The lichen-associated microbiome: taxonomy and functional roles of lichen-associated bacteria

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Dissertation submitted in partial fulfillment of a
Philosophiae Doctor degree in Biology

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The biodiversity of bacterial lichen-associates (Short title)
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Abstract

Although lichens are generally characterized as the symbiotic association of a fungus (mycobiont) and a photobiont (green algae and/or cyanobacteria), species-specific communitites of other endolichenic bacteria, typically dominated by Proteobacteria, can also be found within the symbiosis. The roles that the non-phototrophic microbiota of lichens play in the symbiotic association is, however, not fully understood. This thesis describes studies that aim to characterize the non-phototrophic microbiota of lichens and elucidate some of their roles in the symbiotic association. Firstly, bacteria were cultured from four saxicolous seashore lichens; *Hydropunctaria maura, Verrucaria ceuthocarpa, Caloplaca verruculifera* and *Lecanora helicopis*, which are all commonly found along the Icelandic seashore. A total of 168 strains were isolated and selected isolates were further analysed. Secondly, lichen thalli from the membranous dog lichen, *Peltigera membranacea*, were investigated using culture-independent techniques, as well as characterizing cultured isolates and some of the roles the constituent bacteria play in the symbiotic association elucidated. Total DNA was extracted and a metagenomic library of 150 thousand contigs constructed and binned against 15 reference genomes. The BlastX algorithm was used for analyses of the selected contigs against the protein database. Taxonomically, the majority of the bacterial associates belong to *Alphaproteobacteria*, although members of *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and *Verrucomicrobia* are also present. Analysis of 28,000 bacterial contigs from the *Peltigera membranacea* metagenome yielded multiple hits on several genes involved in lichen secondary metabolite resistance, inorganic phosphate mobilization, biopolymer degradation and several other potentially important functions in thallus colonization and symbiosis. Samples of *P. membranacea* thalli were plated on various media types and 113 bacterial strains isolated. Phenotypic analyses of selected strains indicates that hydrolytic activity is common among these bacteria, as is inorganic phosphate mobilization and nitrogen fixation. Scavenging of organic nutrients may also constitute an important role for *Peltigera*-associated bacteria, with many of the strains producing strong biosurfactants and being able to degrade naphthalene.

Útdráttur

Fléttur eru sambýli sveppa og ljóstillífandi lífvera, ýmist grænþörunga eða blábaktería. Í þeim er þó einnig að finna stórt og fjölbreytt samfélag annarra baktería, en hin ljósóháða bakteríubíóta fléttna hefur afar lítið verið rannsökuð þar til á allra síðustu árum. Í fyrri hluta verkefnisins var sýnum af fjórum íslenskum strandfléttum (*Hydropunctaria maura*, *Verrucaria ceuthocarpa*, *Caloplaca verruculifera* and *Lecanora helicopis*) safnað og ræktanleg bakteríubíóta þeirra greind. Einangraðir voru 168 bakteríustofnar og valdir stofnar teknir til greiningar. Í síðari hluta verkefnisins var sýnum af himnuskóf, *Peltigera membranacea*, safnað. Erfðaefni fléttunnar var einangrað og raðgreint. Gerður var gagnagrunnur samfellna (*contigs*) sem flokkaðar voru úr rúmlega 150 þ. samfella samkvæmt einsleitni við 15 valin bakteríuerfðamengi. BLASTx algóriþmi var notaður til að leita gegn þekktum röðum í prótein gagnabanka. Niðurstöður sýna að meirihluti bakteríubíótunnar tilheyrir *Alphaproteobacteria* en jafnframt er þar að finna *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* og *Verrucomicrobia*. Einnig sést að þar er að finna gen sem taka þátt í grunnefnaskiptum, áreitissvörun (*stress response*), boðferlum (*signal transduction*), niðurbroti fjölliða, nýmyndun próteina og DNA og fleira. Að auki var sýni úr *P. membranacea* sáð á mismunandi ætistegundir. Einangraðir stofnar úr himnuskóf voru samtals 113 og voru framkvæmd ýmis greiningapróf á þeim, t.a.m. niðurbrot fjölliða og olíuefna, og fleiri lífefnafræðilegir þættir.

To my family and my friends…

…for their incredible support over the years

List of papers

The thesis is based on four papers of which two are published and two are submitted to scientific journals. The papers will be referred in the text by their respective numbers as following:

Paper I: Sigurbjörnsdóttir, M. A., Heiðmarsson, S., Jónsdóttir, A. R. and Vilhelmsson, O. 2014. Novel bacteria associated with Arctic seashore lichens have potential roles in nutrient scavenging. *Canadian Journal of Microbiology*, **60:** 307-317.

Paper II: Sigurbjörnsdóttir, M. A., Andrésson, Ó. S. and Vilhelmsson, O. 2015. Analysis of the *Peltigera membranacea* metagenome indicates that lichen-associated bacteria are involved in phosphate solubilization. *Microbiology*, **161:** 989-996. doi: 10.1099/mic.0.000069

Paper III: Sigurbjörnsdóttir, M. A. & Vilhelmsson, O. (SUBMITTED). Culturable phosphate-mobilizing, biosurfactant-producing and biodegradative bacteria associated with a sub-Arctic, terricolous lichen, *Peltigera membranacea*. Submitted to *FEMS Microbial Ecology* November 24th 2015.

Paper IV (Invited mini-review): Sigurbjörnsdóttir, M. A., Andrésson, Ó. S., Grube, M. & Vilhelmsson, O. (IN PRESS). Nutrient scavenging activity and antagonistic factors of non-photobiont lichen-associated bacteria: a review. Accepted for publication in *World Journal of Microbiology Biotechnology*.

Peer reviewed paper not included in the thesis:

Paper V: Vilhelmsson, O, Sigurbjörnsdóttir, M. A., Grube, M. & Höfte, M. (IN PRESS). Are lichens natural reservoir for plant pathogens? Accepted for publication in *Molecular Plant Pathology*.

Declaration of contribution

Paper I

Jónsdóttir and Vilhelmsson designed the study and were responsible for collecting samples with Heiðmarsson. Jónsdóttir was responsible for the isolation procedure and analysing the data. Sigurbjörnsdóttir was responsible for sample collection when repeating the study and wrote the first draft of the paper, with supervision from Vilhelmsson and Heiðmarsson. Vilhelmsson and Heiðmarsson critically revised the manuscript, edited it and approved the final version of manuscript.

Paper II

Andrésson designed the study, collected samples, and was responsible for DNA isolation. Snæbjarnarson constructed the metagenomic database with assistance from Andrésson and Vilhelmsson. Sigurbjörnsdóttir analysed the bacterial metagenome obtained from the larger metagenomic dataset, with supervison from Vilhelmsson. Sigurbjörnsdóttir wrote the first draft of the paper, with supervision from Vilhelmsson and Andrésson, which critically revised the manuscript. Sigurbjörnsdóttir worked on the final version of the manuscript. All authors gave final approval of the manuscript to be published.

Paper III

Sigurbjörnsdóttir designed the study and was responsible for collecting samples, with supervision from Vilhelmsson. Ásgeirsdóttir, Jónsdóttir, Þorsteinsdóttir, and Kjartansdóttir assisted with strain isolation and phenotypic analysis, with supervision from Sigurbjörnsdóttir. Sigurbjörnsdóttir was responsible for analysing the data and writing the first draft of paper, with supervision from Vilhelmsson. Vilhelmsson critically revised the manuscript and edited it. Sigurbjörnsdóttir worked on the final version and Vilhelmsson approved the final version of manuscript to be published.

Paper IV

Sigurbjörnsdóttir was responsible for writing the paper. Vilhelmsson and Andrésson critically revised the manuscript and edited it. Sigurbjörnsdóttir worked on the final version and all authors approved the final version submitted.

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1 Introduction

1.1 Symbiosis

The term symbiosis is derived from the Greek word for “living together”. It was initially coined by the German mycologist, Heinrich Anton de Bary, in 1879 as; “the living together of unequally named organisms” (“Das Zusammenleben ungleichnamiger Organismen“) and thus applies to the association between two different species that may last for the lifetime of one or all partners (de Bary, 1879). This includes mutualistic (where all partners benefit), commensalistic (where one partner benefits but the other remains unharmed), or parasitic association (where one partner benefits to the detriment of the other). However, many symbiotic systems are too complex and too poorly understood to fit them in one category rather than another (Douglas, 1994). Symbiotic associations exist all over the world and are found in both marine and terrestrial environments. These interactions can occur either between two distantly related organisms or between two or more closely related species (Dimijian, 2000). Among the most ancient and most commonly encountered prevalent symbiotic associations on Earth is the relationship between fungi and plant root (Relman, 2008). Arbuscular mycorrhiza fungi, where part of the fungal hyphae of Glomeromycota grows inside plant cells, are among the most widespread terrestrial symbiosis (Fitter, 2005) and is formed by 70-90% of land plant species. Most of the fungal hyphal network is in the soil next to the plant roots, thus assisting the plant in water absorbtion, phosphate, nitrogen, and other nutrients uptake through the arbuscular mycorrhiza (Parniske, 2008). Symbiotic associations can therefore allow organisms to explore new niches and to survive in conditions where they would otherwise would be non-competitive.

1.2 Lichens

Lichens are a fascinating example of symbiotic relationships in which two or three organisms form unique and easily recognizable structures, the vegetative thalli. The symbiosis yields a small biotope that can be considered a small ecosystem and is usually composed of a fungal partner, the mycobiont, and one or more photosynthetic partners, the photobiont,

which generally is either a green alga or cyanobacterium (Nash, 2008a). Lichens can thrive in practically any terrestrial environment, and are among the earliest colonizers of recently exposed land, such as lava fields, glacial moraines and nunataks (Gadd, 2007). They are dominant in some parts of the planet's landscape and have been estimated to cover up to 8% of the total land surface (Ahmadjian, 1995). Lichens are perennial and slow-growing organisms that present a variety of colorful forms and maintain fairly uniform, long-lived morphologies, reaching ages at up to several thousand years. Generally, lichens are broadly distributed and are found in a wide range of habitats, including extreme environments such as the Antarctic (Øvstedal & Smith, 2001), the Arctic (Printzen, 2008), on mountain summits, as components of desert crusts (Buedel et al., 2009) and tropic regions (Morley & Gibson, 2004). Lichens are usually highly tolerant of harsh environmental conditions such as extremes in temperature, low water content, low nutrient availability and high UV light intensities. They are thus often found in environments where few other macroscopic organisms would thrive (R.P. Beckett, Kranner, & Minibayeva, 2008). Despite their resistance to abiotic stresses in their dormant stage, lichens are adapted to their habitat and can be vulnerable to even slight changes in environmental conditions that can disrupt the integrity of the symbiotic association (Erlacher et al., 2015).

