichen symbiosis, but their role has remained largely elusive in comparison to the well-known functions of the fungal and algal partners. We have explored the metabolic potentials of the microbiome using the lung lichen *Lobaria pulmonaria* as the model. Metagenomic and proteomic data were comparatively assessed and visualized by Voronoi treemaps. The study was complemented with molecular, microscopic and physiological assays. We have found that more than 800 bacterial species have the ability to contribute multiple aspects to the symbiotic system, including essential functions such as (i) nutrient supply, especially nitrogen, phosphorous and sulfur, (ii) resistance against biotic stress factors (that is, pathogen defense), (iii) resistance against abiotic factors, (iv) support of photosynthesis by provision of vitamin B_{12}, (v) fungal and algal growth support by provision of hormones, (vi) detoxification of metabolites, and (vii) degradation of older parts of the lichen thallus. Our findings showed the potential of lichen-associated bacteria to interact with the fungal as well as algal partner to support health, growth and fitness of their hosts. We developed a model of the symbiosis depicting the functional multi-player network of the participants, and argue that the strategy of functional diversification in lichens supports the longevity and persistence of lichens under extreme and changing ecological conditions.

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Introduction

Symbiosis, one of the most common lifestyles on earth, is a long-term interaction, which acts as source of evolutionary innovation (Margulis and Fester, 1991; Martin and Schwab, 2013). The term symbiosis was introduced by Frank (1877) in a study of lichens, which are today considered a classic example for self-sustaining partnerships of species belonging to different kingdoms of life (Nash, 2008). Lichens represent one of most diversified and oldest symbiotic lifestyles of fungi, with more than 18 000 recognized fungal species and a typical stratified morphology that evolved at least 415 million years ago (Honegger *et al.*, 2013). The light-exposed lichen thalli are shaped by outer layers of fungal hyphae, which shelter internalized phototrophic partners. Lichen thalli develop only with proper combinations of fungal and algal species. Once the symbiotic phenotype is established, lichens may reach indeterminate ages and may even survive even the harshest conditions on earth (Øvstedal and Lewis-Smith, 2001). One reason for the ecological success of this fungal-algal partnership is the mutually enhanced ability to survive oxidative stress by suspended animation and rapid resumption of metabolism under permissive conditions (Kranner *et al.*, 2005). However, additional and hitherto unidentified forces might have helped lichens to adapt to nutrient-poor and hostile habitats with strong fluctuation of abiotic parameters.

Most biology textbooks characterize lichens as an association solely between a fungal (mycobiont) and

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an algal (photobiont) partner. Our recent studies, however, revealed a high diversity of bacteria that are also associated with lichens. We have visualized biofilm-like communities, dominated by *Alphaproteobacteria* on the surfaces of thalli (Cardinale *et al.*, 2008, 2012a, b). Their structure suggests some degree of host specificity of the bacterial communities (Grube *et al.*, 2009; Hodkinson *et al.*, 2011; Bates *et al.*, 2011). The ubiquity and abundance of lichen-associated bacteria challenge the classic view of lichens as a two-tier partnership and support an extended concept that also includes the universally present bacterial communities. With this new perspective in mind, we have now explored the potential functions of the bacterial communities with the goal of understanding their symbiotic context in a more holistic way. Functional analysis of the culturable fraction of the lichen-associated bacteria suggested their involvement in several roles, including iron and phosphate mobilization, hormone production, nitrogen fixation as well as several lytic activities (Liba *et al.*, 2006; Grube *et al.*, 2009). The culturable fraction usually represents only a minor part of the total lichen-associated microbiome and therefore most likely does not cover the host-dependent majority of the bacterial species (Cardinale *et al.*, 2008). Recently developed omics approaches and subsequent bioinformatics tools are therefore required for a more comprehensive understanding of the role of the microbiome.

The objective of our study was therefore to investigate the function and metabolic potential of the bacterial lichen microbiome using a combined omics approach, together with a comprehensive spectrum of molecular, microscopic and physiological assays. The results were comparatively assessed and visualized using bioinformatics tools. For our study, we chose the lung lichen *Lobaria pulmonaria* (L.) Hoffm., a lichen which is considered to be endangered in many areas. *L. pulmonaria* serves as an indicator of primeval forest ecological continuity (Scheidegger and Werth, 2009). The mycobiont of *L. pulmonaria* engulfs a green-algal photobiont (*Dictyochloropsis reticulata*; found in 90% of the lichen surface) and a minor cyanobacterial partner, *Nostoc* sp. (Cornejo and Scheidegger, 2013). Schneider *et al.* (2011) provided an initial insight into the *L. pulmonaria* proteome and suggested *Lobaria* as an ideal model to study symbiotic processes. In our study, we extended this work and now provide results from the comparison of metagenomic and metaproteomic data.

Material and methods

Sampling strategy and preparation

L. pulmonaria was sampled from a rich population on maple tress (*Acer* spp.) in the Alps (Johnsbach, Austria; N 47°32′35″, E 14°37′38″) after visual inspection to avoid contamination by lichenicolous fungi and other organisms (Supplementary Figure S1). Using integrated sampling, a total amount of 176.3 g lichen was shock frozen with liquid nitrogen and immediately ground with mortar and pestle. The sample was homogenized in 360 ml 0.85% NaCl and filtered using a 63 μm mesh sieve; larger lichen parts were retained and colonizing bacteria were enriched in the filtrate. The filtrate was centrifuged at 8,000 r.p.m. at 4 °C for 20 min and the pellet was resuspended in 16×1.5 ml 0.85% NaCl aliquots. After a subsequent centrifugation step at 13 000 r.p.m. at 4 °C for 20 min, the supernatant was discarded and the pellets were used for DNA isolation (PowerSoil DNA Isolation Kit, MO BIO Laboratories Inc., Carlsbad, CA, USA). Following the DNA isolation, an aliquot containing 22 μg of metagenomic DNA was sent to GATC Biotech (Konstanz, Germany) for Illumina sequencing (HiSeq 2000 paired-end runs, Illumina Inc., San Diego, CA, USA).

Quality control and assembly of Illumina reads

Illumina HiSeq 2000 paired-end metagenomic DNA sequencing reads (GATC Biotech) were initially quality-checked using the FastQC program. The adapter sequence (5′-GATC GGAA GAGC ACAC GTCT GAAC TCCAG TCAC GTCC GCAC ATCT CGTAT-3′: identified as part of the Illumina TruSeq Indexed Adapter) was found in over 1% of the reads in set 1, and diminishing quality scores were observed towards the end of reads. Based on this observation, quality trimming and filtering was performed on each raw read set, using a custom-developed Perl script (written in-house by JS). The adapter sequences were removed and the sequence ends were trimmed, when the base quality score was smaller than 20. After the trimming step, reads were filtered out (i) if the length was shorter than 75 bp; (ii) or the read contained one or more ambiguous (N) bases; or (iii) the average quality score overall bases of the read was <25. After quality control, we did not observe overrepresented sequences, significant degradation of base qualities, or any other major quality issues. The sequence quality was confirmed again using the FastQC program. After quality control, some reads from set 1 did not have matching reads from set 2 due to read filtering, and vice versa. Therefore, to use paired-end information during assembly, only those reads that formed a matching pair were retained for further analysis.

Assembly of the reads into contiguous sequences (contigs) was performed using the Velvet *de novo* assembly software (Zerbino and Velvet, 2008; http://www.ebi.ac.uk/~zerbino/velvet/). Multiple trial assemblies with different *k*-mer lengths (57, 61, 65, 67, 69, 71 and 73) were performed and assembly statistics were compared with find the best *k*-mer length. All assemblies were conducted with an insert length of 350. The final *k*-mer length chosen was 71, which resulted in the maximum N50 value (2411 nts).

Taxonomic and functional analysis of assembled Illumina reads
The number of actual contigs used for this analysis was 368 424 (out of 503 528 overall contigs), based on the default Velvet minimum contig-length threshold of two times the *k*-mer length and the default coverage cutoff of half the median coverage. The Tera-BLASTN program (www.timelogic.com/documents/TeraBLAST_2009.pdf) was run on the 368 424 contigs, using TimeLogic (Active Motif, Carlsbad, CA, USA) DeCypher boards against the 'nt' database from NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db). The blastn results were imported into MEGAN (Metagenome Analyzer, v4.70.4; Huson *et al.*, 2011) to produce several taxonomy profiles. For assembly-based functional analysis, we used a similar approach as above but using BLASTX (www.timelogic.com/documents/TeraBLAST_2009.pdf), which was run against the 'non-redundant protein sequence' database from NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db). The blastx results were imported into MEGAN (v4.70.4) for functional analysis. Both SEED and KEGG functional analyses were conducted with MEGAN.

MG-RAST analysis of Illumina reads
Unprocessed Illumina HiSeq 2000 reads were uploaded on the MG-RAST v3 public server (Meyer *et al.*, 2008) to undergo paired-end reads joining and quality filtering (with default settings). 67 731 962 (88.8%) out of 76 310 051 sequences passed quality control; therein 60 015 088 (78,7%) sequences contained predicted proteins of either known or unknown function, while 1 788 100 (2.3%) sequences contained ribosomal RNA genes. SEED Subsystems Annotation was conducted with a maximum e-value cutoff of $1e^{-5}$ and a minimum 60% identity cutoff. Rarefaction analysis based on identified ribosomal RNA genes was done using best-hit classification and the Greengenes database (http://greengenes.lbl.gov) as the annotation source (with a minimum e-value cutoff of $1e^{-5}$; Supplementary Figure S2). The *Lobaria* metagenome was compared within a Principal Coordinates Analysis (annotation source: subsystems) with 20 publicly available datasets on MG-RAST. The functional abundance of eight particular habitats was compared using a minimum e-value cutoff of $1e^{-5}$. A table with all compared habitats together with their MG-RAST accession numbers is provided in the Supplementary Material (Supplementary Table S1). The metagenomic dataset is available under MG-RAST ID 4530091.3.

Quantitative real-time PCR
Quantification of *nifH* genes in the lichen DNA extract was conducted with primer pair *nifH*-F/*nifH*-R, as described by Hai *et al.* (2009). Standards containing the *nifH* fragments were prepared according to Bragina *et al.* (2013). Briefly, the gene fragments from *Erwinia carotovora* subsp. *atroseptica* SCRI1043 were cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) and later re-amplified with vector-specific primers. Amplification-grade DNase I (Sigma-Aldrich, St Louis, MI, USA) treated total DNA extract was used to determine inhibitory effects of co-extracted substances. Based on this experiment, the total community DNA was diluted to 1:25 and target genes were amplified using KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Woburn, MA, USA). Two independent runs, with three replicates for each sample, were performed on the Rotor Gene 6000 (Corbett Research, Mortlake, VIC, Australia), according to Bragina *et al.* (2013). The specificity of the amplicons was confirmed with both melting-curve analysis and gel electrophoresis of the qPCR products, respectively. Gene copy numbers for *nifH* were calculated per gram of lichen fresh weight.

Sample preparation for protein extraction
The collected thalli (Johnsbach, Austria; N 47°32′35″, E 14°37′38″) were cleaned with sterile tweezers from moss, bark and other visible contaminations. Samples from different thalli were pooled to a total amount of 2 g. Liquid nitrogen was added to the pooled thalli, which were subsequently ground to a fine powder using mortar and pestle. Proteins were extracted as described by Wang *et al.* (2006).

Gel electrophoresis with extracted proteins
One-dimensional SDS-polyacrylamide gel electrophoresis was performed as described earlier (Laemmli, 1970), and by loading 50 µg of extracted lichen protein mixture per lane. The sample was analyzed in three technical replicates (three lanes). Electrophoresis was carried out at 150 V and 250 mA for 45 min, afterwards proteins were fixed by shaking the gel in an aqueous solution containing 40% ethanol and 10% acetic acid for 30 min. Finally, proteins were stained with 25 ml of colloidal Coomassie Brilliant Blue G (Sigma-Aldrich, Steinheim, Germany), as described earlier (Neuhoff *et al.*, 1988). The gel was scanned on a standard light scanner (Microtek, Hsinchu, Taiwan) for documentation. Afterwards each of the three lanes was cut into 20 pieces, as shown in the Supplementary Material (Supplementary Figure S3).

In-gel digestion after SDS-polyacrylamide gel electrophoresis
Each of the 20 gel pieces from the three technical replicates was cut into small cubes (1 mm^3), which were destained by adding 700 µl of 30% acetonitrile containing 0.2 M NH_4HCO_3, and shaking for 15 min at 37 °C and 1500 r.p.m. This step was repeated

twice. After destaining, the pieces were dried in a vacuum concentrator (Eppendorf, Hamburg, Germany). In-gel digestion was performed with 50-μl-modified sequence-grade trypsin (Promega), with an enzyme concentration of $2\,\mu g\,ml^{-1}$ overnight at 37 °C. Subsequently, peptides were eluted by sonication for 15 min (Ultra sonic cleaner, VWR, Darmstadt, Germany). The eluted peptides were transferred into vials, dried in a vacuum concentrator (Eppendorf) again, and finally dissolved in 10 μl of ultrapure water.

Mass spectrometry analysis

Peptide mixtures resulting from in-gel tryptic cleavage were subjected to liquid chromatography-tandem mass spectroscopy measurements, using an EASYnLC 1000 (Thermo Scientific, Odense, Denmark), with self-packed columns (Luna 3 μ C18(2) 100 A, Phenomenex, Aschaffenburg, Germany) in a one-column setup online, coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher, Bremen, Germany). Samples were loaded and desalted in 0.1% acetic acid, with a flow rate of $700\,nl\,min^{-1}$, followed by peptide separation achieved by a binary non-linear 170 min gradient from 5–50% acetonitrile in 0.1% acetic acid at a flow rate of $300\,nl\,min^{-1}$. Mass spectrometric measurement was performed in the Orbitrap Elite (Thermo Fisher Scientific) at a spray voltage of 2.4 kV applied to the emitter. After a survey scan in the Orbitrap ($R = 30\,000$) tandem mass spectroscopy data were recorded for the 20 most intensive precursor ions in the linear ion trap. Singly charged ions were not taken into account for tandem mass spectroscopy analysis. The lock mass option was enabled throughout all analyses.

Database construction for metaproteome analysis, database search and metaproteome data analysis

Due to the lack of translated metagenomic sequences of *L. pulmonaria,* a synthetic metagenomic-based database was created. This database contained all protein sequences available in the NCBI NR protein database (state of 13.09.26) which have been predicted to be present in the sample based on similarity searches of the metagenomic reads by BLAST (Altschul *et al.*, 1990). Because neither the fungal nor the algal symbiont genomes of *L. pulmonaria* have been sequenced yet, all available NCBI protein sequences from fungi and algae were added to the database, together with a set of typical contaminations (for example, porcine trypsin, human keratin). Entries with the same protein sequence, but different headers were combined into one entry by an in-house php script (written by SF). The final database contained 2 473 550 protein sequences, pointing to 2 581 850 GenBank identifiers (bacteria: 1 564 300; algae: 226 723; fungi: 513 152; archaea: 26 952; other unclassified: 250 723).

The raw files were converted to mgf-files by MSconvert (www.Proteowizard.org), and searched with the Mascot search engine (version 2.2.04, Matrix Science Inc., Boston, MA, USA) with the following parameters: parent mass tolerance 10 ppm, fragment mass tolerance 0.5 Da, maximum missed cleavages 2; charge state 1+; variable modifications and oxidation of methionine. The Mascot search was followed by an X-tandem analysis in Scaffold (version 4.0.7, Proteome Software Inc., Portland, OR, USA). This analysis was performed as a 'MudPit experiment' to merge the individual mascot result files into a single file. The results were filtered as follows: 99% peptide probability, 1 peptide, 99% protein probability. Only proteins detected in two out of three technical replicates were considered for further analyses.

For functional classification and taxonomical distribution the in-house developed metaproteome analyses pipeline 'Prophane 2.0' was used (http://www.prophane.de; Schneider *et al.*, 2011). Briefly, peptide to protein matches were clustered in groups by the Scaffold software. To standardize functional annotation all peptide to protein matches were functionally characterized based on TIGRFAMs (release 12; Haft *et al.*, 2013) using HMMER3 (e-value $\leqslant 1E-10$; Haft *et al.*, 2013; Eddy, 2011). Functional data were transferred to the protein group if the members share the same predicted function. If these proteins share multiple predicted functions the function with the lowest overall e-value were assigned to the respective protein group. Groups of proteins sharing no functional prediction were named heterogeneous on functional level.

Moreover, protein groups were taxonomically classified based on the annotation of the respective protein members. Protein quantification was based on normalized spectral abundance factor values (Zybailov *et al.*, 2006), however, only exclusive spectral counts were considered.

Voronoi treemaps

Voronoi treemaps were generated using Paver (Decodon, Greifswald, Germany, http://www.decodon.com/).

Fluorescence *In Situ* Hybridization and Confocal Laser Scanning Microscopy samples were collected from the same *Lobaria* population used for the metagenomics and metaproteomics analysis. Lichen thalli were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) (v/v, 3:1) at 4 °C for at least 4 h, followed by three washing steps with ice-cold PBS. The samples were stored at −20 °C in ethanol absolute/PBS (v/v, 1:1). Before the hybridization step of the thallus, 30-μm thick cryosections were made. Fluorescence *In Situ* Hybridization was carried out in tubes, as outlined in Cardinale *et al.*,

(2008) and samples were analyzed using a Leica TCS SPE microscope (Leica Microsystems, Heidelberg, Germany), as well as image analysis and three-dimensional modeling, using the software Image Surfer (http://imagesurfer.cs.unc.edu/) and Imaris 7.0 (Bitplane, Zurich, Switzerland), respectively.

Isolation of lichen-associated bacteria
Lichen thalli of *L. pulmonaria* were sampled from three different locations in Austria (Tamischbachgraben, N 47°32′40″, E 14°37′35″, Johnsbach, N 47°32′35″, E 14°37′38″, and St. Oswald, N 46°44′50″, E 15°04′26″). After grinding lichen samples with mortar and pestle, a homogenate was prepared using sterile 0.85% NaCl in a 1:10 (w/v) ratio, together with a lab stomacher (BagMixer; Interscience, St Nom, France). Diluted fractions were plated on R2A agar (Carl Roth, Karlsruhe, Germany), R2A agar with 25 μg ml^{-1} cycloheximide, starch casein agar (Kuester and Williams, 1964) and ISP2 agar (Shirling and Gottlieb, 1966). Bacterial colonies were randomly picked within 5 days of incubation at room temperature.

Screening of isolates for in vitro antagonistic activity towards particular bacteria and fungi
Dual-culture experiments were carried out as confrontation assays, using different media and target organisms according to Berg *et al.* (2002) and Opelt *et al.* (2007). Lichen-associated isolates were spotted on solid media pre-inoculated with *E. coli* XL1 and *S. aureus* ATCC 25923 and assessed for inhibition zones after 4 days of incubation at 30 °C. Antagonistic activity against the fungus *Botrytis cinerea Pers.* (TU Graz culture collection, Graz, Austria) was tested by dual culture on Waksman agar, according to Berg *et al.* (2002) and assessed after 5–7 days of incubation at 20 °C. Cultures of the lichen-colonizing fungus *Rhinocladiella* sp. (TU Graz culture collection) were homogenized and resuspended in sterile 0.85% NaCl. Aliquots from one batch (50 μl) were used to inoculate each well of 24-well plates, containing solid potato dextrose agar (Carl Roth, Karlsruhe, Germany). Subsequently, 100-μl culture filtrate obtained from each lichen-associated isolate was added to particular wells. After 3 weeks of incubation, the wells were checked for growth reduction. All experiments were conducted with replicates and carried out twice.

Functional assays with Lobaria-associated bacteria
Altogether, 388 randomly selected bacterial cultures were subjected to functional assays based on different growth media. Protease, β-glucanase and chitinase activity were analyzed according to Berg *et al.* (2002), and phosphate solubilization of strains as described by Nautiyal (1999). *Chromobacterium violaceum* CV026 was used to detect C4–C6 AHL-mediated quorum sensing by visualizing purple pigmentation of the reporter strain (McClean *et al.*, 1997). *Pseudomonas putida* F117 pAS-C8 and *P. putida* F117 pAS-C12 (Steidle *et al.*, 2001) were used to detect C8 AHLs and C12 AHLs, respectively. Visualization of the green fluorescent protein-based AHL sensor was achieved through epifluorescence imaging, using an Universal Hood III (Bio-Rad, Hercules, CA, USA). All strains were incubated at 30 °C for 48 h.

Results

Taxonomic structure of the bacterial lichen microbiome
The analysis of 368 424 contigs revealed the taxonomic profile represented in Figure 1 that shows the overall composition of the metagenome and a more detailed structure of the dominant bacterial taxa within. Among the *Proteobacteria*, *Alphaproteobacteria* was the prominent taxon, with *Rhizobiales* and *Sphingomonadales* as the most frequently called orders. Within *Rhizobiales*, *Methylobacteriaceae* and *Bradyrhizobiaceae* are prominent, with *Rhizobiaceae*, *Beijerinckiaceae*, *Xanthobacteriaceae* and *Phylobacteriaceae* in minor quantities. Almost all of the *Sphingomonadales* belonged to the *Sphingomonadaceae*, and we estimate that a total of more than 800 bacterial species represent the diversity of the associated bacterial community, according to the rarefaction analysis (Supplementary Figure S2). Results derived from the present metaproteomic analysis presented an outmost similar community structure to the metagenome analysis. *Proteobacteria* were the predominant phylum and accounted for 361 distinct database hits, followed by *Cyanobacteria* with 47 hits and *Acidobacteria* with 28 hits. A complementary Fluorescence *In Situ* Hybridization visualization with *Alphaproteobacteria* and *Betaproteobacteria* specific probes, together with unspecific eubacteria probes illustrated and generally confirmed the taxonomic distribution observed by the metagenome analysis. *Alphaproteobacteria* were predominant and widespread on both, the upper and lower surface of the leaf-like lichen thallus, respectively, while *Betaproteobacteria* were less abundant and locally restricted (Figure 2a).

Metagenome and proteome of the bacterial lichen microbiome
Functional analyses of the lichen metagenome focused on bacterial contigs, as the sampling procedure was designed to enrich the bacterial metagenome, using both SEED and KEGG functional analyses. SEED functional analysis was used for finding a set with functions of interest (that is, carbohydrate, virulence, cofactors and so on), many of which are standard SEED functional terms. Out of the 368 424 contigs, 69 823 were assigned to a functional term and the breakdown of the assignments at the top level is shown as a bar graph

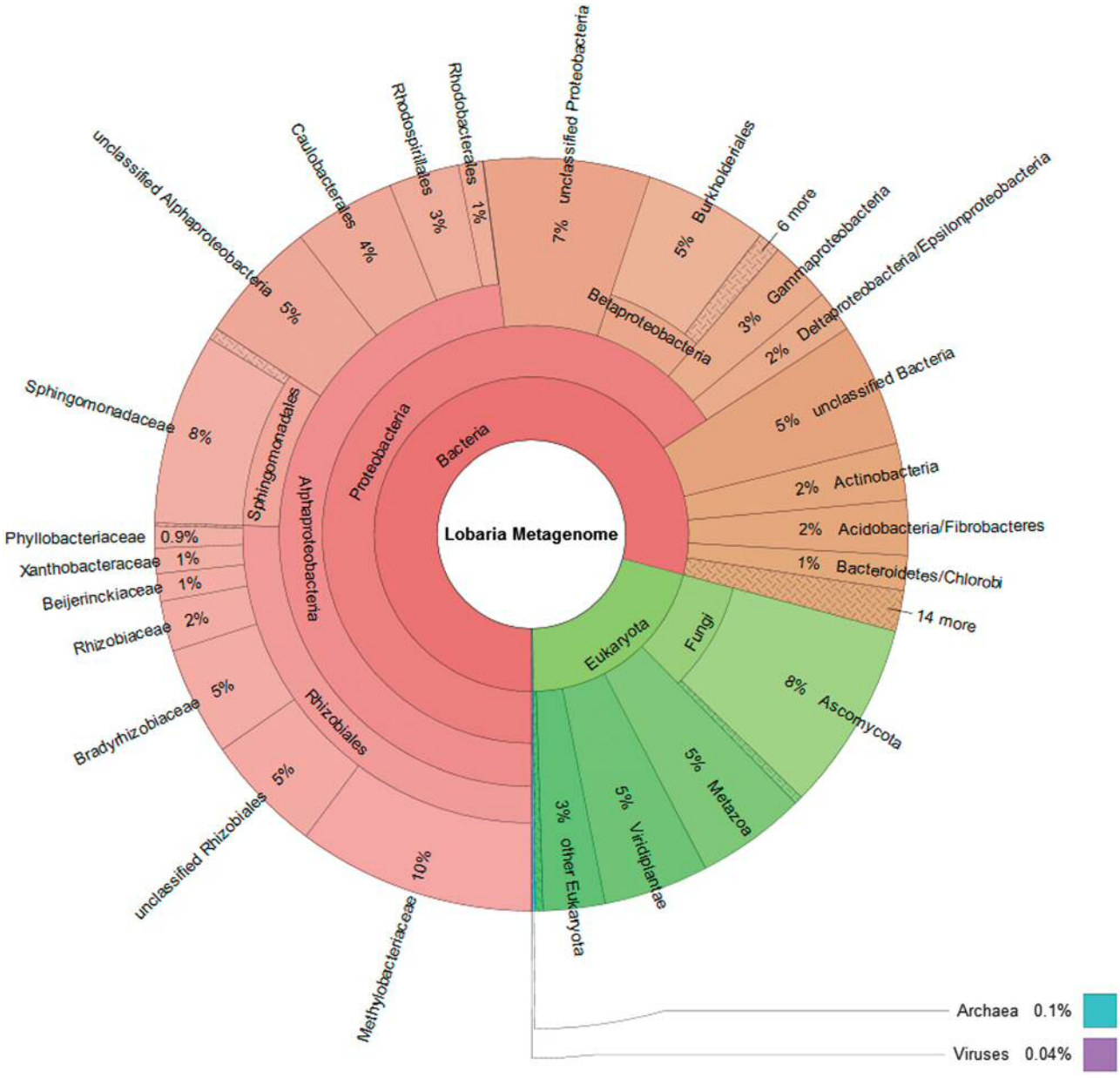

Figure 1 Taxonomic spectrum visualized with Krona (www.krona.sourceforge.net/) of contigs in the metagenome for all domains of life. Circles represent taxonomic classifications in ascending order up to the family level (outermost circle). Less-abundant taxa are listed outside the charts together with their relative abundance.

(Supplementary Figure S4). A significant number of contigs represented primary metabolic functions of bacteria (amino acids and derivatives: 6440; central carbohydrate metabolism: 2770; fatty acids, lipids and isoprenoids: 2721). Taxonomic separation of functional assignments revealed that up to 50% of different functional terms were covered by *Alphaproteobacteria*, while less-abundant bacteria account for the remaining contigs (Figure 3). Moreover MG-RAST visualization of functional abundance with SEED subsytems annotation showed presence of all level 4 functional terms. Out of the 368 424 contigs, 66 739 were assigned to KEGG pathways. Although the KEGG pathway assignment is primarily developed using mammalian genome information, it covers central capabilities of universal primary metabolism (carbohydrate metabolism: 12 823, energy metabolism: 7616, lipid metabolism: 5301, nucleotide metabolism: 5909, amino acid metabolism: 12 784, metabolism of other amino acids: 4068, glycan biosynthesis and metabolism: 2181 and genetic information processing: 12 258). Principal Coordinates Analysis carried out with MG-RAST revealed a unique functional distribution most similar to the one found on the plant phyllosphere (Figure 4; Supplementary Table S1).

Lichen samples used for metagenome analyses were investigated in parallel on metaproteomics

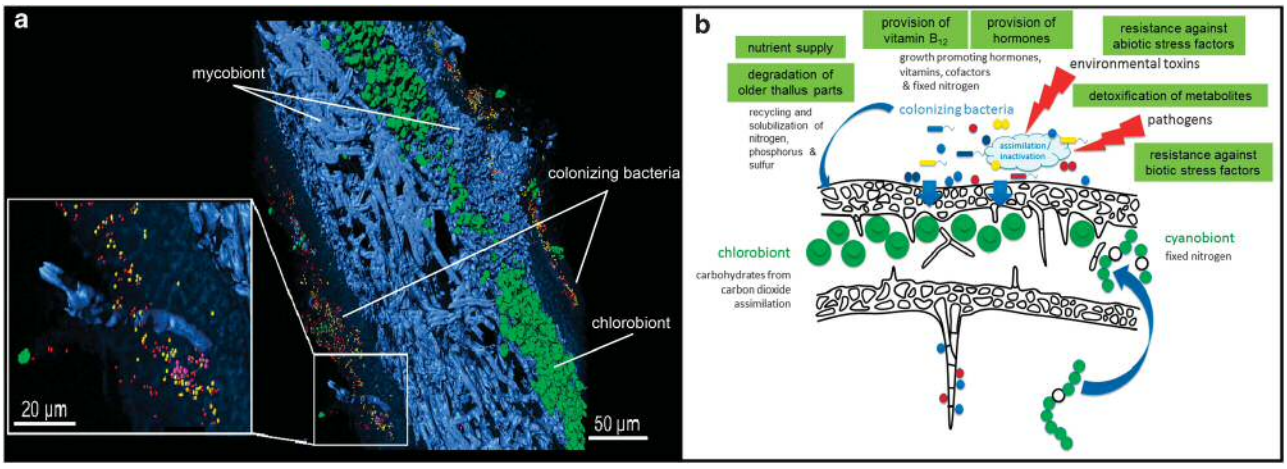

Figure 2 (**a**) Leaf-like thallus visualization of bacteria on a cross-section by 3D reconstruction of FISH image stacks. *Eubacteria* (red) and *Alphaproteobacteria* (yellow) were found widespread on both, the upper and the lower cortex, while *Betaproteobacteria* (pink) were less abundant and locally contained. Fungal hyphae (blue) and algae located under the upper cortex (green) were visualized without specific FISH probes, due to the naturally occurring fluorescence of the internal structures. (**b**) Model of the lichen symbiosis depicting the functional network of the participants. The model includes relevant functions of the colonizing bacteria, which are derived from metagenomic/metaproteomic analysis, as well as cultivation-dependent experiments.

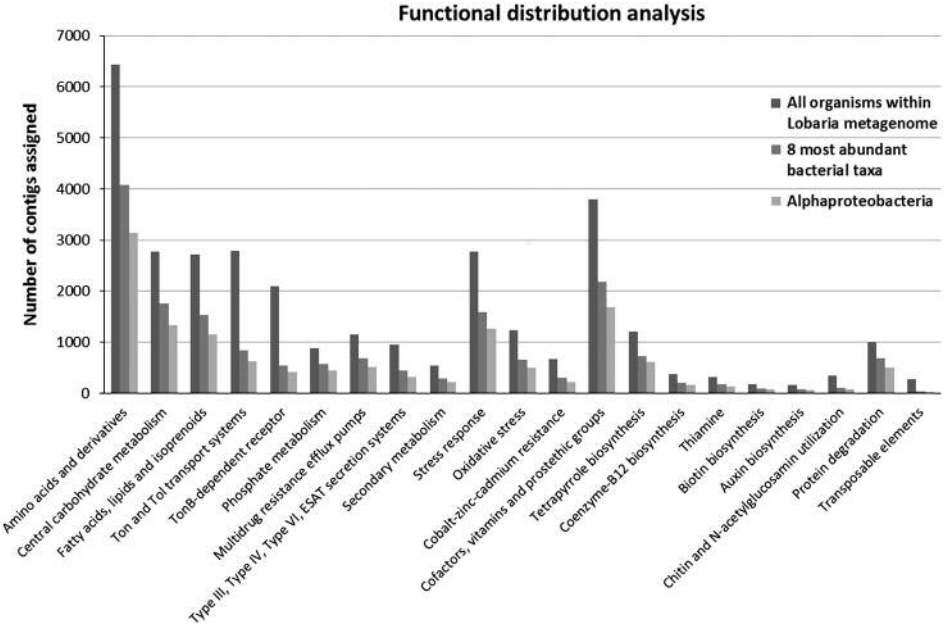

Figure 3 Distribution of particular functions prior and after exclusion of less-abundant taxa. The number of contigs assigned to all organisms within the sample (blue bars) is visualized in contrast to assigned contigs of the eight most-abundant taxa (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes* and *Firmicutes*; red bars) and *Alphaproteobacteria* (green bars). The full colour version of this figure is available at *ISME* Journal online.

level. For this a in-gel tryptic digestion followed by liquid chromatography-tandem mass spectrometry based metaproteomics approach was used as recently described (Schneider *et al.*, 2011) combined with a database containing all BLAST protein hits of the metagenomic reads (best hit per read). In total, our metaproteome analyses revealed 4405 different proteins, which were covered by at least one peptide-to-spectrum match (99% peptide probability, 99% protein probability) in at least two of three replicates. All proteins were divided into 3226 groups, based on shared peptide-to-spectrum matches by the Scaffold software (Koskinen *et al.*, 2011). Out of these, 2676 and 541 groups could be unambiguously assigned to a eukaryotic and eubacterial origin, respectively, employing the Prophane workflow (Schneider *et al.*, 2011). As our study aims for a better understanding of structure and functionality of the bacterial lichen microbiome, further functional analyses based on TIGRFAMs (e-value $\leqslant 1E-10$) focused exclusively on protein groups of bacterial origin. Functional

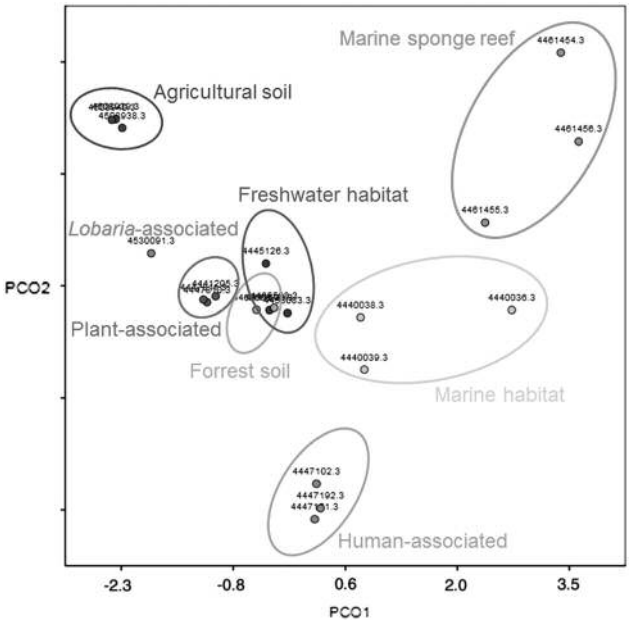

Figure 4 Principal Coordinates Analysis (PCoA) including 20 publicly available metagenomic datasets from MG-RAST and the *Lobaria* metagenome (red dot). All datasets were compared with subsytems and calculated using normalized values and the Bray-Curtis distance matrix. Single metagenomes from different biomes are labeled with their MG-RAST accession numbers and grouped in colored ellipses. The full colour version of this figure is available at *ISME* Journal online.

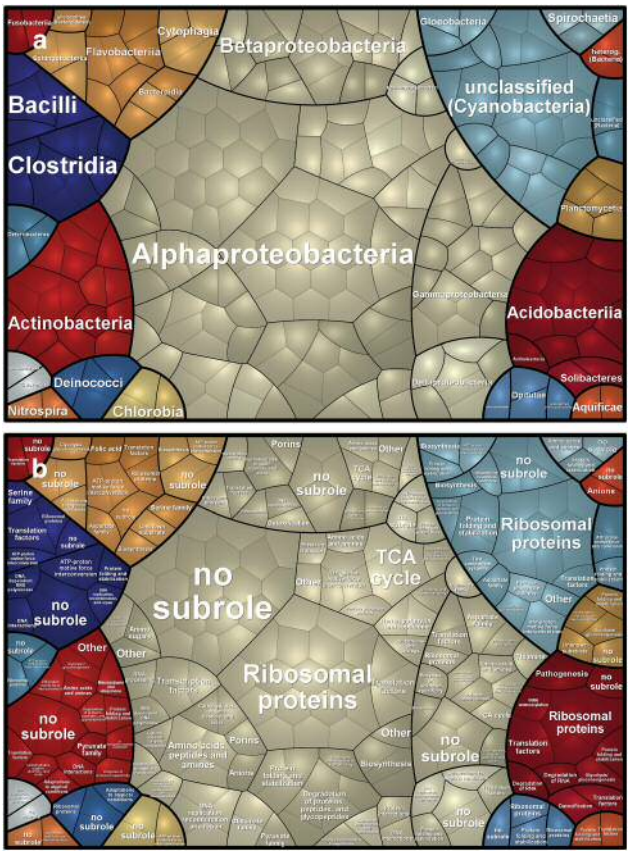

Figure 5 Voronoi Treemap visualization of the prokaryotic metaproteome on the taxonomic (**a**) and functional (**b**) level. The taxonomic and functional distributions were carried out by Prophane 2.0. Taxonomic groups are indicated by different colors (**a** and **b**) and the functional classes are separated by black lines (based on TIGRRoles). Each cell represents at least one protein (or protein group) assigned to the respective phylum.

data were only transferred to bacterial protein groups whose members share the same functional prediction (424 out of 541, see material and methods for details). To gain a view on the role of bacterial proteins, the data were visualized with Voronoi treemaps, which also show the participation of different eubacterial groups in functions (Figure 5). In addition, we integrated the metaproteomic data with metagenomic data using the Voronoi treemap (Figure 6). Generally, most of the functional classes predicted by the metagenome analysis are also represented by at least one protein. As expected, numerous proteins assigned to functions involved in protein synthesis, DNA replication, recombination and repair were found in the metaproteome. Moreover, a significant number of proteins involved in protein fate and central energy metabolism, for example, tricarbonic acid cycle, were recovered from the bacterial lichen microbiome.