1.3 General description of the lichen symbiosis

By definition, lichens are the symbiotic association of lichen-forming fungi and a photoautotrophic partner. They represent a classic example of self-sustaining symbiotic interactions that today are recognized to be widespread in nature, occurring in different kingdoms of life (Nash, 2008a). Lichenized fungi, i.e. fungi that have acquired fixed carbon from algae and/or cyanobacteria, comprise about 20% of all extant fungi (Hawksworth, 1988). The number of classified lichen species varies from 13.500 to approximately 17.000 (Nash, 2008a), although numerous lichenized fungi are still undescribed at the species level (Lawrey & Diederich, 2003). Ascomycetes are by far the most common mycobionts whereas less than 1% belong to the Basidiomycota (Honegger, Edwards, & Axe, 2013; Lutzoni et al., 2004). As a heterotrophic organism, the mycobiont requires carbohydrates, which are produced by the photobiont; sugar alcohols by algal photobiont and glucose by cyanobionts (Nash, 2008a). The majority, or approximately 85% of lichens, are symbiotic with green microalgae. The

genera *Trebouxia* and *Trentepohlia* are the most frequent photobionts, although about 40 genera have been found in lichens. About 10% of the lichen-forming fungi interact with cyanobacteria, where the genus *Nostoc* is the most common, and 4% associate with both cyanobacteria and green algae, namely tripartite lichens (Honegger et al., 2013). For a long time, it has been acknowledged that the lichen symbiotic association often involves other organisms than the two typical partners. In addition to the primary photobiont, tripartite lichens can acquire cyanobacteria within their thalli. This incorporation of cyanobacteria leads to the formation of special structures, cephalodia, wherein the cyanobacteria reside. The gall-like structure is located inside the thallus in some lichens, but on the outer surface on others (Cornejo & Scheidegger, 2013). In tripartite lichens, the cyanobacteria are apparently focused on supplying fixed nitrogen used by symbionts, as higher rates of nitrogen fixation have been reported in cephalodia than in bipartite cyanolichens (Rikkinen, 2002). The nitrogen fixation has important effects on the lichen ecology, including colonization in special ecological niches, such as extremely oligotrophic habitats (Büdel & Scheidegger, 2008).

Lichens are poikilohydric, meaning that the water status is controlled by the availability of water in their habitat. Therefore, the lichen thallus is often subjected to dramatic fluctuations in water content (Honegger, 1998). It is generally assumed that lichen's carbon and nitrogen supplies are modulated by the environment through photosynthetic activity. In addition, the transport and distribution of photosynthetic products may be controlled by the environment, depending on repeated drying and wetting of the thallus (Nash, 2008a)

1.4 Lichen morphology

In general, lichens are divided into three morphological groups, based on their overall habitat: crustose, foliose and fruticose lichens. In addition to those three main groups, special types are also known, including gelatinous thallus morphologies restricted to some cyanobacterial lichens (Büdel & Scheidegger, 2008).

Regardless of the morphological group, the developmental process of a lichen thallus starts primarily when the fungus encounters a suitable photobiont. In general, the mycobiont determines the appearance of a lichen thallus albeit a few cases are known where the photobiont determines the habitat. The photobiont however influences the lichen morphogenesis and the thallus is not fully developed unless both partners are present (Büdel & Scheidegger, 2008). The partners establish a tangled cell mass in their early stages, later developing the thallus which is primarily shaped by fungal mycelia and the majority of biomass composed of the mycobiont (Gilbert, 2000). The thallus is usually a distinctly layered structure (Fig. 1) and involves upper, and in some cases, lower cortex, an algal layer, a medulla and rhizinae (Jahns, 1973). The symbiotic partners launch their contact within the algal layer but the relation between algae and fungal hyphae varies.

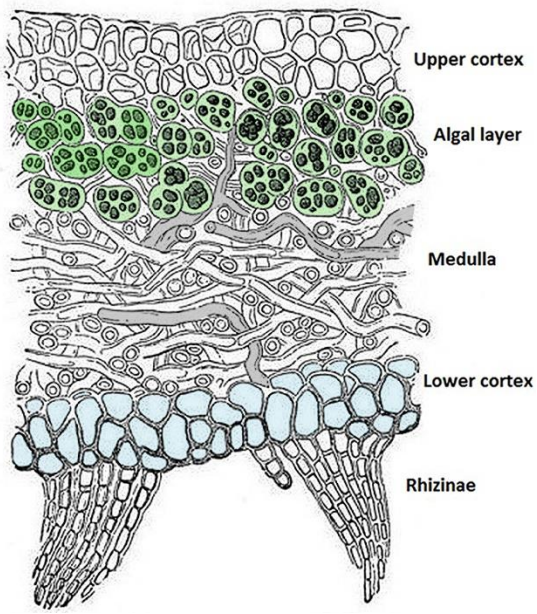

Figure 1. A schematic section through a typical foliose lichen thalli. Figure adapted from Meyer, 2009.

The upper cortex involves tightly packed cells which covers the upper side of the thallus. This cortical layer can be up to several hundred micrometers thick but can also appear as a thin layer (Büdel & Scheidegger, 2008). The upper cortex has multiple functions, including providing protection against high radiation, such as UV light (Solhaug, Gauslaa, Nybakken, & Bilger, 2003), modifying energy budgets and defending against lichenivorous animals (Büdel & Scheidegger, 2008).

The algal layer is situated in the upper part of the medulla and receives sufficient light for the photobiont to photosynthesize (Gilbert, 2000). Although the fungal hyphae surround the algal cells, they are lightly interwoven and leave enough space for optimum light intensity. Nevertheless, the photobionts are seldom located deep within the thallus and there are no unattached algae in the lichen thallus (Jahns, 1973).

The medullary layer makes up a large part of the internal thallus in most foliose or fruticose lichens; this layer consists of long and loosely packed fungal hyphae, forming a cottony layer with high internal airspace, facilitating gas exchange within the thallus (Honegger, 1993). In some lichens, particularly fruticose lichens, supporting tissue is often formed within the medulla, making irregular forms which can be observed in genera such as *Cladonia* and *Usnea* (Büdel & Scheidegger, 2008). In most cases, the hyphal cell walls of both the algal layer and the medulla are encrusted with crystalline secondary products, making the medullary hyphae hydrophobic. Thus, the medulla and algal layer are able to remain air-filled during moist periods which prevents water from accumulating in internal parts of the lichen by capillary action. Furthermore, the hydrophobic activity of the medulla promotes gas exchange for the photobiont (Honegger, 1998). The mycobiont cell walls are suggested to be responsible for water transport to the photobionts (Büdel & Scheidegger, 2008). The medullary layer can hold more water than other lichen tissues and also serves as a reservoir for carbohydrates produced by the algae (Jahns, 1973). In crustose lichens, the medullary layer serves to attach crustose lichens to the substratum as they lack the lower cortex that foliose lichens have.

In some lichens, the medulla directly forms the outer, lower layer of thallus. However, foliose lichens typically contain a lower cortex. It can include root-like structures (rhizines) that can serve to attachment the lichen to XYZ (Jahns, 1973). The lower cortex is often strongly pigmented and can directly absorb water (Büdel & Scheidegger, 2008).

1.4.1 Crustose lichens

The majority of lichens (~50%) can be described as crustose lichens (microlichens) and their growth and development is not as well-known as of foliose and fruticose lichens (Armstrong & Bradwell, 2010; Honegger, 1993). Crustose lichens lack a lower cortex and are attached to soil, rock or trees by the hyphae of the medullar layer, making them practically

inseparable from the substrate without destruction (Büdel & Scheidegger, 2008). Crustose lichens overgrow the photobiont cells on the uppermost part of the substratum, forming microfilamentous or crustose thalli (Honegger, 1993). Crustose lichens profit from surface water flow when growing on inclined rock surfaces and water loss is restricted to the upper cortex. Variations of the basic crustose lichen are found in nine subtypes; powdery, endolithic, endophloeodic, squamulose, peltate, pulvinate, lobate, effigurate, and suffruticose crusts. Furthermore, the thallus may be organized as either homoiomerous or heteromerous (Büdel & Scheidegger, 2008). The simplest type is found in the powdery crusts where an organized thallus structure is lacking; clusters of photobiont cells are within the fungal hyphae but distinct medulla, or algal layer are not present. The epilithic lichens form the most common crustose growth type and grow entirely inside their substrate, either wood or stone (Jahns, 1973). These types are generally more organized and in some lichens, even an upper cortex is developed. The most complex subtype of crustose lichens is the squamulose type, forming flat scales of squamulose thalli which are attached in the center of the lower surface. The squamulose type of crusts is most often encountered generally apparent in lichens colonizing soils or rock surfaces in warm climates (Büdel & Scheidegger, 2008).

Figure 2. Growth forms, crustose lichens. Fig. 2A Lecidea lapicida on a boulder in Vaglaskógur forest. Fig. 2B Caloplaca verruculifera on a bare rock at the seashore, near Akureyri, Iceland. Photos by Oddur Vilhelmsson.

1.4.2 Foliose lichens

Foliose lichens have a leaf-like thallus, formed of flattened lobes, and are only partially attached to the substrate. Foliose lichens develop in a variety

of thallus morphologies and sizes, although laciniate and umbilicate are the most common growth forms (Büdel & Scheidegger, 2008). The laciniate lichens present the archetypal foliose lichens. They have lobes and the lower surface is either wholly in contact with the substrate or the margin is free, bending upwards (Jahns, 1973). The laciniate group consists of extremely polymorphous lichens and their anatomy is the most complex of all lichens. Whereas some appear similar to large plants, others are only a few mm in diameter. The lower cortex of many laciniate lichens is covered by rhizinae, cilia or a tomentum, often attaching the lichen to its surface (Büdel & Scheidegger, 2008).

The thallus of umbilicate lichens is disklike, either one single, unbranched lobe or multilobate. They are attached to the surface with a central holdfast. (Büdel & Scheidegger, 2008).

Figure 3. Foliose growth form. Fig. 3A. Peltigera membranacea in grassland near Reykjavík, Iceland. Fig 3B. Peltigera malacea in grassland near Reykjavík, Iceland. Photos by Ólafur S. Andrésson

1.4.3 Fruticose lichens

Fruticose lichens have strap-shaped or thread-like thallus lobes that may be flat or cylindrical and are attached to the substrate by a holdfast. All fruticose lichens stand out from the surface and the majority have radial symmetric thalli (Büdel & Scheidegger, 2008).

Figure 4. Fruticose growth forms. Fig. 4A. Cladonia borealis in Vaglaskógur forest, near Akureyri, Iceland. Fig. 4B. Stereocaulon alpinum in Vaglaskógur forest, near Akureyri, Iceland. Photos by Oddur Vilhelmsson.

1.5 Lichen growth and resources

Although lichens, like all higher organisms, have a limited lifespan, they are among the longest-living organisms on earth. Many species are extremely slow-growing, with reported growth rates ranging from ≤0.9 mm/year (Karlén & Black, 2002), up to 35 mm/year (Oksanen, 2006). In general, crustose lichens have substantially lower growth rates as compared to foliose lichens (Armstrong & Bradwell, 2010). Individual lichen growth is dependent on resource acquisition and the subsequent biosynthesis of cellular compounds, minus losses of thallus material through dispersal, fragmentation, grazing. and necrosis (Palmqvist, 2000). Being poikilohydric organisms, unable to control their water status, environmental conditions have a large effect on lichen growth. However, when wet and metabolically active, lichens can convert incident light energy into new biomass just as efficiently as vascular plants. The dominant biomolecule component of lichens is composed of carbohydrate ($(CH_2O)_n$) equivalents (Palmqvist & Sundberg, 2000), thus factors that limit lichen photosynthesis also limit lichen growth. Temperature can affect lichen productivity, especially in the tropics, resulting in decrease in net carbon gain (Zotz, Budel, Meyer, Zellner, & Lange, 1998). Additional mineral nutrients, particularly nitrogen and phosphorous, are also essential for lichen growth necessitating a balance between carbon acquisition and mineral availability (Palmqvist, Dahlman, Jonsson, & Nash, 2008). Lichens efficiently extract mineral nutrients from recalcitrant surfaces although the thallus rhizinae can also participate in nutrient uptake (Oksanen, 2006).

1.5.1 Water

Lichens are dependent on periods of rain, dew, fog, and even relative humidity to maintain their metabolic activity. Therefore, they often undergo drying and wetting cycles, causing their water per thallus dry weight to fluctuate between <20 and >160% (Honegger, 1993). Lichens in coastal deserts are dependent on non-precipitation water sources, where fog or dew occur almost daily but precipitation is minimal (Palmqvist et al., 2008). Water is absorbed when the lichen's water potential is below the atmospheric water potential, which generally happens at relatively low temperatures (<20°C) and high relative humidity (>75-95%) (Palmqvist et al., 2008). On the other hand, when humidity decreases and temperature increases, the atmospheric water potential becomes negative, causing desiccation of the lichen thallus. Other environmental factors can also cause desiccation, for instance high solute and ion concentrations in marine environments. (Palmqvist et al., 2008). During desiccation, the lichen mycobiont and its associated photobiont´s cells, lose water, and soluble compounds but recover rapidly when water becomes available (Honegger, 1993). The frequent drying/wetting cycles affect the carbon and nitrogen budget of lichens. Although they generally recover quickly, too short and infrequent wet periods might lead to high rates of respiration, incomplete photosynthetic recovery, and carbon leakage from the thallus (Dudley & Lechowicz, 1987). Water uptake is restricted to the fungal hyphae in the medullary layer and moves passively within the apoplastic continuum between the symbiosis partners (Honegger, 2006). Since lichens lack both a cuticle and stomata, both uptake and loss of water are physical processes that can vary among lichen species (Palmqvist, 2000). For instance, filamentous and fruticose species can take up and lose water more rapidly than flat, foliose species, due to their larger ratio of surface area to volume (Palmqvist et al., 2008). The difference in thallus thickness also affects the water holding capacity, e.g. thick foliose lichens equilibrate slower than thinner lichens (Gauslaa & Solhaug, 1999)

1.5.2 Carbon

About 40-50% of a lichen's dry mass is composed of carbon, which they mainly gain from the photobiont's photosynthesis (Green, Nash, & Lange, 2008). The process of photosynthesis involves the conversion of light

energy into chemical energy which autotrophic organisms use for their maintenance, growth and reproduction. This process reduces CO_2 into carbohydrates, further used by the organism (Palmqvist et al., 2008). The basic principles of photosynthesis are the same regardless of the photosynthetic partner. However, at least three major types of CO_2 acquisition modes have been distinguished, depending on the photobiont species (Palmqvist, 2000). These different modes involve: a) genera that lack pyrenoids, e.g. *Coccomyxa*, b) genera that have pyrenoids, e.g. *Trebouxia* and c) cyanobacterial primary photobionts (Palmqvist, 2000). The poikilohydric characteristic of lichens in general dominates their CO_2 exchange behavior. The rate of gas exchange dramatically changes in contrast to the thallus water content; when the water content is low, CO_2 diffuses but when hydrated, diffusion of CO_2 is blocked (Honegger, 1991). The CO_2 exchange rates can vary among lichen species or lichen type and environmental conditions can also affect the exchange rates (Green et al., 2008).

1.5.3 Elements

Just like plants, lichens require elements such as hydrogen, carbon, oxygen, nitrogen, potassium, calcium, magnesium, phosphorus, sulfur, chlorine, boron, iron, manganese, zinc, copper and molybdenum (Nash, 2008c). Nitrogen is involved in many processes for both the photobiont and the mycobiont and although it is an important nutrient, nitrogen can cause stress if supplied in excess (Johansson, Nordin, Olofsson, & Palmqvist, 2010). The nitrogen acquisition mode can vary among lichen species, depending on the associated photobiont. Green algal lichens depend on direct nitrogen deposition on the thallus surface whereas cyanobacterial lichens (either bi- or tripartite) have access to atmospheric nitrogen via their cyanobacterial symbiont (Dahlmann, Persson, & Palmqvist, 2004). Lichens can assimilate nitrogenous compounds as nitrate (NO_3^-), ammonium (NH_4^+) or organic N (Johansson, Olofsson, Giesler, & Palmqvist, 2011; Nash, 2008b).

The effects of nitrogen limitation on lichen productivity have been relatively well studied (Palmqvist et al., 2008) but the effect of phosphorus limitation is not fully understood. However, recent studies have shown that addition of phosphorus may increase growth rate, abundance and species richness of lichens, especially N_2-fixing species (McCune & Caldwell, 2009; Benner, Conroy, Lunch, Toyoda, & Vitousek, 2007; Benner &

Vitousek, 2007). Although most studies on phosphorus limitation have been focused on cyanolichens, the effect on green algal lichens following increased nitrogen has been demonstrated (Hogan, Minnullina, Smith, & Crittenden, 2010; Johansson et al., 2010, 2011). Only trace concentrations of phosphorus are present in the atmosphere as dust from soil erosion and rock weathering, and thus lichens have developed uptake pathways for their phosphate capture (Farrar, 1976; Smith, 1960).

1.6 Lichen metabolites and their uses

Among the most visual characteristics of lichens are their colorful thalli, prominently on display wherever they grow in abundance. The distinct colors are due to accumulation of small but chemically complex molecules, 'the lichen substances'. These secondary metabolites are primarily produced by the mycobiont and can either be deposited in the upper layer of the lichen thallus, resulting in visible pigmentation, or as colorless substances found in the internal parts of the thallus (Boustie & Grube, 2005). Lichens and their metabolites have been used by humans for centuries. In 1907, one of the first works on the chemical and pharmacological aspects of lichens was published (Zopf, 1907). Since then, lichenologists have studied lichen chemistry quite extensively and to date over 800 lichen metabolites have been identified (Muller, 2001). Lichens produce their compounds via diverse biosynthetic pathways, such as the polymalonate, shikimic acid and mevalonic acid pathways. In addition, sugar alcohols are produced by photosynthetic pathways of the phycobiont (Huneck, 1999). On the basis of their biosynthesis, lichen metabolites are classified into following groups: aliphatic lichen substances, aromatic lichen substances, and carbohydrates (Zambare & Christopher, 2012). The majority of these secondary metabolites have been identified as phenolic compounds (e.g. orcinol derivatives), vulpinic acid and other pulvinic acid dervatives, depsides (e.g. diffractic acid), lactones (e.g. protolichessterinic acid), and usnic acid (Boustie, Tomasi, & Grube, 2011; Zambare & Christopher, 2012). The possible roles of these diverse substances have been elucidated in numerous publications. Due to the slow growth of lichens, antibiotics are needed for their protection. Some lichen metabolites can serve as such but other suggested possible roles are involved in UV absorption, providing minerals from the substrate, and as toxins to defend against insects, snails, and nematodes (for a review, see Huneck, 1999).

For centuries, lichens have been used for various purposes. Lichen-based remedies have been used in folk medicines, pigments produced by various lichens are used as dyes and even as acid-base indicators, and lichen-derived compounds with antioxidant capacity have been used as preservatives in cosmetics (Muller, 2001). Past and current studies have also shown that lichen secondary metabolites have various biological activities of potential biotechnological potential including antibacterial, antifungal, antiviral, anticancer, and anti-inflammatory activities, inhibitors or plant growth and enzymes, and cytotoxic activities (for a comprehensive overview, see Zambare & Christopher, 2012).

Moreover, lichens have been used for monitoring atmospheric pollution since the late 1960s (Doğrul Demiray, Yolcubal, Akyol, & Çobanoğlu, 2012). As lichens do not have any roots, they take up contaminants directly from the atmosphere and accumulate them in the lichen tissues at high concentrations (Garty, 2001). The lichen thallus is slowly growing and long-lived and their morphology does not have seasonal changes. Thus, accumulation of pollutants can occur over a long period of time and the concentration of trace elements in the lichen tissues reflecting environmental level of these elements (Bari, Rosso, Minciardi, Troiani, & Piervittori, 2000; Kularatne & De Freitas, 2013).

1.7 Lichens in Iceland

Lichens are very prominent members of Icelandic vegetation, often growing in dense mats and crusts covering large areas of ground and rock. To date, more than 750 lichen species have been identified among Icelandic vegetation, whereas there are only 450 species of vascular plants. More than 400 Icelandic lichens are classified as crustose lichens with the remaining being foliose and fruticose lichens. The lichen flora is similar to areas such as Scotland, the Faroe Islands, Greenland, and Svalbard, although it also seems to be related to the Macaronesian flora (Svane & Alstrup, 2004). Lichens have been investigated in all parts of Iceland, so the native lichen flora is considered fairly well-known. Although about 750 lichens have already been listed, additions to the flora have been made recently, suggesting that additions may be yet to come, especially from remote areas that can be difficult to access (Hansen, 2009; Kristinsson & Heidmarsson, 2009).