Selected functions of the bacterial lichen microbiome

Nutrient supply. About 2793 contigs suggested the presence of Ton and Tol transport systems, some of which are also involved in iron uptake. The TonB-dependent receptor, a family of beta-barrel proteins from the outer membrane of Gram-negative bacteria and responsible for siderophore transport into the periplasm, was present in 2094 contigs. Metaproteome analysis indicated at least four different types of TonB-dependent receptors, which were derived from two different bacterial phyla (*Proteobacteria* and *Bacteroidetes*, respectively). Phosphate metabolism is represented in 885 contigs, as well as in two PFAM classifications within the metaproteome and also includes proteins involved in solubilization of phosphates. Corresponding to this finding, 19.6% of all bacterial isolates from *Lobaria* formed clearing zones on NBRIP agar and thus underscored the potential role of lichen-associated bacteria in the solubilization of phosphates. Moreover, we utilized a quantitative real-time PCR approach, based on total community DNA, to evaluate the bacterial potential for nitrogen fixation. Therewith $\log_{10}$ 5.0 ± 0.1 *nifH* copies were identified per gram of lichen fresh weight.

Resistance against biotic stress factors (pathogen defense). Virulence functions are common among the lichen-associated bacteria. About 1152 contigs were assigned to multidrug resistance efflux pumps, and multiple genes that code for resistance against antibiotics were also found (fluoroquinolone, vancomycin, methicillin, penicillin and cephalosporine) in addition to the 955 contigs that contribute

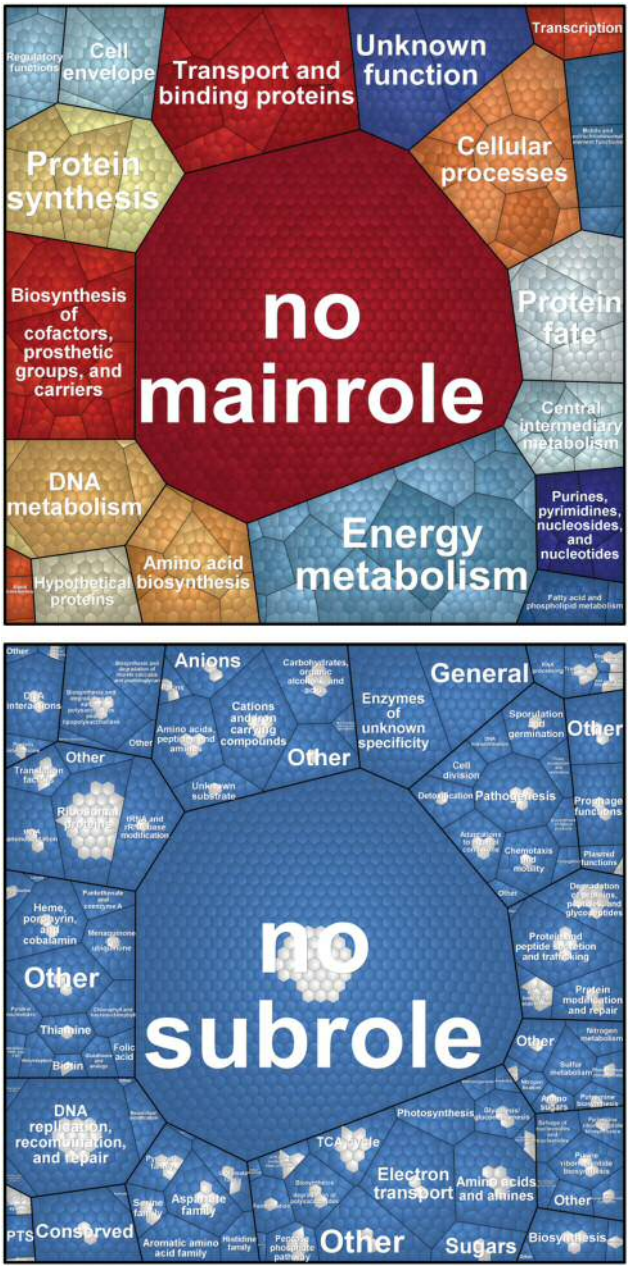

Figure 6 Functional recovery of the bacterial lichen microbiome metagenome on the metaproteomic level using Voronoi treemaps. The protein sequences were compared with metagenomic contigs by BLAST and then functionally characterized using HMMER3 and TIGRFAMs. Upper panel shows all functions covered by the metagenome (third level) and the respective subroles (second level) and main roles (first level). The mainrole labels are shown. On the lower panel, metaproteomic coverage is shown (blue cells: functions present only in the metagenome; grey cells: functions present in both metagenome and metaproteome).

to Type III, Type IV, Type VI and ESAT secretion systems. Type VI secretion systems were represented by three different variants in the metaproteome data, as well as one specific hit for an acriflavin-resistance protein. A moderate number of genes were also found which contribute to secondary metabolism, according to SEED analysis (548 contigs); some of the secondary metabolites are known as antibacterials or antifungals, but are represented by a fairly low number of contigs (phenazine biosynthesis protein PhzF: 26 contigs, clavulanic acid biosynthesis: 11 contigs). Furthermore, results from cultivation-dependent studies involving *Lobaria*-associated bacteria combined with taxonomic data from the present metagenomic approach suggest that around 7% of the taxa present have an antagonistic potential. The majority of these showed antagonism against fungi (data not shown).

Resistance against abiotic stress factors. Stress-related functions are distinct in the bacterial microbiome (2769 contigs; oxidative stress: 1238). About 891 contigs indicated genes conferring resistance to metals (copper, cobalt-zinc-cadmium, silver, mercury and arsenic). Interestingly, a similar proportion of oxidative-stress protectants to heavy metal efflux pumps was observed in the metaproteome (3 and 1, respectively). In addition, we looked for genes involved in dormancy and sporulation, as we expected this to be an important aspect of bacteria adapted to the poikilohydric life on lichens. However, only 13 contigs were related to this function.

Photosynthesis support by vitamin B_{12}. Numerous genes are involved in the metabolism of cofactors, vitamins and prostethic groups (3799 contigs), according to SEED functional analysis, and a relatively high number of 1203 contigs was annotated to tetrapyrrole biosynthesis. Among these, for example, 365 contigs coded for coenzyme-B_{12} biosynthesis, 312 contigs for thiamine and 174 contigs indicated biotin biosynthesis. In the KEGG analysis, 5479 contigs were linked with the metabolism of cofactors and vitamins. Metaproteome analysis resulted in 2 hits that support the presence of enzymes involved in cobalamin biosynthesis, while folic acid and thiamine biosynthesis were represented by 1 hit each, respectively.

Hormone production. For the potential production of hormones, we found that auxin biosynthesis was represented by 156 contigs.

Detoxification of metabolites. In the KEGG analysis, many of the contigs that indicate xenobiotics biodegradation and metabolism (5482 contigs) seem to also be involved in the degradation of phenolic compounds. The presence of enzymes involved in degradation of phenolic compounds was reinforced by three hits in the metaproteome data.

Lytic activities. Degradation of older thallus parts could provide nutrients for the young and growing thallus parts. Such function is enhanced by bacterial enzymes involved in specific degradation procedures. 341 contigs within the metagenome are connected to chitin and N-acetylglucosamine utilization. Additionally, 1000 contigs were found for

protein degradation of which 957 contigs were derived from bacteria. The cultivation-based functional screening underlines that *Lobaria*-associated bacteria are key-players in the recycling of old lichen parts: 33% of the isolates demonstrated protease activity, while 6.7 and 12.9% have shown chitinase and glucanase activity, respectively.

Other functions. In the KEGG analysis, the biosynthesis of polyketides and terpenoids was suggested in 2975 contigs, and the biosynthesis of other secondary metabolites is suggested by a further 2012 contigs. This set also includes known antibiotics such as betalain, penicillin, streptomycin, butirosin, neomycin and novobiocin, as well as phenylpropanoids, stilbenoids and alkaloids. A substantial number of contigs in the KEGG analysis was linked with pathways of environmental information processing (12 053 contigs), and of these, membrane transport forms a fraction of 7629 contigs. Some bacteria also undergo CO_2-fixation, of which about 757 bacterial contigs were assigned. Notably, polyol utilization is represented in 301 contigs (ribitol-, xylitol-, arabitol-, mannitol- and sorbitol-utilization).

Quorum sensing was rather poorly represented in our dataset with only 25 contigs. Metaproteome analysis supports the under-represented role of quorum sensing within the *Lobaria* microbiome, no relevant sequences were identified. It was therefore not surprising, that cultivation-dependent experiments involving lichen-associated bacteria revealed that $<1\%$ of tested isolates produced detectable N-acyl homoserine–lactone-derived quorum sensing molecules. In addition, phages, prophages and transposable elements were not particularly common among the lichen-associated microbiome (275 contigs).

Comparison with an algal partner. To confirm that the selected functions noted above are truly dominant in the lichen-associated bacteria, we have compared the SEED function analysis of the *Lobaria* metagenome with that of a transcriptome of *D. reticulata*, the green-algal photobiont of *L. pulmonaria*. The transcriptome data were obtained from the Joint Genome Institute repository (sequencing of three cultured partners of *L. pulmonaria*, http://genome.jgi.doe.gov/Lobpulcupartners/Lobpulcupartners.info.html). There are a total of 102 102 contigs in the assembly (about 27.71% of the 368 424 contigs of the lichen metagenome). The SEED analysis for the *D. reticulata* data was performed in exactly the same way as was done for the lichen metagenome. Supplementary Table S2 shows a comparison of the selected microbiome function assignments between these two datasets, whereas Supplementary Table S3 shows the same kind of comparison of top-level SEED functions. As Supplementary Table S2 clearly shows, for every selected function discussed until now, the lichen data has far more contigs assigned than the *D. reticulata* data has. Although a genomic/transcriptomic comparison with both fungal and algal partners is currently not feasible, some bacterial functions, which contribute greatly to the overall symbiosis, have been confirmed.

Discussion

The analysis of our data has revealed metabolic capacities and potential roles of the lichen-associated bacteria, especially in the areas of stability and survival of the overall symbiosis. Using our multiphasic approach, combining omics technologies and physiological assays, we found diverse and previously unknown potential functions of the microbiome, such as nutrient supply, resistance against biotic and abiotic stress, support for the photosynthesis and for the growth of the two eukaryotic partners, as well as detoxification and thallus degradation abilities. Supportive roles of associated microbiomes are well-known from humans, animals and plants (Berg, 2009; Bragina *et al.*, 2013; Cho and Blaser, 2012). With our present results, we have found new hints that a similar helper effect can be present in lichen symbioses. This supports our concept of lichens as complex microbial ecosystem (Grube *et al.*, 2009). Combining metagenomic analysis of a bacteria-enriched *Lobaria* sample with a metaproteomic approach provided an additional insight into the functional and structural diversity of bacterial inhabitants. The identified potential functions of the lichen microbiome suggest interactions with the algal as well as with the fungal partner. Metagenomic and proteomic experiments have provided evidence for the capability of production of vitamin B12 and other cofactors supporting the beneficial algae–bacteria interaction. Many algae are auxotroph for vitamin B12, which is often synthesized by prokaryotes in symbiotic interactions (Croft *et al.*, 2005). Other functions such as nutrient supply and resistance against biotic and abiotic stress factors indicate interactions with the fungal partner. Lichens are exposed to abiotic stress and a well-known target for parasitic fungi (Grube *et al.*, 2012). In addition, hormones such as auxin produced by bacteria can support the growth of algae as well as fungi (Gutjahr, 2014). Although results confirmed by both metagenomics and proteomics data corroborated functionalities, a more detailed view is often limited by the availability of annotated data and the lack of completely sequenced genomes, for comparison. Some differences between the two approaches might also be explained by variation of the actual metabolic activity of the involved microorganisms at the time of sample preparation. However, the present results agree with a previous metaproteomic approach of an independent sample of the same lichen, covering the entire lichen holobiome. Schneider *et al.* (2011)

showed that algal proteins are involved in energy production and a diverse set of functions of the fungal proteins relates to the role of the mycobiont in energy consumption and symbiotic control. Similar findings resulted from the analysis of the eukaryotic metatranscriptome in another lichen symbiosis (Juntilla and Rudd, 2012; Juntilla *et al.*, 2013). The present study extends the previous studies in resolution of bacterial functionality. Focusing on the bacterial fraction, the comparison of the new proteomic and metagenomic datasets revealed an overall similarity in the taxonomic representation of bacterial organisms. In both datasets, *Proteobacteria* are the most prominent phylum, with *Alphaproteobacteria* as the most prevalent class. In contrast, cyanobacterial proteins were much more abundant as expected from the contig counts; they might be more active than other phylogenetic groups. As potential carbon and nitrogen fixing organisms, they are known for a long time as substantial part of lichen symbioses (Honegger *et al.*, 2013).

Bacterial communities on long-living lichen thallus surface remain largely constant over seasons (Grube *et al.,* 2009), despite they are exposed to substantial periodicity of abiotic factors in the habitat. Our data show that bacteria living on the surfaces of lichens are well-adapted to abiotic stress, in particular osmotic and oxidative stress. These properties match with the general property of lichens to tolerate periodic drought in their natural habitats. The periodic desiccation and rehydration cycles lead to repeated oxidative bursts at the surfaces of the fungal textures. Release of free radicals under these circumstances has been demonstrated and was interpreted as pathogen defense mechanism of lichens (Minibayeva and Beckett, 2001; Beckett *et al.*, 2013). Thus, thallus-colonizing thalli bacteria without pronounced tolerance to oxidative stress and other selective conditions barely survive. By oxidative degradation of these non-adapted bacteria, a broad spectrum of additional nutrients is accessible. This source might be more important for oligotrophic lichens than for soil-provisioned plants (Paunfoo-Lonhienne *et al.*, 2010; White *et al.*, 2012). Consequently we hypothesize that periodic hydration acts as selective pressure for enrichment of specific and stress-tolerant bacterial communities, which can contribute to longevity and persistence of lichens under extreme and changing ecological conditions.

As we found little evidence of quorum sensing, we hypothesize that bacterial colonization of the thallus is mostly regulated by the fungal partner in the symbiotic community. It is well-established that the secondary metabolites of diverse lichen species have broad antibacterial properties (Boustie and Grube, 2005). The surprising abundance of bacteria on the surfaces and between crystals of secondary metabolites in lichens (for example, *Lecanora polytropa*; Grube *et al.*, 2009) can only be explained by differences in the susceptibility to antibiotic compounds, which may be considered another factor of bacterial selection in lichens. Because we found significant numbers of multidrug resistance efflux pumps, the phylogenetically old lichen symbiosis could represent a natural reservoir of bacterial resistance mechanisms. Moreover, some of the adapted bacteria are potentially involved in the degradation of fungal secondary metabolites, as indicated by contigs of genes whose products may process complex and cyclic carbohydrates. These genes might also be interesting for biotechnological approaches, aiming at the degradation of xenobiotics. The presence of genes for the metabolism of typical bacterial antibiotics in our dataset suggests potential competition among bacterial strains on the lichen surfaces or a defense against other strains entering the microbial surface community. The ecological significance of these functions is pending further experimentation.

The morphological design of lichen structures could have a profound effect on the organization of the symbiotic networking. Bacterial communities primarily colonize the (hydrophilic) lichen surfaces, yet this pattern is strikingly different from the internalized symbionts in lichens, such as the green algae (*D. reticulata* in *L. pulmonaria*), which primarily contribute to the provision of photosynthetically produced carbohydrates. The green-algal strain is massively enriched within the fungal structures, while cyanobacterial *Nostoc* strains are acquired from the surfaces of *L. pulmonaria* recurrently during the life-time of the thallus to form internal organs devoted to nitrogen fixation in lichens (Hyvärinen *et al.*, 2002, Cornejo and Scheidegger, 2013). Conversely, the external presence of other bacteria in the lichen symbiosis recalls the helper bacteria of mycorrhizal symbioses, which provide multiple functions to mutually support and stabilize the root symbioses, including exchange of carbohydrates and vitamin provision (Frey-Klett *et al.*, 2007; Deveau *et al.*, 2010). The long-living lichen thallus is formed by tightly packed fungal hyphae, which are conglutinated by their cell walls. Lichen-adapted bacteria benefit from the persistent cell walls for nutrition, and in return provide multiple helper functions for the longevity of lichen thalli (Figure 2b) to enhance fitness of the holobiome (symbiome). Although we have found a lot of supportive facts for this symbiosis model within our datasets, the evidence for fulfilled contribution of bacterial communities to the lichen symbiosis can only be found by additional experiments, for example, using isotope-labeled compounds and/or comparative physiological analysis between lichens with and without the bacterial microbiota. Both are currently difficult to establish due to slow metabolism and high diversity of bacteria. Despite these facts, we consider lichens as an interesting model for multi-biont symbioses, with different distributions of functions among

the symbionts. It appears that partners with narrowly specified functions, such as carbohydrate or nitrogen fixation, are preferentially internalized by fungal structures and massively enriched in the lichen thallus. Internalization of a partner may help to provide a more uniform and stable environment for these partners. This symbiotic design has evolved as a convergence in unrelated fungal lineages (Grube and Hawksworth, 2007; Honegger, 2012). It has been optimized not only for the association with carbon-providing algal photobionts (Kranner *et al.*, 2005), but also for the enrichment of bacterial supporters. Genome sequences of the eukaryotic partners, which are now becoming available (for example, Wang *et al.*, 2014), will further help to address the intricacies of one of the oldest known symbiosis and its interactions with their bacterial helpers.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)

Publication I – Supporting Material

Exploring functional contexts of symbiotic sustain within lichen-associated bacteria by comparative omics

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Fig. S1. *Lobaria pulmonaria* thalli before sampling. All lichen thalli were checked for infection with lichenicolous fungi and other contaminants.

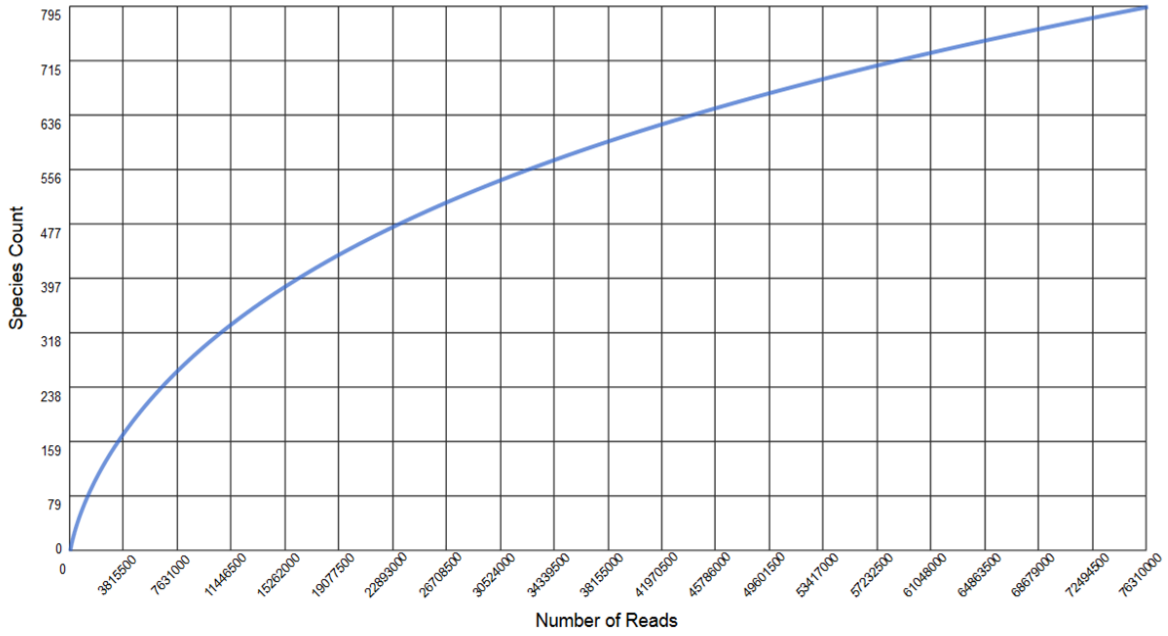

Fig. S2. Rarefaction analysis indicating species richness that can be expected in the *Lobaria* metagenome. The data was compared to Greengenes using standard MG-RAST terms for RNA databases.

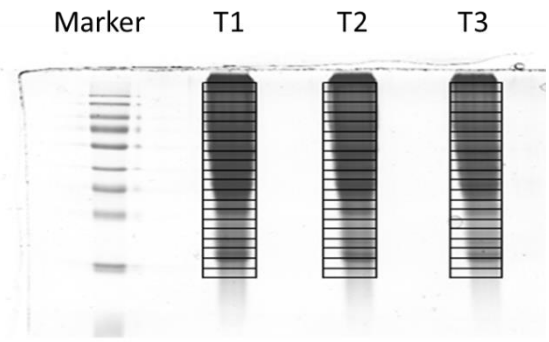

Fig. S3. Colloidal Coomassie-stained 1D SDS-PAGE of three technical replicates T1 – T3 of proteins extracted from *L. pulmonaria*. The cutting of the lanes into 20 individual gel pieces is indicated by the lines.

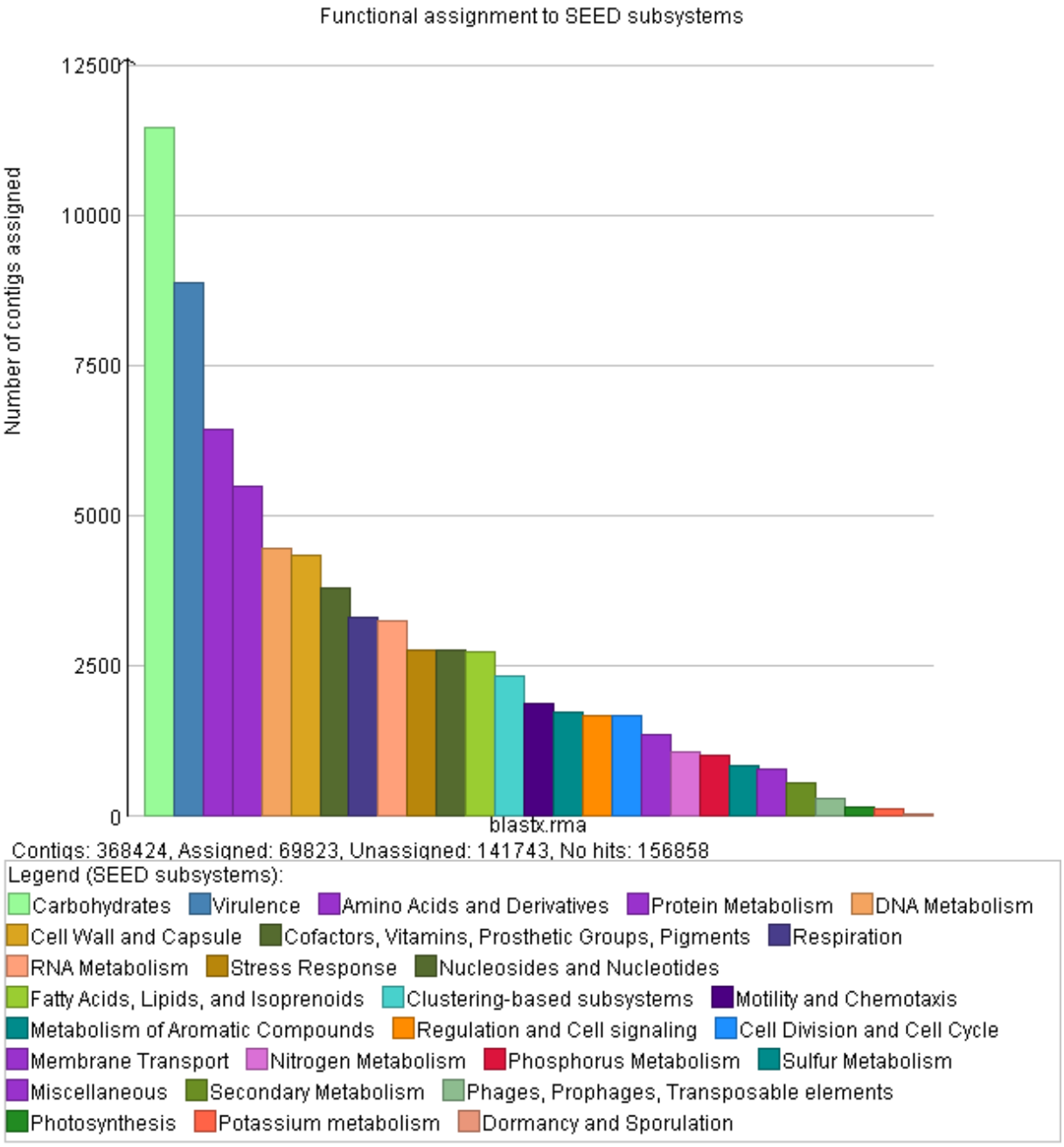

Fig. S4. Functional assignments processed with MEGAN (v4.70.4) and SEED Subsystems analysis. SEED functions were arranged in a decreasing sequence based on the total contig count assigned to certain functions. The most prominent assignments are connected to functions involved in primary metabolism, but also to virulence-related genes, biosynthesis and transport of vitamins, cofactors, prosthetic groups and pigments as well as genes involved in stress response.

Table S1. Brief sample description and corresponding MG-RAST metagenome IDs for all datasets used in the PCoA.

Metagenome ID	Habitat	Country or location	# of sequences	Alpha-diversity
4530091.3	*L. pulmonaria*	Austria	76,310,051	563.852
4447810.3	*A. thaliana* phyllosphere	Spain	1,098,311	470.804
4447811.3	Clover phyllosphere	Switzerland	1,028,814	421.794
4441205.3	Soybean phyllosphere	Switzerland	523,769	381.345
4453083.3	Freshwater	Australia	409,743	1030.286
4441590.3	Freshwater	Panama	296,355	785.38
4445126.3	Freshwater	Canada	256,849	585.456
4447101.3	Human oral cavity	Spain	295,072	299.147
4447102.3	Human oral cavity	Spain	244,881	217.806
4447192.3	Human oral cavity	Spain	204,218	217.793
4461456.3	*A. brasiliensis*	Brazil	64,933	440.293
4461455.3	*A. brasiliensis*	Brazil	167,475	521.743
4461454.3	*A. brasiliensis*	Brazil	85,431	489.784
4440036.3	Seawater	Kingman Atoll	94,915	432.738
4440038.3	Seawater	Christmas Atoll	283,390	250.197
4440039.3	Seawater	Palmyra Atoll	351,205	358.14
4508939.3	Agricultural soil	USA (IN)	16,923,988	641.538
4508938.3	Agricultural soil	USA (IN)	9,999,775	678.281
4508940.3	Agricultural soil	USA (IN)	17,442,824	621.506
4465558.3	Forrest soil	Spain	257,697	715.306
4465556.3	Forrest soil	Spain	412,302	621.136

Table S2. Comparison of the numbers of contigs assigned to selected functions of the lichen microbiome (described in the Results section), between *Lobaria pulmonaria* metagenome and *Dictyochloropsis reticulata* transcriptome (Transcriptome assembly downloaded from http://genome.jgi.doe.gov/Lobpulcupartners/Lobpulcupartners.info.html).

SEED function	*Lobaria pulmonaria* contigs assigned (total=69,823)		*Dictyochloropsis reticulata* contigs assigned (total=28,072)	
	Number	Percentage	Number	Percentage
Ton and Tol transport systems	2,793	4.00	8	0.03
TonB-dependent receptor	2,094	3.00	0	0.00
Phosphate metabolism	885	1.27	11	0.04
Multidrug resistance efflux pumps	1,152	1.65	1	0.00
Type III, Type IV, Type VI, ESAT secretion systems	955	1.37	0	0.00
Secondary Metabolism	548	0.78	53	0.19
Stress Response	2,769	3.97	155	0.55
Oxidative stress	1,238	1.77	63	0.22
Cofactors, Vitamins, Prosthetic Groups, Pigments	3,799	5.44	258	0.92
Tetrapyrroles	1,203	1.72	107	0.38
Coenzyme B12 biosynthesis	365	0.52	3	0.01
Thiamin biosynthesis	312	0.45	11	0.04
Biotin biosynthesis	174	0.25	5	0.02
Auxin biosynthesis	156	0.22	15	0.05
Chitin and N-acetylglucosamine utilization	341	0.49	5	0.02
Protein degradation	1,000	1.43	111	0.40

Table S3. Comparison of the numbers of contigs assigned to top-level SEED functions (as depicted in Fig. S4) between *Lobaria pulmonaria* metagenome and *Dictyochloropsis reticulata* transcriptome (Transcriptome assembly downloaded from http://genome.jgi.doe.gov/Lobpulcupartners/Lobpulcupartners.info.html).

Top-level SEED functions	*Lobaria pulmonaria* contigs assigned (total=69,823)		*Dictyochloropsis reticulata* contigs assigned (total=28,072)	
	Number	Percentage	Number	Percentage
Carbohydrates	11,462	16.42	329	1.17
Virulence	8,861	12.69	70	0.25
Amino Acids and Derivatives	6,440	9.22	264	0.94
Protein Metabolism	5,481	7.85	527	1.88
DNA Metabolism	4,446	6.37	68	0.24
Cell Wall and Capsule	4,325	6.19	44	0.16
Cofactors, Vitamins, Prosthetic Groups, Pigments	3,799	5.44	258	0.92
Respiration	3,299	4.72	70	0.25
RNA Metabolism	3,248	4.65	121	0.43
Stress Response	2,769	3.97	155	0.55
Nucleosides and Nucleotides	2,761	3.95	92	0.33
Fatty Acids, Lipids, and Isoprenoids	2,721	3.90	111	0.40
Clustering-based subsystems	2,328	3.33	112	0.40
Motility and Chemotaxis	1,880	2.69	15	0.05
Metabolism of Aromatic Compounds	1,734	2.48	48	0.17
Regulation and Cell signaling	1,673	2.40	21	0.07
Cell Division and Cell Cycle	1,669	2.39	36	0.13
Membrane Transport	1,361	1.95	27	0.10
Nitrogen Metabolism	1,060	1.52	30	0.11

Phosphorus Metabolism	1,011	1.45	11	0.04
Sulfur Metabolism	836	1.20	17	0.06
Miscellaneous	767	1.10	23	0.08
Secondary Metabolism	548	0.78	53	0.19
Phages, Prophages, Transposable elements	275	0.39	7	0.02
Photosynthesis	139	0.20	27	0.10
Potassium metabolism	103	0.15	5	0.02
Dormancy and Sporulation	13	0.02	1	0.00

Publication II

Rhizobiales as functional and endosymbiontic members in the lichen symbiosis of *Lobaria pulmonaria* L.

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Rhizobiales as functional and endosymbiontic members in the lichen symbiosis of *Lobaria pulmonaria* L.

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Rhizobiales (*Alphaproteobacteria)* are well-known beneficial partners in plant-microbe interactions. Less is known about the occurrence and function of *Rhizobiales* in the lichen symbiosis, although it has previously been shown that *Alphaproteobacteria* are the dominating group in growing lichen thalli. We have analyzed the taxonomic structure and assigned functions to *Rhizobiales* within a metagenomic dataset of the lung lichen *Lobaria pulmonaria* L. One third (32.2%) of the overall bacteria belong to the *Rhizobiales*, in particular to the families *Methylobacteriaceae*, *Bradyrhizobiaceae,* and *Rhizobiaceae*. About 20% of our metagenomic assignments could not be placed in any of the *Rhizobiales* lineages, which indicates a yet undescribed bacterial diversity. SEED-based functional analysis focused on *Rhizobiales* and revealed functions supporting the symbiosis, including auxin and vitamin production, nitrogen fixation and stress protection. We also have used a specifically developed probe to localize *Rhizobiales* by confocal laser scanning microscopy after fluorescence *in situ* hybridization (FISH-CLSM). Bacteria preferentially colonized fungal surfaces, but there is clear evidence that members of the *Rhizobiales* are able to intrude at varying depths into the interhyphal gelatinous matrix of the upper lichen cortical layer and that at least occasionally some bacteria also are capable to colonize the interior of the fungal hyphae. Interestingly, the gradual development of an endosymbiotic bacterial life was found for lichen- as well as for fungal- and plant-associated bacteria. The new tools to study *Rhizobiales*, FISH microscopy and comparative metagenomics, suggest a similar beneficial role for lichens than for plants and will help to better understand the *Rhizobiales*-host interaction and their biotechnological potential.

Keywords: *Rhizobiales*, lichen symbiosis, *Lobaria pulmonaria*, metagenomics, *Rhizobiales*-specific FISH probe, endosymbiont

INTRODUCTION

Lichen symbioses are estimated to cover up to 8% of the global land surface. Many habitats colonized by lichens are characterized by unfavorable environmental conditions, such as low nutrient availability and/or high temperature fluctuations (Ahmadjian, 1995). Although lichens are able to resist extreme environmental conditions via a dormant stage, they are highly specialized for their habitats and vulnerable to slight changes in the microclimate (or air pollution), which can easily disrupt the integrity of the fine-tuned symbiotic interplay. Lichen symbioses appear as composite organisms with a shape-forming fungus (the mycobiont) and a photosynthetic partner (the photobiont), which is often sheltered by complex fungal structures, into which a complex, stable and thallus-specific microbiome is incorporated. Recently, a bacterial microbiome was identified as a third component of this symbiosis (Grube et al., 2009). Lichens are densely colonized by diverse and host-specific communities of bacteria that occur in specific ecological niches of their hosts (Cardinale et al., 2008, 2012a,b; Grube et al., 2009). Specific above-ground niches in higher plants comprize for example the phyllo- and rhizosphere, or the endosphere (Ryan et al., 2008; Berg et al., 2014). However, lichens do not produce the same organs as found in plants, which develop their organs from meristems. Lichens instead produce a thallus of densely conglutinated fungal hyphae which can form foliose, filamentous, crustose, leprose, squamulose, gelatinous, or fruticose shapes (Grube and Hawksworth, 2007), each hosting specific sets of ecologically specific niches, which can be occupied by bacteria.

Rhizobiales are well-studied associates of plants; they commonly exert beneficial functions for their hosts by providing various nutrients, phytohormons as well as precursors for essential plant metabolites (Ivanova et al., 2000; Delmotte et al., 2009; Verginer et al., 2010). The order contains many genera of nitrogen-fixing, methanotrophic, legume-nodulating, microsymbiotic bacteria (Jourand et al., 2004; Garrity et al., 2005). Recently, nitrogen-fixation was shown not to be limited

to *Rhizobiales* in leguminous plants, but also to be expressed within various endophytic compartments of non-leguminous plants (Fischer et al., 2012). Besides their almost ubiquitous presence with higher plants, *Rhizobiales* are also found associated with mosses and lichens (Lundberg et al., 2012; Vorholt, 2012; Erlacher et al., in press). Pink-pigmented-facultative-methylotrophs (PPFMs) are a specific group of *Rhizobiales*, which can affect the host metabolism including production of vitamins and phytohormones, such as auxines and cytokinines (Ivanova et al., 2000; Delmotte et al., 2009). *Methylobacterium* spp. can utilize methanol emitted by the plants, methylamine and further C2, C3, and C4 compounds as solely carbon and energy source (Green and Bousfield, 1983; Lidstrom and Chistoserdova, 2002). Schauer and Kutschera (2013) suggest that ferns, liverworts and moss protonemata have an intimate association with methylobacteria, and they argue that the haploid phases of cryptogames are preferred host organisms of these pink-pigmented microbial phytosymbionts. However, less is known for lichen-associated *Rhizobiales,* including methylobacteria. We postulate that they also play a beneficial role in the lichen symbiosis.

According to recent publications, *Rhizobiales* are a particularly common order on lichens (Bates et al., 2012; Cardinale et al., 2012b). Although there are several reports about endofungal bacteria in ascomycetous fungi (Bertaux et al., 2005; Sharma et al., 2008), until know there is no evidence for them in lichens (Grube and Berg, 2009). While *Alphaproteobacteria* have been detected by fluorescence *in situ* hybridization and confocal laser scanning microscopy in lichens (Cardinale et al., 2008; Grube et al., 2009), there are no suitable FISH probes available to specifically stain the order *Rhizobiales,* except RHIZ1244 (according to Probebase) (http://www.microbial-ecology.net/probebase; accession nr. pB-02665; Thayanukul et al., 2010). *In silico* analysis using probematch (https://rdp.cme.msu.edu/probematch/) revealed that the RHIZ1244 probe detection spectrum is incomplete and fails to recognize important families such as *Methylobacteriaceae*, *Bradyrhizobiaceae,* or *Beijerinckiaceae*.

The lung lichen, *Lobaria pulmonaria* L., is a tripartite lichen, with one ascomycete fungus hosting both a dominant green algal partner (*Dictyochloropsis reticulata*) and a minor cyanobacterial partner (internal herds of *Nostoc*). This lichen is known as a sensitive biological indicator of air pollution that experienced a massive decline in Europe during the twentieth century (Scheidegger and Goward, 2002). Nonetheless, it may develop prolific populations in suitable cool and humid habitats, both by the efficient spread with symbiotic propagules and by its growth rate, which is one of the highest among all lichens (**Figure 1**). In this work, we have investigated *L. pulmonaria* collected in the high montane forest zone in Alps. We pursued a metagenomic approach to assess functional diversity of *Rhizobiales* associated with this lichen species and used *in situ* visualization to localize and reveal colonization strategies of this bacterial order. For this purpose, we designed a novel FISH probe to efficiently and specifically target members of *Rhizobiales*.