In the studies presented in this thesis, the focus was on five different lichens: *Lecanora helicopis*, *Verrucaria ceuthocarpa*, *Hydropunctaria maura*, *Caloplaca verruculifera*, and *Peltigera membranacea*.

1.7.1 *Lecanora helicopis*

Lecanora represents one of the largest genera of lichenized fungi with about 300 species (Blaha & Grube, 2007; M. Grube, Baloch, & Arup, 2004). Although the genus is large and heterogeneous, it is generally characterized by crustose, colorless, non-septate ascosporic, green-algal photobionts, generally *Trebouxia*, and a lecanorine margin (Pérez-Ortega, Spribille, Palice, Elix, & Printzen, 2010). *L. helicopis* lichens have grey-ish, rimose thalli and are commonly found on coastal rocks in the mesic-supralittoral zone in Europe. This species is widespread on coastal rocks around Iceland, however, it has not been found on the sandy coast of S-Iceland. Secondary metabolites have not been detected for this species (Edwards, Aptroot, Hawksworth, & James, 2009).

1.7.2 *Verrucaria ceuthocarpa*

There are approximately 200 accepted species belonging to the genus *Verrucaria*. *Verrucaria* lichens have crustose thalli, immersed or superficial and the cortex is usually brownish. In most cases, the photobiont belongs to *Chlorophyta* although *Phaeophyceae* is also known (Orange, Hawksworth, McCarthy, & Fletcher, 2009). *V. ceuthocarpa* has brown to black, deeply cracked thallus and is found on littoral siliceous rocks on the sea shore of Iceland, especially the North- and East-coast (Kristinsson & Heidmarsson, 2009). Secondary metabolites have not been detected for this species (Orange et al., 2009)

1.7.3 *Hydropunctaria maura*

The genus of *Hydropunctaria* represents four lichen species. They belong to the family of *Verrucariaceae* and were recently described as a new genus (Gueidan et al., 2009). The thallus of *H. maura* is crustose, subgelatinous when wet and often forms a black basal layer. The upper surface is often unevenrough. *H. maura* is a hydrophilic lichen species and is restricted to sites with inundation, either periodic or constant (Gueidan et al., 2009). This lichen species is commonly found on the seashore around Iceland (Kristinsson & Heidmarsson, 2009).

1.7.4 *Caloplaca verruculifera*

Regarding thallus morphology and ascoma anatomy, *Caloplaca* is an extremely heterogeneous taxon. *Caloplaca* lichens are often recognized by their yellow to orange color, caused by the presence of anthraquinones in the thallus, and/or ascomata combined with teloschistaceous ascus type and polarilocular ascospores (Grube & Hawksworth, 2007). Some species of *Caloplaca* have, however, reduced septum, presenting simple ascospores. In addition to numerous anthraquinone present in the genus, other lichen metabolites have also been detected (Sochting & Lutzoni, 2003). *C. verruculifera* is rather commonly found on the seashore around Iceland but hardly ever grows far from the sea (Kristinsson & Heidmarsson, 2009).

1.7.5 *Peltigera membranacea*

The genus *Peltigera* is composed of foliose lichens. The thalli has rhizines or tomentum on the lower side and colorless or brown ascospores (Miadlikowska & Lutzoni, 2004). Lichens of this group are often found in humid, shaded habitats, in forests and along roadsides. They predominantly live on soil or mosses (are terricolous or muscicolous) but are hardly ever found on rocks or tree bark (Zuniga, Leiva, Ramirez-Fernandez, Caru, & Orlando, 2014). *Peltigera* lichens generally form association with the cyanobacterium *Nostoc* although a few species additionally have the green alga *Coccomyxa*, thus comprising tripartite lichens (Miadlikowska et al., 2014). The genus has an estimated number of 60-75 species where most of them have been recorded from Europe and North America although they have also been found in southern parts of Earth, such as New Zealand, Australia and Southern Chile (Zuniga et al., 2014). *P. membranacea* is commonly found all over Iceland, particularly on forest-floors, in grass- and heathlands. It is closely related to *P. canina*, also commonly found in Iceland, and they have only recently been distinguished. Often the thalli of *P. membranacea* are large compared to other lichens, up to 15-20 cm in diameter. No secondary metabolites have been detected in the lichen (Kristinsson & Heidmarsson, 2009).

1.8 Bacterial communities

Although lichens are generally defined only as bipartite or tripartite mycobiont-photobiont symbiosis, the presence of other microorganisms in

the lichen thalli has long been known. Following Johannes Uphof's early report on "purple bacteria" in lichens, suggesting that non-cyanobacterial prokaryotes were present (Uphof, 1925), a series of papers were published, further documenting the association of bacteria with lichens, in the following years and decades (Henkel & Plotnikova, 1936; Iskina, 1938; Panosyan & Nikogosyan, 1966). Throughout most of the 20th century, however, the focus was primarily on the symbiotic components of lichens and their ability to produce secondary metabolites with biological activities (Huneck, 1999; Muller, 2001) and little was known about the endolichenic microbial communities, their function or diversity.

1.8.1 Bacterial diversity of the culturable biota

Some 10 years ago, the first modern-era study on the non-phototrophic lichen-associated microbiota was published (González, Ayuso-Sacido, Anderson, & Genilloud, 2005). That study focused on *Actinobacteria*, isolated from 25 terrestrial lichen samples collected in Alaska, Hawaii, and the Reunion islands. The lichens sampled were not identified in the paper but were described as saxicolous (rock-dwelling) and arboricolous (tree-dwelling). A standard medium (YME agar) was used for actinobacterial cultivation from all lichen samples, resulting in a large number (337) of strains which were identified on the basis of DNA fingerprinting and fatty acid analysis. The majority of isolates belonged to the families *Micromonosporaceae* (142) and *Streptomycetaceae* (110), although *Pseudonocardiaceae* (30), *Nocardiaceae* (4), *Streptosporangiaceae* (2), *Thermomonosporaceae* (7), and *Geodermatophilaceae* (1) were also detected. The isolates were further screened for the presence of genes involved in polyketide, polypeptide, and isoprenoid biosynthesis and antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. At least one biosynthetic cluster was observed in a large part of the isolates (over 60%) and 27% showed antimicrobial activity against at least one of the targeted microorganisms.

The following year, two papers were published on lichen-associated bacterial communities (Cardinale, Puglia, & Grube, 2006; Liba et al., 2006). Cardinale and coworkers analyzed 11 different lichens, belonging to eight species and sampled at five different sites in Austria and France. The lichen sampled belonged to *Cladonia, Pseudevernia, Hypogymnia,* and *Roccella*. Bacteria from external and internal surface of the lichen thalli were isolated, using TY and sugar-rich/N-free medium, resulting in 34 morphologically distinct bacteria. Liba et al. (2006) sampled 5 foliose cyanolichens from

rainforests in Brazil and isolated acetylene-reducing strains on nitrogen-free medium. The presence of *nifH* genes, involved in nitrogen fixation, was confirmed with dot-plot hybridization of genomic DNA from the isolates and the microbiota was suggested to be involved in nitrogen fixation for the lichen symbiosis. The isolates (17) all belonged to different genera of *Gammaproteobacteria* and further analysis of their functions showed them to be involved in phosphate solubilisation, indole-3-acetic acid (IAA) production and amino acid secretion.

Since these studies, a number of other publications on lichen-associated bacteria via cultivation have appeared (Cardinale et al., 2006; Grube et al., 2009; Kim et al., 2014; Lee et al., 2014; Liba et al., 2006). While many of them have targeted individual isolates or species, others have focused on special interest or biotechnological potential of the microbiota. Overall, the taxonomic bacterial diversity has been relatively well established and in number of studies, possible roles of the associated microbiota have been suggested (see Table 1 for overview).

Table 1. A summary of culture dependent studies of lichen associates

Lichen species	Lichen habitat	Taxonomic diversity of the isolated microbiota	Roles of microbiota within the symbiosis/potential biotechnological applications	Reference
Unidentified	Terrestrial	*Actinobacteria*	None suggested/ Bioactive	González et al., 2005
Cladonia digitata, C. rangiferina, C. coniocracea, C. pyxidata, C. coccifera, Pseudevernia furfuraceae, Hypogymnia physodes, Rocella phycopis, R. fuciformis	Terrestrial	*Firmicutes, Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria*	N-fixing/ Diazotrophic bacteria	Cardinale et al., 2006
Canoparmelia caroliniana, C. crozalsiana, C. texana, Parmotrema sanctiangeli, P. tinctorum	Cyanolichens from rain forest in Brazil	*Gammaproteobacteria*	N-fixing, phosphate solubilisation, IAA production, nutrition contribution via amino acid release	Liba et al., 2006
Cladonia arbuscula, Lecanora polytropa, Umbilicaria cylindrica	Saxicolous above tree line in Austria	*Alphaproteobacteria, Actinobacteria, Firmicutes, Betaproteobacteria, Gammaproteobacteria*	N-fixing, proteolysis, chitionlysis, glucanolysis, phosphate solubilisation, IAA production, antagonistic	Grube et al., 2009
Acarospora flavocordia, Lecanora fuscobrunnea, Lecidea cancriformis, Rhyzocarpon sp., *Umbilicaria decussata, Usnea antarctica, Xanthoria elegans*	Antartic	*Actinobacteria, Gammaproteobacteria, Deinococcus-Thermus, Firmicutes*	None suggested	Selbmann et al., 2010

Cladonia sp. *Cladonia rangifernia*, *Sphaerophorus globose*	Epigeal bog in N-Russia (Karelia and Arctic tundra)	*Actinobacteria, Acidobacteria, Alphaproteobacteria, Betaproteobacteria*	None suggested	Pankratov, 2012
Ochrolechia sp.	Arctic region	*Alphaproteobacteria, Betaproteobacteria*	None suggested/ antibacterial and antioxidant activity	Kim et al., 2014
Usnea sp., *Cladonia borealis*, *Psoroma* sp., *Stereocaulon* sp., *Umbilicaria* sp., *Cetraria* sp., *Cladonia* sp., *Ochrolechia* sp.	Antarctic and Arctic regions	*Actinobacteria, Bacteroidetes, Deinococcus-Thermus, Firmicutes, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria*	Nutrient supply (proteolysis and lipase activity)	Lee et al., 2014
Lobaria pulmonaria	Maple trees in the Alps		Nutrient supply phosphate solubilisation, antagonistic activity	Grube et al., 2015
Lobaria pulmonaria	Three different locations in Austria		Volatile organic compound production, spermidine production, hydrogen cyanide production	Cernava et al., 2015a
Lobaria pulmonaria	Three different locations in Austria	*Betaproteobacteria, Gammaproteobacteria, Actinobacteria, Firmicutes*	Antagonistic activity	Cernava et al., 2015b

In the first study published on bacterial associates of Antarctic lichens, a number of psychrotolerant strains were identified (Selbmann et al., 2010). Of the thirty bacterial isolates, a new species of *Deinococcus-Thermus* was reported and several other strains indicated a number of possibly new taxa. The bacterial diversity of Antarctic and Arctic lichens was further studied by Kim et al. (2014) and Lee et al. (2014). Although no potential role of the microbiota within the symbiosis was suggested, the strains isolated from the crustose lichen *Ochrolechia* sp. had antimicrobial and antioxidant activity (Kim et al., 2014). The isolates were found to belong to *Sphingomonas* and *Burkholderia*, both of which genera were also abundantly found in the Antarctic and Arctic lichens (Lee et al., 2014). Lee et al. studied the microbiota of nine different lichens, revealing isolates affiliating with *Actinobacteria, Bacteroidetes, Deinococcus-Thermus, Firmicutes, Alpaproteobacteria, Betaproteobacteria*, and *Gammaproteobacteria*. Although previous studies using cultivation methods represented *Alphaproteobacteria* poorly in the culturable fraction (Cardinale et al., 2006; Selbmann et al., 2010), they were found to be predominant in Lee's study. The majority of the alphaproteobacterial isolates were affiliated with the genus *Sphingomonas* and isolated from lichens collected from the Arctic and Antarctica. In addition to *Sphingomonas*, bacterial members of the genera *Frondihabitans, Hymenobacter,* and *Burkholderia* were isolated across lichen samples from both the Arctic and Antarctica and at lower abundancies, members of *Nakamurella, Streptomyces, Deinococcus, Paenibacillus, Aurantimonas, Methyloferula, Psycrobacter, Pseudomonas,* and *Rhodanobacter* were recovered. Based on functional tests, the overall microbiota of the Arctic and Antarctic lichens were found to have protease and lipase activity and suggested to be involved in nutrient supply for the symbiosis as a whole.

In summary, cultivation-based studies have in general revealed that the culturable non-phototrophic microbiota of lichens tends to be dominated by members of the phyla *Actinobacteria, Firmicutes* and *Proteobacteria*. Some studies also yielded members of *Deinococcus-Thermus, Bacteroidetes,* and *Acidobacteria*. While some of the studies did not consider potential roles of the lichen associates, we can surmise from these studies that the culturable fraction of lichen-associated bacteriomes are involved in nitrogen fixation, phosphate solubilisation, hormone production, nutrient supply via various lytic activity and play roles in defense mechanisms through their antagonistic activity.

1.8.2 Bacterial diversity via culture-independent methods

While the diversity of bacterial lichen-associates has been described, new species published or bioactive products discovered in many studies (Davies et al., 2005; Yamamura et al., 2011), the culturable fraction is nevertheless believed to represent only a small part of the total bacterial biota in environmental samples (Amann, Ludwig, & Schleifer, 1995). Several methods can be used to expose the unculturable bacterial fraction including microbial fingerprinting, where PCR product yield analyzable banding patterns (Portillo, Villahermosa, Corzo, & Gonzalez, 2011). Different primers can be used, either universal or group specific, and the bands can further be excised from the gel matrix and characterized. Such fingerprinting methods have been used in a number of studies, revealing the taxonomic diversity of the total bacterial biota of lichens (see Table 2 for overview). Although fingerprinting methods have in general many advantages, such as comparatively low cost, are less time consuming, and their banding patterns can directly be compared between samples, these methods generally lack the resolution required for a thorough phylogenetic inference (Grube & Berg, 2009). Instead, the analysis of 16S rDNA libraries is commonly used to identify the total bacterial community in lichens.

Table 2. A summary of culture in-dependent studies of lichen associates

Lichen species	Lichen habitat	Technique(s) applied	Taxonomic diversity of the associated microbiota	Potential activity of the associated microbiota	Reference
Cladonia digitata, C. rangiferina, C. coniocraea, C. pyxidata, C. coccifera, Pseudevernia furfuracea, Hypogymnia physodes, Rocella phycopsis, R. fuciformis		ITS fingerprinting, sequencing of bands	*Betaproteobacteria, Gammaproteobacteria,* Actinobacteria	N-fixing	Cardinale et al., 2006
Cladonia arbuscula	Mountain ridge in Styria, Austria	FISH, SSCP	*Alphaproteobacteria,* Actinobacteria, *Betaproteobacteria*	None suggested	Cardinale et al., 2008
Cladonia cristatella, C. cryptochlorophaea, C. sobolescens, C. peziziformis, C. subtenuis, Flavoparmelia caperata, Parmotrema perforatum, Peltigera phyllidiosa	Virginia and N-Carolina, USA	Sanger sequencing (direct of PCR products using universal primers)	*Alphaproteobacteria,* Acidobacteria, *Gammaproteobacteria*	None suggested	Hodkinson & Lutzoni, 2009
Cladonia arbuscula, Lecanora polytropa, Umbilicaria cylindrica	Saxicolous above tree line in Styria, Austria	FISH, SSCP	*Alphaproteobacteria,* Actinobacteria, *Betaproteobacteria*	High abundance of *nifH* genes, diazotrophic bacteria	Grube et al., 2009

Hydropunctaria maura, Ophioparma ventosa, Pertusaria corallina, Rhizocarpon geographicum	Saxicolous lichens from SW-Norway	DGGE, clone libraries	*Alphaproteobacteria, Betaproteobacteria,* Actinobacteria, Acidobacteria, Firmicutes, Chloroflexi, Bacteroidetes	None suggested	Bjelland et al., 2011
Xanthoparmelia plittii, X. somloensis	Foliose lichens sampled in Massachusetts, USA	Pyrosequencing of 16S rRNAs	*Alphaproteobacteria,* Acidobacteria, Bacteroidetes, *Gammaproteobacteria, Deltaproteobacteria*	None suggested	Mushegian et al., 2011
Lobaria pulmonaria	Foliose lichen, arboicolous	FISH, pyrosequencing	*Alpaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria,* Bacteroidetes, Actinobacteria, Firmicutes, Verrucomicrobia	Nutrient availability (various lytic activity), resource reallocation (amino acid release)	Schneider et al., 2011
Parmelia sulcate, Rhizoplaca chrysoleuca, Umbilicaria americana, U. phaea	Foliose lichens from granite rock outcrops, Colarado, USA	Pyrosequencing of 16S rRNAs	*Alphaproteobacteria,* Acidobacteria, *Gammaproteobacteria*, Firmicutes, Verrucomicrobia, Planctomycetes, Actinobacteria, *Betaproteobacteria,* Bacteroidetes, *Deltaproteobacteria*	N-fixing, phosphate solubilisation	Bates et al., 2011
Lobaria pulmonaria	Styria, Austria	FISH, SSCP	*Alphaproteobacteria*	N-fixing	Cardinale et al., 2012a

Cetraria islandica, Lobaria pulmonaria, Lecanora polytropa, Cladonia arbuscula, Umbilicaria cylindrical, Cladonia coccifera	Different parts of thalli sampled (e.g. old, whole, young), Styria, Austria	FISH, SSCP	*Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria,* Actinobacteria	None suggested	Cardinale et al., 2012b
Solorina crocea	Styria, Austria	Pyrosequencing of 16S rRNAs	Acidobacteria, Proteobacteria, Planctomycetes, Actinobacteria	N-fixing	Grube et al., 2012
Cladonia sp., *Flavocetraria* sp., *Ophioparma* sp., *Umbilicaria* sp., *Usnea* sp., *Dictyonema* sp., *Leptogium* sp., *Peltigera* sp., *Sticta* sp.	Tropical or arctic latitudes	Pyrosequencing of 16S rRNAs	*Alphaproteobacteria,* Acidobacteria, *Betaproteobacteria, Gammaproteobacteria,* Actinobacteria, Verrucomicrobia, Planctomycetes, Bacteroidetes, Deltaproteobacteria, Firmicutes	None suggested	Hodkinson et al., 2012
Cetraria aculeata	Collected from different places; Antarctica, Spain, Germany and Iceland	PCR with group specific primers, clone libraries	*Alphaproteobacteria*	None suggested	Printzen et al., 2012
Arthrocaphis citronella, Baeomyces placophyllus, B. rufus, Icmadophila ericetorum, Psora decipiens, Trapeliopsis granulosa	Styria, Austria	FISH	*Alphaproteobacteria,* Acidobacteria	None suggested	Muggia et al., 2013

Lobaria pulmonaria	Maple trees in the Alps	Pyrosequencing of 16S rRNAs	*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Epsilonbacteria*, Actinobacteria, Acidobacteria, Bacteroidetes	Nutrient supply, pathogen defence, abiotic stress resistance, detoxification of metabolites, lytic activity	Grube et al., 2015
Lobaria pulmonaria	Styria, Austria	SSCP, pyrosequencing of 16S rRNAs, FISH	*Alphaproteobacteria*, Bacteroidetes, Verrucomicrobia, *Deltaproteobacteria*, Actinobacteria, *Betaproteobacteria*, *Gammaproteobacteria*, Acidobacteria, Planctomycetes	None suggested	Aschenbrenner et al., 2014
Lobaria pulmonaria	Three different locations in Austria	Pyrosequencing of 16S rRNAs	*Betaproteobacteria*, *Gammaproteobacteria*, Actinobacteria, Firmicutes	Antagonistic activity	Cernava et al., 2015b
Lobaria pulmonaria	Two mountain forests	Pyrosequencing of 16S rRNAs, FISH	*Alphaproteobacteria*	N-fixing, auxin and vitamin production, stress protection	Erlacher et al., 2015

The location of bacteria in the symbiotic structure has been established in a number of studies, using fluorescent *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM). In the first published study where FISH-CLSM was used to analyze lichen-associated bacteria, a protocol for cryosections of lichen fragments was optimized (Cardinale et al., 2008). In cryosected specimens of a *Cladonia arbuscula* thallus, about 6×10^7 bacteria per gram were found. Dense colonization of bacteria was mainly found on the internal thallus surfaces. The universal FISH probe EUB338 was used to detect the abundancy of total bacteria and about 86% cells were stained. Group-specific FISH probes were then further used in the study, revealing the prominence of *Alphaproteobacteria* (>60% of all bacteria). *Actinobacteria* and *Betaproteobacteria* were also detected although at much lower abundances. Few members of Firmicutes were found and *Gammaproteobacteria* were not detected. In this pioneering study, lichens were found to host more bacteria than previously thought and the method has since been used in several studies. Grube et al. (2009) studied the bacterial diversity of three lichen species, *C. arbuscula, Lecanora polytropa,* and *Umbilicaria cylindrica,* collected from alpine environments. The number of bacteria per gram specimen of *C. arbuscula* were comparable to the previous study of Cardinale et al. (2008) but lower numbers were obtained for *L. polytropa* and *U. cylindrica*, 7×10^5 and 9×10^5, respectively. Using FISH group-specific probes, *Alphaproteobacteria* were revealed to form 45 – 75% of the total bacterial biota but other groups were much fewer. In this work, bacterial fingerprints from the three lichen species were compared for universal bacterial and group-specific fingerprints (*Alphaproteobacteria, Pseudomonas* and *Burkholderia*) and the bacterial biota found to be species-specific (Grube et al., 2009).