FIGURE 1 | *Lobaria pulmonaria* from a mountain maple in an Abieti-Fagetum forest in Johnsbach (Austria) at an altitude of 1200 m above sea level. (Styria, Austria, 47°32′29.7″N 14°37′36.6″E).

MATERIALS AND METHODS

SAMPLING

L. pulmonaria samples were collected in two mountain forests from mountain maple (*Acer pseudoplatanus* L.) in Austria (Styria, Gstatterboden, 47°34′20″N, 14°35′4.4″E and Styria, Johnsbach, 47°32′29.7″N 14°37′36.6″E). Ten individual lichen thalli were collected and stored in sterile plastic bags on ice.

LOBARIA PULMONARIA METAGENOME AND ANALYZES

All metagenome-based analyzes were carried out on the assembled dataset described in a previous study by Grube et al. (2015). The number of actual contigs used for the *in silico* FISH-probe evaluation was 368,424, while 28,526 contigs assigned to the taxon *Rhizobiales* were used for the functional analysis of *Rhizobiales*. CLUSTER CONTROL (Stocker et al., 2004) was used to search with the blastn algorithm for FISH-probe matches within the dataset (*e*-value cutoff = 1.6). The assembled metagenomic dataset is publicly available on MG-RAST (http://metagenomics.anl.gov; project ID: 4529136.3). To obtain taxonomic assignments, the Tera-BLASTN program (www.timelogic.com/documents/TeraBLAST2009.pdf) was run on the 368,424 contigs, using TimeLogic (Active Motif, Carlsbad, CA, USA) DeCypher boards against the "nt" (nucleotide sequence) database from NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db). The blastn results were imported into MEGAN (Metagenome Analyzer, v4.70.4) (Huson et al., 2011) to produce several taxonomy profiles. For functional analysis, we used a similar approach as above but used Tera-BLASTX (www.timelogic.com/documents/TeraBLAST2009.pdf), which was run against the "nr" (non-redundant protein sequence) database from NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db). The blastx results were imported into MEGAN (v4.70.4) as well for functional analysis. The 28,526 contigs that were previously assigned to *Rhizobiales* were used for assignment of SEED functions (Overbeek et al., 2005) within MEGAN. SEED-based analysis allows hierarchical organization of complete and partial gene sequences allocated within the utilized contig collection and thus quantification of specific functions on different levels.

IN SILICO ANALYSIS OF rRNA-TARGETED OLIGONUCLEOTIDE PROBES

ProbeBase (http://www.microbial-ecology.net/probebase; Loy et al., 2007) was used to screen for available FISH probes targeting the order *Rhizobiales*. RDP Probe Match (http://rdp.cme.msu.edu/probematch; Cole et al., 2005) and the Silva RNA database using TestProbe 3.0 (http://www.arb-silva.de/search/testprobe; Quast et al., 2013) with taxonomy browser were utilized to

evaluate the amplitude and coverage of available and designed FISH probes. The Probe sequences (5′–3′) were aligned (reversed and complement search allowed) to RDP and Silva SSU r119 databases with the REFNR sequence collection.

FLUORESCENCE *IN SITU* HYBRIDIZATION COMBINED WITH CONFOCAL LASER SCANNING MICROSCOPY (FISH-CLSM)

FISH-CLSM was applied on *L. pulmonaria* samples to investigate colonization patterns of *Rhizobiales* and all bacteria. Within 3 h after collection, samples were fixed with 4% paraformaldehyde and 1x phosphate-buffered saline (PBS) (3:1 ratio, respectively) for 6 h at 4°C. Fixed *Lobaria* thallus samples were cut with a cryotome.

We designed FISH probe RHIZ3r (**Table 1**) specific to our metagenomic data. The oligonucleotide sequence based on the Primer 3r (Nishio et al., 1997) was synthetized and labeled with a Cy5 fluorochrome (Biomers, Wiener Neudorf, Austria). FISH was applied according to Cardinale et al. (2008) to visualize and decipher the nature of the correlations detected by metagenomics. Briefly, the cryosections were transferred into 1.5 ml Eppendorf tubes and rinsed with 1x PBS. Lysozyme (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) treatment was applied and incubated at RT for 10 min. After an ethanolic series (50–70–96% EtOH solutions; 3 min each) samples were rinsed and further washed for 3 min with ice-cold 1x PBS. All hybridizations were performed at 43°C for 2 h in a buffer containing 0.9 M NaCl, 0.02 M Tris-HCl, 0.01% sodium dodecyl sulfate, 10–50% (**Table 1**) ultrapure formamide (FA; Invitrogen), and 5.0 ng of each FISH probe μl^{-1} (pH 8). An equimolar mixture of Cy3-labeled EUB338, EUB338-II and EUB338-III probes (Amann et al., 1990; Daims et al., 1999) was used for staining all Bacteria. RHIZ3r and RHIZ1244 (Thayanukul et al., 2010) was used to stain taxa within the bacterial order *Rhizobiales*. NONEUB probes (Wallner et al., 1993) labeled to fluorochromes analogous to the positive probes were used as negative controls. The hybridization buffer was replaced by a prewarmed (44°C) washing buffer [20 mM Tris-HCl, 450/46/18 mM NaCl (10/40/50% FA), and 5 mM EDTA (for 40% and 50% FA)] and incubated for 15 min in a water bath (44°C). The hybridization and washing step were repeated sequentially for the utilized FISH-probes in dependency of the specific FA requirements. After eliminating the washing buffer the sections were again rinsed with ice-cold double-distilled H_2O in order to remove residual salt crystals. FISH stained samples were transferred on optical slides, dried and mounted with SlowFade Gold antifadent (Molecular Probes, Eugene, USA). For visualization a Leica TCS SPE confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany) was used. Autofluorescence and additional calcofluor white (Sigma-Aldrich) staining of the lichen tissues was used for imaging the host structures. The fluorescent dyes Cy3, and Cy5 labeling the FISH probes were sequentially excited with 532 and 635 nm laser beams. Autofluorescence and calcofluor staining was excited with a 405 nm laser beam. The confocal stacks were acquired with a LeicaACS APO 40x oil CS objective lens (NA, 1.15) and a Leica ACS APO 63x oil CS objective lens (NA, 1.30) and for each field of view, an appropriate number of optical slices were acquired within a Z-step ranging from 0.15 to 0.5 µm. The software Imaris 7.3 (Bitplane, Zurich, Switzerland) was used for imaging and post-processing of the confocal stacks and maximum projections. Adobe Photoshop (Adobe Systems Inc., USA) was used to label the final figures.

RESULTS

ANALYSIS OF *LOBARIA*-ASSOCIATED *RHIZOBIALES*

An assembled *Lobaria*-associated metagenome consisting of 362,424 contigs was utilized for taxonomic and functional studies of assigned *Rhizobiales*. In total, 88,602 contigs were assigned to bacteria. *Alphaproteobacteria* was the most frequently identified bacterial phylum in the metagenome and comprized 53,688 contigs (46.8% of all identified cellular organisms and 60.6% of identified bacteria). Of these, 28,526 assigned contigs belong to the predominant *Rhizobiales* (32.2% of identified bacteria), with families *Methylobacteriaceae* (11,421 contigs or 12.9% of identified bacteria), *Bradyrhizobiaceae* (5230 contigs or 5.9% of identified bacteria), and *Rhizobiaceae* (2403 contigs or 2.7% of identified bacteria). *Methylobacterium* was the only identified genus of *Methylobacteriaceae* and *Methylobacterium radiotolerans* (8% of all *Rhizobiales*) the most frequent species. Less frequent species were identified as *M. nodulans*, *M. populi* and members of the *M. extorquens* group. A total of 21% of present *Rhizobiales* was assigned to the cluster *Methylobacterium* sp. 4–46 or remained unclassified. Identified genera within the family of *Bradyrhizobiaceae* were more diverse and represented by four distinctive genera: *Bradyrhizobium* (7% of all *Rhizobiales*), *Rhodopseudomonas* (6% of all *Rhizobiales*), *Nitrobacter* (1% of all *Rhizobiales*) and *Oligotropha* (0.5% of all *Rhizobiales*).

Table 1 | Oligonucleotide probes utilized for FISH in this study.

Name	Sequence (5′-3′)	Fluorochrome	Target	Formamide (% at 43°C)	References
EUB338*	GCTGCCTCCCGTAGGAGT	Cy3	Most bacteria	10	Amann et al., 1990
EUB338II*	GCAGCCACCCGTAGGTGT	Cy3	*Planctomycetales*	10	Daims et al., 1999
EUB338III*	GCTGCCACCCGTAGGTGT	Cy3	*Verrucomicrobiales*	10	Daims et al., 1999
NONEUB**	ACTCCTACGGGAGGCAGC	Cy5 or Cy3	/	**	Wallner et al., 1993
RHIZ3r	GGCTTATCACCGGCAGTCTCC	Cy5	*Rhizobiales*	40	Nishio et al., 1997
RHIZ1244	TCGCTGCCCACTGTCACC	Cy5	*Rhizobiales*	50	Thayanukul et al., 2010

*Probes were used in equimolar concentration.

**NONEUB was applied as negative control; formamide concentrations were analog to the positive FISH probes.

Three percent of all *Rhizobiales* remained unclassified genera of the *Bradyrhizobiaceae* family. The most abundant species within *Bradyrhizobiaceae* was identified as *Rhodopseudomonas palustris* (6% of all *Rhizobiales*). *Rhizobiaceae* included the *Rhizobium/Agrobacterium* group (6% of all *Rhizobiales*) and the *Sinorhizobium/Ensifer* group (2% of all *Rhizobiales*). Less abundant *Rhizobiales* families were assigned to the genera of *Beijerinckiaceae* (5% of all *Rhizobiales*), *Xanthobacteraceae* (4% of all *Rhizobiales*), *Phyllobacteriaceae* (4% of all *Rhizobiales*) and *Brucellaceae* (0.4% of all *Rhizobiales*). A detailed taxonomic composition of *Rhizobiales* up to species level was visualized with Krona ((Ondov et al., 2011); **Figure 2**).

SEED-based functional analysis (**Figure 3**) focused on *Rhizobiales* and its three most abundant families. Included clustering and hierarchical organization of identified functions was utilized to retrieve quantitative information for highly abundant taxa. The abundance of function-related genes assigned to specified taxonomic ranks was compared to their overall occurrence in the entire metagenome. Thereby we obtained a comprehensive overview of functions with high relevance to the symbiotic system (Table S1). *Lobaria*-associated *Rhizobiales* were shown to be involved in all candidate functions (except biosynthesis of plant alkaloids). They were found to be involved in the biosynthesis of auxins and plant octadecanoids. Notably *Bradyrhizobiaceae* accounted for 24 contigs assigned to biosynthesis of plant octadecanoids, while *Methylobacteriaceae* only accounted for one contig containing this function. Nitrogen fixation was represented by 5 contigs assigned to *Rhizobiales*, and one assigned to either *Bradyrhizobiaceae* or *Methylobacteriaceae*. Type III secretion systems were found in 35 contigs, with 16 assigned to *Methylobacteriaceae*, 7 assigned to *Bradyrhizobiaceae*, one assigned to *Rhizobiaceae* and 11 without assignment to a specific family. One-carbon metabolism and carbon dioxide fixation were represented within *Rhizobiales* by 224 and 286 contigs, respectively. Biosynthesis of cofactors, vitamins, prosthetic groups and pigments was particularly frequent and represented by 848 contigs within *Rhizobiales*. Notably *Bradyrhizobiaceae* were found to contribute to chlorophyll biosynthesis (59 contigs), synthesis of folate and pterines (46 contigs) and coenzyme B12 biosynthesis (99 contigs). Conversely, *Rhizobiaceae* were rather underrepresented with 5 contigs assigned to synthesis of folate and pterines and 7 contigs assigned to coenzyme B12 biosynthesis. Overall stress response was found within 632 contigs associated with *Rhizobiales*. *Methylobacteriaceae* accounted

FIGURE 2 | *Rhizobiales* taxa within the *Lobaria*-associated metagenome are presented in a multi-level chart. Taxonomic information was retrieved through blastn assignments of assembled contigs, followed by processing with MEGAN. Circles represent taxonomic classifications in ascending order up to the species level (outermost circle). Less abundant taxa are listed outside the charts together with their relative abundance.

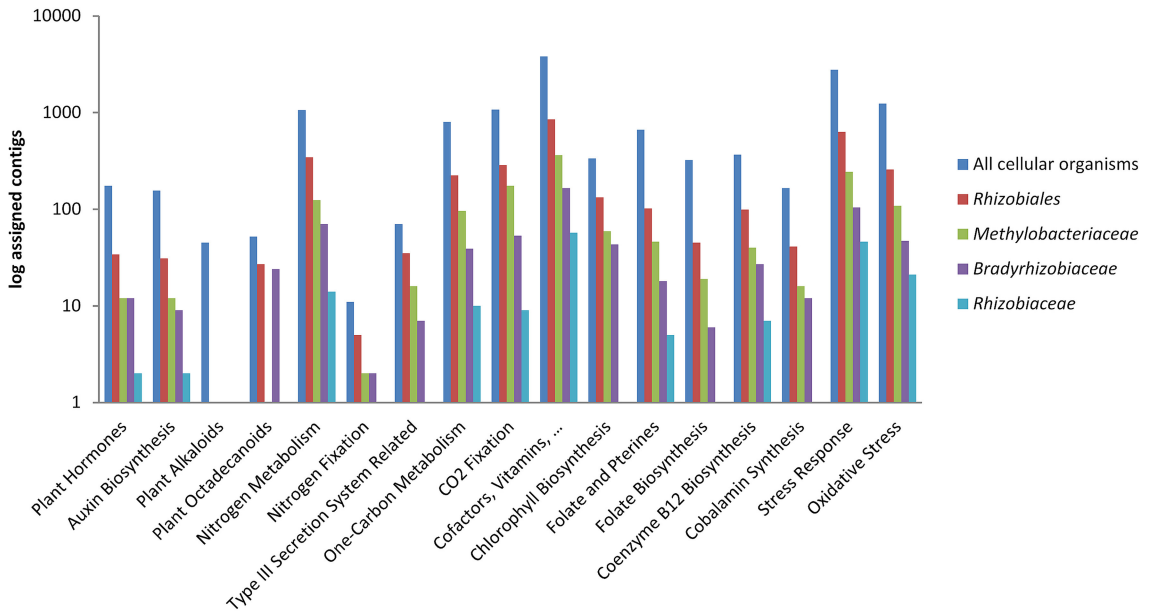

FIGURE 3 | Selected functions within the *Lobaria*-associated metagenome were assigned to taxa at order (*Rhizobiales*) and family (*Methylobacteriaceae, Bradyrhizobiaceae* and *Rhizobiaceae*) level together with hits without assignment to a specific taxon. Taxonomic information was retrieved through blastx assignments of assembled contigs, followed by processing with MEGAN.

for 243 contigs, while *Bradyrhizobiaceae* and *Rhizobiaceae* accounted for 104 and 46 contigs, respectively. Response to oxidative stress was present with 257 contigs (108 assigned to *Methylobacteriaceae*, 47 to *Bradyrhizobiaceae* and 21 to *Rhizobiaceae*).

EVALUATION OF THE RHIZ3r FISH PROBE FOR *RHIZOBIALES* STAINING

Alignments to sequences of the Silva (Quast et al., 2013) and Probematch databases (Cole et al., 2005) revealed that the only available FISH probe RHIZ1244 targeting *Rhizobiales* was not suitable to label taxa retrieved in the *Lobaria* microbiome (**Figure 4**; Table S2). The designed FISH probe RHIZ3r, based on the primer 3r (Nishio et al., 1997), was therefore evaluated and we could demonstrate a high coverage for specific taxa, including the most abundant families *Methylobacteriaceae* and *Bradyrhizobiaceae* (**Figure 4**). According to the Probematch analysis (Table S3), RHIZ3r (11166 hits) shows slightly reduced coverage in the order *Rhizobiales* compared to RHIZ1244 (14312 hits). However, the latter probe does not match well with the highly abundant families *Bradyrhizobiaceae* (7/11453) and *Methylobacteriaceae* (5/9098 hits), whereas RHIZ3r performs much better in this respect (*Methylobacteriaceae*: 3821/9098 hits; *Bradyrhizobiaceae:* 5586/11453 hits; Table S3). *In silico* alignment of the sequence to genus level shows that the FISH probe RHIZ3r targets bacteria belonging to the order *Rhizobiales*, families *Methylobacteriaceae* (genus *Methylobacterium*, coverage: 86%) and *Bradyrhizobiaceae* (genus *Bradyrhizobium*, coverage: 99%; genus *Afipia*, coverage: 100%; genus *Nitrobacter*, coverage: 100%; genus *Oligotropha,* coverage: 100%; genus *Rhodoblastus,* coverage: 100%; genus *Rhodopseudomonas*, coverage: 92%). The results of the *in silico* analysis were confirmed by identification of excised SSCP bands amplified with primer RHIZ3r (Erlacher et al., in press) and with the metagenomic data.

IN SILICO EVALUATION OF POTENTIAL FISH PROBES FOR *RHIZOBIALES* IN THE METAGENOME

We used Blastn to search for FISH probe binding sites within the entire assembled *Lobaria*-associated metagenome, and found different *Rhizobiales* taxa, including members of the families *Rhizobiaceae, Beijerinckiaceae, Bradyrhizobiaceae, Methylobacteriaceae, Methylocystaceae,* and *Phyllobacteriaceae*. Hits for non-targeted taxa included mostly unspecific chloroplast and plastid DNA as well as two hits for *Xanthomonadaceae* and one hit for *Pseudomonadaceae* (Table S2).

VIZUALISATION OF *LOBARIA*-ASSOCIATED *RHIZOBIALES*

Fluorescence *in situ* hybridization with both the RHIZ3r and the EUB338-MIX probes resulted in unambiguously strong signals. Image analysis and three-dimensional reconstructions of confocal stacks showed that most of the bacteria colonize *L. pulmonaria* at the outer surface of the lichen cortex (**Figures 5A–C**). Mixed colonies formed by putative *Rhizobiales* and other bacteria were frequently detected (**Figures 5A–C,E**), and morphological diversity of bacteria was apparent (**Figure 5E**). The autofluorescent fungi and algae in *Lobaria* allowed us to reconstruct the host structure (**Figures 5A,B,D**). Close co-existence between the bacteria and the hydrophilic fungal cortex were observed (**Figure 5D**), whereas intra-thalline hydrophobic spaces as well as photobionts were not colonized by bacteria (**Figures 5A,C**). Free

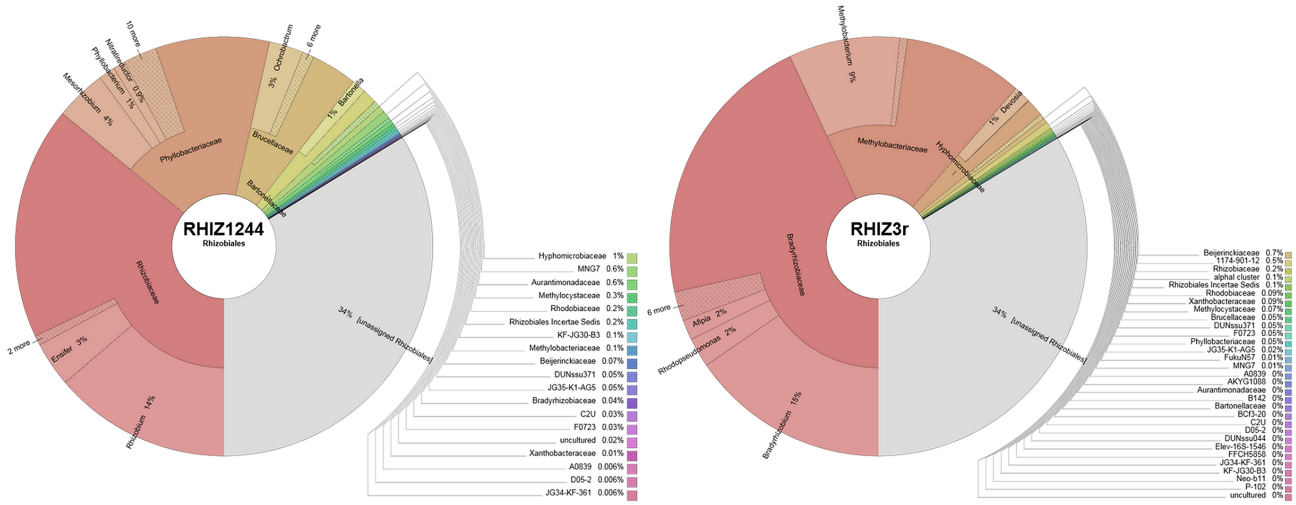

FIGURE 4 | TestProbe 3.0 (Silva ribosomal RNA database) analysis showing the targeted coverage of bacterial taxa in a comparison of RHIZ3r- and RHIZ1244- FISH Probes. The maximum amount of mismatches was set to 0 (see also Table S3 for RDP Probmatch comparison).

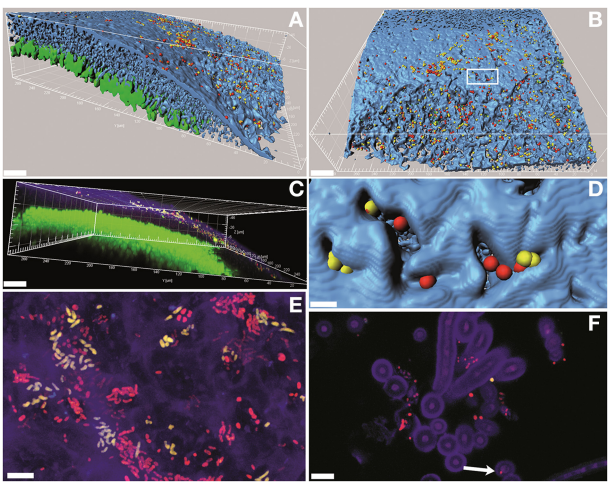

FIGURE 5 | Confocal laser scanning microscopy (CLSM) micrographs showing the bacterial colonization of the lichen *L. pulmonaria* stained by fluorescence *in situ* hybridization (FISH). Green, algae; blue/purple, fungi; yellow, *Rhizobiales*; red, other bacteria. (**A,B,D**), three-dimensional models made of isosurfaces and spheres; **(C)**, volume rendering; **(E)**, maximum projection; **(F)**, single optical slice. In **(B)**: white box indicates the region shown in **(D)**. Arrow in **(F)** indicates an endophytic bacterial cell (see also Figure S1). Scale bars: **(A-C)** (30 μm); **(D-F)** (5 μm).

hyphae protruding from the lower surfaces were often covered by bacteria. In rare cases, bacteria colonized the hyphae internally (**Figure 5F**; Figures S1, S2).

DISCUSSION

Our new study provides a first insight into the functional potential of *Rhizobiales,* which are the predominant order of bacteria associated with the lichen symbiosis. *Rhizobiales* are responsible for more than one third of all bacterial taxonomic assignments. About 20% of our metagenomic assignments could not be placed in any of the *Rhizobiales* lineages, which indicates that there might be numerous yet undescribed bacterial diversity colonizing lichens. One taxonomically undescribed phylogenetic lineage of *Rhizobiales*, not present in our dataset, was detected in diverse lichens from North America, and named LAR1 (Hodkinson and Lutzoni, 2009). Most of the classified bacteria in our dataset belong to the families *Methylobacteriaceae, Bradyrhizobiaceae* and *Rhizobiaceae*, which are therefore expected to play an important role within the lichen symbiosis.

Because *Rhizobiales* are common in growing lichen parts, we argue that they could play a role in development and growth of lichens. This hypothesis is well supported by the potential functions encoded in the metagenomic contigs of *Alphaproteobacteria* and *Rhizobiales* in our dataset. SEED-based functional analysis revealed functions supporting the symbiosis, including auxin and vitamin production, nitrogen fixation and stress protection. Taxonomical assignments showed high proportions of beneficial nitrogen fixing at species level. However, we think that nitrogen-fixation is not a required rhizobial function in the *L. pulmonaria* symbiosis, because fixed nitrogen is provided by the associated cyanobacterial partners (which is located in clustered colonies, in so-called internal cephalodia), and because excessive nitrogen (e.g., agricultural contamination) is rather a problem affecting the survival of *L. pulmonaria* in many localities. It is therefore interesting to observe a significant number of contigs that is assigned to nitrogen metabolism. Metabolism related to cofactors and vitamin production is also well represented in our dataset, suggesting that the corresponding products are valuable to support the growing lichen thallus. In addition, the high abundance of *Methylobacterium* species might be a promising source to find novel compounds or bioconversion as in higher plants (Verginer et al., 2010). In comparison with a study of *Methylobacterium* spp. on mosses by Erlacher et al. (in press), using fingerprinting methods, we

detected higher species diversity in the *L. pulmonaria* microbiome, which is also confirmed by the metagenomic data. Stress protection for the symbiosis by bacteria was detected, which seem to play a unique and important function of host-associated microbiomes. Stress protection was already detected for mosses (Bragina et al., 2014) but also for plant-associated bacteria (Alavi et al., 2013). The biotechnological potential of stress-protecting bacteria was already shown (Alavi et al., 2013; Berg et al., 2013), which shows new solutions for agriculture in a changing climate.

So far, colonization of lichens was mostly shown on surfaces of lichens (e.g., Cardinale et al., 2008). The present study clearly shows that *Rhizobiales* members are not restricted to the thallus surface. It is thus tempting to consider endobiotic life style of bacteria, similar to endophytism in plants. However, there are marked differences to an endophytic lifestyle of higher plants. Plants are typically characterized by a protective cuticula which forms a clear boundary between the plant and the external environment. By the cuticula internal tissues, plants are protected against uncontrolled water loss or contamination from external water, dirt, and from invasion of microorganisms. Such a layer is missing in thalline organisms such as mosses or lichen thalli (which are also known as "lower plants" or "cryptogams"). Both mosses and lichens belong to poikilohydric organisms, desiccating with atmospheric drought. Without a cuticula it is also more difficult to differentiate between endosphere and phyllosphere. The present data confirm that there is no clear external border of the lichen surface. We have already observed a depletion of bacterial abundance in other lichens but no qualitative differences when we analyzed bacterial associates of lichens after increasing duration of surface sterilization (unpublished data). By studying lichens we uncover interesting new insights about the endophytic strategies. In some lichens, the internal parts of lichens can be colonized. This is clearly shown in *Cladonia*, where hollow thalli are internally colonized by biofilm like bacterial communities (Cardinale et al., 2008). In cases of crust-forming lichens we observed that bacteria can partly enter the lateral parts of neighboring thallus segments (areoles; e.g., *Lecanora polytropa*, Grube et al., 2009). The case of *Lobaria* now shows that the external polysaccharide matrix between the hyphae of the lichens can, at varying depths, be penetrated by *Rhizobiales*. The loose aggregation of hyphae and the lack of a cuticula found in higher plants facilitate mutualistic bacterial colonization which gradually develops from ecto- to endo-symbiotic lifestyles. We have not observed bacteria so far in the algal layer or in the aerated medulla part beneath the algal layer. We suppose this is due to the fact that particularly the medulla layer of lichens has strongly hydrophobic surfaces (due to a hydrophobin cell layer, which enwrap the cells of the eukaryote partners). However, we also found first indications of endohyphal occurrences of bacteria in *L. pulmonaria* (**Figure 5F**; Figures S1, S2). While our findings of intracellular colonization of lichenized-fungal hyphae still require additional methodological prove to be validated, the endohyphal bacterial occurrence in non-lichenized fungi has repeatedly been found in very different lineages (e.g., Bertaux et al., 2005; Partida-Martinez and Hertweck, 2005; Sharma et al., 2008). Recent work sheds light on their diverse functions (Ghignone et al., 2012), and also revealed new details regarding how bacteria penetrate fungal hyphae (Moebius et al., 2014). We argue that occasional endohyphal bacteria in lichens might be particularly efficient strains to digest the rather thick fungal cell walls in lichens. This observation may also spur new interest in lichens as a bioresource for biotechnological applications.

Beneficial plant-microbe interactions were extensively studied in the past and reviewed by Berg (2009). Such interactions include diverse and important functions including the suppression of pathogens and the increase in plant growth and fitness. While the traits involved in bacterial adaption and exchange of particular metabolites to higher plants are partially deciphered (Vorholt, 2012), less is known about microbe-lichen interactions. Functional assignments from the metagenome suggest *Rhizobiales* as a vital component supporting the lichen symbiosis. Results indicate that they are able to supply auxiliary as well as essential metabolites to their host. This study is the first to relate the abundance of bacteria with potential functions of their representatives within the lichen structure. Our present study also provides first indications for lichen endosymbiosis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00053/abstract

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Publication II – Supporting Material

Rhizobiales as functional and endosymbiontic members in the lichen symbiosis of *Lobaria pulmonaria* L.

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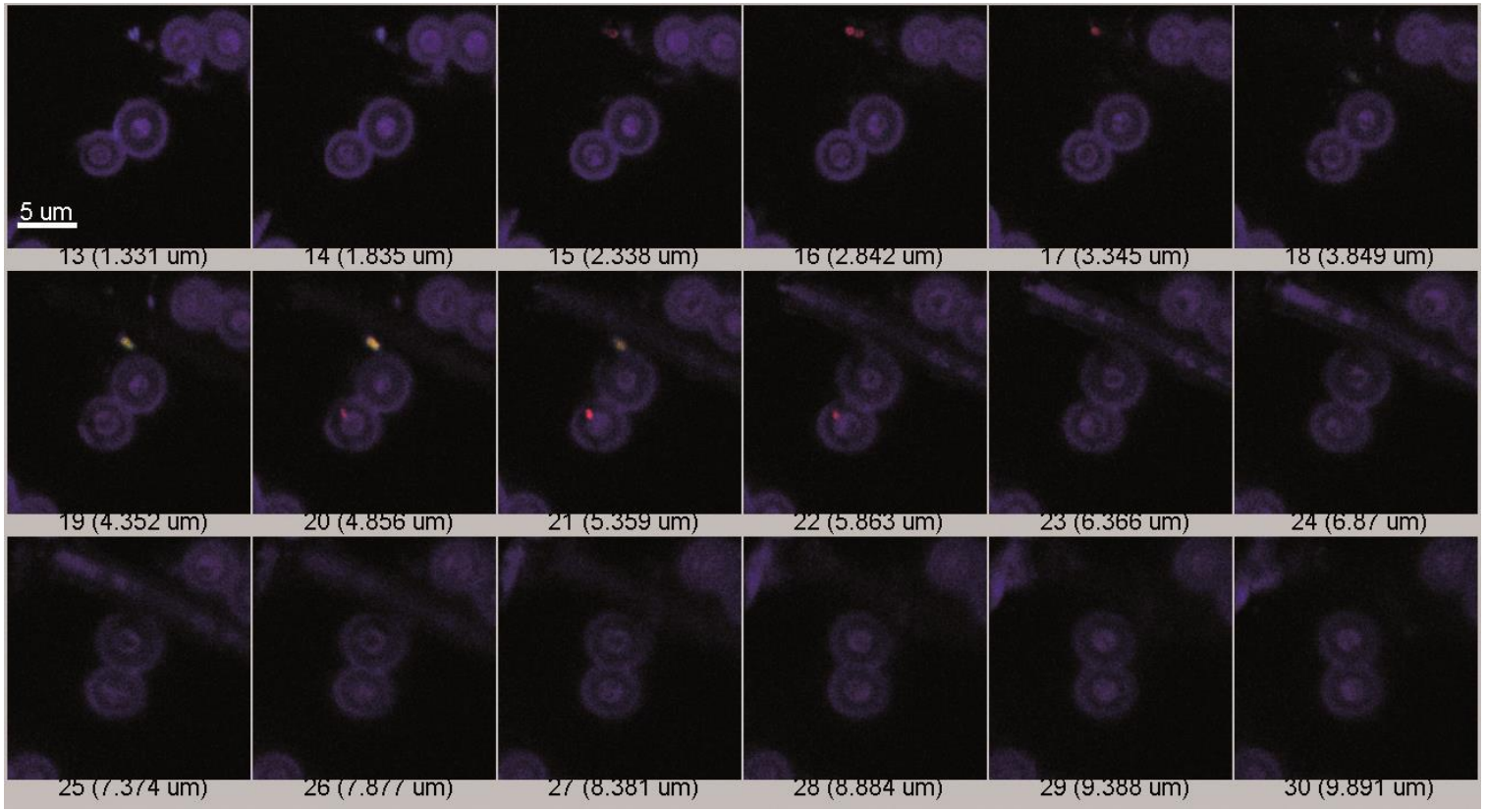

Figure S1 Endohyphal bacterial occurrence in *Lobaria pulomonaria*. Confocal microscopy image series showing a FISH-stained bacterial cell endophytically colonizing a fungal hypha. The distance between the confocal planes is 0.5 µm. Blue/purple = fungi (autofluorescence); yellow = RHIZ3r-FISH+EUB338-MIX stained bacteria; red = only EUB338-MIX stained bacteria.

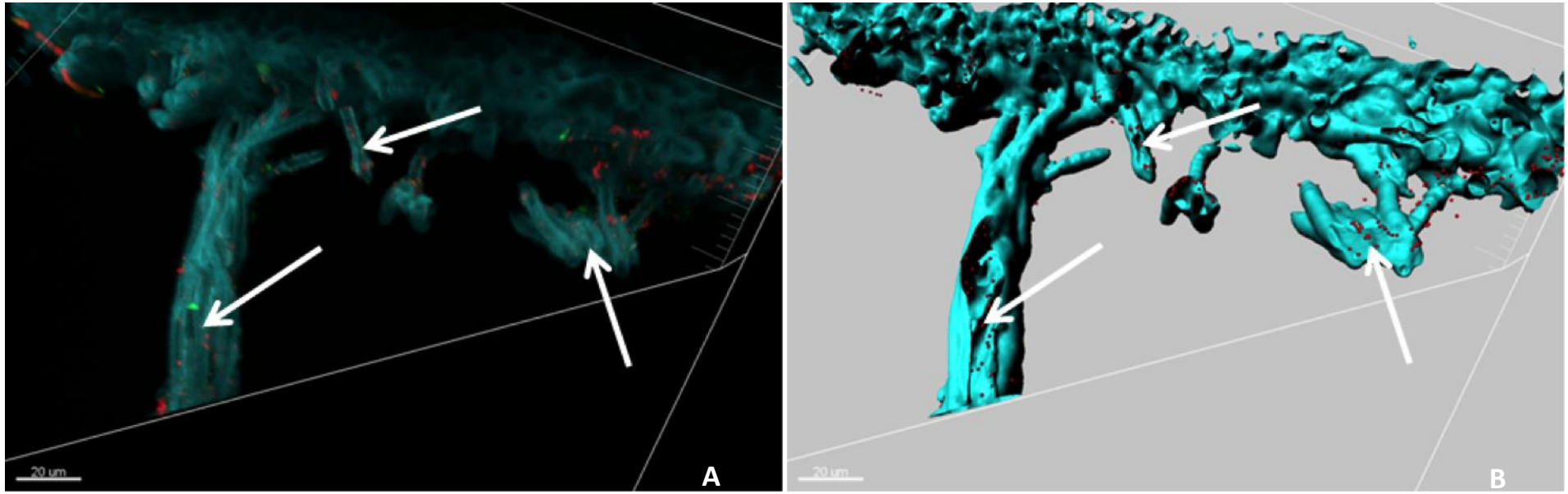

Figure S2 Endohyphal bacterial occurrence in *Lobaria pulomonaria*. Confocal microscopy volume (A) and iso-surface model (B) showing FISH-stained bacterial cells (red, arrows) endophytically colonizing fungal hyphae. The distance between the confocal planes is 0.5 µm.

Table S1 Assignment of SEED functions to bacterial taxa within the *Lobaria* metagenome. Functions were assigned to overall *Rhizobiales* and the three most abundant families therein. Higher SEED level functions are highlighted in grey with more specific functions thereunder (not highlighted).

	Assignment of SEED functions to specific bacterial taxa				
	All cellular organisms	Rhizobiales	Methylobacteriaceae	Bradyrhizobiaceae	Rhizobiaceae
Contigs assigned to specified taxon	**114821**	**28526**	**11421**	**5230**	**2403**
SEED functions of interest					
Plant Hormones	175	34	12	12	2
Auxin Biosynthesis	156	31	12	9	2
Plant Alkaloids	45	0	0	0	0
Plant Octadecanoids	52	27	1	24	0
Nitrogen Metabolism	1060	344	124	70	14
Nitrogen Fixation	11	5	2	2	0
Type III Secretion System Related	70	35	16	7	1
One-Carbon Metabolism	802	224	96	39	10
CO2 Fixation	1072	286	175	53	9
Cofactors, Vitamins, Prosthetic Groups, Pigments	3799	848	361	165	57
Chlorophyll Biosynthesis	334	133	59	43	0
Folate and Pterines	662	102	46	18	5
Folate Biosynthesis	323	45	19	6	0
Coenzyme B12 Biosynthesis	365	99	40	27	7
Cobalamin Synthesis	165	41	16	12	0
Stress Response	2769	632	243	104	46
Oxidative Stress	1238	257	108	47	21

Table S2 *In silico* evaluation of the FISH probe with the whole *Lobaria*-associated metagenome and blastn analysis.