Interestingly, relatively few studies thus far have focused on the non-phototrophic bacteria of crustose lichens. In one of the few published work, samples of *Hydropunctaria maura, Ophioparma ventosa, Pertusaria corallina,* and *Rhizocarpon geographicum* were analysed (Bjelland et al., 2011). In this study, DGGE and clone library sequencing were used to determine the composition and abundance of bacteria. *Acidobacteria, Proteobacteria* (*Alpha-* and *Betaproteobacteria*), and *Chloroflexi* classes dominated the bacterial biota in *O. ventosa, P. corallina,* and *R. geographicum* although *O. ventosa* was slightly different and more often associated with *Beta-* and *Gammaproteobacteria.* In terms of abundance, the samples in this study had similar numbers of bacteria per cell lichen thallus as previously reported by Cardinale et al. (2008) and Grube et al. (2009). *H. maura* had however higher number, 1.8×10^{11} g^{-1} and the

bacterial community reflected marine influence which was not detected in the other three lichen samples. Moreover, *Archaea* were, for the first time, found to be associated with lichens in this study.

Many studies have used culture independent methods to elucidate the functional role of the lichen-associates. In Grube's recent work (Grube et al., 2015) the functional gene composition of the lung lichen *Lobaria pulmonaria* was revealed as well as the taxonomic structure. Of the obtained bacterial contigs, *Proteobacteria* were dominant with *Alphaproteobacteria* the prominent taxon but members of *Beta-*, *Gamma-* and *Deltaproteobacteria* also present. Members of the taxa *Actinobacteria*, *Acidobacteria*, and *Bacteriodetes* were found in 2, 2, and 1% of the total bacterial contigs, respectively. Functional analyses of the lichen genome was carried out by using SEED and KEGG functional analyses. The analyzed contigs were found to be involved in primary metabolism such as carbohydrate, energy, lipid, nucleotide and amino acid metabolism, glycan biosynthesis and genetic information processing. The metagenome was further screened for selected functions where contigs involved in e.g. nutrient supply, resistance against biotic and abiotic stress factors, hormone production and various lytic activities were detected.

1.8.3 Functional roles of lichen associates

Nitrogen is one of the essential elements lichens need for maintaining growth. In cyanolichens, the photobiont can gain nitrogen from the atmosphere thus nitrogen fixation has been suggested to be one of the important role of the non-cyanobacterial prokaryotes of lichens. Nitrogen is abundant in nature, however, it is not always in an available form for assimilation. It must thus be fixed to usable form and the transformation is mediated by nitrogenase enzymes (Shridhar, 2012). Nitrogen fixing bacteria, often referred to as diazotrophs, might deliver nitrogen for the symbiotic partners of lichens (Kneip, Lockhart, Voß, & Maier, 2007) which would especially be an important role in non-cyanobacterial lichens and in the case of nitrogen limiting conditions (Grube & Berg, 2009).

Phosphorus (P) is another essential macronutrient for plants and lichens and is involved in all major metabolic pathways (Hayat, Ali, Amara, Khalid, & Ahmed, 2010; Sharma, Sayyed, Trivedi, & Gobi, 2013; Zhao et al., 2014). In soil, P exists as organic and inorganic phosphates and needs to be converted to soluble form for uptake by plants (Richardson, Hadobas, Hayes, O'Hara, & Simpson, 2001). Phosphate solubilizing bacteria (PSB)

have the ability to release P and make it available to plants (Chhabra et al., 2013; Richardson et al., 2001; Sashidhar & Podile, 2010; Sharma et al., 2013; Zhao et al., 2014). The pathways involved in phosphate solubilation are complex and several key enzymes are needed, including alkaline and acid phosphatases, phytases, and phosphonatases (Rodríguez, Fraga, Gonzalez, & Bashan, 2006; Sharma et al., 2013). Organic acids, especially gluconic acid, are essential for acidification, a key step in the dissolution of many poorly soluble mineral phosphatase. Goldstein (1995) proposed the direct oxidation of glucose to gluconic acid (GA), and in some cases 2-ketogluconic acid, to be a key step of the mineral phosphate solubilization (MPS) in some Gram-negative bacteria. Membrane-bound glucose dehydrogenase mediates the pathway and requires pyrroloquinolline quinone (PQQ) as a cofactor (Goldstein, 1995). Bacteria of various genera have been reported to be efficient in phosphate solubilization, such as members of *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aerobacter, Flavobacterium,* and *Erwinia*. The bacteria can solubilize mineral phosphate (inorganic compounds), including tricalcium phosphate, hydroxyl apatite ($Ca_{10}(PO_4)_6(OH)_2$), and rock phosphate (Rodríguez et al., 2006; Rodríguez & Fraga, 1999).

In many of the recent functional and metagenomics studies, one of the suggested trait of the bacterial microbiota of lichens is their wide range of lytic activities including chitinolysis, proteolysis, and glucanolysis (Grube et al., 2015, 2009; Lee et al., 2014; Schneider et al., 2011). Those extracellular enzymes are relatively abundant among lichen associates and are suggested to be involved in nutrient availability within the lichen symbiosis and contribute to the hydrolysis of organic compounds. The mycobiont comprises the major part of a lichen thallus and as fungal cells are mixtures of polysaccharides and proteins, bacterial strains with lytic activity are likely to degrade older parts of the thallus where antibacterial compounds are no longer present. Many foliose and fruticose lichens grow in cellulose-rich environments and are often in close proximity to mosses and other vegetation, thus cellulolysis might be particularly important for many lichen symbiosis albeit lichens generally obtain their carbon from the photosynthetic partner (Palmqvist et al., 2008) it has been suggested that cellulase systems, found within lichens, are used for saprophytic activity which could be beneficial for the symbiosis, e.g. when lichens are covered by snow (Beckett, Zavarzina, & Liers, 2013).

Plant growth-promoting rhizobacteria (PGPR) can influence plant growth by synthesizing and exporting phytohormones which may act as regulators in plant growth and development (Hayat et al., 2010). One of the physiologically most active phytohormones in plants is indole-3-acetic acid (IAA) and is produced by several lichen associates (Grube et al., 2009; Liba et al., 2006). In plants, production of IAA is known to stimulate root elongation and long-term (e.g. cell division and differentiation) responses (Hayat et al., 2010; Spaepen, Vanderleyden, & Remans, 2007). IAA has been shown to effect fungal growth (Gryndler, Hršelová, Chvátalová, & Jansa, 1998) and cell division in unicellular algae (Lau, Shao, Bock, Jürgens, & De Smet, 2009). Therefore, IAA producing bacteria have been suggested to alter morphogenetic processes of lichen symbiosis and influence both the mycobiont and algal partner (Grube & Berg, 2009).

Bacteria of the phylum *Actinobacteria* are known for their antimicrobial properties and biosynthetic potential (Jose & Jebakumar, 2013) and have commonly been isolated or detected using culture independent methods, from various lichen species. It has thus been suggested that lichen associates are involved in the defense mechanism of lichens (Cernava, Muller, et al., 2015; Grube et al., 2015, 2009).

1.8.4 Lichen *in vitro* cultures

Effort to culture the fungal and algal partners of lichens can be traced back to as early as the 1880s where Bonnier (1887, 1889) and Stahl (1877) published their successful results in culturing lichen fungi (Stocker-Wörgötter, 2001). Since then, several reports are of culture of the fungal partners of lichens (Stocker-Wörgötter, 2001), however, only a few cultures of lichen-forming fungi are available and the culture conditions for the majority of mycobiont species are as of yet unknown (McDonald, Gaya, & Lutzoni, 2013). Axenic cultures of lichen symbionts have been suggested to be successful for investigating the interaction between the lichen partners, although separating them might be a difficult process due to their obligate relationship in the symbiosis (Joneson & Lutzoni, 2009). With *in vitro* techniques, it is possible to investigate the initial stages of lichenization process and moreover, acquaint different mycobionts with different photobionts, as developed by Ahmadjian (1993). In one of the recent studies on *in vitro* lichenization, the *Trebouxia* photobionts and mycobiont from *Protoparmeliopsis muralis* thalli were isolated and re-lichenized. Additionally, the green algea *Asterochloris* was introduced to

the mycobiont, which established primordial stages of lichenization (Guzow-Krzemińska & Stocker-Wörgötter, 2013).

2 Aims

Several authors have suggested that non-phototrophic lichen-associates play important, but so far poorly defined, roles within the lichen symbiosis. The main objective of this project was thus to address the hypothesis that lichen-associated bacteria play important roles in the symbiosis. The hypothesis was addressed by:

- Isolating and characterizing the diversity of non-phototrophic bacteria of four different seashore lichens, commonly found alongside the Icelandic coast.

- Isolating and characterizing the non-phototrophic bacterial diversity of *Peltigera membranacea*-associates and isolate key members in pure cultures.

- Characterizing the functional roles of non-phototrophic *P. membranacea*-associated bacteria, based on the presence of key bacterial genes in the *P. membranacea* metagenome.

3 Paper summary

In the following chapter, the work presented in the research papers will be summarized. Where appropriate, unsuccessful methodologies, and thus not included in the papers/manuscript, will be mentioned.