Contig ID	Score (bits)	E-value	Closest BLASTn hit
NODE_342079_length_71_cov_5.422535	42	4,00E-04	*Methylobacterium* sp.
NODE_215355_length_99_cov_22.181818	42	4,00E-04	Uncultured bacterium/Chloroplast DNA
NODE_13033_length_932_cov_5.468884	42	4,00E-04	*Afipia* sp.
NODE_1705_length_71_cov_25.323944	42	4,00E-04	Uncultured bacterium/Plastid DNA
NODE_48805_length_113_cov_49.150444	40	0,002	*Microvirga* sp.
NODE_500503_length_5127_cov_5.032768	38	0,006	Plastid/Chloroplast DNA
NODE_245160_length_460_cov_4.478261	38	0,006	*Methylocella* sp.
NODE_469663_length_91_cov_3.560440	36	0,025	*Mesorhizobium* sp.
NODE_419997_length_203_cov_4.650246	36	0,025	Uncultured bacterium
NODE_419761_length_91_cov_9.362638	36	0,025	*Rhodanobacter* sp./*Luteibacter* sp.
NODE_398947_length_86_cov_3.941860	36	0,025	Uncultured *Rhizobiales* bacterium
NODE_398053_length_107_cov_4.093458	36	0,025	*Rhodanobacter* sp.
NODE_239641_length_131_cov_18.465649	36	0,025	*Pseudomonas* sp.
NODE_79526_length_86_cov_16.546511	36	0,025	*Methylocystis* sp.
NODE_400044_length_11426_cov_5.588395	34	0,1	Uncultured *Rhizobiales* bacterium
NODE_81818_length_110_cov_3.336364	34	0,1	Chloroplast DNA
NODE_3958_length_3680_cov_154.217941	34	0,1	*Rhizobiales* sp.
NODE_367687_length_1456_cov_5.146978	30	1,6	*Beijerinckia* sp.
NODE_345763_length_80_cov_6.550000	30	1,6	*Rhizobum* sp.
NODE_305066_length_80_cov_10.550000	30	1,6	Chloroplast DNA

Table S3 RDP Probematch analysis showing the comparison of RHIZ3r- and RHIZ1244-FISH Probes. Bold letters indicate taxa occurring in the *Lobaria* metagenome; Asterisks indicate the two most dominant groups *Methylobacteriaceae* and *Bradyrhizobiaceae*. The maximum amount of mismatches was set to 0.

RHIZ3r	Root (13311/3122829) ; Bacteria (13311/2879170) ; "Proteobacteria" (13126/944978) ; Alphaproteobacteria (12664/215572)
	order Rhizobiales (11166/71864) (hits/total searched)
	family "Aurantimonadaceae" (1/1437)
	family Bartonellaceae (0/475)
	family Beijerinckiaceae (169/1791)
	* **family Bradyrhizobiaceae (5586/11453)**
	family Brucellaceae (0/2902)
	family Cohaesibacteraceae (0/112)
	family Hyphomicrobiaceae (236/6126)
	* **family Methylobacteriaceae (3821/9098)**
	family Methylocystaceae (7/1575)
	family Phyllobacteriaceae (2/5718)
	family Rhizobiaceae (6/12475)
	family Rhodobiaceae (6/1269)
	family Xanthobacteraceae (5/1813)
	family Rhizobiales_incertae_sedis (1/290)
	unclassified_Rhizobiales (1326/15330)
RHIZ1244	Root (16831/3122829) ; Bacteria (16831/2879170) ; "Proteobacteria" (16777/944978) ; Alphaproteobacteria (16398/215572)
	order Rhizobiales (14312/71864) (hits/total searched)
	family "Aurantimonadaceae" (33/1437)
	family Bartonellaceae (395/475)
	family Beijerinckiaceae (11/1791)
	* **family Bradyrhizobiaceae (7/11453)**
	family Brucellaceae (1670/2902)
	family Cohaesibacteraceae (0/112)
	family Hyphomicrobiaceae (143/6126)
	* **family Methylobacteriaceae (5/9098)**
	family Methylocystaceae (13/1575)
	family Phyllobacteriaceae (3475/5718)
	family Rhizobiaceae (7736/12475)
	family Rhodobiaceae (26/1269)
	family Xanthobacteraceae (4/1813)
	family Rhizobiales_incertae_sedis (20/290)
	unclassified_Rhizobiales (774/15330)

Publication III

Analyzing the antagonistic potential of the lichen microbiome against pathogens by bridging metagenomic with culture studies

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Analyzing the antagonistic potential of the lichen microbiome against pathogens by bridging metagenomic with culture studies

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Naturally occurring antagonists toward pathogens play an important role to avoid pathogen outbreaks in ecosystems, and they can be applied as biocontrol agents for crops. Lichens present long-living symbiotic systems continuously exposed to pathogens. To analyze the antagonistic potential in lichens, we studied the bacterial community active against model bacteria and fungi by an integrative approach combining isolate screening, omics techniques, and high resolution mass spectrometry. The highly diverse microbiome of the lung lichen [*Lobaria pulmonaria* (L.) Hoffm.] included an abundant antagonistic community dominated by *Stenotrophomonas, Pseudomonas,* and *Burkholderia.* While antagonists represent 24.5% of the isolates, they were identified with only 7% in the metagenome; which means that they were overrepresented in the culturable fraction. Isolates of the dominant antagonistic genus *Stenotrophomonas* produced spermidine as main bioactive component. Moreover, spermidine-related genes, especially for the transport, were identified in the metagenome. The majority of hits identified belonged to *Alphaproteobacteria*, while *Stenotrophomonas*-specific spermidine synthases were not present in the dataset. Evidence for plant growth promoting effects was found for lichen-associated strains of *Stenotrophomonas.* Linking of metagenomic and culture data was possible but showed partly contradictory results, which required a comparative assessment. However, we have shown that lichens are important reservoirs for antagonistic bacteria, which open broad possibilities for biotechnological applications.

Keywords: lichen, antagonistic bacteria, plant growth promotion, *Stenotrophomonas,* spermidine

Introduction

Plant pathogens and the diseases they cause are major threats to humanity. Each year we globally lose over one third of the total harvest to bacterial and fungal pathogens. The past two decades have seen an increasing number of virulent infectious diseases in plants (Fisher et al., 2012), and human activity is intensifying pathogen dispersal as well as reducing diversity in agricultural systems (Schmid et al., 2011). However, microbial diversity is a key factor in avoiding pathogen outbreaks (Mendes et al., 2012; van Elsas et al., 2012). Therefore, biocontrol of plant pathogens is

a promising solution to control plant pathogens (Berendsen et al., 2012; Berg et al., 2013; Berg, 2015) because it was also shown that it enhances general microbial diversity (Erlacher et al., 2015a). Naturally occurring antagonists toward plant pathogens play an important role for biocontrol approaches. In natural ecosystems, which often contain a high proportion of antagonistic microorganisms, such antagonists potentially function in stabilizing the community, but might also protect the community against pathogen outbreaks (Opelt et al., 2007; Zachow et al., 2008; Grube et al., 2015). However, the ecology of naturally occurring antagonistic microorganisms is only partly understood and not yet exploited.

Lichens, which are classic examples of self-sustained symbioses, are interesting models for antagonism studies because within these mini-ecosystems the cooperation between microbial partners facilitates stability and longevity under extreme ecological conditions although they are often attacked by allochthonous bacteria and fungi (Lawrey and Diederich, 2003; Bates et al., 2011; Mushegian et al., 2011). While the lichen-specific structure is provided by fungal symbionts, which also is the naming component of the symbiosis, green algae, and/or cyanobacteria are incorporated into specific layers or compartments and contribute with photosynthetically fixed carbohydrates to the symbiosis (Nash, 2008). Lichen-associated bacteria were only recently shown to be highly diverse and omics approaches have indicated that they are functional contributors to robustness of the lichen holobiome (Grube et al., 2009, 2015). The intricate association of members of different organismal kingdoms in well-delimited and long-living symbiotic structures – as symbiotic hotspots of terrestrial life – highlights lichens as a veritable treasure chest for interorganismal communication, regulation, and bioactivity in general (Boustie and Grube, 2005; Boustie et al., 2011). Conditioned by the slow growth of many lichens and difficulties in culturing the symbionts, biotechnological exploitation of lichens was lagging behind other natural resources. With the advent of modern technologies, however, the secondary metabolism and antagonistic potentials in lichens receive new impulses, and this will particularly apply to culturable bacterial partners. Although, lichens are equipped with various secondary compounds with antagonistic effects (Oksanen, 2006; Lawrey, 2009; Boustie et al., 2011), we hypothesize that only a diverse protective microbiome can efficiently maintain stability over longer periods to prevent pathogen attacks.

The objective of this study was to analyze the antagonistic potential of the lichen microbiome against model pathogens by a novel approach bridging metagenomic with culture techniques. Model pathogens associated with human, lichen and plant diseases were accessed to screen for a broad spectrum of antagonistic activity. Furthermore, we utilized the lung lichen *Lobaria pulmonaria* (L.) Hoffm., which is one of the fastest growing leaf-like lichens (MacDonald and Coxson, 2013) and used as indicator species of undisturbed forests and air pollution (Rose, 1976; Scheidegger, 1995). We also characterized the most active as well as the most abundant lichen-associated antagonists *Stenotrophomonas,* which were already identified as versatile antagonists from plant origin (Ryan et al., 2009; Alavi et al., 2014; Berg and Martinez, 2015). Beneficial *Stenotrophomonas* strains produced osmoprotectans and spermidine in response to eukaryotic hosts (Alavi et al., 2013). In our study we applied multidisciplinary techniques to link metagenomic data with those obtained from bacterial cultures. Moreover, we could show that lichens are important reservoirs for antagonistic bacteria, which can also be used for biological control approaches to protect plants against biotic and abiotic stress.

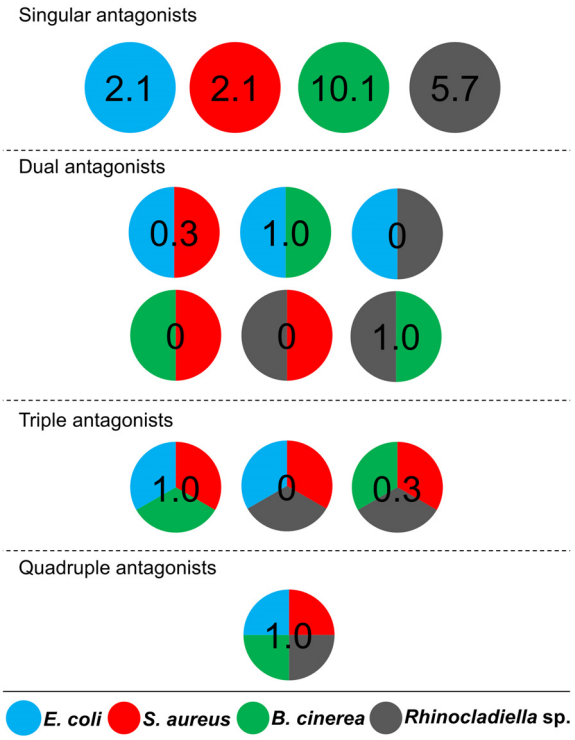

FIGURE 1 | Distribution of antagonistic bacteria isolated from multiple *Lobaria pulmonaria* thalli. Dual-culture experiments with 388 bacterial cultures were used to identify antagonists against four different model pathogens. *Escherichia coli* and *Staphylococcus aureus* were used as models for human pathogens, *Botrytis cinerea* and *Rhinocladoniella* sp. were used as models for plant and lichen pathogens, respectively. Results are presented in a schematic illustration of antagonists, targeting a specific pathogen (indicated by relative numbers and different colors, respectively). Divided circles indicate particular combinations of pathogens, which were inhibited by an assigned percentage of *Lobaria*-associated isolates. Singular antagonists: *Lobaria*-associated isolates that inhibited only one model pathogen; Dual antagonists: isolates that inhibited two distinct model pathogens; Triple antagonists: isolates that inhibited three distinct model pathogens; Quadruple antagonists: isolates that inhibited four distinct model pathogens.

Materials and Methods

Sampling Strategy and Isolation of Lichen-Associated Bacteria

Lichen thalli of *L. pulmonaria* were sampled from three different locations in Austria (Tamischbachgraben, N47°32′40″, E14°37′35″, Johnsbach, N47°38′07″, E14°44′45″, and St. Oswald

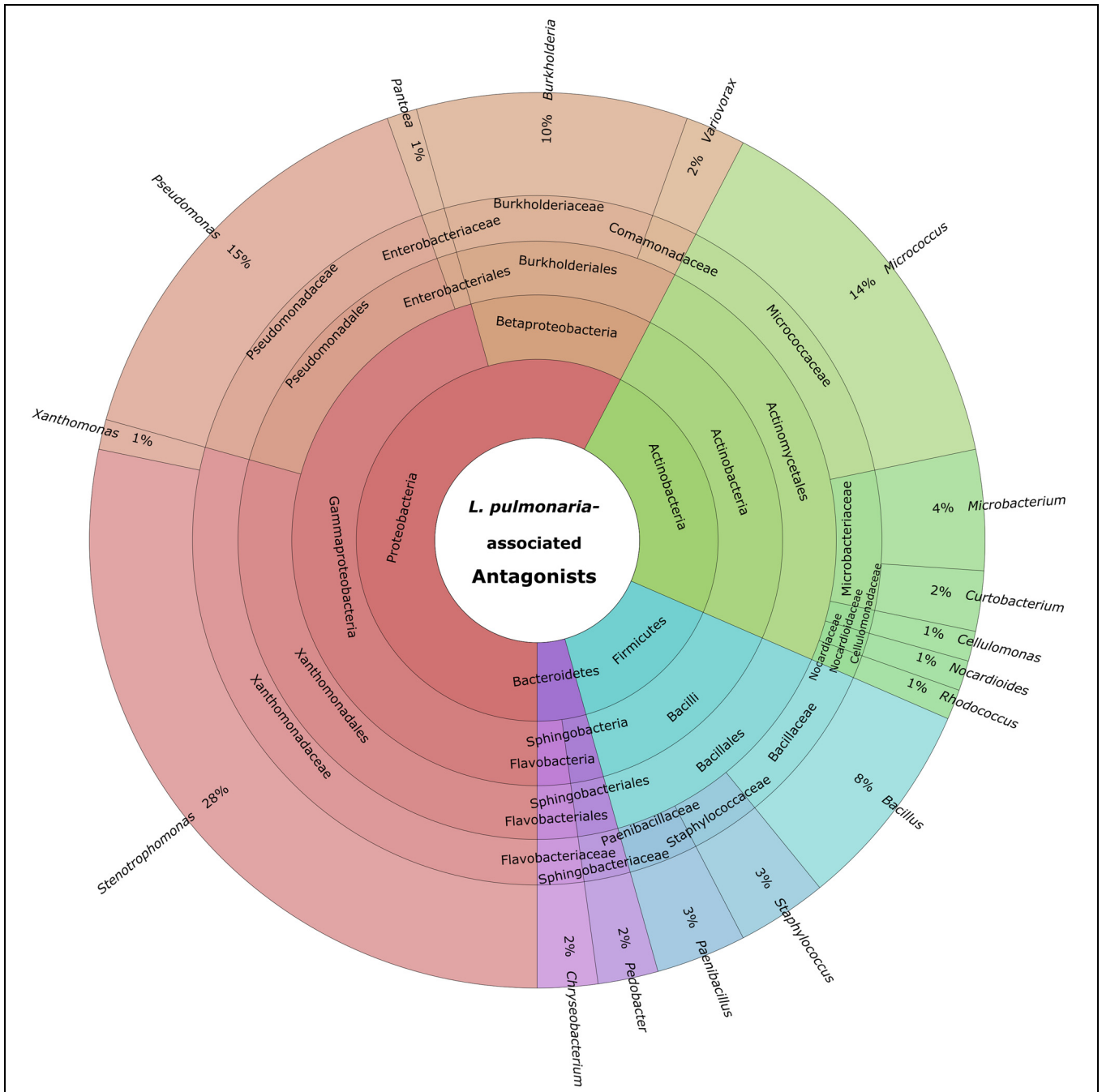

FIGURE 2 | Summary of identified antagonistic bacteria in a multi-level chart (http://sourceforge.net/p/krona). The displayed taxa were shown to inhibit growth of one or more of the utilized model pathogens: *B. cinerea*, *E. coli*, *S. aureus,* and *Rhinocladionella* sp. The inner circles represent higher taxonomic ranks, while more detailed taxonomic ranks (up to genus level) are presented in the outer circles.

ob Eibiswald, N46°44′ 50″, E15° 04′ 26″) after visual inspection to avoid contamination by lichenicolous fungi and other organisms. Five separate lichen thalli were sampled from each sampling site. The samples were stored on dry ice and were, shortly after, ground with mortar and pestle. A homogenate was prepared using sterile 0.85% NaCl in a 1:10 (w/v) ratio, together with a lab stomacher (BagMixer; Interscience, St Nom, France). Diluted fractions were plated on R2A agar (Carl Roth, Karlsruhe, Germany), R2A agar with 25 μg ml^{-1} cycloheximide, starch casein agar (SCA; Küster and Williams, 1964) and ISP2 agar (Shirling and Gottlieb, 1966). Bacterial colonies were randomly picked within 5 days of incubation at room temperature (RT) and a total of 388 isolates was obtained. The isolates were stored in glycerol stocks at −70°C prior to cultivation-based experiments.

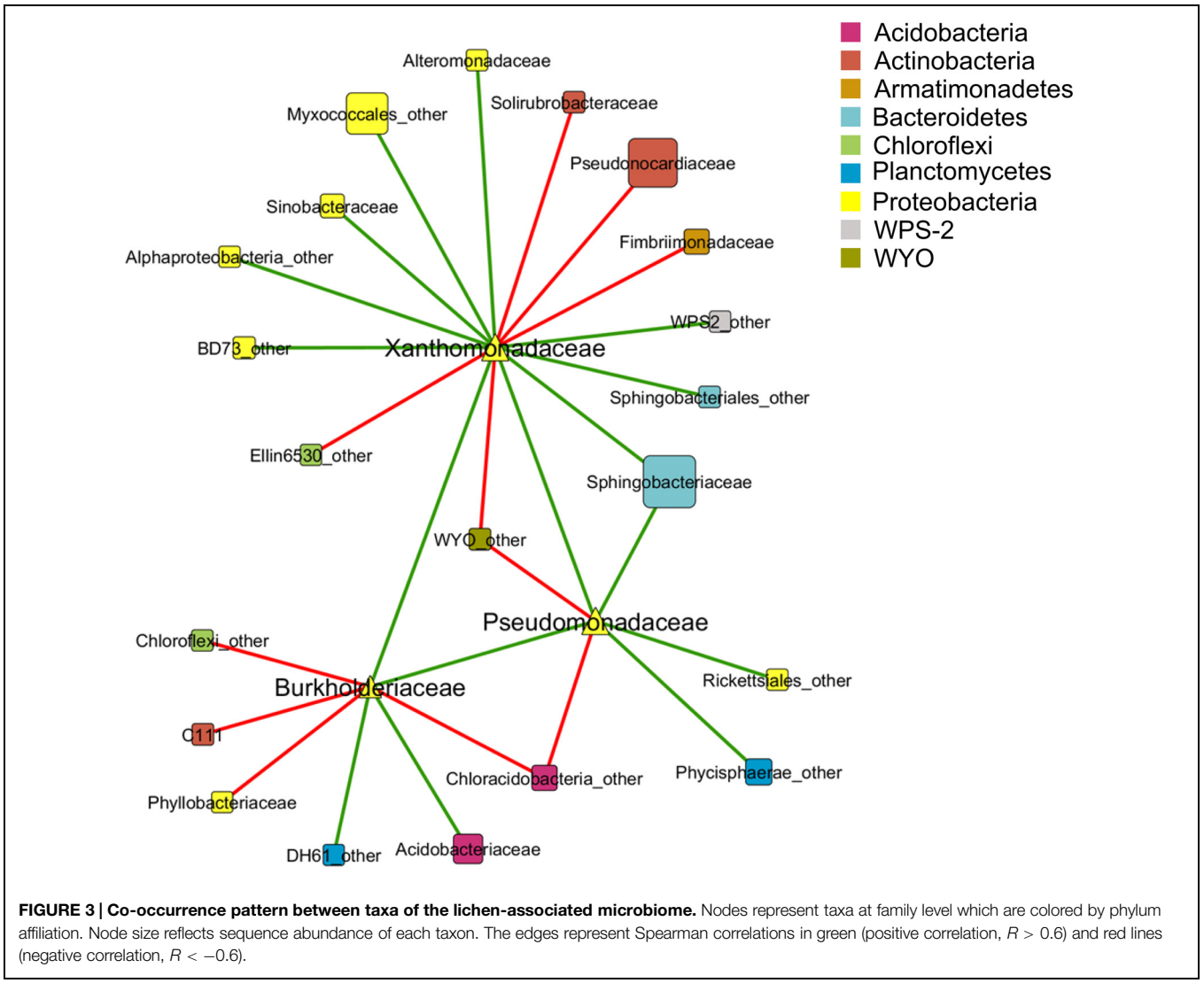

FIGURE 3 | Co-occurrence pattern between taxa of the lichen-associated microbiome. Nodes represent taxa at family level which are colored by phylum affiliation. Node size reflects sequence abundance of each taxon. The edges represent Spearman correlations in green (positive correlation, $R > 0.6$) and red lines (negative correlation, $R < -0.6$).

Screening of Isolates for *In Vitro* Antagonistic Activity Toward Particular Bacteria and Fungi

Dual-culture experiments were carried out as confrontation assays, using different media and target organisms according to Berg et al. (2002) and Opelt et al. (2007). Lichen-associated isolates were spotted on solid media pre-inoculated with *Escherichia coli* XL1 and *Staphylococcus aureus* ATCC 25923 and assessed for inhibition zones after 4 days of incubation at 30°C. Antagonistic activity against the fungus *Botrytis cinerea* Pers. (in-house culture collection) was tested by dual culture on Waksman agar (WA), according to Berg et al. (2002) and assessed after 5–7 days incubation at 20°C. Cultures of the lichen-pathogenic fungus *Rhinocladiella* sp. (culture collection of Lucia Muggia; Institute of Plant Sciences, University of Graz) were homogenized and re-suspended in sterile 0.85% NaCl. In the following step, 50 μL aliquots from one batch were used to inoculate each well of 24-well plates which contained solid potato dextrose agar (PDA; Carl Roth, Karlsruhe, Germany). Subsequently, 100 μL culture filtrate obtained from each lichen-associated isolate was added to particular wells. After 3 weeks of incubation, the wells were checked for growth reduction. All experiments were conducted with three independent replicates.

Amplicon Library Preparation and Co-Occurrence Analysis

Amplicon libraries obtained by Aschenbrenner et al. (2014) were used to extract distinct taxa for additional studies. The utilized 454-pyrosequencing data was obtained from lichen samples from the same sampling sites that were used for isolation of lichen-associated bacterial cultures. Out of the 454-amplicon dataset 15 thallus samples (five for each sampling site) were used for a co-occurrence analysis. Therefore OTUs (Operational Taxonomic Units) were clustered with UCLUST (Edgar, 2010) at 95% similarity (correlates with the taxonomic genus level). Mitochondrial, chloroplast, and *Nostoc* sequences were excluded as well as all OTUs with less than three sequences. Co-occurrence patterns were created with calculated Spearman correlations

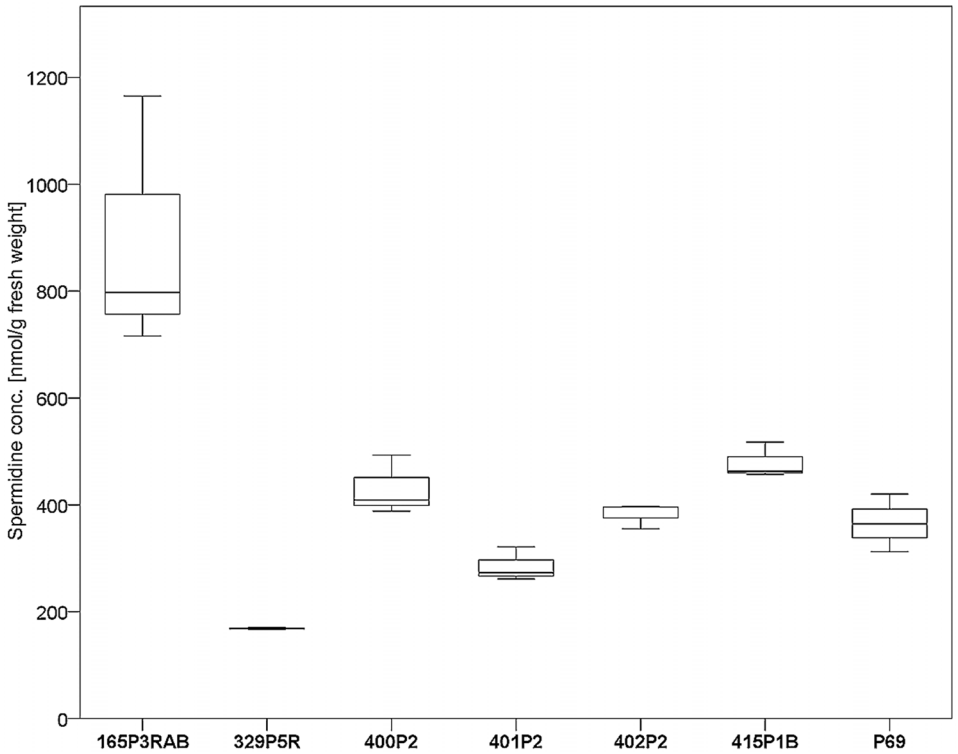

FIGURE 4 | Analysis of internal spermidine concentrations and lichen-associated *Stenotrophomonas* sp. isolates. The isolates were cultivated on solid cultivation media followed by cell disruption and quantification of internal spermidine levels ($n = 21$). A total of six *Lobaria*-associated isolates were utilized together with one plant-associated isolate (P69) in a comparative approach. *Stenotrophomonas* sp. 165P3RAB was shown to contain the highest internal spermidine concentration ($p < 0.1$) compared to all other isolates, while isolate 329P5R had the lowest concentration ($p < 0.01$).

between taxa at family level (>0.6 and <−0.6; R environment version 3.1.2[1]). Only families, which showed a correlation to *Pseudomonadaceae, Xanthomonadaceae,* and *Burkholderiaceae* were considered for further analysis and visualized as network with Cytoscape (organic layout; version 3.2.1; Saito et al., 2012). Node size within the network reflects the sequence abundance of each taxon and nodes were colored according to phylum affiliation.

16S rRNA-Based Identification of Antagonistic Bacteria and Phylogenetic Analysis

Primer pair 27F/1492r was used to amplify specific 16S rRNA gene fragments from antagonistic bacterial cultures. Subsequent sequencing and BLASTn searches within the 16S ribosomal RNA sequence database (NCBI) were conducted for identification of antagonists. These sequences were later trimmed to the hypervariable V4 region to allow alignments with 454-pyrosequencing data of the same rRNA region. QIIME 1.6.0 (Quantitative Insights Into Microbial Ecology, Caporaso et al., 2010) and the implemented Greengenes database (DeSantis et al., 2006) was used to search for bacterial OTUs in the corresponding amplicon dataset (Aschenbrenner et al., 2014) that were assigned to the three most dominant proteobacterial genera within prior identified antagonists. OTUs which could not be assigned to any particular phylum or to species level within *Proteobacteria* were additionally analyzed with Seqmatch (RDP database) for taxonomic assignment. The representative sequences of these additionally identified OTUs were used for further phylogenetic analyses. The phylogenetic tree was constructed with V4-trimmed 16S rRNA sequences from antagonistic bacteria cultures and an amplicon subset. Sequences were aligned with MEGA6 (Tamura et al., 2013) and processed for bootstrapped neighbor-joining with PHYLIP work package v.3.695[2]. Confidence levels for the internal branches were assessed by bootstrap analysis with 100 re-samplings. FigTree v.1.4.0[3] was used for annotation and final graphic visualization of the phylogenetic tree. All utilized 16S rRNA gene fragment sequences from isolate and amplicon sequencing were deposited at GenBank[4] (accession numbers: KP739786–KP739797 and KR611621–KR611709).

Metagenomic Mining for Specific Genes of Interest

All metagenome-based analyzes were carried out on the assembled dataset described in a previous study by Grube

[1]http://www.r-project.org

[2]http://evolution.genetics.washington.edu/phylip

[3]http://tree.bio.ed.ac.uk/software/figtree

[4]http://www.ncbi.nlm.nih.gov/genbank

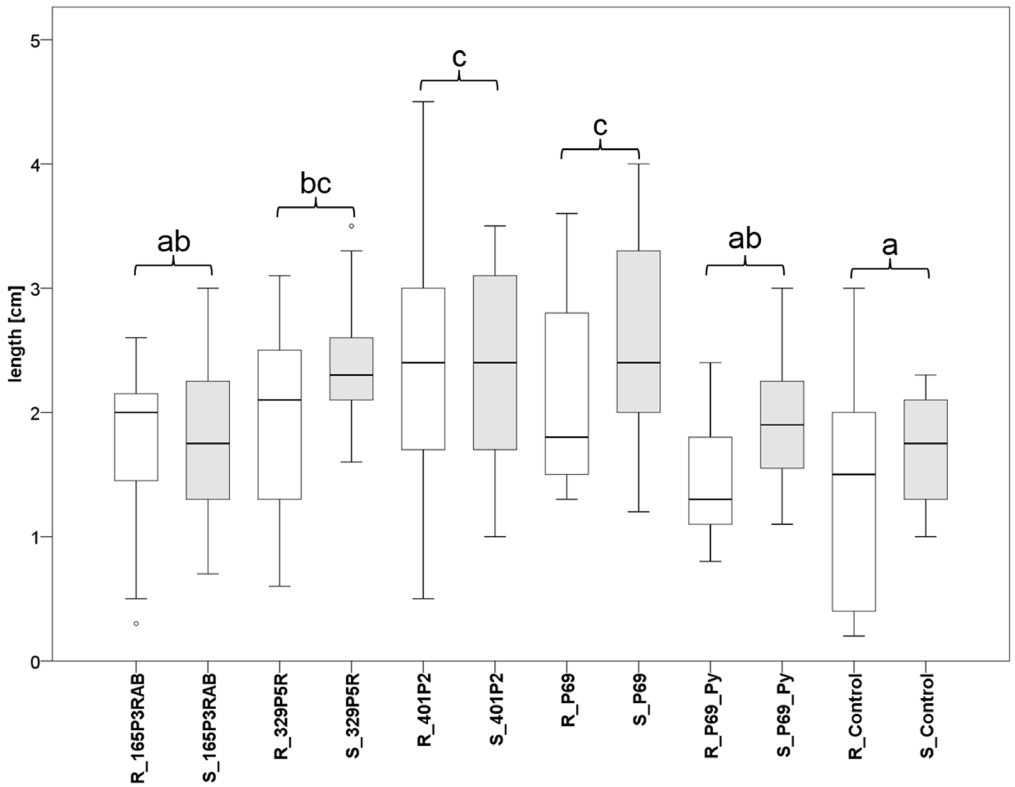

FIGURE 5 | Greenhouse experiment with *Stenotrophomonas*-primed tomato seeds and two distinctive controls (P69_Py and Control). Three *Lobaria*-associated isolates (165P3RAB, 329P5R, and 401P2) were utilized together with a plant-associated isolate (P69). Root (R; $n = 64$) and stem (S; $n = 66$) lengths were measured after 2 weeks plant growth under limited water supply. White (root lengths) and gray (stem lengths) plot pairs represent the treatments with specific isolates and complementary controls. Statistical analysis was employed to identify significant ($p < 0.05$) differences in combined root and stem lengths. Different letters were used to differentiate between statistically discriminative groups.

et al. (2015). CLUSTER CONTROL (Stocker et al., 2004) was used to search with the blastn algorithm for specific spermidine synthase matches (NCBI accession numbers: NC_010943.1, NC_011071.1, NC_015947.1, and NC_017671.1) within the dataset (368,424 contigs). MEGAN (v4.70.4) was used to retrieve taxonomic classification and relevant SEED functions.

Metabolite Extraction from Bacterial Cultures Grown on Solid Medium

Bacterial cultures were washed from several densely colonized Nutrient Agar (NA; Sifin, Berlin, Germany) plates after 48 h incubation at 30°C and homogenized in 9 mL 0.85% NaCl solution. The homogenate was centrifuged for 20 min, 2,000 *g* at 4°C. This step was repeated two times to remove residual media from bacterial cells. The pellet was re-suspended in 2 mL ddH_2O followed by centrifugation for 15 min, 18,000 *g* at 4°C. Precooled 90% methanol at −70°C was used for reproducible extraction and to avoid further degradation of metabolites. Subsequently, 1 mL was added to each pellet and the bacterial cells were mechanically disrupted with glass beads for 2 × 45 s at 6 m/s. Followed by a final centrifugation step for 15 min, 18,000 *g* at 4°C, 100 µl of each supernatant was collected and immediately placed in a deep freezer at −70°C until further analysis. Three independent biological replicates were prepared for each isolate.

Preparation of Culture Supernatants for Spermidine Quantification

Bacterial cultures were used to prepare overnight cultures (ONC) in fluid Nutrient Broth II (NBII; Sifin, Berlin, Germany) medium. These ONCs were used to inoculate 50 mL NBII flasks, which were then incubated at 30°C, 120 rpm for 48 h. In the following step, 2 mL aliquots were taken from the cultures and centrifuged for 20 min, 18,000 *g* at 4°C. The supernatants were filtered with 0.25 µm filters and immediately placed in a deep freezer at −70°C until further analysis. Three independent biological replicates were prepared for each isolate.

Quantification of Specific Bacterial Metabolites with High Resolution Mass Spectrometry

Samples were analyzed in nine biological/technical replicates with a combined HPLC-hybrid quadrupole-orbitrap mass spectrometer (Q Exactive; Thermo Scientific, Bremen, Germany). A Luna 5u NH2 100A 250 × 4.6 column (Phenomenex,

Aschaffenburg, Germany) was used to separate different metabolites from the cell extracts. Formic acid (0.1%, v/v) in acetonitrile was used as solvent A and aqueous formic acid (0.1%, v/v) as solvent B. Starting conditions for the gradient elution were 10% A and 90% B. The conditions were gradually changed to 80% A and 20% B within 15 min. This step was followed by 5 min at 10% A and 90% B for readjustment to initial conditions. The eluent flow was maintained at 0.8 mL/min together with a column temperature of 25°C. Sample analysis was carried out with negative ion ESI detection. ESI conditions were set to 3.2 kV spray voltage and 350°C capillary temperature. Scans were recorded in the range 100.0–300.0 m/z with the AGC target set to 500,000 and maximal accumulation time of 200 ms. The resolution was adjusted to 200,000. Altering full MS-SIM and targeted MS^2 cycles were employed and a specific inclusion mass of 146.16517 amu was selected. Standard calibration was obtained with 0, 0.02, 0.03, 0.04, 0.05, 0.1, and 0.2 μM spermidine standard (Duchefa Biochemie, Haarlem, The Netherlands) diluted in 0.2 mM HCL.

Plant Growth Experiments with *Stenotrophomonas*-Primed Seeds

Overnight cultures with selected *Stenotrophomonas* isolates were used to inoculate main cultures in fluid NBII. In addition to the lichen-associated isolates, a plant-associated isolate *Stenotrophomonas rhizophila* P69 (Minkwitz and Berg, 2001; Wolf et al., 2002) was also utilized for comparisons. After 2 h of growth at 30°C and 120 rpm the fluid cultures were diluted to 5×10^6–1×10^7 cells per mL in sterile 0.85% NaCl solution. Tomato (*Solanum lycopersicum* L. cv. Kremser Perle; Austrosaat, Graz, Austria) seeds were surface sterilized with 4% NaHClO for 10 min followed by drying at RT. The sterilized seeds were put into the respective bacterial suspensions and incubated for 4 h at 120 rpm and RT. The control samples were put in 0.85% NaCl solution without bacteria. Ground and homogenized seeds from each inoculum were plated on NA to test priming efficiency after the incubation time. The remaining seeds were planted in sterile soil (150 g/tray) with vermiculite (1:3 ratio) and watered with 30 mL sterile H_2O. Beside the control with non-primed seeds an additional control with P69-primed seeds was added. Therefore, 60 μL 5-sec-butyl-2,3-dimethylpyrazine (Sigma–Aldrich, Steinheim, Germany) was supplemented into 30 mL sterile H_2O used for irrigation after planting. The closed trays were placed without further irrigation for 2 weeks into a greenhouse with 12 h day/night cycles and a constant temperature of 24°C. Root ($n = 64$) and stem ($n = 66$) lengths of the plantlets were assessed separately for all samples.

Statistical Analysis

Statistical analyses were performed with SPSS v.20.0.0 (SPSS Inc, Chicago, IL, USA). Data were tested for normal distribution with the Kolmogorov–Smirnov test. Sets with normally distributed data were analyzed with univariate ANOVA and Duncan tests at $p < 0.05$. The *t*-test was employed for statistical analysis of data that was not normally distributed ($p < 0.05$ and $p < 0.1$).

Results

Antagonistic Bacteria within the *Lobaria* Microbiome

Cultivable bacteria, which have been isolated from 15 *L. pulmonaria* samples, were tested in dual-culture assays against the bacterial model pathogens *E. coli*, *S. aureus*, the plant pathogen *B. cinerea* and the lichenicolous fungus *Rhinocladoniella* sp. to determine the general antagonistic potential. In these experiments, lichen-associated antagonists were shown to primarily target lichen and plant pathogenic fungi (20.1% of all isolates) while a lower proportion was directed against bacterial model pathogens (7.7% of all isolates). A total of 95 isolates (24.5%) showed inhibition of growth of at least one of the model pathogens (**Figure 1**). Singular antagonists (active against only one target microorganism) were dominated by *Stenotrophomonas* spp. (31% of singular antagonists) followed by *Pseudomonas* spp. (19%) and *Burkholderia* spp. (12%; Supplementary Figure S1). Dual antagonists (active against two microorganisms) comprised nine isolates. *Stenotrophomonas* and *Micrococcus* were represented by three isolates and *Chryseobacterium*, *Microbacterium,* and *Paenibacillus* by only one isolate, respectively. Nine bacterial strains inhibited the growth of either three or four model pathogens simultaneously. These cultures were identified at genus level as *Bacillus* (five isolates), *Micrococcus* (one isolate), and *Paenibacillus* (three isolates). A complete taxonomic breakdown for all identified antagonists was visualized in **Figure 2**.