3.1 Paper I

The work presented in paper I was focussed on bacterial associates of crustose lichens, generally found along the Icelandic seashore. We sampled the four lichen species, *Lecanora helicopis, Verrucaria ceuthocarpa, Hydropunctaria maura* and *Caloplaca verruculifera*, from a rocky promontory near Akureyri, Iceland, in June 2009. *Ascophyllum nodosum* was sampled at the same location and used as a non-lichen control. Standardized isolation methods were used and all lichen samples were surface sterilized with 3% H_2O_2 prior to isolation of endolichenic bacteria. In total, we isolated 93 strains, representing 17 different colony types as determined by eye and under the dissection microscope. The final colony count varied among the lichen samples where *L. helicopis* yielded significantly lower numbers than the other lichens. In January 2012, we repeated the isolation and re-collected lichen samples from the same location as in June 2009. The isolation conditions were the same as previously, in terms of media, incubation temperature and time, and the results were in a good accordance to the previous results. Of the total of 93 isolates obtained from the seashore lichens, we identified 55 by partial 16S rRNA gene sequence analysis. The collection was found to contain members from 7 classes: *Alphaproteobacteria, Bacilli, Actinobacteria, Flavobacteria, Cytophagia, Sphingobacteria,* and *Gammaproteobacteria* and the similarity to entries in GenBank ranged from 86-100%. The overall microbial diversity differed between lichen species and *L. helicopis* yielded the lowest CFU g^{-1}, although more taxonomic diversity was observed from that sample than the other three species. We expect unculturable bacteria to be present although we believe the majority was culturable as the final counts presented in our study were in good accordance other recent studies. We screened a total of 27 isolates, selected on the basis of taxonomy and colony morphology, for their production of several extracellular enzymes. The majority of isolates displayed proteolytic, glucanolytic and/or amylolytic activity. Furthermore, a fairly large number of isolates showed phosphate-solubilizing activity but nitrogen fixation was observed for a small number of isolates.

3.2 Paper II

In our second paper we analyzed the microbiome of *Peltigera membranacea*, a foliose lichen commonly found all over Iceland. Fairly large lichen thalli (8-10 cm long) were sampled in September 2008 and cleaned; ephiphytes and extraneous material removed and samples then extensively washed. We used standardized methods for DNA extraction which was then processed for sequencing via Roche 454 technology. The 454 data resulted in approximately 1.74 Gb reads which were assembled into contigs and then further analyzed. Firstly, we sorted the contigs into three bins based on scores with tblastn against protein databases of filamentous ascomycetes and *Nostoc*. The remaining contigs were mainly bacterial. Secondly, we assigned the bacterial contigs to taxonomic groups by Infernal and SeqMatch matching against the RDP database. We selected 15 reference genomes to use as the database the contigs were compared to, using the blastn algorithm, and the metagenomic library then constructed. We extracted contigs with *E*-values below 10^{-3} from the library and composed the final set of data we then further analyzed. The contigs (a total of 30,033; 95 Mb) were imported into CLC Genomic Workbench and the blastx algorithm run against the nr-protein sequence database from NCBI. The prominent fraction of contigs belonged to *Proteobacteria* (86%), with *Actinobacteria* (8%), *Bacteroidetes* (3%), *Firmicutes* (1%), *Acidobacteria* (1%), and *Verrucomicrobia* (1%) present as well. The majority of proteobacterial coding sequences were from *Alphaproteobacteria* (55%), *Betaproteobacteria* (31%) and *Gammaproteobacteria* (13%). Furthermore, we manually mapped the contigs to metabolic pathways according to KEGG orthology groups and Cluster of Orthologous Groups (COG) categories. Nearly half of the sequences were mapped to metabolic contigs, about one-third were classified as enzymes participating in cellular processes and signaling or information storage and processing. The remaining contigs were poorly characterized. In this study, we hypothesized that phosphate solubilization is an important role of lichen associates. We, therefore screened, our dataset for enzymes participating in phosphate metabolism and found about 1335 contigs of which inorganic phosphatases were particularly prominent although other types, such as organic and alkaline phosphatases, were also present. Moreover, genes encoding pyrroloquinoline quinone (PQQ)-associated proteins were found in 27 contigs in our dataset and further supported our hypothesis for this study.

3.3 Paper III

The focus on our work presented in Paper III was on bacterial strains, isolated from *P. membranacea* thalli. Lichen thalli were sampled from a scrub heath environment in eastern Iceland in August 2012, April 2013 and April 2013. In each case, the lichen thalli were surfaced sterilized and endolichenic bacterial strains isolated. We used nine types of isolation media, focusing on obtaining a collection of bacterial isolates with certain biodegrative and other nutrient scavenging properties. All plates were incubated at three different temperatures; 4°C, 15°C and 22°C, resulting in a total of 110 bacterial strains isolated from the *P. membranacea* thalli. *Proteobacteria* dominate the culturable microbiota but representatives of *Bacteroidetes*, *Actinobacteria*, and *Firmicutes* were also isolated. As in paper I, we screened our isolates for secretion of various enzymes, including proteases and glycanaes. We found protease activity to be common among the isolates but glycanase activity (cellulase, glycanase and xylanase) was less common. In paper II we suggested phosphate solubilisation to be among the most important roles of lichen associates. In paper III we found nearly half of tested isolates from *P. membranacea* thalli forming clearing zones on NBRIP, underscoring the importance of phosphate solubilisation by lichen associates. Nitrogen fixation and degradation of naphtalene were found among a small part of the tested isolates. About half of the bacterial isolated were positive for bio surfactant activity.

We also wanted to investigate the location and abundance of bacteria directly in the *P. membranacea* thalli, using CLSM and the protocol optimized by Cardinale et al. (2008). We used FISH probes, specific for *Alphaproteobacteria, Burkholderiales, Rhizobiales, Gammaproteobacteria, Actinobacteria* and the universal probe EUB338, detecting all bacteria. Unfortunately, the background was too high from all in tube FISH, leading to no useful data obtained from the CLSM. Thus, the protocol needs to be adapted for *P. membranacea* thalli, adding washing steps and even optimize the hybridization further.

3.4 Paper IV

In Paper IV we review the current status on lichen associates, based on previous culture and culture in-dependent studies.

4 Conclusions and future prospects

A great many conclusions can be drawn from the work presented in this thesis. Among those are the following:

- The crustose seashore lichens *Lecanora helicopis, Verrucaria ceuthocarpa, Hydropunctaria maura,* and *Caloplaca verruculifera,* harbor high numbers of bacteria, culturable on MA agar at 15°C.
- The seashore lichen-associates were found to belong to 7 classes: *Alphaproteobacteria, Bacilli, Actinobacteria, Flavobacteria, Cytophagia, Sphingobacteria,* and *Gammaproteobacteria*.
- Seashore lichens harbor bacteria specifically associated with their lichen host.
- Seashore lichen-associates have several biodegradative properties, including proteolytic, glucanolytic and amylolytic activities, they can fix nitrogen from the atmosphere and are phosphate solubilizing.
- The bacterial metagenome of the terricolous lichen *Peltigera membranacea* suggests the non-phototrophic microbiota to consist of *Proteobacteria* as the major part, with *Actinobacteria, Bacteriodetes, Acidobacteria, Firmicutes,* and *Verrucomicrobia* also present.
- Many genes contributing to phosphate solubilization were found within the metagenome, suggesting the importance of bacterial phosphate solubilization for the lichen symbiosis.
- Non-phototrophic bacteria from *P. membranacea* thalli were culturable on various media types at 4°C, 15°C and 22°C.
- The culturable microbiota from *P. membranacea* is composed of *Proteobacteria, Bacteroidetes, Actinobacteria,* and *Firmicutes*.
- Bacteria, isolated from *P. membranacea* thalli, secreted biodegradative enzymes such as proteases, xylanases, cellulases, and chitinases. Furthermore, they are able to fix atmospheric nitrogen, solubilize phosphate, degrade naphthalene, and are biosurfactant producing.
- Our findings in Paper I are in general agreement with other recent findings; the culturable microbiota differs among lichen species although the bacterial biota mainly consists of *Proteobacteria*. Among the isolates from the four seashore lichens sampled, six isolates may represent novel taxa and need further attention. Our

findings from papers II and III also agree with previous studies on lichen associates. We used metagenomics methods to identify the associated microbiota of *P. membranacea,* to our knowledge, for the first time. Although phosphate solubilization among lichen associates has been identified as among likely roles for lichen-associated bacteria in recent studies, specific genes encoding proteins involved in phosphate solubilization have not been published previously (Paper II). In Paper III we isolated and characterized a number of bacteria from *P. membranacea* thalli, where several isolates may represent novel taxa. As we suggest in Paper IV, it is likely that bacteria can pass from lichen to the surrounding environment and vice versa, although previous studies have indeed stated that lichens harbor a specific bacterial biota (Paper I), distinct from the surrounding environment. A number of the isolates in paper III were gammaproteobacterial and are similar to bacteria known as plant associates; endophytic, plant growth-promoting or phytopathogenic bacteria. A search for several genes involved in lichen secondary metabolite resistance and genes likely to participate in plant pathogenicity, within the metagenome of *P. membranacea,* yielded several hits. Plant virulence genes were found additionally found, including homologs of type III and type IV secretion systems from diverse bacteria (Appendix 1). From the work presented in this thesis, not only the role of the associated microbiota of lichens can be in part elucidated, but a suggestion has emerged that lichens host several bacteria which may be phytopathogenic and, thus, lichens may serve as non-host reservoirs of these economically and environmentally important organisms. These speculations have thus far not been investigated at all and, require further attention.

- Lichens may be considered as potential reservoirs for phytopathogenic bacteria, such as *Pseudomonas syringae, Burkholderia glathei* and several xanthomonads, all of which have been observed in recent studies, either molecular or culture-based, in or on lichen thalli. Among facets that need to be studied in more detail before the role of lichens in plant pathogen ecology and evolution can be elucidated are systematic data mining for known virulence factors, identification and quantification of prophages in lichen-associated microbiomes, assessment of potential population exchange between lichens and plants and rigorous characterization of potentially plant pathogenic isolates.

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ARTICLE

Novel bacteria associated with Arctic seashore lichens have potential roles in nutrient scavenging

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Abstract: While generally described as a bipartite mutualistic association between fungi and algae or cyanobacteria, lichens also host diverse and heretofore little explored communities of nonphototrophic endolichenic bacteria. The composition and possible roles of these bacterial communities in the lichen symbiotic association constitute an emerging field of research. Saxicolous (rock-dwelling) seashore lichens present an unusual environment, characterized by rapid fluctuations in temperature, salinity, exposure to solar radiation, etc. The present study focuses on the bacterial biota associated with 4 species of crustose, halophilic, saxicolous seashore lichens found in northern Iceland. A denaturing gradient gel electrophoresis based characterization of the composition of the lichen-associated microbiotas indicated that they are markedly lichen-species-specific and clearly distinguishable from the environmental microbiota represented by control sampling. A collection of bacterial strains was investigated and partially identified by 16S rDNA sequencing. The strains were found to belong to 7 classes: *Alphaproteobacteria*, *Bacilli*, *Actinobacteria*, *Flavobacteria*, *Cytophagia*, *Sphingobacteria*, and *Gammaproteobacteria*. Several isolates display only a modest level of similarity to their nearest relatives found in GenBank, suggesting that they comprise previously undescribed taxa. Selected strains were tested for inorganic phosphate solubilization and biodegradation of several biopolymers, such as barley β-glucan, xylan, chitosan, and lignin. The results support a nutrient-scavenging role of the associate microbiota in the seashore lichen symbiotic association.