Comparison of the hypervariable V4 rRNA region from the most abundant proteobacterial antagonistic isolates: *Stenotrophomonas* spp., *Pseudomonas* spp., and *Burkholderia* spp. with filtered OTUs from an amplicon library constructed with *Lobaria* samples from the same sampling sites, revealed high homology of sequences within the same genus (Supplementary Figure S2). Specific branches (bootstrap values > 70%) were detected for amplicon- and isolate-based sequences. OTUs that were assigned to *Stenotrophomonas* sp., *Pseudomonas* sp., and *Burkholderia* sp. comprised, respectively, 0.06, 0.56, and 0.09% of all analyzed OTUs in the amplicon library. Interestingly three of the highly active antagonistic genera (*Bacillus*, *Micrococcus,* and *Paenibacillus*) were not substantially represented in the amplicon library.

A co-occurrence pattern between different taxa at family level was created with the calculated Spearman correlations based on 15 lichen thallus samples. In total, 24 correlations between the families *Pseudomonadaceae*, *Burkholderiaceae,* and *Xanthomonadaceae* to other taxa within the microbiome could be detected and were visualized as a co-occurrence network (**Figure 3**). Most correlations (10 out of 24) were found within the phylum *Proteobacteria* followed by *Actinobacteria* (3). The strongest positive correlations (Spearman correlation > ±0.7) showed *Xanthomonadaceae* with *Pseudomonadaceae* and *Alteromonadaceae*, all assigned to *Gammaproteobacteria*, whereas the strongest negative correlations were found between *Burkholderiaceae* (*Betaproteobacteria*) and *Phyllobacteriaceae* (*Alphaproteobacteria*) and a family within the class

Chloracidobacteria (*Acidobacteria*) which was not further classified in the utilized database.

In addition, the abundance of antagonistic taxa was extracted from the *Lobaria* metagenome. The proportion of retrievable antagonistic genera was determined for *Stenotrophomonas* (0.22% of all bacteria within the metagenome), *Pseudomonas* (1.14%), *Burkholderia* (2.81%), *Xanthomonas* (0.43%), *Nocardiodes* (0.10%), *Rhodococcus* (0.18%), *Bacillus* (0.08%), and *Staphylococcus* (0.02%). The remaining antagonistic taxa could not be retrieved at genus level. However, they comprised at family level together 2.14% of all bacteria. Altogether, antagonistic taxa comprised 7.12% of the total bacterial community. Neither the genus *Cellulomonas* nor the family *Cellulomonadaceae* was present in the assembled metagenomic dataset.

Spermidine Production *In Vitro* and Spermidine-Related Genes within the *Lobaria*-Associated Metagenome

Genes coding for spermidine synthases were analyzed from the *Lobaria* metagenome and taxonomically assigned. In addition, spermidine production and secretion was analyzed *in vitro*.

Seven antagonistic *Stenotrophomonas* sp. isolates were cultivated on solid agar plates and in liquid media prior to the extraction of spermidine. The detection limit for spermidine on the utilized instruments was determined to be <30 nM. Externalized spermidine levels detected in liquid cultivation media were in the range between 8.2 and 10.5 μmol/g fresh weight. Extracellular spermidine concentration differences between utilized *Stenotrophomonas* isolates were not statistically significant (Supplementary Figure S3). Conversely, the same isolates were shown to contain different internal spermidine concentrations after cultivation on solid media (**Figure 4**). The lowest internal spermidine concentration was found to be 168 nmol/g fresh weight, which was around fourfold lower than the highest observed concentration. Isolate 165P3RAB was found to contain significantly higher spermidine concentrations than all other isolates. In contrast, *Stenotrophomonas* isolate 329P5R contained the lowest spermidine concentrations.

For BLASTn searches, reference sequences for spermidine synthases from four different *Stenotrophomonas* strains were utilized. Additionally, SEED assignments were searched for related functions. *Stenotrophomonas*-specific contigs that contain known spermidine synthases were not found in the utilized metagenome, while three other bacteria-derived contigs were present. Two spermidine synthase contigs that were retrieved with SEED-based analysis were assigned to *Proteobacteria*. One contig was assigned to *Burkholderiaceae* and the other contig to *Acetobacteraceae*. A third contig could not be assigned to any taxon. Conversely, spermidine putrescine transporter permeases were more abundant in the metagenome. A total of 50 contigs were assigned to this specific transporter protein (Supplementary Figure S4). More than a half of these contigs were assigned to bacteria (58%), while 42% remained unassigned to a specific kingdom. The hits were predominantly associated with *Proteobacteria* (52%) and more specifically to *Rhizobiales* (28%).

Stenotrophomonas Treatments Increased Plant Growth of Tomato Under Greenhouse Conditions

Tomato (*Solanum lycopersicum* L.) seeds were inoculated with three lichen-associated and one plant-associated *Stenotrophomonas* isolate and stress protecting agent as reference (Alavi et al., 2013) to analyze the effect of bacterial inoculants on plants. The primed seeds were grown with limited irrigation for 2 weeks in sterile soil. Two control types were implemented to evaluate growth promotion effects by the inoculants. One control (P69_Py) was supplemented with 60 μL 5-sec-butyl-2,3-dimethylpyrazine per tray during the initial irrigation of *Stenotrophomonas* P69-primed seeds. This heterocyclic compound was found to limit the growth of *Stenotrophomonas* isolates in previous experiments. Correspondingly, the growth of P69-primed samples that were treated with 5-sec-butyl-2,3-dimethylpyrazine (R_P69_Py and S_P69_Py), was similar to non-primed control samples (**Figure 5**). Also, the plant growth was not enhanced by isolate 165P3RAB, which was shown to contain the highest internal spermidine concentrations when compared to both implemented controls. *Stenotrophomonas* isolates 329P5R, 401P2, and P69 enhanced the plant growth significantly when compared to both controls. These isolates were shown to produce low internal spermidine concentrations in previous experiments. Treatments with the lichen-associated isolate 401P2 and the plant-associated isolate P69 resulted in similar plant growth.

Discussion

The lichen symbiosis was discovered as reservoir for antagonistic bacteria. Interestingly, it was possible to transfer selected isolates from lichens to cultivated plants while maintaining beneficial effects. In addition, we have shown the usability as well as limits of various applied techniques to efficiently screen for specific characteristics and how to reasonably couple classic microbiology with high-end techniques in a comprehensive approach. Starting from a culture collection and dual-culture experiments to screen for active antagonists, the approach was expanded with detailed specification of continuously filtered isolates.

The microbiome involved in the lichen symbiosis is highly diverse (Aschenbrenner et al., 2014) and was identified as bioresource for antagonistic bacteria. *L. pulmonaria* is predominately colonized by *Alphaproteobacteria* (Grube et al., 2015), in particular by various members of *Rhizobiales* (Erlacher et al., 2015b), which harbor many bacterial genera known for a beneficial host-microbe interaction especially with plants. Interestingly, all antagonistic genera identified for lichens – *Stenotrophomonas, Pseudomonas, Burkholderia, Micrococcus, Chryseobacterium*, *Microbacterium,* and *Paenibacillus* – are well-known from plant studies (Haas and Défago, 2005; Ryan et al., 2009; Rybakova et al., submitted). This is an interesting finding because it shows that these bacteria have the same redundant function independent of the habitat. This observation is underlined by the greenhouse experiments, which have shown

that lichen-associated antagonists are active on plants. This also supports the hypothesis that natural ecosystems are interesting reservoirs for biotechnologically relevant bacteria. The present study depicts that cultivable bacterial taxa with lower occurrence on lichens are mainly responsible for the protection against biotic disturbance. A highly diversified bacterial microbiome enhances the available functional repertoire, which might play a crucial role for the stability and longevity of the lichen symbiosis. Comparison of isolate-derived 16S rRNA gene fragments and amplicon-based sequences of abundant antagonists has indicated that a high proportion of *Burkholderia* spp., *Pseudomonas* spp., and *Stenotrophomonas* spp. can be retrieved from lichen symbioses by cultivation experiments on conventional growth media. It was also demonstrated that several isolated antagonists, such as *Bacillus, Paenibacillus*, and *Micrococcus*, were not detectable in the amplicon library but partially in the metagenomic dataset. The most reasonable explanation is that these antagonistic species occur with low abundance within this lichen microbiome and therefore these species might be below the detection limit of the utilized 454 pyrosequencing approach. Other methods with higher coverage might be more suitable to uncover all present bacterial colonizers. Further studies that address this question should preferably subject less multiplexed samples to high-throughput sequencing platforms to obtain a higher read number per sample. This would allow more accurate characterizations of the rare microbial population.

The majority of the antagonistic isolates was assigned to the genus *Stenotrophomonas*. These bacteria have been reported to protect plants against unaffordable conditions like drought and elevated salinity by exudation of protective compounds like spermidine and different osmolytes (Berg et al., 2010; Alavi et al., 2013). Corresponding to this, polyamines which also include spermidine were shown to be involved in plant response to abiotic stress in prior studies (Alcazar et al., 2006; Liu et al., 2007). Environmental strains of *S. maltophilia* and *S. rhizophila* were reported to exert a certain degree of tolerance toward salinity of up to 9% (w/v) NaCl which was correlated with the ability to produce the osmolytes trehalose and glucosylglycerol (Ribbeck-Busch et al., 2005). Even though *Stenotrophomonas*-specific spermidine synthases were not present in the analyzed metagenome, it was evident that utilization of spermidine is widely distributed among various detected organisms. Since lichens presents a habitat that is frequently subjected to drought, the association with bacteria having protective properties appears favorable. However, lichens themselves have mechanisms to account for desiccation, which also includes osmolytes (Green et al., 2011). Moreover, protection mechanisms against oxidative stress-related damage act in a mutual manner among the eukaryotic partners (Kranner et al., 2005). Therefore, we consider stress-protective functions of lichen-associated bacteria to act as an enhancer, which might react more flexibly to local fluctuations of the conditions than their hosts.

Although synthesized and excreted in different amounts, for all selected *Stenotrophomonas* isolates, *in vitro* spermidine production was detected. According to the result, we assume that the function of lichen-associated bacteria includes assistance in the protection against pathogens as well as against damage caused by desiccation. *Stenotrophomonas* is a well-known antagonist of plant-associated origin (Ryan et al., 2009) and connected with a beneficial effect on plant hosts (Berg et al., 1996; Egamberdieva et al., 2011; Alavi et al., 2013). *Stenotrophomonas* strains might also have the same function independent of the habitat. However, in the last two decades, they have received additional attention for opportunistic infections in humans (Berg and Martinez, 2015). It is difficult to identify specific factors of pathogenicity of the virulent isolates but the ability of persistence, resistance and survival – also essential to colonize lichens – allowed a colonization of immunocompromised patients with predisposition. Similar to the plant rhizosphere (Berg et al., 2005), lichens may act as a reservoir for facultative human pathogenic bacteria, or close relatives thereof.

The host-microbiome balance as well as indigenous diversity is essential for functional stability in ecosystems. This balance also depends on the mutual effects among bacteria within the microbiome. In our co-occurrence analyses we found indications for positive and negative correlations among bacterial groups on samples of the same host lichen symbiosis in the same habitat, which is a strong indication of antagonistic and synergistic effect in the lichen habitat. Further analyses are required for clarifying the mechanisms responsible for these effects, as these might involve direct interactions or diffusible metabolites in the system or both. The present results have already shown that lichen symbioses are valuable bioresources to discover bacteria with antagonistic potential and we suggest that a systematic screening of a broader range of lichens may be useful for finding biocontrol solutions that are specifically tailored for ecologically different plant habitats.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00620

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publication III – Supporting Material

Analyzing the antagonistic potential of the lichen microbiome against pathogens by bridging metagenomic with culture studies

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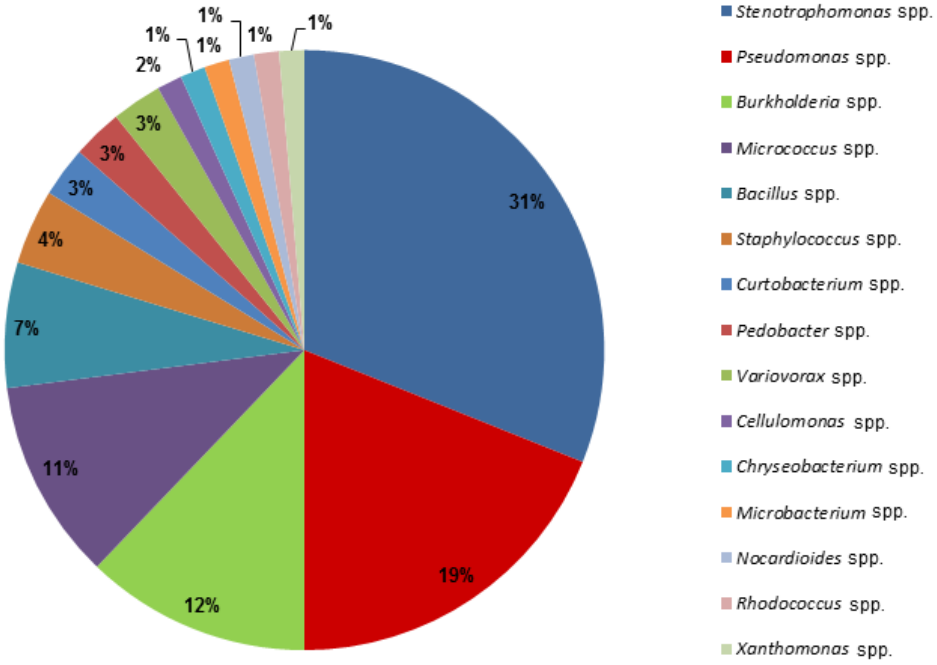

Figure S1 Visualization of lichen-associated antagonistic bacteria which targeted only one utilized model pathogen. The depicted taxa inhibited growth of either *B. cinerea*, *E. coli*, *S. aureus* or *Rhinocladionella* sp.

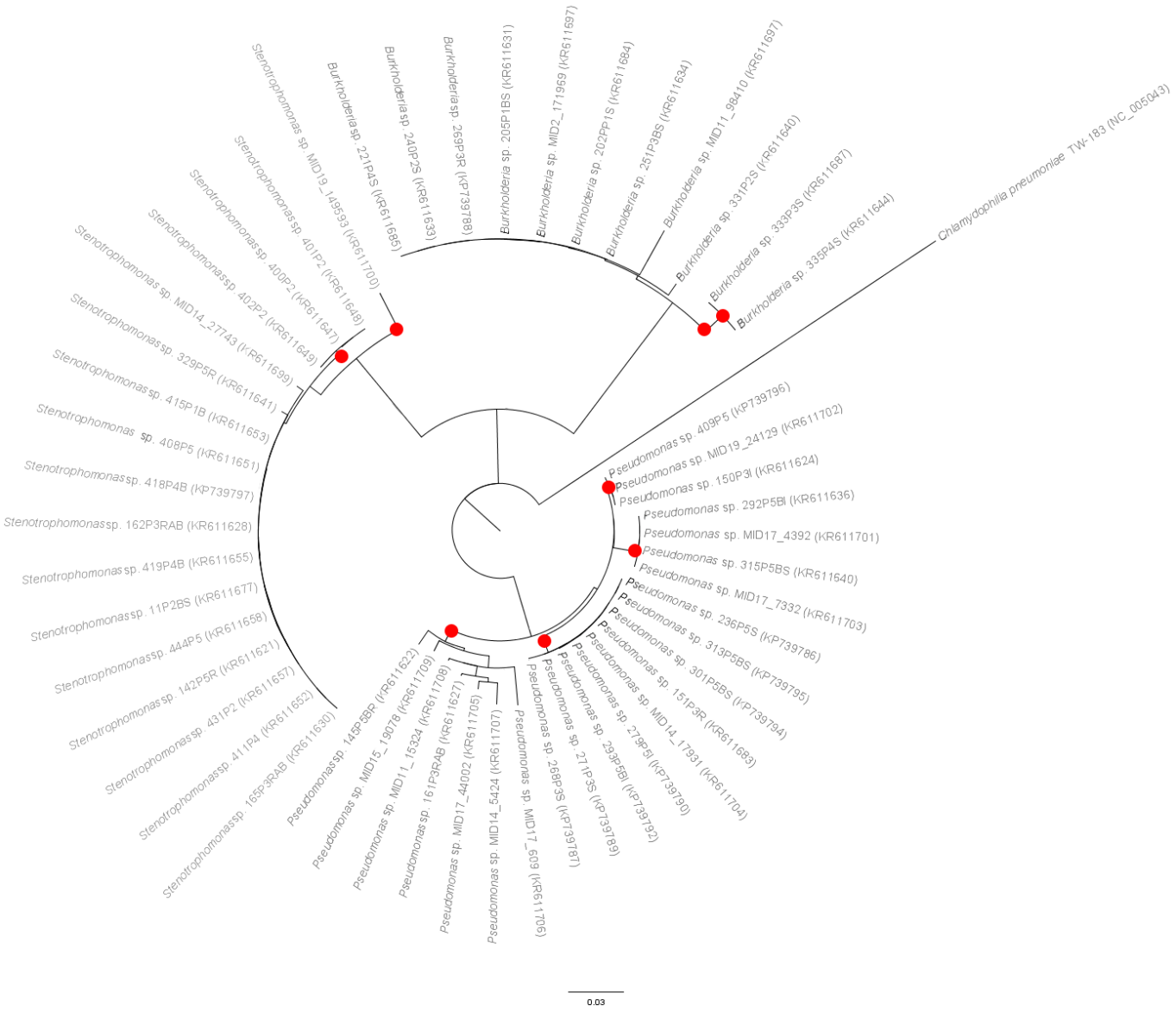

Figure S2 Neighbor-joining tree based on isolated-derived 16S rRNA gene fragments and specific amplicon sequences from the V4 region. Amplicon-based sequences are labelled with 'MID' in the respective sequence names. *Chlamydophila pneumoniae* TW-183 (NC_005043.1) was used as an out-group. Red dots mark nodes with bootstrap values > 70%. Distance bar: 0.03 substitutions per site.

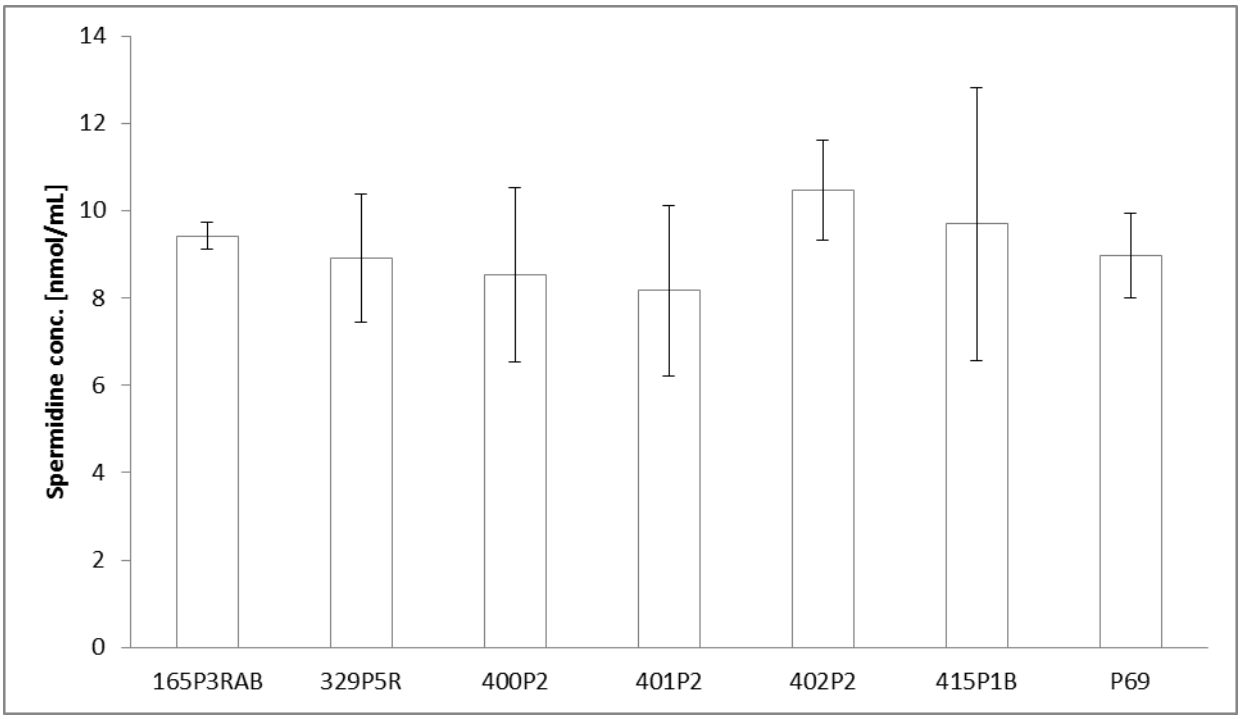

Figure S3 Analysis of extracellular spermidine concentrations and lichen-associated *Stenotrophomonas* spp. isolates. The isolates were cultivated in fluid cultivation media followed by cell removal and quantification of extracellular spermidine levels. HPLC-MS analysis was utilized to quantify spermidine concentrations. A total of six *Lobaria*-associated isolates was utilized together with one plant-associated isolate (P69) in a comparative approach. ANOVA and Tukey's HSD post hoc tests did not show significant differences between the samples.

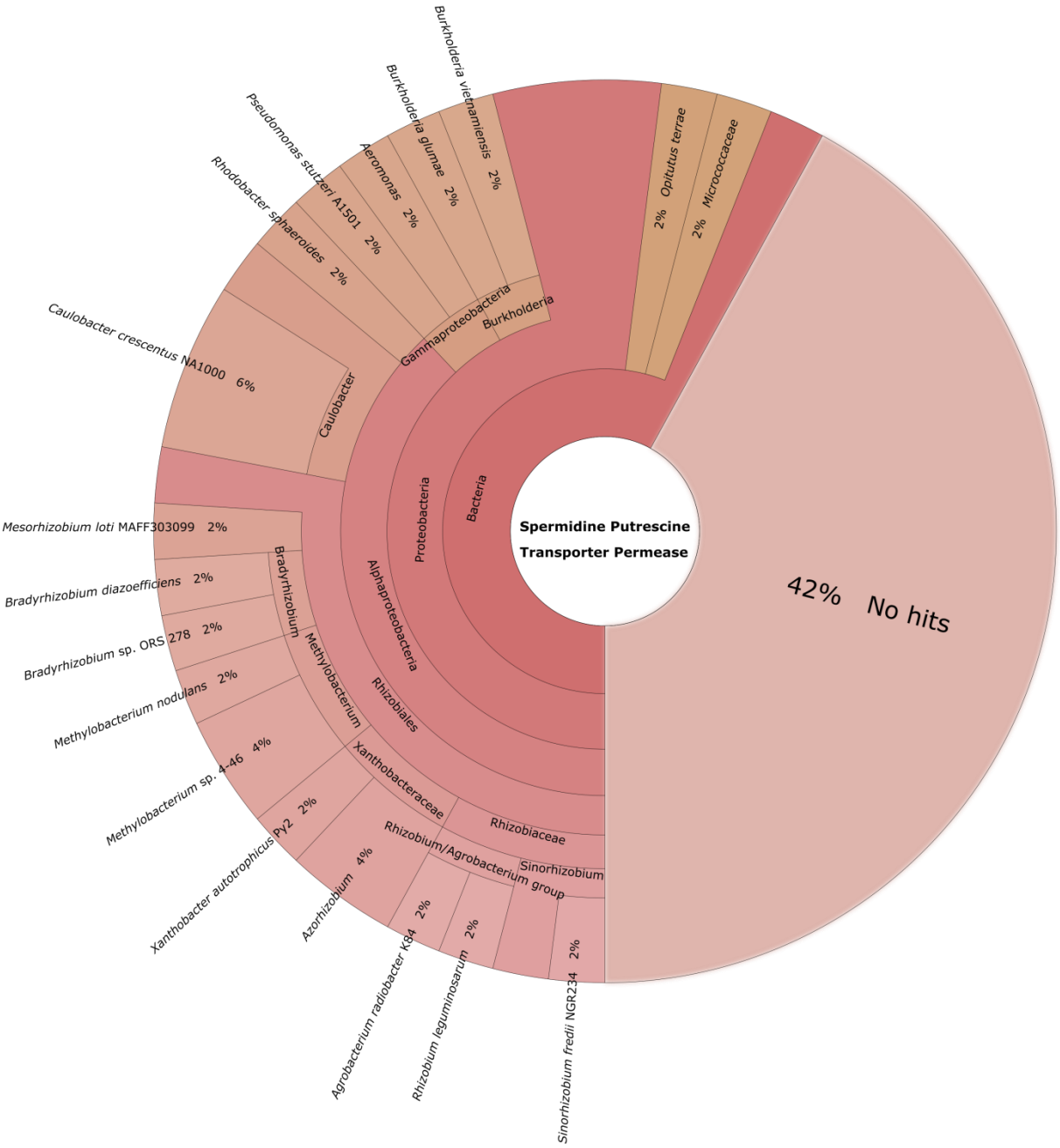

Figure S4 Visualization of taxonomic assignments of spermidine putrescine transporter permease hits within a *L. pulmonaria* metagenome. The data is visualized in a multi-level chart (http://sourceforge.net/p/krona) with different taxonomic resolutions.

Publication IV

A novel assay for the detection of bioactive volatiles evaluated by screening of lichen-associated bacteria

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METHODS
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A novel assay for the detection of bioactive volatiles evaluated by screening of lichen-associated bacteria

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Volatile organic compounds (VOCs) produced by microorganisms are known both for their effect on pathogens and their role as mediators in various interactions and communications. Previous studies have demonstrated the importance of VOCs for ecosystem functioning as well as their biotechnological potential, but screening for bioactive volatiles remained difficult. We have developed an efficient testing assay that is based on two multi-well plates, separated by a sealing silicone membrane, two tightening clamps, and variable growth media, or indicators. The experiment design as presented here is a novel and robust technique to identify positive as well as negative VOC effects on the growth of a target organism and to test for specific substances e.g., hydrogen cyanide which can be detected with a suitable indicator. While the first pre-screening assay is primarily based on indicator color change and visible growth diameter reduction, we also introduce an advanced and quantitatively precise experiment design. This adaptation involves qPCR-based quantification of viable target cells after concluding the treatment with VOCs. Therefore, we chose preselected active isolates and compared the partial 16S rRNA gene copy number of headspace-exposed *E. coli* with non-treated controls. Separately obtained headspace SPME and GC/MS-based profiles of selected bacterial isolates revealed the presence of specific and unique signatures which suggests divergent modes of action. The assay was evaluated by screening 100 isolates of lung lichen-associated bacteria. Approximately one quarter of the isolates showed VOC-based antibacterial and/or antifungal activity; mainly *Pseudomonas* and *Stenotrophomonas* species were identified as producers of bioactive volatiles.

Keywords: volatiles, VOCs, antifungal, antibacterial, lichen symbiosis

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Introduction

Volatile organic compounds (VOCs) are organic compounds that have a high vapor pressure at ordinary room temperature. VOCs are produced by the majority of organisms and they often function as communication molecules (Effmert et al., 2012). The most notable characteristic of all VOCs is the extent of their range of influence as compared to non-volatile substances. While other secreted metabolites rely on close contact between interacting organisms or diffusion through

separating matter, VOCs can overcome much greater distances. Bacterial as well as fungal strains produce a broad spectrum of bioactive VOCs with multi-functional effects, which are not restricted to the same species. Their action across organismal kingdoms was shown by Ryu et al. (2003) who demonstrated that bacterial volatiles promoted growth in *Arabidopsis thaliana*. In contrast, some bacterial isolates were also shown to reduce the growth of *A. thaliana* through emission of bioactive volatiles (Vespermann et al., 2007; Blom et al., 2011; Weise et al., 2013). Moreover, bacterial VOCs were shown to be able to suppress the growth of soil-borne pathogenic fungi, e.g., *Rhizoctonia solani* (Kai et al., 2007). Bacteria are able to communicate over long distances within the root system, both among bacteria and with plant hosts, where they elicit induced systemic resistance (ISR) and growth promotion (Farag et al., 2013). VOCs emitted by different soil bacteria can affect the growth, antibiotic production, and gene expression of soil bacteria (Garbeva et al., 2014). Owing to these multi-functional roles of VOCs, they have an enormous potential for biotechnological applications (Strobel, 2006; Schalchli et al., 2014). Currently, there is no efficient testing assay that allows for rapid screening of bioactive volatile metabolites in interrelation between two different microorganisms within the same headspace.

Host-associated microbiomes are important reservoirs for VOC-producing organisms because communication and pathogen defense are essential functions of the microbiome, and recently shown to be integral for healthy plant and human life (Blaser et al., 2013; Philippot et al., 2013). For the rhizosphere microbiome located on/in plant roots, the proportion of VOC producers is often high because in plants the root-associated microbiome acts as a primary protection shield against soil-borne pathogens (Cook, 1990; Weller et al., 2002). A similar role was also attributed to bacteria in the self-sustaining lichen symbiosis (Grube et al., 2015). In each microbiome a certain proportion of microorganisms with antagonistic activity against pathogens is involved in this function. Using a combination of metagenomic, -proteomic, and cultivation approaches, a proportion of 7% antagonists was identified for the lung lichen (Grube et al., 2015). Identification of antagonistic microorganisms is still a challenge (Berg et al., 2013), but nevertheless important for a more profound understanding of ecosystem functioning and also a necessary tool for bioprospecting in biotechnology (Strobel, 2006). The discovery of novel bioactive compounds facilitates improvement in disinfection strategies and drug discovery, both of which are in high demand due to the increasing rates of resistance to antibiotics (Woolhouse and Farrar, 2014). Antagonistic microorganisms harbor a vast potential to produce active biomolecules for direct activity against pathogens but also for mediators in various interactions, e.g., pathogen defense, quorum sensing, microorganism-host-interaction. Some of these biomolecules are highly active modifications of known antimicrobial substances and are therefore less susceptible to existing resistance mechanisms. In the past, most efforts focused on antibiotics for which high-throughput screening strategies were already developed (Conery et al., 2014; Seyedsayamdost, 2014). Although previous studies have demonstrated promising effects of bacterial and fungal volatile compounds, they are difficult to detect as well as to identify. Due to their inspiring odors, lichen extracts are used as raw materials in perfumery (Joulain and Tabacchi, 2009). As the specificity of bacterial communities in this phylogenetically old symbiosis was only recently detected (Grube et al., 2009), nothing is yet known about VOCs produced by the abundant lichen-associated bacteria.

The objective of our study was to develop a well plate-based and cost-effective testing assay for the emission of bioactive VOCs. We chose lichen-associated bacteria for evaluation purposes. One hundred lichen-associated bacterial isolates were tested for volatile antagonistic activity in order to evaluate our assay. A noteworthy screening assay for biological hydrogen production developed by Schrader et al. (2008) was used as the basis for developing our testing system. The assay is based on two micro-well plates, separated by a sealing silicone membrane, two tightening clamps, and variable growth media or indicators. The suggested experiment design can be used to differentiate between target organism inhibition or growth promotion by a pure substance and also for the same effects caused by volatile mixtures emitted by living microorganisms. This, as well as an increased throughput compared to classic single plate-tests illustrates the novelty of the presented assay in comparison to already described experiment setups. In addition, it can be employed to test for specific substances which can be detected with a suitable indicator (**Figure 1**). Using this design we identified 30 out of 100 lichen-associated bacterial isolates, which produced bioactive volatiles and induced growth inhibition in two distinct target organisms. Since many lichenicolous organisms are characterized by slow growth rates and difficult or impossible to grow on media, two classic model targets were employed for evaluation purposes. *E. coli* was used in this experimental approach as a model for a typical human pathogen and *B. cinerea* as a model for a plant pathogen. Additional GC/MS-based headspace analysis with different lichen-associated isolates was applied to demonstrate the occurrence of isolate-specific VOC profiles.

Material and Methods

Isolation of Lichen-Associated Bacteria

Lobaria pulmonaria lichen thalli were sampled from three different locations in Austria (Tamischbachgraben, N 47°32′40″, E 14°37′35″, Johnsbach, N 47°38′07″, E 14°44′45″, and St. Oswald ob Eibiswald, N 46°44′50″, E 15°04′26″). The lichen samples were ground with mortar and pestle, and subsequently combined with a ratio of 1:10 0.85% sterile NaCl in a lab stomacher to form a homogenate (BagMixer; Interscience, St Nom, France). The diluted fractions were then plated onto agars R2A (Carl Roth, Karlsruhe, Germany), R2A with 25 μg ml^{-1} cycloheximide, starch casein agar (SCA; Küster and Williams, 1964), and ISP2 (Shirling and Gottlieb, 1966). Distinctive bacterial colonies were transferred onto R2A plates for sub-cultivation within 5 days of incubation at room temperature. After subsequent testing for antagonism against different pathogens among other physiological tests, 100 lichen-associated bacterial isolates were selected out of 388 available isolates from the in-house culture collection. All of these isolates met at least one of the following criteria: (i) antagonistic activity against *E. coli* K12, (ii) antagonistic

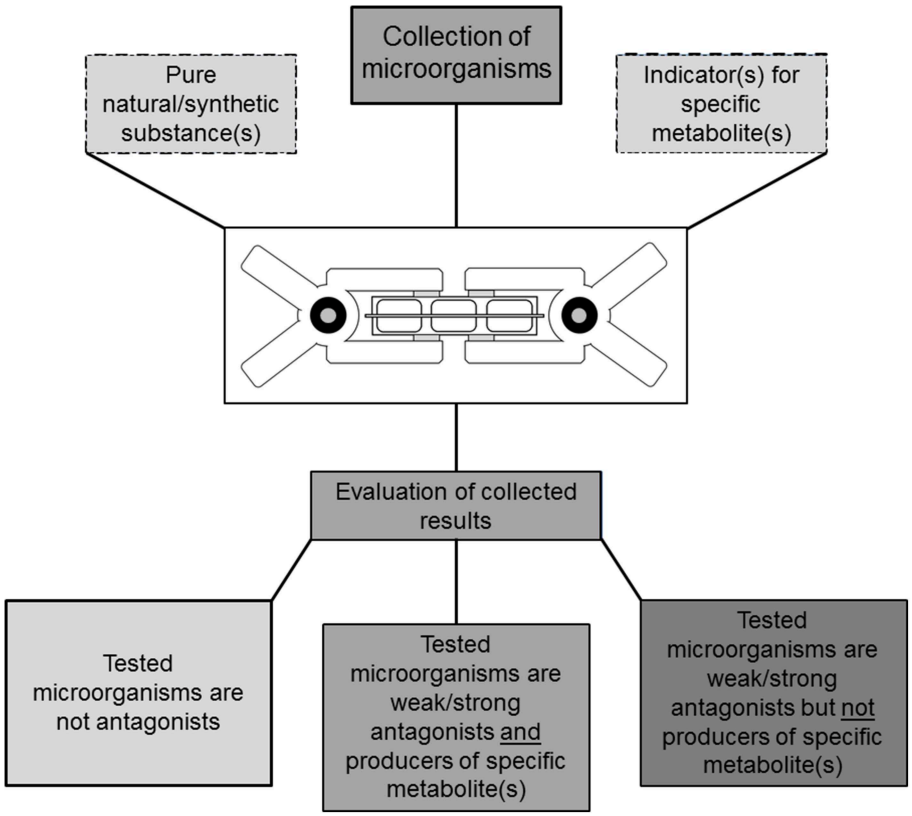

FIGURE 1 | Illustration of a TCVA-based screening approach for bioactive volatile compounds. The experiments are based on microorganism cultures suitable for cultivation in well-plates. Parallel setup of different experiments facilitates selectivity and can be used to eliminate producers of predefined compounds e.g., HCN if indicator substances are available. Utilization of additional reference substances enables gradual evaluation of visually inspected experiments.

activity against *Staphylococcus aureus* ATCC 25923, (iii) antagonistic activity against *Botrytis cinerea* (SCAM, culture collection of the institute of Environmental Biotechnology, Austria), (iv) antagonistic activity against *Rhinocladiella* sp. (culture collection of the Institute of Plant Sciences, University of Graz) in dual-culture experiments, (v) chitinase activity on chitin agar and in chitin-RBV assay, (vi) β-glucanase activity with chromogenic AZCL-Barley β-glucan.

Two Clamp VOCs Assays (TCVAs)

Depending on the experiment type, 6-, 12-, and 24-well plates (Greiner Bio-One, Frickenhausen, Germany) were used together with a perforated (0.5 cm ø) 1 mm silicone foil (detailed specifications are presented in Table S1) for tightening connected wells and usual clamps for fixation. Sterile plates were acquired and the silicone foils used were washed and autoclaved at 121°C (holding time of 20 min). 6-, 12-, and 24-well plates were filled with respectively 5, 3.5, and 1.5 mL sterile media per well. The preparation steps and the final setup are pictured sequentially in Figure S1.

TCVA with *B. cinerea* and Lichen-Associated Bacteria

The bacterial isolates were streaked onto Nutrient Agar (NA; Sifin, Berlin, Germany) in 6-well plates and pre-incubated for 24 h at 30°C. Next, 5 mm diameter plugs were cut from a donor plate evenly covered with *B. cinerea*. These plugs were placed in the center of each well in the 6-well plates containing Synthetic Nutrient-Poor Agar (SNA). After the inoculated plates were checked for sufficient growth, silicone foils were placed between plate pairs containing lichen isolates and *B. cinerea,* respectively. The plates were then clamped together; the lichen-associated bacteria plate was placed upside-down over the *B. cinerea* plates on the bottom. The plates were incubated in the dark at 21°C for 4 days and subsequently visually inspected for mycelium growth and compared to untreated controls (**Figure 2A**). Two types of controls were implemented; one containing NA wells without any bacteria and one inoculated with *E. coli* K12 instead of lichen-associated isolates.

TCVA with *E. coli* and Lichen-Associated Bacteria

The bacterial isolates were streaked onto NA in 12-well plates and pre-incubated for 24 h at 30°C. Following the incubation time, a fluid Nutrient Broth (NB; Sifin, Berlin, Germany) culture of *E. coli* K12 was grown to an OD_{600} = 0.4–0.6. 6 mL aliquots were then sequentially transferred to 200 mL NA (20%) supplemented with 0.2 mg/mL 2–(4–iodophenyl)–3–(4–nitrophenyl)–5–phenyltetrazolium chloride (INT; Sigma-Aldrich, St. Louis, MO, USA) and immediately pipetted into sterile 12-well plates.

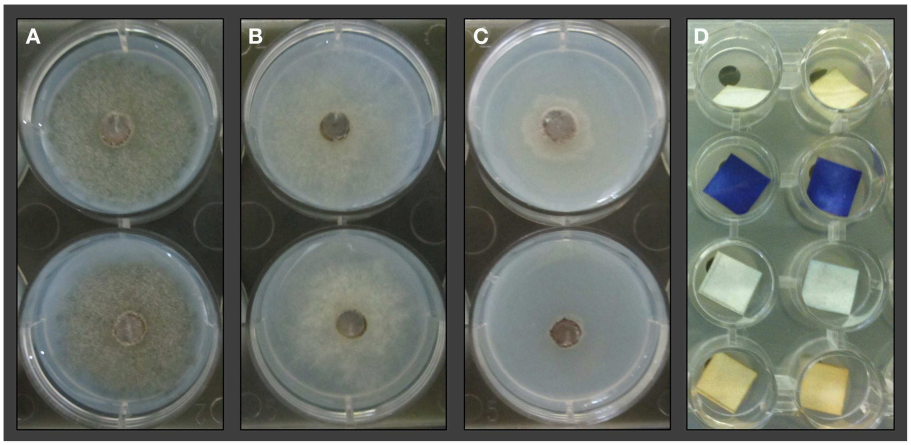

FIGURE 2 | TCVA with lichen-associated bacterial isolates and *Botrytis cinerea*. Mycelium growth and sporulation was compared to untreated controls **(A)** after 4 days of co-incubation. Inhibition of sporulation was recorded for wells where discoloring of the mycelium was not observable **(B)**. Inhibition of mycelial growth was recorded for wells with 50% or less mycelium proliferation **(C)** compared to negative controls. TCVA with HCN indicator **(D)** based on copper(II) ethyloacetoacetate and 4,4'-methylenebis(N,N-dimethylaniline). The second row shows positive reactions where bacterial isolates from counterpart wells secreted HCN into the headspace which led to the color change of indicator strips.

INT can be utilized to detect dehydrogenase activity due to visible color changes. Hence, approximate differences in bacterial abundance can be correlated to the grade of visible discoloring of INT-supplemented growth medium. After solidification of the *E. coli* K12 containing plates, silicone foils were placed between plate pairs containing lichen isolates and *E. coli* K12, respectively. The plates were then clamped together; the lichen-associated bacteria plate was placed upside-down over the *E. coli* K12 plates on the bottom. After 24 h incubation at 21°C, the plates were checked for differences in indicator color change and compared to positive and negative controls. Positive controls were obtained using different commercial disinfectants to determine sufficient OD_{600} values (0.4–0.6) for *E. coli* K12 and an adequate concentration of INT (Figure S2). Two types of negative controls were implemented: one containing NA wells without any bacteria, and one inoculated with *E. coli* K12 instead of lichen-associated isolates.

qPCR Validation of TCVA Results

This experiment is an adaption of the aforementioned TCVA with *E. coli* K12, the only modification being that semi-solid 0.3% NA was used instead of solid 1.5% NA in the initial steps of the experiment. After the incubation time, 500 µL of the semi-solid medium with *E. coli* K12 and INT was transferred into 2 mL reaction tubes with 1 mL 0.85% NaCl and subsequently dissolved via vortex. Each tube was supplemented with 10 µL (1:100 solution) propidium monoazide (PMA; GenIUL, Barcelona, Spain) and incubated on ice in the dark while shaking at 100 rpm for 50 min. The tube lids were then opened after incubation and placed under a LED light source for activation of PMA with an emission maximum of 520 nm for 10 min. PMA forms covalent bonds with available DNA but cannot pass through undisrupted cell membranes. This step was performed to exclusively detect gene fragments from living *E. coli* K12 in the qPCR-based quantification. The suspension was then transferred to glass bead containing tubes and mechanically disrupted for 2 × 45 s at 6 m/s with a FastPrep®-24 Instrument (MP Biomedicals Europe, Illkirch, France) and centrifuged at 3000 × g for 2 min to sediment beads and residual Agar. DNA was subsequently extracted from 500 µL of supernatant using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA, USA). Quantification of 16S rDNA fragments from the DNA extract was conducted with primer pair Unibac-II-515f/Unibac-II-927r as described by Lieber et al. (2003), and standards containing the Unibac-II fragments were prepared according to Köberl et al. (2011). For standard preparation, the gene fragments from *Bacillus subtilis* subsp. *subtilis* Sd3-12 were cloned into the pGEM®-T Easy Vector (Promega, Madison, WI, USA) and later re-amplified with vector specific primers. Total DNA extract treated with amplification-grade DNase I (Sigma-Aldrich, St. Louis, MO, USA) was used to determine the inhibitory effects of co-extracted substances. Based on these results, the extracted DNA was then diluted 1:10 and the target regions were amplified using KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Woburn, MA, USA). Two independent runs with three replicates for each sample were performed on the Rotor Gene 6000 (Corbett Research, Mortlake, Australia) according to Bragina et al. (2013). The specificity of the amplicons and qPCR products was confirmed using melting-curve analysis and gel-electrophoresis, respectively.

TCVA with a Hydrogen Cyanide (HCN) Indicator and Lichen-Associated Bacteria

The bacterial isolates were streaked onto NA in 24-well plates and pre-incubated for 24 h at 30°C. Indicator strips were prepared using blotting paper submerged in 10 mL chloroform (Carl Roth, Karlsruhe, Germany) solution with 50 mg copper(II) ethylacetoacetate (Sigma-Aldrich, St. Louis, MO, USA) and 50 mg 4,4-methylenebis(N,N-dimethylaniline) (Sigma-Aldrich, St. Louis, MO, USA) and left to air dry. After the pre-incubation time,

1 × 1 cm HCN indicator strips were placed in each well of a 24-well plate. Silicone foils were placed between the upside-down lichen-associated bacteria plates and those containing the HCN indicators. The plate pairs were then clamped together and incubated for 48 h at 30°C. Lastly, the indicator plates were checked for an intense blue color change in the corresponding upper wells. Negative controls were conducted with non-inoculated NA wells.

Identification of Active Isolates by 16S rDNA Sequencing

Isolated DNA from pure cultures was amplified with primer pair 27F/1492r according to Lane (1991). The PCR product was purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison; WI, USA) followed by Sanger sequencing (LGC Genomics, Berlin, Germany). The sequences were aligned with BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and 16S ribosomal RNA sequences database. Identification of the closest match was applied for the retrieved results.

Headspace SPME and GC/MS Analysis of Bacterial VOCs

The utilized GC/MS SPME headspace method was adapted with minor changes from Verginer et al. (2010). For sample preparation from bacterial isolates, single colonies were transferred with an inoculating loop on 10 mL NA slope agar (1.5%) in 20 mL headspace vials (75.5 × 22.5 mm; Chromtech, Idstein, Germany). The isolates were streaked out in 3 parallel lanes to ensure similar bacterial lawn density after incubation. Following 48 h of incubation at 30°C the vials were sealed with adequate crimp seals and incubated for additional 2 h. Solid phase micro extraction (SPME) was performed with an automated sampler and 50/30 μm Divinylbenzen/CarboxenTM/ Polydimethylsiloxane (PDMS) 2 cm Stableflex/SS fiber (Supelco, Bellefonte, PA, USA). Volatile compounds were enriched for 30 min at 30°C. Compound separation and detection was performed on a system combining a GC 7890A with a quadrupol MS 5975C (Agilent Technologies, Waldbronn, Germany). Samples were run through a (5%-phenyl)methylpolysiloxane column, 60 m × 0.25 mm i.d., 0.25 μm film thickness (DB-5MS; Agilent Technologies, Waldbronn, Germany), followed by electron ionization (EI; 70 eV) and detection (mass range 25–350). The inlet temperature was adjusted to 270°C. For the temperature gradient the GC column was kept at 40°C for 2 min, raised to 110°C at a rate of 5°C/min, then to 280°C at 10°C/min and finally maintained at 280°C for 3 min. The helium flow rate was set to 1.2 mL/min. Serial analysis was done with up to 12 samples per run. Obtained spectra were compared with NIST Mass Spectral Database 08 entries. Specific compounds were identified based on their retention indices and comparison to reference substances (Sigma-Aldrich, St. Louis, MO, USA). Origin 8.5 (OriginLab, Northampton, MA, USA) was applied for visualization of total ion chromatograms (TICs). Background-subtracted mass spectra were used for the depiction of unidentified substances.

Statistical Analysis

The statistical analysis was conducted with ANOVA within RStudio (version 0.97.551) and one-sided *t*-test ($P < 0.001$). Gene copy numbers of the UniBac-II fragment from TCVA-exposed samples ($n = 36$) were compared to untreated controls ($n = 12$). The gene copy numbers were obtained from two biological samples and three qPCR repeats respectively.

Results

Testing Volatile Activity Against *E. coli* and Botrytis Cinerea

The here presented Two Clamp VOCs Assay (TCVA) made it possible to detect bioactive VOC producers within 100 lichen-associated bacterial isolates. Sporulation reduction (**Figure 2B**) was demonstrated for five isolates; four isolates reduced sporulation of *B. cinerea* in three out of four replicate experiments, while one isolate reduced sporulation in all four trials. *B. cinerea* growth was repeatedly reduced after exposure to 21 different lichen-associated bacterial isolates in the TCVA, and mycelium proliferation was visibly affected (**Figure 2C**) for these isolates when compared to negative controls. Moreover, 16 isolates reduced proliferation in three out of four replicate experiments, while five isolates reduced proliferation in all four trials. Only one of the identified growth-reducing isolates was later shown to release HCN into the headspace. TCVAs with *E. coli* allowed identification of 10 lichen-associated isolates that are associated with the exertion of antagonistic activity through headspace. Low INT-based growth media discoloring indicating a reduced number of metabolically active bacteria was observed in all three replicate experiments. Comparison to the corresponding HCN TCVAs showed that two of the growth-reducing isolates did not release HCN into headspace. Only one isolate inhibited the growth of both target organisms and was later identified as *Pseudomonas umsongensis* 313P5BS. From all identified antagonists we have selected the 15 most active isolates against one or both target organisms and one non-inhibiting isolate for Sanger sequencing (**Table 1**).

Screening for HCN Producers

All lichen isolates were tested for HCN production in a modified TCVA by imposing indicator strips to the headspace. Nine bacterial isolates induced dark blue discoloring of the indicator strips in all three replicate experiments. Eight of the identified HCN-producing isolates also reduced growth of *E. coli* in previous experiments. These isolates were later assigned to *Pseudomonas* spp., while the non-antagonistic HCN producer had the highest sequence similarity to a *Bacillus pumilus* isolate.

Validation of TCVA Results by Quantitative PCR

DNA extracts from wells containing *E. coli* that had shown low discoloring of INT after exposition to lichen-associated bacteria were used to determine the gene copy number of the Unibac-II fragment. DNA from dead or disrupted cells was blocked by PMA which enabled a correlation between gene copy number and living cells. A significantly lower gene copy number compared to controls was shown for all samples exposed to the headspace of highly active antagonists that were pre-screened based on INT discoloring. An approximately 4-fold decrease of the gene copy

TABLE 1 | Overview of identified isolates including corresponding activity in TCVAs.

Strain ID	Closest BLASTn match	GenBank accession #	Inhibition of *E. coli*	Inhibition of *B. cinerea*	HCN producer
43P2BR	*Bacillus pumilus*	KP739785		✓	
236P5S	*Pseudomonas umsongensis*	KP739786	✓		✓
268P3S	*Pseudomonas umsongensis*	KP739787	✓		✓
269P3R	*Burkholderia sordidicola*	KP739788	✓		
271P3S	*Pseudomonas umsongensis*	KP739789	✓		✓
279P5I	*Pseudomonas umsongensis*	KP739790	✓		✓
288P4R	*Burkholderia sordidicola*	KP739791	✓		
293P5BI	*Pseudomonas umsongensis*	KP739792	✓		✓
300P5BR	*Chryseobacterium piscium*	KP739793		✓	
301P5BS	*Pseudomonas umsongensis*	KP739794	✓		✓
313P5BS	*Pseudomonas umsongensis*	KP739795	✓	✓	✓
409P5	*Pseudomonas lini*	KP739796	✓		✓
418P4B	*Stenotrophomonas rhizophila*	KP739797		✓	
439P1B	*Stenotrophomonas rhizophila*	KP739798		✓	
460P5B	*Stenotrophomonas rhizophila*	KP739799		✓	
471P3B	*Bacillus pumilus*	KP739800			✓

Listed species represent the closest match of BLASTn searches within the 16S ribosomal RNA sequences database (NCBI). The 16S gene fragment sequences were deposited at GenBank (http://www.ncbi.nlm.nih.gov/genbank).

number was observed with the least inhibiting antagonist *Pseudomonas* sp. 279P5I, while the most effective antagonist *Pseudomonas* sp. 236P5S decreased the gene copy number of *E. coli* approx. 15-fold (**Figure 3**).

Taxonomic Assignment of Active Lichen-Associated Isolates

Sanger sequencing revealed multiple occurrence of some dominant genera. Furthermore, sequencing of 16S rRNA gene fragments from the 15 most active isolates and a non-antagonistic HCN producer revealed the majority belonging to the genus *Pseudomonas* (8 isolates), followed by *Stenotrophomonas* (3 isolates) and three other genera with lower occurrence: *Bacillus*, *Burkholderia,* and *Chryseobacterium.* Utilization of the TCVA demonstrated that *E. coli* inhibition was mostly observed after exposure to the headspace of *Pseudomonas* sp., while *B. cinerea* growth reduction was mostly observed after exposition to the headspace of *Stenotrophomonas rhizophilia*. Moreover the sequencing approach revealed that different *Pseudomonas* sp. inhibited *E. coli* growth accompanied by HCN release into headspace. Identified isolates are presented together with corresponding TCVA results in **Table 1**.

GC/MS-Based Headspace Analysis with Selected Isolates

Three representative isolates which were shown to inhibit growth of headspace-exposed target microorganisms and which were taxonomically assigned to reoccurring genera were used for subsequent GC/MS headspace SPME profiling. Isolate-specific VOCs were identified by overlays of total ion chromatograms (TIC; **Figure 4**). A total of 21 compounds (Table S2) were found to be unique and only present in TICs of a specific isolate. *Bacillus pumilus* 43P2BR emitted nine distinctive volatile compounds (compound IDs: 2, 5, 8, 10, 13, 14, 15, 18, and 21), followed by *Pseudomonas umsogensis* 313P5BS with eight distinctive compounds (compound IDs: 1, 6, 7, 9, 16, 17, 19, and 20). *S. rhizophila* 418P4B was shown to emit only four distinctive compounds (compound IDs: 3, 4, 11, and 12). Compound identification indicated that *B. pumilus* 43P2BR emitted 1-butanol, 3-methyl-2-pentanone and seven unidentified substances (Figures S3-18). 2-butanol, 2-methyl-1-propanol and two unidentified substances were found within spectra of *S. rhizophila* 418P4B. Conversely, *P. umsogensis* 313P5BS emitted methyl thiocyanate as well as seven unidentified substances.

Discussion

The results of the screening for bacteria-derived bioactive VOCs demonstrated the applicability of a novel testing system, which is not restricted to bacteria associated with lichens, but can be widely applied with microorganisms sampled in other habitats. With the presented setup several 100 isolates can be tested simultaneously for VOCs-driven effects on target microorganisms and occurrence of specific metabolites. This facilitates screening programs for strain-specific biological effects. Thus, the method is also not limited to observations of growth inhibition such as demonstrated in the present study, but could also be used to identify growth promoting effects. The method, however, reveals the effect of the total "volatilome" of a bacterial strain and does not dissect the effect of individual substances. The composition of the mixture of volatile substances must still be assessed by chemical analysis, after which individual compounds might be tested separately.

We have selected 100 isolates for evaluation purposes that met predefined criteria such as antagonism in classic dual-culture experiments. Thus, we expected to identify a sufficient number of isolates which emit bioactive VOCs to validate the experimental design and the presented workflow. Utilization of different TCVA variations allowed the identification of 30 growth inhibiting bacterial isolates with a novel and reliable technique. Subsequent analysis of the headspace from taxonomically dissimilar bacterial isolates by employing headspace SPME GC/MS demonstrated the presence of isolate-specific TIC profiles and unique compounds in each sample. We have identified five out of 21 unique compounds, emitted by three distinct bacterial strains, to exemplify the presented workflow. While some compounds most likely originate from bacterial degradation of growth media

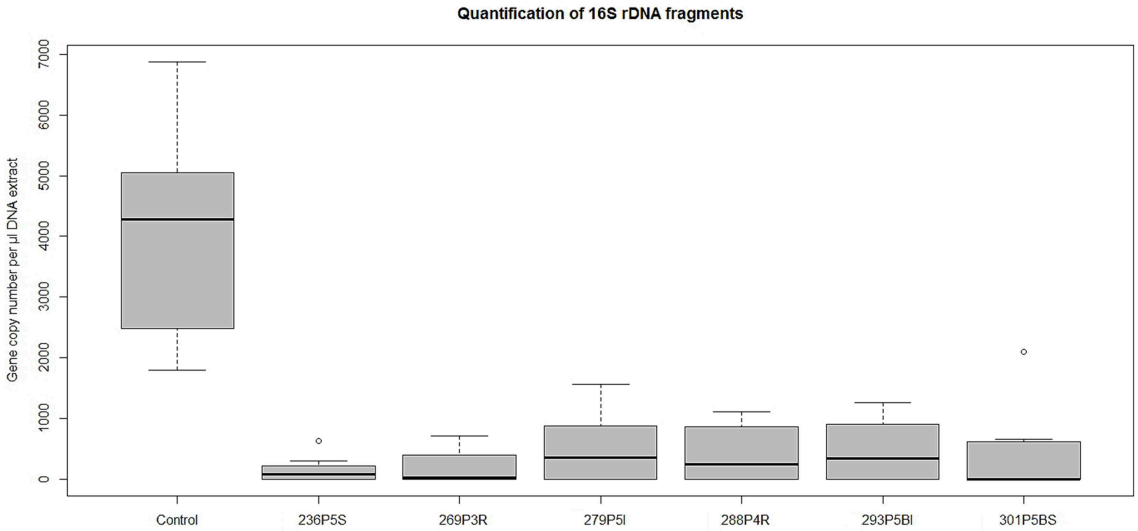

FIGURE 3 | A qPCR approach was used to determine gene copy numbers of *E. coli* K12 after exposition to strong VOCs antagonists. TCVA-exposed samples were treated with PMA and gene copy numbers of viable cells were quantified with Unibac-II primers. Values for the treated samples were obtained from respectively six qPCR runs and additional 12 qPCR runs for untreated controls. Statistical analysis with ANOVA and one-sided *t*-test confirmed a highly significant decrease of gene copy numbers of the treated samples compared to the control group ($P < 0.001$).

(waste products of bacterial metabolism) and therefore do not target pathogens specifically, other compounds might either be involved in molecular signaling or inhibition of competing organisms. These differences and their significance in natural systems merit further exploration. It might be hypothesized that bacterial bioconversion of the natural substrate may result in volatile compounds with signaling effect. Specifically, the odor of lichens, which may attract reindeer or is part of perfumes, could be influenced not only by the genuine compounds produced by the fungal or algal symbiont, but possibly also by VOCs produced by the bacteria themselves or by compounds released from the fungal matrix due to the metabolic activity of associated bacteria.

The presented workflow includes a pre-incubation of the tested isolates to minimize their inhibition by volatiles emitted by the target organisms. Due to intended growth advantage of the tested organisms, emitted volatiles from the target organisms might not play an important role during the co-incubation. Still, such effects cannot be completely avoided with the presented setup. An inverted approach where the "target" is pre-incubated and subsequently tested against the respective isolate collection could be implemented to obtain a more holistic view on occurring interactions.

Interestingly, *P. umsongensis,* a bacterial species isolated from soil as well as from fungal hyphae and described as "fungiphilic" (Warmink et al., 2009), was the only representative of *Pseudomonas* isolates that consistently inhibited *B. cinerea* growth. This may have resulted from better and faster growth on solid medium compared to the other utilized strains and therefore a higher accumulation of cyanide in the headspace. Various *Pseudomonas* species are known to be cyanogenic bacteria and therefore enhanced toxicity toward various prokaryotes and eukaryotes can be expected even if they are not in close contact. While the employed headspace SPME GC/MS method was not suitable for detection of hydrogen cyanide, we were able to detect methyl thiocyanate above cultures of *P. umsongensis* 313P5BS. Conversion of cyanide to thiocyanate is accomplished by bacterial rhodanese and these co-occurring molecules can be extracted simultaneously from headspace above living cultures (Broderick et al., 2008). Weise et al. (2013) have highlighted the importance of bacterial ammonia production and demonstrated accompanied growth inhibition of *Arabidopsis thaliana*. Specific indicator stripes in the TCVA would allow to test for ammonia in the headspace, which would also imply an unspecific inhibition of target organisms.

Some bacteria are well known for pronounced antifungal effects against phytopathogenic fungi. This effect is typical for several strains belonging to *Stenotrophomonas* (Wolf et al., 2002), which was also observed with headspace experiments (Ryan et al., 2009). *Bacillus* species were shown in prior studies not only to produce antifungal VOCs (Fiddaman and Rossall, 1993), but also volatiles that promoted growth in *A. thaliana* (Ryu et al., 2003). Our study demonstrates such antagonistic effects with a robust well plate-based approach and provides various options for modifications to study further effects e.g., growth promotion of bacteria on plants in an adaptable testing system. Moreover, this approach could also be applied to study the prevalence of similar bioactive effects across entire bacterial genera and to correlate volatile effects with the occurrence of strains in particular ecological niches.

As we are convinced that bacterial volatiles might play an important role to modify the composition of host-associated communities, future research needs to focus on the, possibly context-dependent, effects of such small molecules. We anticipate

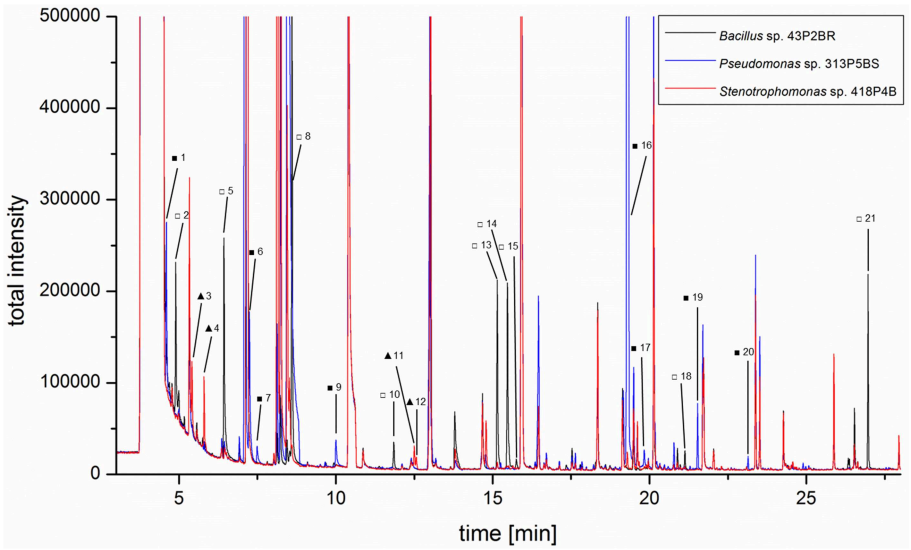

FIGURE 4 | Three lichen-associated bacterial isolates were subjected to headspace SPME GC/MS analysis to identify isolate-specific VOC profiles. An overlay of the respective TIC chromatograms illustrates the presence of specific metabolites in the headspace of *Bacillus pumilus*43P2BR (empty squares), *Pseudomonas umsongensis* 313P5BS (filled squares), and *Stenotrophomonas rhizophila* 418P4B (filled triangles). Identified *Bacillus*-specific compounds were: 1-butanol (5□) and 3-methyl-2-pentanone (8□). Methyl thiocyanate (7■) was identified as *Pseudomonas*-specific compound. *Stenotrophomonas*-specific compounds were: 2-butanol (3▲) and 2-methyl-1-propanol (4▲). More details in Table S2.

that this newly developed testing approach will be a major step forward to facilitate such studies.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00398/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publication IV – Supporting Material

A novel assay for the detection of bioactive volatiles evaluated by screening of lichen-associated bacteria

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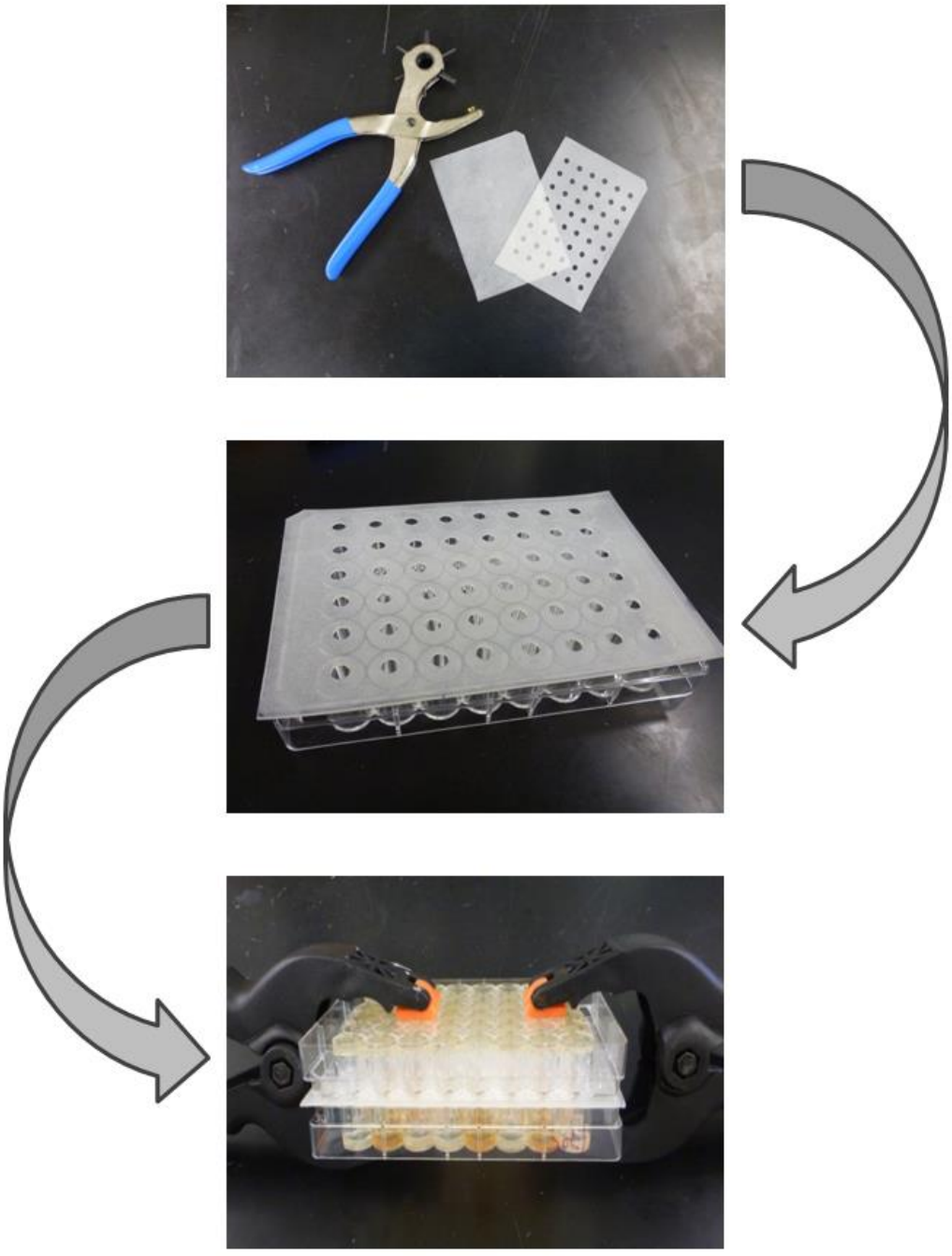

Figure S1. Step by step illustration of a TCVA with two microorganism-containing well-plates. Perforated silicone foil together with two clamps per plate was used to seal adjacent wells and join them into separate chambers. After a microorganism-specific incubation time, the plates were separated again and visually inspected for growth reduction within wells of the target plate.

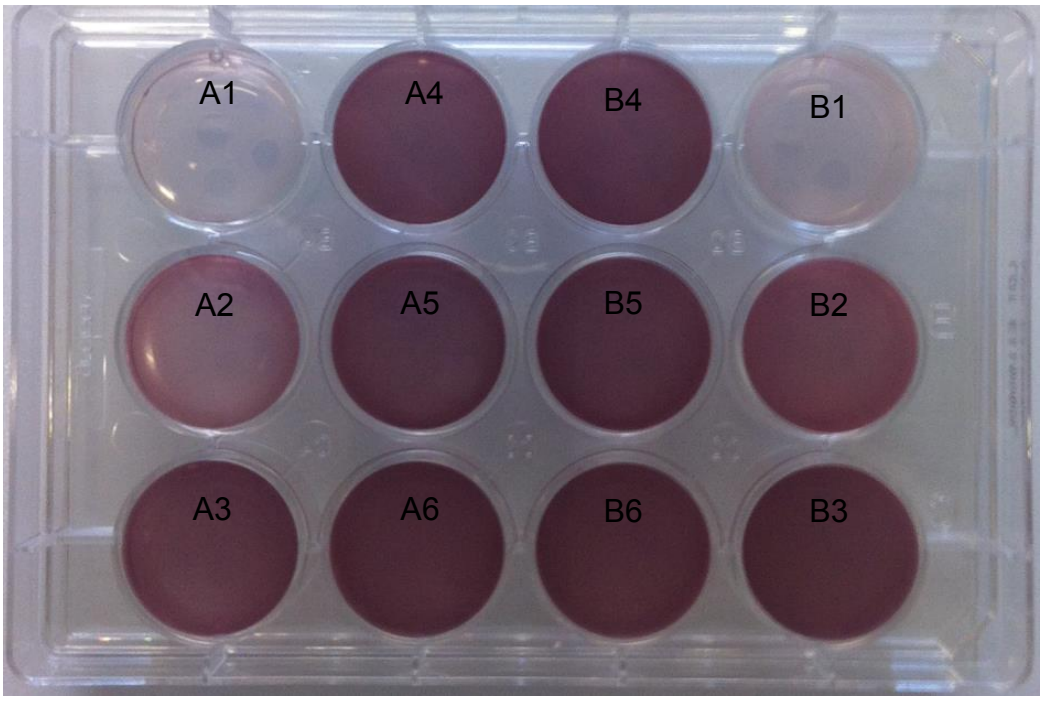

Figure S2. Pretest with *E. coli* K12 and 2–(4–iodophenyl)–3–(4–nitrophenyl)–5–phenyltetrazolium chloride (INT; 0.2 mg/mL) supplemented nutrient agar (NA) 12-well plates. The left plate compartment (A1-A6) was exposed to different Sterilium™ concentrations, while the right plate compartment (B1-B6) was exposed to different Bacilol™ concentrations. High inhibition of *E. coli* was observed with 100 µL Sterilium™ and Bacilol™, respectively (A1 and B1). Lower inhibition was observed with decreasing Sterilium™ and Bacilol™ concentrations (A2-A5 and B2-B5). Two negative controls without any disinfectant were additionally conducted (A6 and B6).

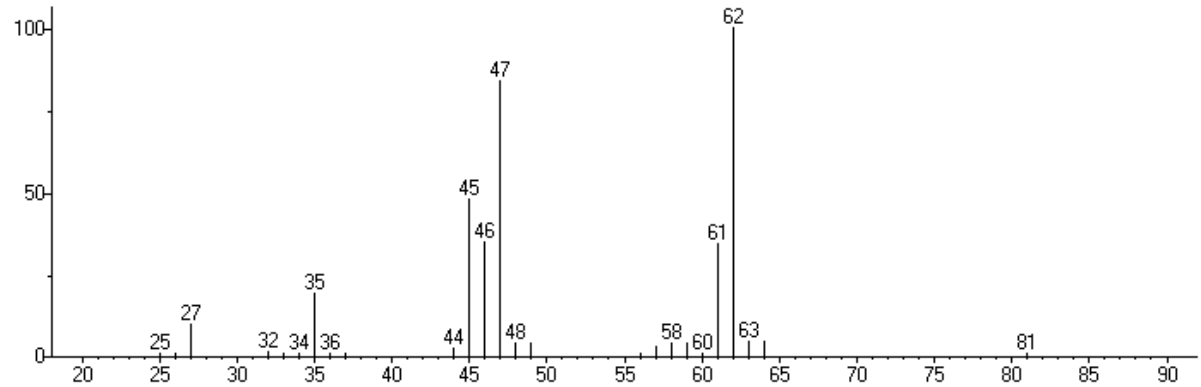

Figure S3. Mass spectrum of unidentified substance (compound ID: 1).

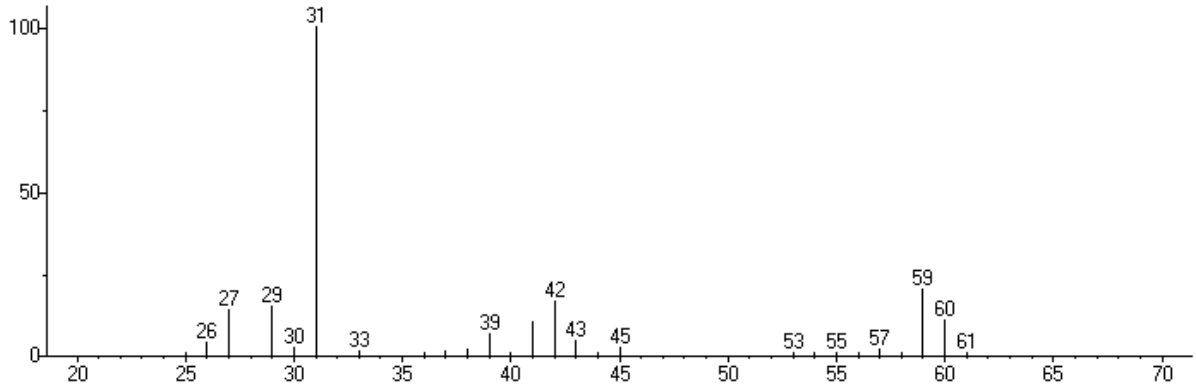

Figure S4. Mass spectrum of unidentified substance (compound ID: 2).

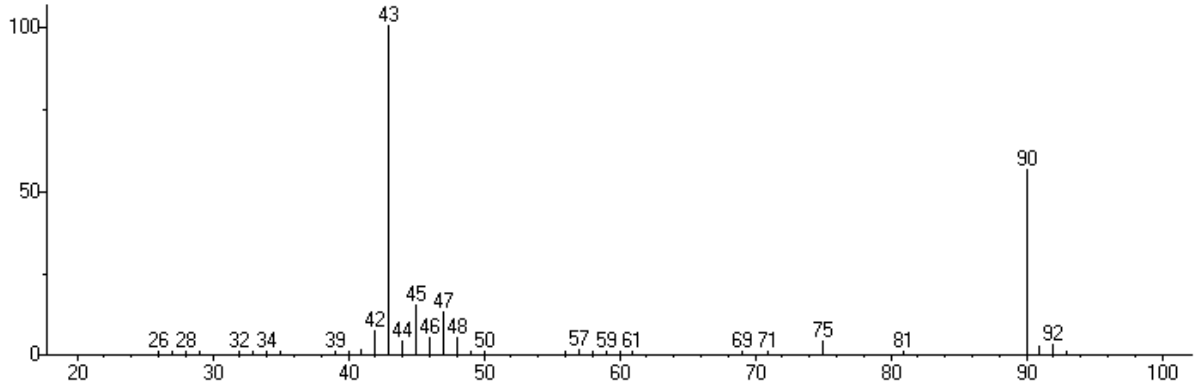

Figure S5. Mass spectrum of unidentified substance (compound ID: 6).

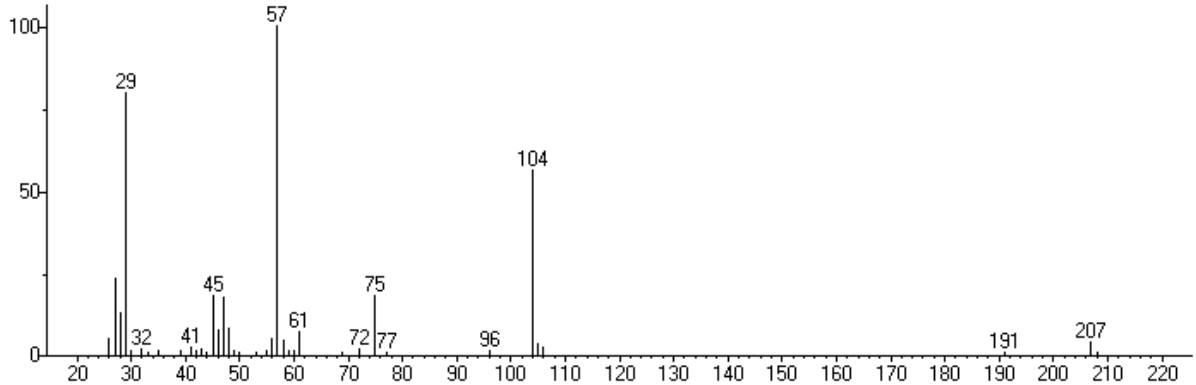

Figure S6. Mass spectrum of unidentified substance (compound ID: 9).

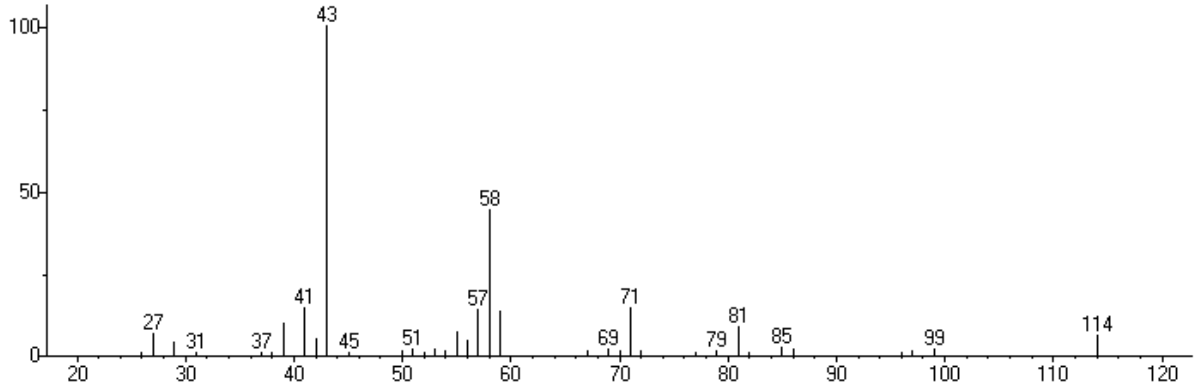

Figure S7. Mass spectrum of unidentified substance (compound ID: 10).

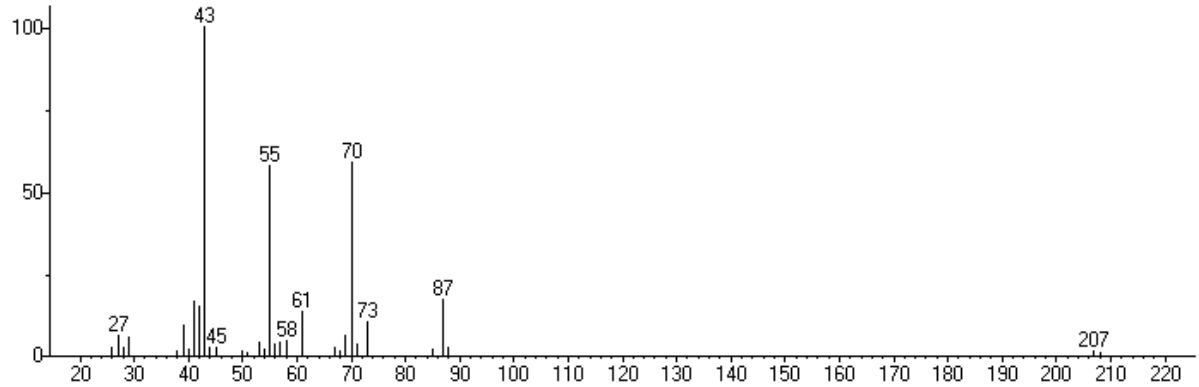

Figure S8. Mass spectrum of unidentified substance (compound ID: 11).

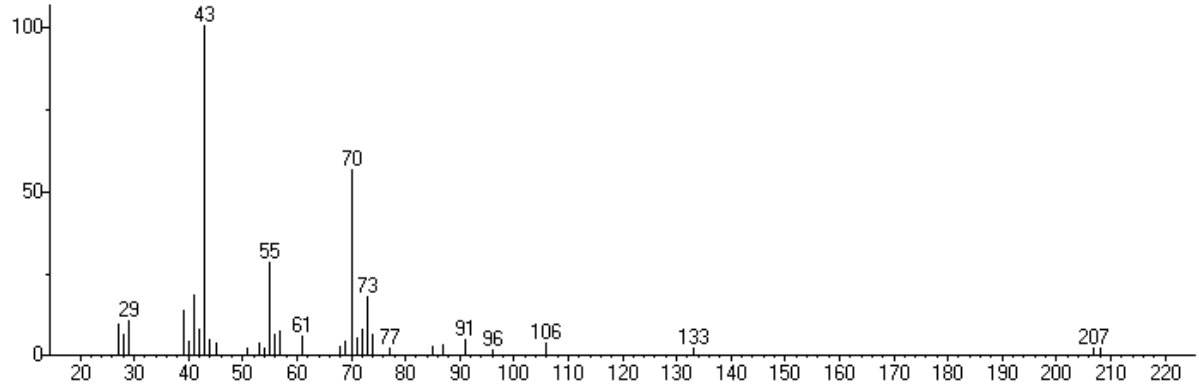

Figure S9. Mass spectrum of unidentified substance (compound ID: 12).

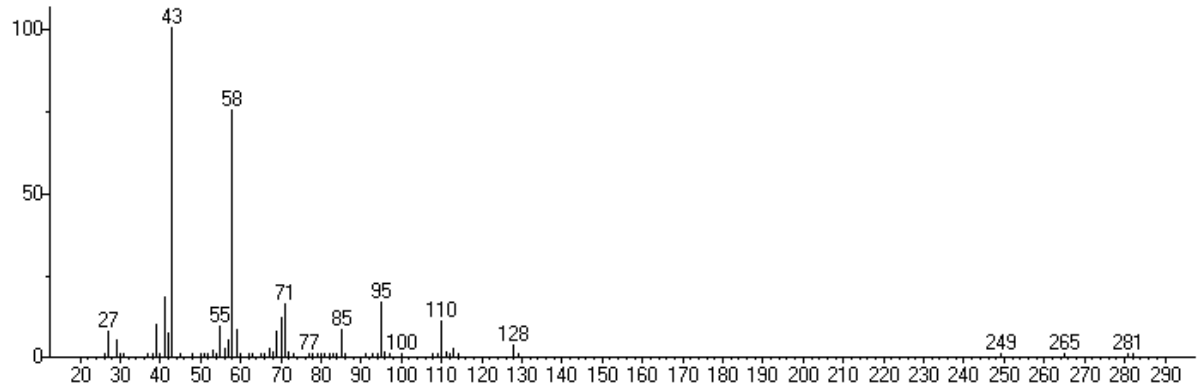

Figure S10. Mass spectrum of unidentified substance (compound ID: 13).

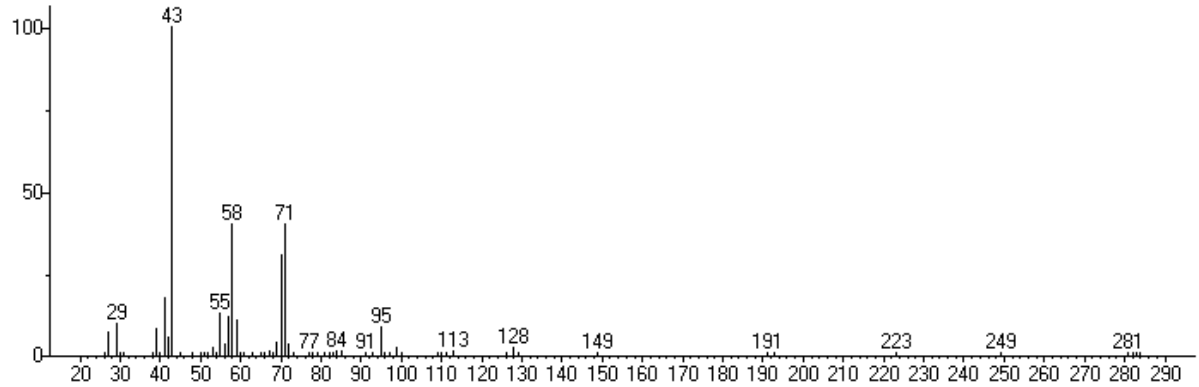

Figure S11. Mass spectrum of unidentified substance (compound ID: 14).

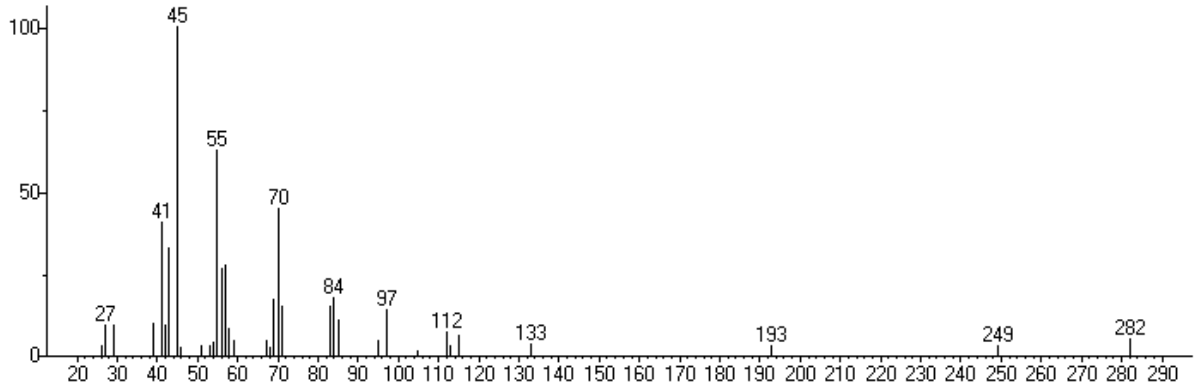

Figure S12. Mass spectrum of unidentified substance (compound ID: 15).

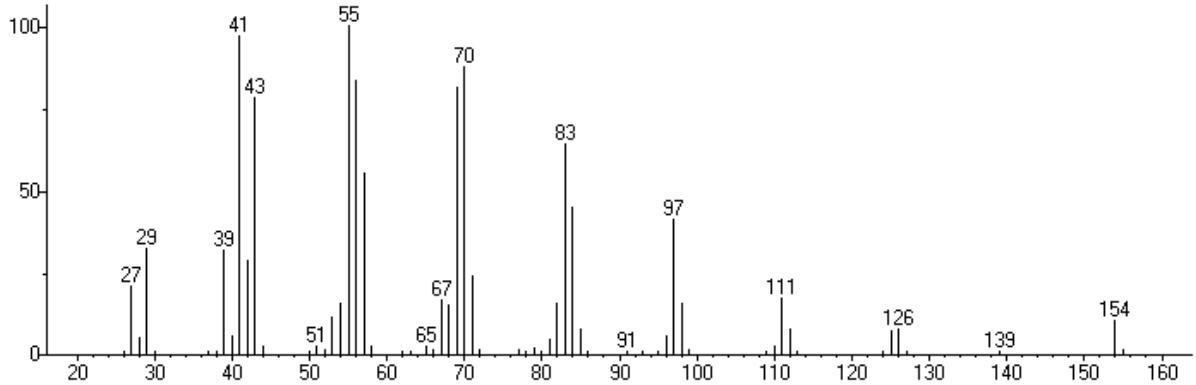

Figure S13. Mass spectrum of unidentified substance (compound ID: 16).

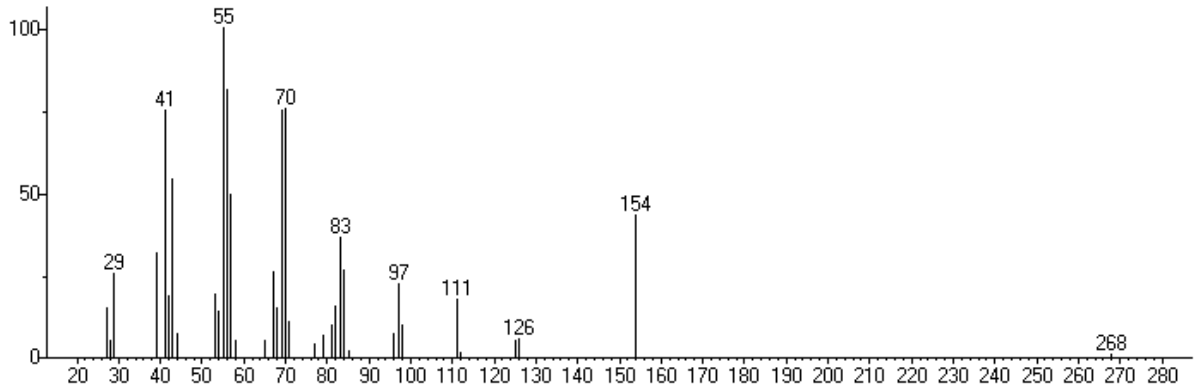

Figure S14. Mass spectrum of unidentified substance (compound ID: 17).

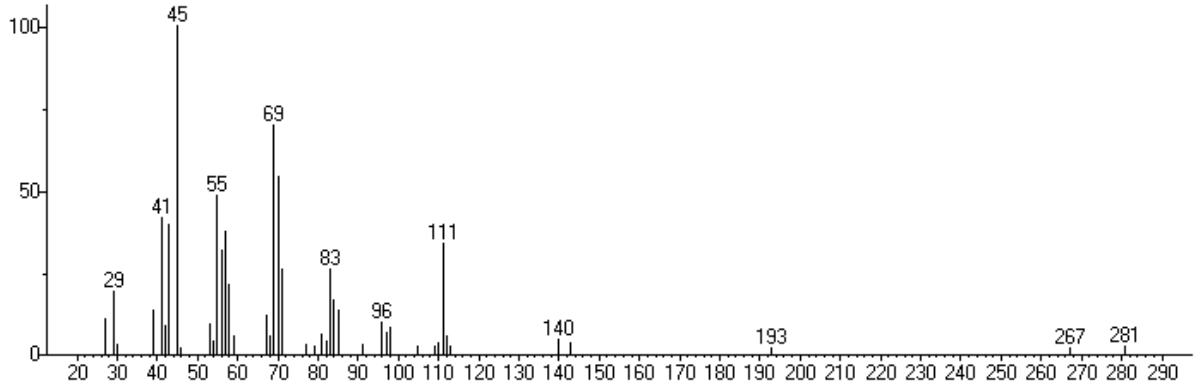

Figure S15. Mass spectrum of unidentified substance (compound ID: 18).

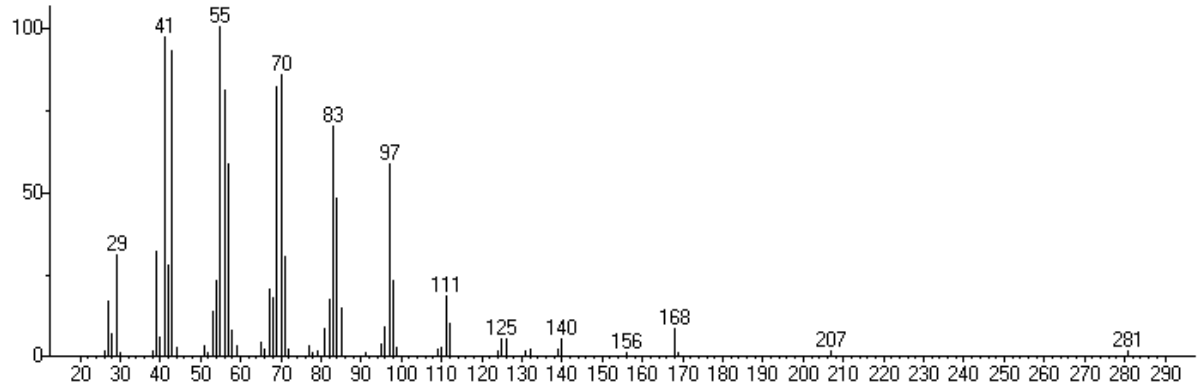

Figure S16. Mass spectrum of unidentified substance (compound ID: 19).

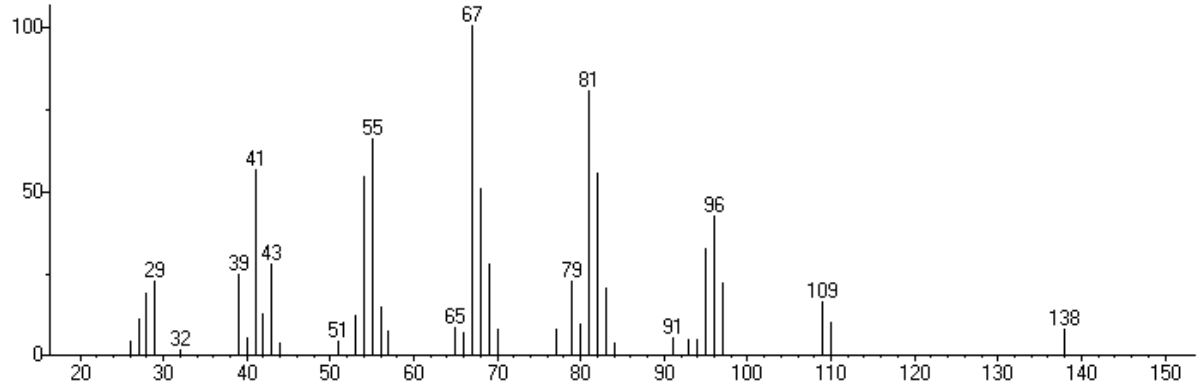

Figure S17. Mass spectrum of unidentified substance (compound ID: 20).

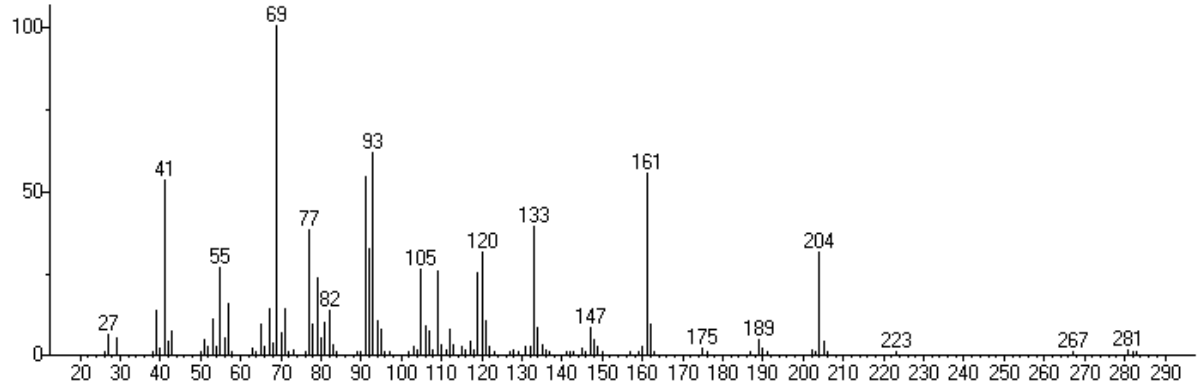

Figure S18. Mass spectrum of unidentified substance (compound ID: 21).

Table S1. The utilized silicone foils were obtained from a local reseller (Hostra GmbH, Graz, Austria). To allow reproducibility of our experiments we have summarized the most important properties.

Detailed properties of the utilized tightening foil	
Material	silicone
Thickness	1 mm
Density	1.14 g/cm^3
Hardness Shore A DIN 53505	55–65 SHA
Temperature Range	-60 – +230 °C
Elongation at Brake DIN 53504	250%
Compression Set ISO 815	24 h, 150 °C = 30%
Color	transparent

Table S2. Unique volatile metabolites in the headspace of three bacterial isolates. VOCs from *Bacillus* sp. 43P2BR, *Pseudomonas* sp. 313P5BS and *Stenotrophomonas* sp. 418P4B were detected and specific peaks for each isolate were extracted from chromatogram overlays. Compounds were identified by comparison with reference substance mass spectra and their respective retention indices.

Strain ID	RI[a]	Substance	Substance ID
43P2BR	n.a.[b]	unidentified substance	2
	645	1-butanol	5
	738	3-methyl-2-pentanone	8
	855	unidentified substance	10
	954	unidentified substance	13
	963	unidentified substance	14
	972	unidentified substance	15
	1175	unidentified substance	18
	1540	unidentified substance	21
313P5BS	n.a.[b]	unidentified substance	1
	680	unidentified substance	6
	691	methyl thiocyanate	7
	798	unidentified substance	9
	1094	unidentified substance	16
	1106	unidentified substance	17
	1193	unidentified substance	19
	1279	unidentified substance	20
418P4B	617	2-butanol	3
	632	2-methyl-1-propanol	4
	875	unidentified substance	11
	876	unidentified substance	12

[a]Calculated Kovats retention indices

[b]The substance was not retained within in the utilized n-alkane series

Additional Publication I

Endophytes-assisted biocontrol: novel insights in ecology and the mode of action of *Paenibacillus*

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REGULAR ARTICLE

Endophytes-assisted biocontrol: novel insights in ecology and the mode of action of *Paenibacillus*

Daria Rybakova · Tomislav Cernava · Martina Köberl · Stefan Liebminger · Mohammad Etemadi · Gabriele Berg

Received: 22 January 2015 / Accepted: 18 May 2015

Abstract
Background Biological control is an environmentally sound and effective means of reducing pathogen-induced damage to agriculture using natural antagonists. *Paenibacillus* is a cosmopolitan and ubiquitously occurring bacterial genus with antagonistic activity against phytopathogens. Many species and strains with promising potential for plant growth promotion and biocontrol of pathogens have been identified since *Paenibacillus* was first described 20 years ago. Nevertheless, important questions regarding the colonization of plants, and the mode of action of *Paenibacillus* remain unanswered.
Scope This review focuses on the occurrence of *Paenibacillus* in microbial metagenomes, the endophytic lifestyle of *Paenibacillus,* and the function of *Paenibacillus*-derived volatile organic compounds (VOCs) combining actual literature with our own results.
Conclusions This review provides new insights into the endophytic lifestyle of *Paenibacillus* and discusses strain-specific and system-dependent growth promotion effects on plants. VOCs, in particular pyrazine derivatives emitted by *Paenibacillus,* showed high activity against other organisms. This suggests that VOCs play an important role in communication and interaction. Overall, *Paenibacillus* strains demonstrate promising potential not only for sustainable agriculture and biological control, but also as a source for novel bioactive volatiles.

Keywords PGPR · Plant growth promotion · Endophyte · Volatiles · Metagenomics

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Daria Rybakova and Tomislav Cernava contributed equally to this work.

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Introduction

Novel high-throughput sequencing techniques in microbial ecology have opened up an immense treasure chest

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of microbial diversity that has been observed in the vast majority of plant-associated habitats (Berg et al. 2014). While the microbiology of the rhizosphere has been thoroughly studied for more than 100 years (Hartmann et al. 2008; Philippot et al. 2013), it has long been assumed that the endosphere was sterile (Smith 1911; Compant et al. 2010). It is now known to be colonized by a variety of microorganisms including beneficial archaea, bacteria and fungi (Hallmann et al. 1997; Hardoim et al. 2015; Müller et al. 2015). A substantial part of the plant-associated microbiome is known for its antagonistic activity against other microorganisms including pathogens (Berg et al. 2013). This functional group of antagonists is a valuable resource in the ongoing development of biological control agents (BCAs), which are applied in agriculture to suppress pathogens. In addition, they often support microbial diversity, which is reduced in intense agricultural ecosystems (Schmid et al. 2011; Van Elsas et al. 2012; Erlacher et al. 2014). Bacterial and fungal antagonists have been especially well studied; *Pseudomonas* and *Trichoderma* are model organisms important for developing an understanding of their mode of action, their intra-specific diversity, and their effects in the field (Weller 2007; Mukherjee et al. 2012). As early as 1999, Emmert and Handelsman suggested *Bacillus* spp. as alternative BCAs due to their unique type of resting cells called endospores appropriate for long-term formulations. Indeed, most of the BCA and bio stimulant products on the market today are *Bacillus*-based (Calvo et al. 2014). *Paenibacillus*, a phylogenetically related genus, was introduced by Ash et al. (1993) to accommodate the "group 3" of the genus *Bacillus*. However, there are only a few specific phenotypical features of *Paenibacillus* distinguishing them from *Bacillus*, e.g. weak reaction with the Gram's stain and differentiation of the cells into ellipsoidal spores that distinctly swell the mother cell (Ash et al. 1993). One of the most likely potential contributing factors accounting for differences between the behavior of *Bacillus* and *Paenibacillus* spp. relates to their ability to fix nitrogen (Jin et al. 2011; Xie et al. 2014). This is a common and widespread feature of *Paenibacillus* but was also shown for several rhizosphere-associated *Bacillus* strains (Ding et al. 2005). In comparison with other bacterial BCAs like *Pseudomonas* or *Bacillus,* less is known about the properties of plant-associated *Paenibacillus* species.

At present, *Paenibacillus* includes 145 published species of facultative anaerobes, endospore-forming, neutrophilic, periflagellated, heterotrophic, and low G+C Gram-positive bacilli, although the taxonomic classification has been debated for a long time (Tindall 2000; Trüper 2005; Keita et al. 2014). The extent and complexity of the *Paenibacillus* taxon are apparent from a phylogram generated from the 16S rRNA gene sequences of 116 species described for this genus (Supplementary Fig. S1). *Paenibacillus* is a cosmopolitan and ubiquitously occurring genus. Although it occurs naturally in soil and marine sediments, plant-associated habitats like the rhizosphere and roots of crop plants are its preferred environments (McSpadden Gardener 2004). In addition, *Paenibacillus* is well-known for its endophytic lifestyle (Hallmann et al. 1997; Krechel et al. 2004). *Paenibacillus* species can be retrieved as epiphytes and endophytes of animals (Pettersson et al. 1999). Several species are the obligate pathogens of honeybees (Genersch 2010) or scarab beetles (Pettersson et al. 1999). Several *Paenibacillus* species are characterized by a unique behavior, and *P. vortex* and *P. dendritiformis* are the most thoroughly studied examples: when grown under stress inducing conditions, they form colonies that behave much like a multi-cellular organism with cell differentiation and task distribution (Ben-Jacob et al. 2004).

Paenibacillus species have been described as promising plant growth promoting bacteria (PGPBs) and/or as BCAs of plant diseases (Berg 2009; Lal and Tabacchioni 2009). Several *Paenibacillus*-based products have been patented and introduced as commercial BCAs (Table 1). The antagonistic potential of *Paenibacillus* spp. against a broad range of phytopathogenic fungi has been well documented *in vitro* as well as *in situ* (Tupinambá et al. 2008; Fürnkranz et al. 2012a, b; Köberl et al. 2013). For example, the biocontrol activity of *P. alvei* K-165 against *Verticillium dahliae* and *Thielaviopsis basicola* was previously demonstrated (Tjamos et al. 2005; Antonopoulos et al. 2008; Schoina et al. 2011). One of the ways how *Paenibacillus* species protect plants from the pathogens is a creation of a biofilm disease shield around the roots (Timmusk et al. 2005). *P. polymyxa* is considered to be the one of the best rhizosphere biofilm formers and is even able to form single species root biofilms under natural conditions (Timmusk et al. 2005, 2011; Timmusk and Nevo 2011). The occurrence of antagonistic *Paenibacillus* in soils and plants of arid zones was reported (Köberl et al. 2011) as well as their potential to compensate plant drought (Timmusk and Wagner 1999; Timmusk et al.

Table 1 Selected patents that are derived from *Paenibacillus* spp. or products thereof applied in plant bioprotection

Patent name (number)	Strain	Target pathogen	Target plant/ disease	Further applications
Paenibacillus terrae biological agent and application thereof in agriculture (CN 103141517 A)	*P. terrae*	*Fusarium oxysporum*	fungal soil-borne diseases, soybean seedling root rot disease	decomposition of mineral phosphorus, potassium and insoluble minerals
Paenibacillus alvei and its applications (CN 103205372 A)	*P. alvei* ZJUB2011-1	*F. oxysporum*	stigma croci bulb rot	–
Biocontrol for plants with *Paenibacillus macerans*, *Pseudomonas putida*, and *Sporobolomyces roseus* (WO 1999005257 A1 and EP 0998554 A1)	*P. macerans*	*Cochliobolus sativus*, *Colletotrichum graminicola*, *F. graminearum*, *F. moniliforme*, *Pyrenophora tritici-repentis*, *Stagonospora nodorum*, *S. avenae* f. sp. *triticea*, *Stenocarpella maydis*	spot blotch/common root rot of cereals, corn, anthracnose, scab of cereals, ear/stalk rot of corn, tan spot of wheat, *Stagonospora nodorum* blotch of wheat, *Stagonospora avenae* blotch of wheat, and stalk/ear rot of corn	PGP; reduces grain contamination by *Fusarium* mycotoxin deoxynivalenol
Paenibacillus alvei strain ts-15 and its use in controlling pathogenic organisms (WO 2012166392 A1)	*P. alvei* TS-15	human foodborne plant pathogens (unspecified)		–
Paenibacillus polymyxa and applications thereof (CN 102851243 A)	*P. polymyxa* JZB120001	broad spectrum pathogenic fungi and pathogenic bacteria, e.g. *Botryosphaeria berengeriana* f. sp. *piricela*, *V. dahliae*, *Monilinia fructicola*, *Rhizoctonia cerealis*, *F. oxysporum* f. sp. *lilii*, *F. oxysporum* f. sp. *conglutinans*; cucumber angular leaf spot fungus, *Agrobacterium tumefaciens*	apple ring rot fungus, peach brown rot, cereal *Rhizoctonia* (*Rhizoctonia cerealis*), Lily base rot pathogen (*Fusarium oxysporum* f. sp. lilii), kale dry outs germs, cucumber angular leaf spot fungus, peach crown thin germs	PGP
Paenibacillus polymyma SHL-1 and application thereof in preventing and controlling stalk rot of *Cymbidium sinense* (CN 102433285 B)	*P. polymyma* SHL-1	Unspecified	*Cymbidium* stem rot	–
Paenibacillus polymyxa for antagonizing *Fusarium oxysporum* in rhizosphere soil of Radix Pseudostellariae (CN 102676435 B)	*P. polymyxa* S960	*F. oxysporum*	*Fusarium oxysporum* in the rhizosphere soil of *Pseudostellaria heterophylla*	–
Anti-microbial agent from *Paenibacillus* sp. and methods and uses thereof (US 20120121543 A1)	*P. polymyxa* JB05-01-1	Gram-negative bacteria	Unspecified	
Novel strains belonging to the genus *Paenibacillus* and method of controlling plant disease by using these strains or culture thereof (EP 1788074 B1)	*P. polymyxa* BS-0105; *Paenibacillus* sp.	Gram-negative bacteria, *F. graminearum*, *F. avenaceum*, *F. oxysporum* f. sp. *cucumerium*, *F. culmorum*, *F. oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *lycopersici*, *V. dahliae*, *Phytophthora capsici*, *Ralstonia solanacearum*	Scab of barley, wheat, oats and rye, Fusarium wilt of cucumber, Fusarium wilt of melon, Fusarium wilt of tomato, Verticillium wilt, brown rot and bacterial wilt of eggplant	Induces resistance to plant diseases

Table 1 (continued)

Patent name (number)	Strain	Target pathogen	Target plant/ disease	Further applications
Paenibacillus polymyxa for preventing and treating plant fungal diseases and production method thereof (CN 101519639 A)	*P. polymyxa* EBL-06	*Botrytis cinerea*, *Cladosporium cucumerinum*	*Botrytis* mold, cucumber scab	–
Endogenous *Paenibacillus polymyxa* (CN 102250815 A)	*P. polymyxa*	*Phytophora palmivora*	Unspecified	–
Biocontrol agent and pesticide (EP 1079692 A1)	*P. polymyxa* PKB1	*Leptosphaeria maculans*, *Sclerotinia sclerotiorum*, *Marasmius oreades*, *Pythium pythioides*, *Rhizoctonia solani*, *Fusarium avenaceum*, *Alternaria brassicae*	Unspecified	–
Peptide antibiotic against *Leptosphaeria*, *Micrococcus*, *Streptomyces*, *Escherichia*; crops, canola (US 6602500 B1)	*P. polymyxa* ATCC 202127	*Leptosphaeria* spp., *Sclerotinia* spp., *Rhizotonia* spp., *Pythium* spp., *Fusarium* spp., *Alternaria* spp., *Aspergillus* spp., *Sporobolomyces* spp., *Penicillium* spp., *Marasmius* spp.	Unspecified	Produces a peptide antibiotic against fungi

2014). While the mechanisms involved in drought tolerance are not well-understood, the production of plant hormones like indole-3-acetic acid (IAA) or cytokinins was often described (Lebuhn et al. 1997; Timmusk et al. 1999; Spaepen et al. 2007; Da Mota et al. 2008). In addition, a long list of *Paenibacillus*-derived antibiotic compounds was identified (reviewed by Raza et al. 2008; Table 2). The antimicrobial potential of *P. polymyxa* and its unique antibiotic spectrum has been known for several decades. The first soluble antibiotic substances showing a remarkable activity against Gram-negative bacteria were isolated as early as 1947 from culture filtrates of a soil isolate of *P. polymyxa* by Stansly and Schlosser. Post 1947, many more peptide antibiotics from various *P. polymyxa* strains were obtained and classified. These strains were primarily isolated from soil and rhizosphere samples (Wilkinson and Lowe 1966; Kimura et al. 1969; Shoji et al. 1977a; Pichard et al. 1995). Currently, polymyxins derived from *Paenibacillus* are again attracting an increasing amount of attention for the treatment of multidrug-resistant Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii, Klebsiella pneumoniae*, and *Stenotrophomonas maltophilia* (Landman et al. 2008; Giamarellou and Poulakou 2009). LI-F antibiotics (gatavalin and fusaricidins) with a broad inhibitory effect against fungi and Gram-positive bacteria were also found (Nakajima et al. 1972; Kurusu et al. 1987; Kajimura and Kaneda 1996, 1997; Deng et al. 2011c; Bionda et al. 2013) as well as iturin-like compunds with activity against dermatophytic fungi (Cotta et al. 2012). Recently, volatile organic compounds (VOCs) from *Paenibacillus* spp. were identified and found to induce resistance in host-plants (Lee et al. 2012). Still, their role in plant-microbe interaction and biocontrol is not holistically understood and requires further exploration.

This review addresses several unanswered questions regarding the ecology and physiology of *Paenibacillus*. The first section is an analysis and assessment of metagenomic datasets with the objective of gaining better insights into the ecology and abundance of *Paenibacillus*. The second section discusses the specific properties of the endophytic lifestyle of *Paenibacillus.* Finally, the third section is an analysis of the potential functions of VOCs derived from *Paenibacillus* combining literature research and actual results.

Table 2 Antimicrobial properties of *Paenibacillus* isolates

Paenibacillus species	Isolation source	Antagonistic potential	Mode of action	Reference
P. polymyxa		antibacterial (*Bacillus subtilis*, *Corynebacterium diphtheria*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi, S. typhimurium*, *Shigella flexneri*, *S. sonnei*, *Vibrio cholera*)	soluble cyclic lipopeptide antibiotics polymyxins	Wilkinson and Lowe (1966), Kimura et al. (1969), Shoji et al. (1977a, b, c)
P. polymyxa		antifungal (*Saccharomyces cerevisiae*, *Torulaspora delbrueckii*); antibacterial (*Bacillus subtilis*, *Micrococcus luteus*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, *P. vulgaris*, *Pseudomonas aeruginosa*, *P. fluorescens*)	soluble cyclic peptide antibiotic jolipeptin	Ito and Koyama (1972)
P. polymyxa L-1129	bulk soil of Odawara City in Kanagawa Prefecture, Japan	antifungal (*Aspergillus flavus*, *A. nidulans*, *A. niger*, *A. oryzae*, *A. versicolor*, *Candida albicans*, *C. guilliermondii*, *C. tropicalis*, *C. parapsilosis*, *C. utilis*, *Cladosporium fulvum*, *C. shaerospermum*, *Crytpcoccus neoformans*, *Fonsecaea pedrosoi*, *Fusarium moniliforme*, *F. oxysporum*, *F. roseum*, *F. solani*, *Geotrichum candidum*, *Gibberella fujikuroi*, *Helminthosporium sesamum*, *Microsporum canis*, *M. gypseum*, *Penicillium expansum*, *Saccharomyces cerevisiae*, *Sporothrix schenckii*, *Trichophyton rubrum*, *T. mentagrophytes*); antibacterial (*Bacillus subtilis*, *Corynebacterium diphtheria*, *Enterococcus faecalis*, *Micrococcus luteus*, *Mycobacterium smegmatis*, *Staphylococcus aureus*, *S. epidermidis*)	soluble cyclic lipodepsipeptide antibiotics LI-F03a to LI-F08a and LI-F03b to LI-F08b	Kurusu et al. (1987)
P. polymyxa KT-8	rhizosphere of *Allium sativum* L.	antifungal (*Aspergillus niger*, *A. oryzae*, *Candida albicans*, *Fusarium oxysporum*, *Penicillium thomii*, *Saccharomyces cerevisiae*); antibacterial (*Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*)	fusaricidin A-D of cyclic lipodepsipeptide antibiotics	Kajimura and Kaneda (1996, 1997)
P. polymyxa PKB1	canola stubble from a field near Edmonton, Canada	antifungal (*Alternaria brassicae*, *Fusarium avenaceum*, *Leptosphaeria maculans*, *Marasmius oreades*, *Neurospora crassa*, *Penicillium roquefortii*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*); antibacterial (*Micrococcus luteus*, *Streptomyces clavuligerus*)	fusaricidins of cyclic lipodepsipeptide antibiotics	Beatty and Jensen (2002)
P. polymyxa SCE2			protease	Alvarez et al. (2006)
P. polymyxa JSa-9	bulk farmland soil of Nanjing in Jiangsu province, China	antifungal (*Aspergillus niger*, *A. oryzae*, *Penicillium expansum*, *P. notatum*, *Rhizopus stolonifer*);	soluble cyclic and linear LI-F type antibiotics, polymyxin B_6	Deng et al. (2011a, b, c)

Table 2 (continued)

Paenibacillus species	Isolation source	Antagonistic potential	Mode of action	Reference
		antibacterial (*Bacillus cereus*, *Micrococcus luteus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *P. fluorescens*)		
P. polymyxa PB71	spermosphere of *Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb., Austria	antifungal (*Didymella bryoniae*); antibacterial (*Pectobacterium carotovorum*, *Pseudomonas viridiflava*, *Xanthomonas cucurbitae*)	unknown soluble and volatile antibiotics	Fürnkranz et al. (2012a)
P. polymyxa M-1	endorhiza of wheat, China	antibacterial (*Erwinia amylovora*, *E. carotovora*)	polymyxin P	Niu et al. (2013)
P. polymyxa Wb2-3, Mc5Re-14	bulk soil of the Sinai desert and endorhiza of *Matricaria chamomilla* L., Egypt	antifungal (*Fusarium culmorum*, *Rhizoctonia solani*, *Verticillium dahlia*); antibacterial (*Escherichia coli*); nematicidal (*Meloidogyne incognita*)	β-1,3-glucanase, siderophores, unknown soluble antibiotics	Köberl et al. (2013)

New insights into *Paenibacillus* ecology

Exploring the occurrence of *Paenibacillus* in plant-associated habitats using metagenomics

Plant-associated habitats like the rhizosphere and endosphere of crop plants are often the environments in which *Paenibacillus* is reported to have been found. In order to gain greater insight into the relative abundance of *Paenibacillus* we have analyzed 17 publicly available datasets (Delmotte et al. 2009; Knief et al. 2011; Köberl et al. 2011; Grube et al. 2015; Moissl-Eichinger et al. 2015) including our own datasets from the metagenomes of seven plant habitats, four soils, and three lichens, and compared them with three indoor habitats (Supplementary Table S1). We used sequence-rich datasets based on 16S rRNA gene amplicon sequencing and whole metagenomic shotgun sequencing. The most common preparation technique for amplicon sequencing includes multiplexing of numerous samples which can reduce the sequencing costs and thus facilitate higher replicate numbers for statistically documented results. Metagenomic shotgun sequencing based on next generation high-throughput sequencing is less applicable for high replicate numbers, but delivers functional information in addition to taxonomic assignments based on 16S rRNA and other marker genes. Interpretation of obtained data requires knowledge about the possibilities, but also constraints of the respective approaches. Both techniques are suitable for taxonomic analysis; however they are known to be error-prone to a certain degree (Logares et al. 2014). On the one hand, amplicon-based sequencing relies on PCR-based amplification of predefined targets, and can therefore be affected by amplification-induced errors. On the other hand, metagenomic sequencing of very complex samples often does not result in sufficient coverage of the analyzed sample necessary to completely describe the present diversity (Roh et al. 2010; Zhou et al. 2015). Rarefaction analyses are useful in estimating the species richness and the coverage within specific datasets. The majority of the datasets analyzed were below saturation due to high bacterial diversity.

Within analyzed biomes, 0–4.06 % of the bacterial sequences were assigned to *Paenibacillus* spp. (Supplementary Table S1). In all rhizosphere samples, *Paenibacillus* was abundant and represented 0.7–2.05 % of the microbiome (Knief et al. 2011). We found no difference in the utilized datasets between plants

grown under humid or arid conditions. Timmusk et al. (2009) employed a qPCR-based approach to quantify *P. polymyxa* in the rhizosphere of wild barley sampled from arid soils. The observed abundance was significantly higher than in the adjoining microclimate, suggesting a possible role in adaptive co-evolution of plants. It was also shown that *P. polymyxa* isolates from the arid soil rhizosphere samples were metabolically different from their counterparts in moderate soils (Timmusk et al. 2011). Interestingly, both the highest and the lowest observed percentages of *Paenibacillus* spp. were found in the plant phyllosphere (Delmotte et al. 2009; Knief et al. 2011), which reflects the extremely changeable conditions of this habitat. The plant phyllosphere is directly exposed to external stress factors, hence more susceptible to fluctuations. Furthermore, host specificity is a key factor which shapes the taxonomic composition and spectrum of the bacterial microbiome on various plant species. *Paenibacillus* was also substantially abundant in all soil samples studied (Köberl et al. 2011). Different lichen species were shown to harbor rather small proportions of *Paenibacillus* (0.01–0.04 %; Grube et al. 2015). In contrast, high *Paenibacillus* abundance was found in cleanrooms (0.1–4.0 %; Moissl-Eichinger et al. 2015). These artificial habitats are repeatedly exposed to decontamination-related stress, and therefore spore-forming bacteria may have better chances of survival or at least be more persistent. While metagenomics is a powerful new technique allowing analyzing and quantifying bacterial species present in different environments, we still have to rely on experimental data in order to speculate on the habitat-specific modes of action of bacterial species. For example, some studies suggest that *Paenibacillus* spp. adapted to harsh environments are potentially good candidates for use as BCAs or PGPBs with plants that are growing under severe conditions (Köberl et al. 2013; Timmusk et al. 2014).

Paenibacillus as a facultative endophyte

Endophytes as microorganisms that live in the internal tissues of plants without altering the normal functioning of the tissues are very promising and popular as PGPBs or BCAs (Bacon and Hinton 1997; Berg et al. 2005; Hardoim et al. 2015). Endophytic *Paenibacillus* isolates obtained from medicinal plants cultivated on a desert farm (*Matricaria chamomilla* L., and *Solanum distichum* Schumach. and Thonn) were identified as being amongst the most efficient broad-spectrum antagonists against the soil-borne pathogens present (Köberl et al. 2013). Endophytic *Paenibacillus* spp. have been found in association with various plants like *Arabidopsis thaliana*, *Pinus* sp., *Coffea arabica* or *Curcuma longa* (Sakiyama et al. 2001; Bent and Chanway 2002; Timmusk et al. 2005; Aswathy et al. 2013). *P. polymyxa* PB71 isolated from the spermosphere of the Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) was able to reduce disease severity of the Styrian oil pumpkin caused by the phytopathogenic fungus *Didymella bryoniae* under greenhouse conditions (Fürnkranz et al. 2012a) and led to an increase in harvest yield and a suppression of powdery mildew under field conditions (Fürnkranz et al. 2012b, Table 2). *Paenibacillus* spp. were found in all parts of plants, even in grape berries (Verginer et al. 2010). Although endophytes are generally harmless, the interaction between endophytic *Paenibacillus* spp. and their hosts can be very diverse. For example, it was shown that beneficial *Paenibacillus* spp. can overgrow other endophytic bacteria in plant cell cultures (Ulrich et al. 2008a). While *Paenibacillus* spp. comprised 1.4 % of the total endophytic bacteria isolates colonizing the aerial parts of trees (Ulrich et al. 2008b), they became the predominant endophytic bacteria in poplar tissue cultures when cultured *in vitro* (Ulrich et al. 2008a). Inoculation of poplar seedlings with the enriched *Paenibacillus* isolate resulted in a significantly higher number of roots per cutting and in increased root length when compared with the control plants after 3 weeks (Ulrich et al. 2008b). It was also shown that an endophytic *Paenibacillus* strain strongly affected the composition of the plant metabolites of *in vitro*-grown poplars free from other culturable endophytic bacteria (Scherling et al. 2009). The shifts in the primary metabolism of the poplar plants indicated a mutualistic interaction between the *Paenibacillus* strain, which was capable of fixing nitrogen, and the host plant with altered nitrogen assimilation patterns.

The main difference between endophytic and non-endophytic bacteria is their ability to enter into the plant tissues. *Paenibacillus* spp. are known to produce high amounts of different hydrolyzing enzymes that facilitate plant tissue colonization (Sakiyama et al. 2001; El-Deeb et al. 2013). Even though *P. polymyxa* is generally considered a free-living rhizobacterium, Timmusk et al. (2005) could detect GFP-labelled *P. polymyxa* cells for the first time inside the root tissue by using confocal

laser scanning microscopy (CLSM). However, not all *P. polymyxa* strains are capable of invading the plant tissue. Two PGP *P. polymyxa* strains Pw-2 and L6-16R isolated from lodgepole pine were studied in depth for their ability to live inside plant tissues (Holl and Chanway 1992; Shishido et al. 1996; Anand et al. 2006). While *P. polymyxa* Pw-2 has been identified as an endophyte of the lodgepole pine, the *P. polymyxa* strain L6-16R was not able to enter into the plant tissue even when co-inoculated with an endophytic organism (Shishido et al. 1995; Bent and Chanway 1998). Both strains possessed similar metabolic capabilities with several potentially important exceptions (Shishido et al. 1995). In contrast to the non-endophytic L6-16R isolate, the endophytic Pw-2 strain showed the capacity to metabolize sorbitol, D-melezitose and D-galacturonic acid. Sorbitol is associated with the bacteria's ability to grow anaerobically on highly reduced, scarce substrates; D-melezitose is a sugar detected in the sap of conifers (Lehninger 1975), and D-galacturonic acid is the primary component of pectin, a major component of the middle lamellae of plant cell walls. The ability of the Pw-2 strain to degrade pectin is especially interesting as it may explain how endophytic bacteria avoid cell defense mechanisms. Endophytic bacteria, in particular those that are found in the plants intracellularly, must destroy plant cell walls in order to enter into plant cells. The breakdown products of cell wall components are known to induce systemic disease responses in plants (Heil and Bostock 2002). It was therefore suggested that endophytic bacteria may be able to avoid plant defense mechanisms by metabolizing degradation products of cell wall components like pectin (Anand et al. 2006).

While the bioprotection and plant growth promotion qualities of *Paenibacillus* spp. are at present undisputed, the opposite effect of endophytic *Paenibacillus* spp. on the plant growth has been reported in several cases. For example, *P. polymyxa* was shown to induce mild biotic stress in *A. thaliana* grown under gnotobiotic conditions (Timmusk and Wagner 1999). The bacterial cells invaded the intracellular space of the plant root causing degradation of the root cup and severe root damage resulting in a 30 % reduction of the growth of the plant and a stunting of root systems. A similar phenotype was observed in oilseed rape and cauliflower seedlings when the respective seeds were bioprimed with *Paenibacillus* strains (Rybakova et al. 2015). The *Paenibacillus* cells were found mainly in the intercellular space of oilseed rape roots. Large colonies were observed in the cavities remaining from destroyed plant cells (Fig. 1). Interestingly, this was only observed for plants grown under gnotobiotic conditions in germination pouches. When seeds treated in the same manner were sown in unsterile soil, they did not impair plant growth significantly, and were even shown to promote enhanced plant growth under sterile soil conditions (Rybakova et al. 2015). This observation substantiates the hypothesis that while defined and functioning as a PGP bacterium, *P. polymyxa* can also act as a deleterious bacterium (Timmusk et al. 2005). Root cell damage of *A. thaliana* seedlings by *P. polymyxa* was observed by Timmusk et al. (2005) not only in a gnotobiotic system, but also in sterile and non-sterile soil. The differences between inoculation methods (biopriming of the seeds versus root dipping of the seedlings) as well as the choice of different plant cultivars (oilseed rape and cauliflower versus *A. thaliana*) used in the two studies may in part explain the variations between observations. Because no data are available on the PGP effect of *P. polymyxa* on *A. thaliana* seedlings, the results of both studies cannot be compared in detail.

It has been reported that morphological changes of the root have been associated with auxin production and excretion by PGP bacteria (Xie et al. 1996; Dobbelaere et al. 1999, 2003; Da Mota et al. 2008). Auxin is a class of plant hormones that play a crucial role in the coordination of many growth and behavioral processes in the plant's life cycle and are essential for plant body development (Davies 2010). On the other hand auxin is essential for bacterial phytopathogenesis (reviewed by Hayat et al. 2010). A variety of studies has shown that auxin promotes the sensitization of the host towards the bacterial pathogen and results in the development of disease symptoms (reviewed by Ludwig-Müller 2014). Because many bacterial pathogens can produce auxin, it can be hypothesized that this feature is a part of the strategy used by the pathogen to bypass the plant defense system. It is therefore speculated that this strategy may be utilized by auxin producing *P. polymyxa,* resulting in a deleterious effect on the host plant grown in gnotobiotic conditions. On the other hand, the very recent study of Timmusk (2015) showed that NRP/PK origin compounds produced by *P. polymyxa* may be the primary reason for its mild deleterious influence. The authors constructed a *P. polymyxa* A26 mutant A26Δ*sfp* with inactivated A26 Sfp-type phosphopantetheinyl transferase. This

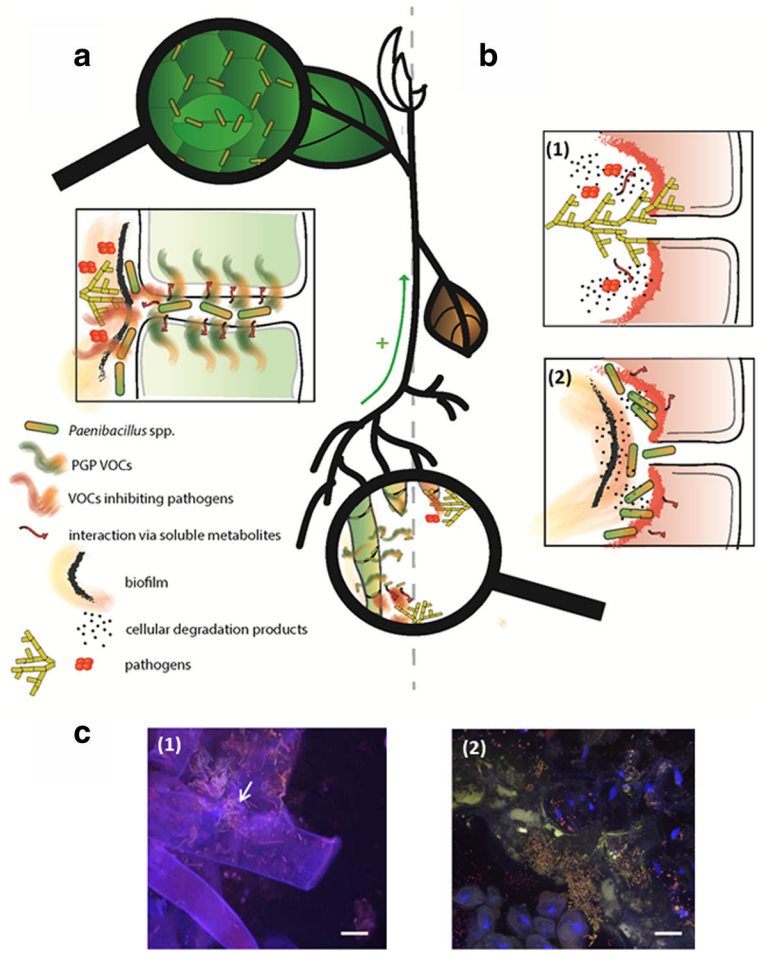

Fig. 1 *Paenibacillus*-plant interactions. *Paenibacillus* spp. are typical soil bacteria that are able to interact with plants and pathogenic microorganisms. They can also colonize plant tissues and thus adopt an endophytic lifestyle (lower magnified image in the illustration (**a**)). The image in **a** illustrates how the interaction with *Paenibacillus* spp. improves plant health. *Paenibacillus* forms a biofilm around the roots to protect root tissues from the pathogens, inhibits the growth of pathogens via soluble and volatile metabolites (indicated as *red arrows* and *orange* volatiles symbols, respectively), and induces systemic resistance (ISR). It also promotes plant growth by solubilizing inorganic elements like phosphorus or nitrogen and produces soluble and volatile metabolites promoting plant growth. The last three factors are summarized in the image as a *green arrow* with a "+" sign. All these factors lead to the development of a plant that is more resistant to pathogens. The image **b** shows how a plant can be exposed to attacks by pathogens in the absence of *Paenibacillus* spp. and/or other beneficial endophytic bacteria (1). Stunting of the root system and inhibition of plant growth may result from degradation of the plant root cells by *Paenibacillus* spp. in the absence of other competing microorganisms and a low nutrient environment (2). The image **c** shows a visualization of *Paenibacillus* colonization patterns by FISH-CLSM using an equimolar ratio of the *Firmicutes*-specific FISH probes LGC354A, LGC354B and LGC354C labeled with the fluorescent dye FITC. *P. polymyxa* Mc2-9 colonies are denoted with arrows. *P. polymyxa* Mc2-9 macrocolonies were detected in the root tissue (1) and cavities of the damaged oilseed rape leaves (2). Bar represents 25 μm. The images are taken from Rybakova et al. (2015)

mutant was characterized by increased biofilm secretion, no lipopeptide antibiotics production and the ability to promote growth and enhance drought tolerance of its host plant *A. thaliana* better than the wild type A26 strain. Of interest, A26 and its extracts caused damage and reduced root growth of the host plant compared to A26Δ*sfp* bacteria and their metabolite extracts suggesting that the presence of NRP/PK polypeptides in A26 metabolites was responsible for the deleterious effect on the plant.

In summary, we propose the following model for the interaction of endophytic *Paenibacillus* spp. with the host plant and pathogens as illustrated in Fig. 1. *Paenibacillus* is a typical soil bacterium that is able to

invade plant roots and live inside plant tissue (Fig. 1a). It builds a biofilm around the roots that functions as a protective layer to prevent access by pathogens (Timmusk et al. 2005, Fig. 1a). Additionally, *Paenibacillus* spp. produce soluble and volatile metabolites that inhibit the growth of pathogens. They also induce plants' defense mechanisms resulting in changes in plant gene expression (Timmusk and Wagner 1999; Fig. 1a). Pathogens may invade plant tissue causing plant diseases in the absence of either *Paenibacillus* spp. or other biocontrol agents (Fig. 1b). Under certain conditions, *Paenibacillus* spp. are capable of degrading plant cells as shown by CLSM (Timmusk et al. 2005; Rybakova et al. 2015, Fig. 1b). This results in stunted root systems and eventually reduced plant growth or even death. We speculate that the aforementioned paradox of the *Paenibacillus*-plant relationship may occur when the balance between *Paenibacillus* spp. and the soil microbiome in gnotobiotic conditions is upset. Under those circumstances *Paenibacillus* spp. are able to overpopulate roots as revealed by CLSM (Fig. 1c). This overpopulation results in nutrient depletion inducing a possible switch in bacterial metabolism that may in turn be mediated by *Paenibacillus* spp. production of auxin and/or in local oversaturation of *Paenibacillus*-derived secondary metabolites that are harmful to the plant. As a result, *Paenibacillus* spp. damage root cells instead of protecting plants from pathogens.

Volatile metabolites mediate extended antagonistic potential

Our studies on volatile organic compounds (VOCs) emitted by cultured *Paenibacillus* spp. have demonstrated that specific metabolites are widely distributed and are furthermore involved in various bacteria-host and pathogen interactions. Moreover, different experimental setups have shown that VOCs-mediated effects not only target other microbes, they also target eukaryotic hosts e.g. higher plants. *Paenibacillus* emitted VOCs were shown to induce systemic resistance in *Arabidopsis* plants and to enhance plant growth at the same time (Lee et al. 2012). For example, *P. polymyxa* strain E681 produced a long chain C13 compound that was found to be partially involved in the observed effects. Additional VOCs were also proposed to be present, but could not be detected within the study. A multitude of interactions of *Paenibacillus* spp. with other organisms can be predicted based on the evidence of the natural occurrence of *Paenibacillus* spp. in different soil types and plant habitats, as well as in the built environment. In a multidisciplinary study, different species were shown to colonize the surface of ripe grapes prior to harvesting. Verginer et al. (2010) demonstrated that *Paenibacillus* spp. were amongst the most abundant bacterial colonizers, and that they have a certain influence on fruit aroma. Different isolates were tested for emitted VOCs in sensory evaluations and solid phase microextraction (SPME) headspace analysis. Not only was a mixture of primarily short-chain alcohols, ketones and aldehydes found in the headspace of living cultures, sulphur-containing and cyclic molecules were also found (Table 3; Verginer et al. 2010; Cernava 2012). In addition to the positive contribution of *Paenibacillus* spp. volatiles to the sensory properties of grapes (and the wine produced from them), these molecules are most likely emitted in order to fulfil vital metabolic functions, and interact with the surrounding species.

While antifungal activities of VOCs were reported in different studies (Liu et al. 2008; Zhao et al. 2011), less is known about volatile *Paenibacillus* metabolites with antibacterial properties. Beck et al. (2003) were able to show that in the polymyxin biosynthesis pathway of a *P. polymyxa* strain, a complex mixture of methyl-branched alkyl-substituted pyrazines was produced. However, the function of these volatiles is not fully resolved, although it is known that pyrazines are involved in bactericidal and chemoprotective activities (Beck et al. 2003). We applied the headspace SPME method developed by Verginer et al. (2010) to specifically screen for alkyl-substituted pyrazines in the headspace of different *Paenibacillus* spp. cultures that were shown to express antagonism against pathogenic bacteria and fungi. The investigated *Paenibacillus* cultures included three plant endophytic bacteria isolated from the Styrian oil pumpkin (Fürnkranz et al. 2012a). In addition, five isolates were obtained from medicinal plants grown in desert farming soils, one isolate from desert farming soils, and one isolate from the adjacent desert soils (Köberl et al. 2013). All of the isolates utilized were shown to emit three distinct pyrazine derivatives into the headspace (Cernava 2012). While 2-methyl-5-(1-methylethyl)-pyrazine and 2-(2-methylpropyl)-3-(1-methylethyl)-pyrazine were emitted in species-specific proportions, 2,3,5-trimethyl-6-propyl-pyrazine was the most prominent molecule in all samples. Bacterial pyrazine emission was shown to be both species-specific and strain-specific. *P. polymyxa*

Table 3 Gas chromatography – mass spectrometry headspace SPME identification of volatile metabolites from *Paenibacillus* spp. cultures grown on slope agar in headspace vials

Substance in headspace	Method of identification
Acetaldehyde[2]	spectral database
Ethanole[2]	spectral database
Hydroxyurea[2]	spectral database
Cycloserine[2]	spectral database
Butanal[2]	spectral database
Ethoxyethene[2]	spectral database
2-butanone[1]	reference substance
1-butanol[2]	spectral database
2-methyl-1-propanol[1,2]	reference substance
Methyl-3-methylbutanoate[1]	reference substance
2-pentanone[1]	reference substance
3-hydroxy-2-butanone[2]	spectral database
2-ethyl-1-butanol[2]	spectral database
3-methyl-1-butanol[1]	reference substance
2-methylbutan-1-ol[1,2]	reference substance
Methoxy-phenyl-oxime[2]	spectral database
Benzaldehyde[2]	spectral database
Dimethyl disulfide[1]	reference substance
2-heptanone[1]	reference substance
6-methyl-5-hepten-2-one[1]	reference substance
Dimethyl trisulfide[1]	reference substance
2-methyl-5-(1-methylethyl)-pyrazine[2]	spectral database
Trimethylpyrazine[1]	reference substance
2,3,4-trimethyl-5-propyl-pyrazine[2]	spectral database
2-(2-Methylpropyl)-3-(1-methylethyl)-pyrazine[2]	spectral database
2-ethyl-1-hexanol[1]	reference substance
Phenylacetaldehyde[1]	reference substance
3-methylbutanoic acid[1]	reference substance
2-phenylethanol[1]	reference substance

[1] Substances identified in the headspace above *Paenibacillus* sp. T2B1c.1-B (Verginer et al. 2010)

[2] Substances identified in the headspace above *Paenibacillus* spp. from desert soils and Styrian oil pumpkin-associated isolates (Cernava 2012)

strains that were isolated from different habitats were shown to produce specific proportions of volatile pyrazines. We have studied the antibiotic effects of *Paenibacillus*-derived pyrazines extensively, and have demonstrated that they suppress the growth of bacteria, fungi and yeast under laboratory conditions (Cernava 2012). Antibiotic effects of the detected metabolites were further demonstrated by the subsequent utilization of synthetic alkyl-substituted pyrazines. Concentrations as low as 0.20 pmol cm^{-3} of synthetic compounds were shown to decrease the viability of the same pathogens that are targeted by *Paenibacillus* spp. in dual-culture experiments (Cernava 2012). More interestingly, all synthetic VOCs were administered through the headspace in order to mimic the natural mode of action, although substances applied directly to the growth media were also shown to be highly effective (Cernava 2012).

Conclusion and outlook

1. In our survey on *Paenibacillus* we found that the global mode of action against pathogens or other microorganisms is very similar to those reported for *Bacillus*, however *Paenibacillus* spp. appear to be generally less studied than its closely related genus *Bacillus*. The main reason for this lack of knowledge on *Paenibacillus* spp. is that *Paenibacillus* spp. and especially *P. polymyxa* are particularly hard to manipulate: only recently techniques were discovered that allowed genetic manipulation of some *Paenibacillus* strains (Kim and Timmusk 2013). Although the extent of research is far greater for *Bacillus*, for both genera the mode of action includes antibiosis, lysis, competition and induced resistance (Emmert and Handelsman 1999; Govindasamy et al. 2011). Nitrogen fixation is reported as being a unique feature of *Paenibacillus* with one exception published by Ding et al. (2005). The detailed comparison of the metabolites produced by *Bacillus* and *Paenibacillus* spp. revealed that the spectrum of soluble and especially volatile metabolites produced by both genera is highly diverse and is rather species- or strain- than genus-specific (Liu et al. 2008; Govindasamy et al. 2011; Lee et al. 2012 and this study). Strains of both genera produce powerful weapons, e.g. lipopetides against plant pathogens (Ongena and Jacques 2008). Therefore, *Paenibacillus* strains have an enormous potential in biotechnology as a source of novel bioactive compounds, and to date this potential has only partially been exploited. The antibacterial effect of pyrazine derivatives (and VOCs in general) opens a new door to developing techniques for the suppression of multi-resistant bacterial

pathogens—one of the most important future challenges facing mankind (Woolhouse and Farrar 2014).

2. Our review demonstrates the huge potential of *Paenibacillus* spp. as PGPBs and/or BCAs. Those strains with an endophytic lifestyle show especially interesting capacities. The easy formulation and high shelf life of *Paenibacillus* strains due to their spore formation abilities is an additionally positive quality. In general there is only a low risk of *Paenibacillus* infection of higher animals or humans. Although several cases of infection by *Paenibacillus* have been reported, this interaction was found to be influenced by the susceptibility of the host. Only hosts with a predisposition were reported to have been infected, and the infection was restricted to several *Paenibacillus* species. The genus *Paenibacillus* was characterized as having high intraspecific diversity, as well as strain-specific modes of action and effects on plants. We also observed system-dependent growth effects, and noticed a much more positive effect on the host plant under natural rather than under gnotobiotic conditions in one particular case study (Rybakova et al. 2015). Therefore, the evaluation and risk assessment should be done at strain level and under natural conditions.
3. In future, we need a much deeper insight into the *Paenibacillus* ecology and physiology. For example, *Bacillus subtilis* was shown to act as a multicellular organism (Aguilar et al. 2007) responsible for biofilm formation on the root surface as well as for biocontrol (Vlamakis et al. 2013). Moreover, this biofilm formation is induced by plant polysaccharides (Beauregard et al. 2013) but also by cannibalism of the *Bacillus* strains themselves (López et al. 2009). In addition, we have no insight into the epigenom of plant-associated *Paenibacillus* strains, which can have an enormous impact on the effect. Such knowledge on *Paenibacillus* can improve their biotechnological applications.

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Additional Publication I – Supporting Material

Endophytes-assisted biocontrol: novel insights in ecology and the mode of action of *Paenibacillus*

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Supplementary Table S1 Relative abundances of *Paenibacillus* spp. within selected biomes

Biome	Sample	Sequencing Method	Country	Number of Analyzed Sequences	Abundance of *Paenibacillus* spp. [%][1]	Study	Additional Information (Accession #)
Plant rhizosphere	*Matricaria chamomilla*	IBMS	Egypt	28,900,898	2.05	Köberl et al.[2]	npa
	Solanum distichum	IBMS	Egypt	29,684,017	0.70	Köberl et al.[2]	npa
	Eruca sativa	IBMS	Austria	23,192,621	1.03	Erlacher et al.[2]	npa
	Oryza sativa	PBMS	Philippines	1,026,982	1.33	Knief et al. (2011)	4449956.3[a]
Plant phyllosphere	*Arabidopsis thaliana*	PBMS	Spain	1,098,311	0.00	Delmotte et al. (2009)	4447810.3[a]
	Eruca sativa	IBMS	Austria	26,534,191	3.04	Erlacher et al.[2]	npa
	Oryza sativa	PBMS	Philippines	2,213,945	4.06	Knief et al. (2011)	4450328.3[a]
Soil	Agricultural soil	IBMS	USA	17,442,824	0.25	Smith et al.[3]	4508940.3[a]
	Home garden soil	IBMS	Austria	19,031,310	1.62	Erlacher et al.[2]	npa
	Desert soil	PBAS	Egypt	19,244	0.19	Köberl et al. (2011)	npa
	Desert farming soil	PBAS	Egypt	33,384	1.40	Köberl et al. (2011)	npa
Lichen thalli	*Lobaria pulmonaria*	IBMS	Austria	76,310,051	0.01	Grube et al. (2015)	4530091.3[a]
	Cladonia furcata	IBMS	Austria	21,711,407	0.02	Cernava et al.[2]	npa
	Peltigera polydactylon	IBMS	Austria	19,711,397	0.04	Cernava et al.[2]	npa
Indoor microbiome	Clean room (ISO-8)	PBAS	Germany	1,895	4.00	Moissl-Eichinger et al. (2015)	npa

Clean room (100K)	IBAS	USA	35,674	0.10	Mahnert et al.[3]	PRJEB8763[b]
Clean room surroundings	IBAS	USA	35,880	2.00	Mahnert et al.[3]	PRJEB8763[b]

[1] Abundances are based on Greengenes database assignments within all bacterial rRNA gene sequences in the specified sample

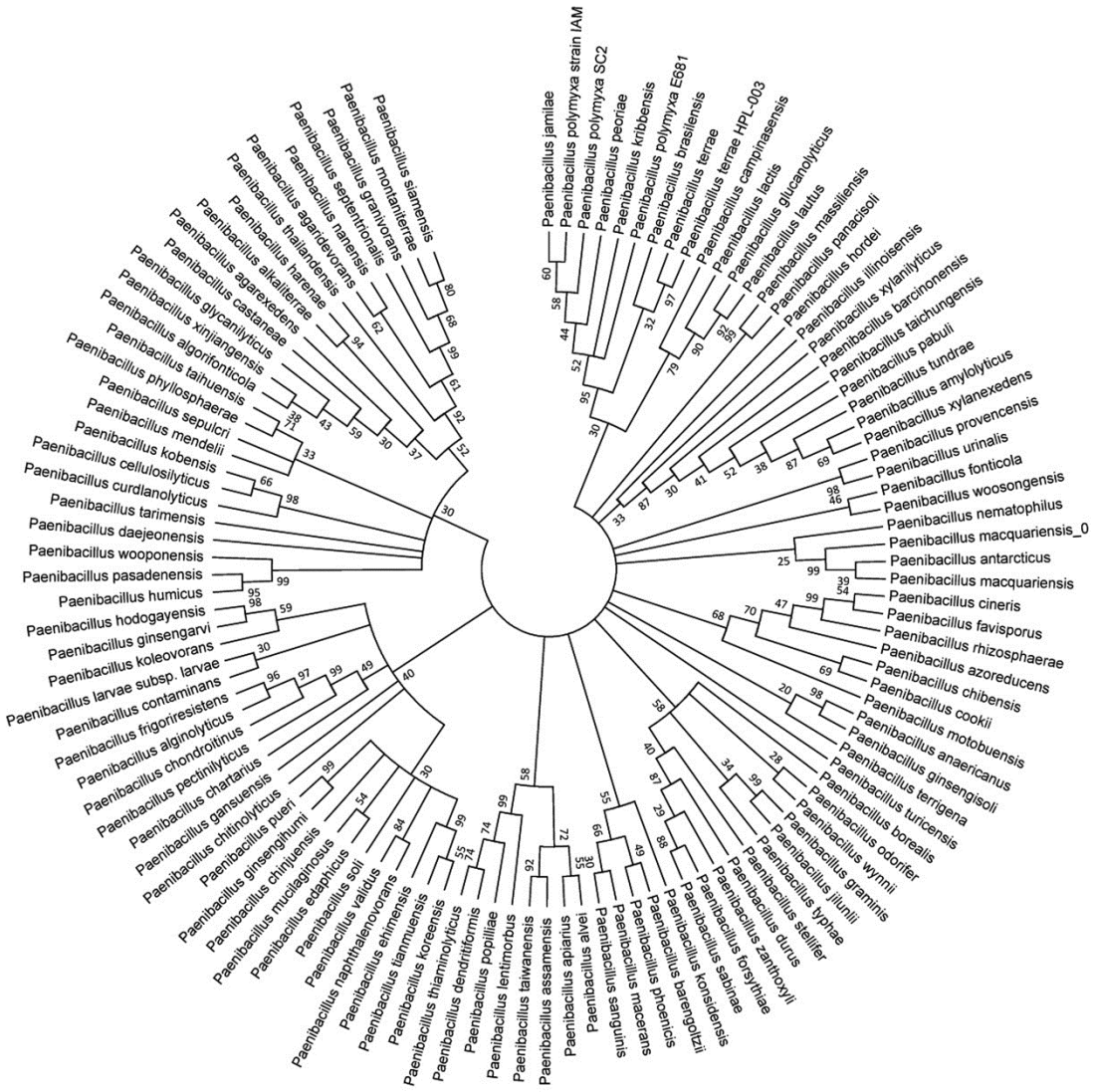
[2] The authors had exclusive access to these datasets to extract required taxonomic information for this review

[3] Unpublished study with data publicly available

[a] MG-RAST ID (http://metagenomics.anl.gov/)

[b] ENA primary accession (http://www.ebi.ac.uk/ena)

IBMS: Illumina-based metagenome sequencing, PBMS: Pyrosequencing-based metagenome sequencing, PBAS: Pyrosequencing-based amplicon sequencing, IBAS: Illumina-based amplicon sequencing, npa: dataset was not publicly available



Supplementary Figure S1 Phylogram of 16S rRNA gene sequences from the type strains of 116 species belonging to the genus *Paenibacillus*.

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Curriculum Vitae

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Education

since July 2012:	PhD student at the Institute of Environmental Biotechnology at Graz University of Technology. PhD Thesis: "*Exploring the functional contributions and the global interactions of the microbiome in an ancient symbiosis"* – supervised by Univ.-Prof. Dr. Gabriele Berg
September-July 2011-2012:	Employment at the Research Center Pharmaceutical Engineering (RCPE) in Graz
February-March 2011-2012:	Master Thesis: "*Identification of volatile compounds from plant-associated bacteria"* in *Biochemistry and Molecular Biomedicine* at the Institute of Environmental Biotechnology at Graz University of Technology in cooperation with the Research Center Pharmaceutical Engineering (RCPE) in Graz
2009-2012:	Master studies in *Biochemistry and Molecular Biomedicine* at Karl-Franzens-University and Graz University of Technology within the NAWI Graz project
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January-May 2009:	Joint Study exchange semester at the University of Arkansas at Little Rock (UALR) in the USA
2006-2009:	Bachelor studies in *Molecular Biology* in Graz at Karl-Franzens-University and Graz University of Technology within the NAWI Graz project
1997-2005:	High School (Realgymnasium) in Klagenfurt/Viktring at BRG Viktring
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Additional Skills

- Good Bioinformatics knowledge
- Good PC hardware-based knowledge
- Assistance in various Bachelor and Master courses at Graz University of Technology
- Multiple seminars on group dynamics and group management
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Foreign Languages

English:	Excellent skills, conversation highly fluent (TOEFL IBT: 106/120)
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Publication List

Peer-reviewed Publications

Cernava, T., Aschenbrenner, I. A., Grube, M., Liebminger, S., and Berg, G. (2015). A novel assay for the detection of bioactive volatiles evaluated by screening of lichen-associated bacteria. *Frontiers in microbiology*, *6*, 398.

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the lichen-associated bacterial microbiome. SGM Annual Conference 2015. Birmingham, United Kingdom 01.04.2015.

Poster Presentations

Cernava, T., Grube, M., and Berg, G. Gaining deeper insight into the lichen microbiome. 1st International Winter School on Evolution. Lisbon; Portugal 11.03.2013.

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