Key words: lichens, bacterial community, biodegradation, symbiosis, saxicolous.

Résumé : Bien que les lichens soient généralement décrits comme une association mutualiste bipartite entre un champignon et une algue ou une cyanobactérie, ils abritent également une flore diversifiée de bactéries endolichéniques non phototrophes aux attributs encore mal connus. La composition de ces communautés bactériennes et leurs rôles possibles au sein de l'association symbiotique du lichen sont au centre d'un domaine de recherche en émergence. Les lichens saxicoles (poussant sur les rochers) des littoraux présentent un environnement peu commun caractérisé par des fluctuations rapides de température, de salinité, d'exposition aux rayons solaires, etc. La présente étude s'intéresse à la flore bactérienne associée à quatre espèces de lichens crustacés, halophiles, saxicoles des littoraux du nord de l'Islande. La composition des microflores associées aux lichens, analysée par DGGE, s'est révélée hautement spécifique au lichen et facilement distinguable de la microflore environnementale de l'échantillon témoin. Un ensemble de souches bactériennes a été examiné et identifié partiellement par séquençage de l'ADNr 16S. On a constaté que les souches appartenaient à 7 classes : *Alphaproteobacteria*, *Bacilli*, *Actinobacteria*, *Flavobacteria*, *Cytophagia*, *Sphingobacteria* et *Gammaproteobacteria*. Sept isolats n'affichaient qu'une similitude modérée avec leur plus proche parent retrouvé dans GenBank, ce qui laisse croire qu'ils représenteraient un taxon nouvellement défini. Chez des souches choisies, on a analysé la capacité de solubilisation du phosphate et de biodégradation de plusieurs biopolymères, notamment le β-glucane d'orge, le xylane, le chitosane et la lignine. Les résultats viennent étayer le rôle de collecte de nutriments que l'on attribue à la microflore liée à l'association symbiotique du lichen littoral. [Traduit par la Rédaction]

Mots-clés : lichens, communauté bactérienne, biodégradation, symbiose, saxicole.

Introduction

Lichens are able to grow in practically any terrestrial surface environment and are thought by many to be among the earliest life-forms to colonize Earth's land surfaces (Gadd 2007). It has been estimated that they may cover up to 8% of the total land surface (Ahmadjian 1995; Haas and Purvis 2006). The lichen body can be regarded as a small ecosystem where at least 2 organisms can be found: an ascomycete or basidiomycete mycobiont and an algal or cyanobacterial photobiont (Tehler and Wedin 2008). Recent studies have shown that lichens host substantial communities of nonphototrophic bacteria in terms of cell density (Grube et al. 2009) as well as phylogenetic diversity (Hodkinson and Lutzoni 2009; Selbmann et al. 2010; Bates et al. 2011). Although the presence of nonphototrophic bacteria within lichen thalli has been known for a long time (Henckel and Yuzhakova 1936; Henckel and Plotnikova 1973), they did not receive much attention from the research community (Banfield et al. 1999) until recently, and are now regarded as significant players in the symbiosis. Many lichens grow on extremely nutrient-poor substrates, suggesting that these nonphototrophic bacteria provide a substantial source of crucial nutrients (González et al. 2005; Liba et al. 2006; Hodkinson et al. 2012). Recent studies have suggested that *Alphaproteobacteria* is the most common taxa in growing parts of lichens, although higher diversity is present in older parts (González et al. 2005; Liba et al. 2006; Cardinale et al. 2008; Hodkinson et al. 2012; Grube et al. 2013).

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Seashore lichens in Iceland are relatively well known. Several species are restricted to seashore rocks while others have broader habitats. There are 26 obligate marine species that show zonation from 5 different zones: littoral, mesic supralittoral, submesic supralittoral, xeric supralittoral, and terrestrial (Baldursdóttir 1985). Several species of the family Verrucariaceae, whose taxonomy and nomenclature have recently been re-examined (Gueidan et al. 2009), are confined to marine habitats and are most prominent in the littoral zone with some of the species such as *Wahlenbergiella mucosa*, even growing below the intertidal zone. Other Verrucariaceae can be found in the intertidal zone, with *Hydropunctaria maura* as the most abundant one being a very prominent part of Icelandic seashore communities often visible as a blackish zone just above the intertidal zone. In the *H. maura* zone, *Lichina confinis* (Lichinaceae) is often abundant. Higher up but still under marine influence is the mesic supralittoral zone, where other species belonging to different families, such as *Caloplaca marina*, *Caloplaca verruculifera* (Teloschistaceae), and *Lecanora helicopis* (Lecanoraceae), can be found.

For this study, 4 saxicolous, crustose lichen species commonly found on seashores in Iceland were sampled at a northern Iceland seashore crag. The main aims were to assess the microbial community diversity in the lichen thalli, to identify selected strains to the genus level by partial 16S rDNA sequencing to determine several key indicators of their potential functional roles in the symbiotic association, and to address the question of whether lichen-associated bacteria contribute to nutrient sequestration in these lichens.

Materials and methods

Sampling

Four lichen species, *L. helicopis* (sample AR01), *Verrucaria ceuthocarpa* (AR02), *H. maura* (AR03), and *C. verruculifera* (AR04) were collected at a small, rocky promontory in Sílastaðatangi, Eyjafjörður, Iceland (lat 65.725, long –18.146) in June 2009. Lichen samples from the intertidal zone were collected at 4 locations within an area of 1 Ha. At each location, fragments of lichen species were collected together with the underlying rock substrate using a sterile hammer and a chisel. In addition, a sample of knotted kelp (*Ascophyllum nodosum*) was collected as a non-lichen control (AR05). The samples were transferred into sterile polyethylene bags until processing, which occurred within 1 h of sampling.

Media, culture conditions, and strain isolation

Samples were either washed with 3.0% H_2O_2 for surface sterilization or extracted without treatment. Samples (100 mg) of both peroxide-treated and nontreated lichens were scraped off the rock substratum using a sterile scalpel or spatula and ground in sterile 1.5% saline using a sterile mortar and pestle. The ground sample was serially diluted in sterile 1.5% saline and plated on marine agar (MA, Becton Dickinson, Franklin Lakes, New Jersey, USA) in duplicate. A sterility check was performed on peroxide-treated samples by pressing the sample against an MA plate. Plates were incubated in the dark at 15 °C for 3 weeks. Growth was monitored periodically by colony counts for up to 8 weeks. The plates were examined for colony morphotypes based on color, texture, opaqueness, concavity, size, and other visible features. Representatives of each morphotype were streaked onto fresh MA to obtain pure cultures. Stocks of purified isolates were prepared by suspending a loopful of growth in 1.0 mL of 28% (*v*/*v*) glycerol and are stored at –80 °C in the culture collection of the University of Akureyri.

Biodegradation properties

Degradation assays were performed on 1.5% agar plates as follows. Plates were incubated for 5 days or until growth was visible. Starch hydrolysis was assayed on nutrient agar (Becton Dickinson) supplemented with 0.2% soluble starch and 5% NaCl. Colonies were rinsed off with sterile water and the plate flooded with iodine. A clear halo was indicative of starch hydrolysis (Tindall et al. 2007). Positive and negative controls were *Bacillus subtilis* DSM 10 and *Escherichia coli* DSM 1103, respectively. Glycanolytic activity was tested against barley β-glucan, birch xylan, and chitosan in half-strength tryptic soy agar (Becton Dickinson), supplemented with 5% NaCl and the appropriate 0.2% (*m*/*v*) azurine cross-linked polymers (Megazyme, Wicklow, Ireland). Appearance of blue halos was indicative of degradation. Negative control was provided by *E. coli* DSM 1103. Positive controls were *Saccharophagus degradans* DSM 17024, *Bacillus* sp. strain KA0902, and *Cellulophaga chitinilytica* DSM 17922 for β-glucan, xylan, and chitosan, respectively. Casein degradation was assayed by the appearance of clear halos on 50% (*v*/*v*) skim milk in nutrient agar. *Bacillus subtilis* DSM 10 and sphingomonad UA-AR0413 were used as positive and negative controls, respectively. Luxuriant growth and clear halos on NBRIP medium (Nautiyal 1999), supplemented with 5% NaCl, and nitrogen-free agar was taken to indicate possible ability to mobilize inorganic phosphates and nitrogen fixation. *Rhodococcus* sp. strain KA1105 and *Bacillus* sp. strain KA0514 were employed as positive and negative controls for both tests, respectively. Degradation of Azure B (trimethyl thionine) was taken to be indicative of partial lignin biodegradative potential (Pangallo et al. 2007) and was indicated by appearance of clear halos on R2A (Becton Dickinson) supplemented with Azure B (0.2 g/L) and 5% NaCl. Positive and negative controls were *Rhodococcus* sp. strain KA0206 and *E. coli* DSM 1103, respectively.

Phylogenetic analysis

Isolated strains were selected for identification based on colony morphology and (or) biodegradative properties. For DNA extraction from pure cultures the Ultra Clean Microbial DNA isolation kit (MoBio Laboratories, Carlsbad, California, USA) was used according to the manufacturer's instructions. Extracted DNA was PCR amplified in an MJR PTC-200 thermocycler (MJ Research Inc., Massachusetts, USA) using the universal primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) at a final concentration of 1.0 μmol/L in a total volume of 25 μL of PCR mixture. The PCR reaction was performed as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 51 °C for 45 s, 72 °C for 30 s, and a final extension was performed at 72 °C for 7 min. PCR products were purified with the NucleoSpin Extract II (Macherey-Nagel GmbH & Co. KG, Düren, Germany) kit according to the manufacturer's protocol. Partial sequencing of the purified PCR products was performed with a BigDye terminator kit and run on Applied Biosystems 3130XL DNA analyzer (Applied Biosystems, Foster City, California, USA) at Macrogen Europe, Amsterdam, the Netherlands. The obtained 16S rDNA sequences were subjected to multiple alignments at the Ribosomal Database Project (Cole et al. 2009). Reference sequences were selected from the database. *Escherichia coli* X80725 provided the outgroup sequence. The evolutionary history was inferred using MEGA5 program with neighbor-joining method (Tamura et al. 2011).

DGGE-based DNA fingerprinting

For the extraction of total DNA from lichen samples, 100 mg was ground in liquid nitrogen, suspended in extraction buffer (1% (*m*/*v*) cetyl trimethyl ammonium bromide, 1 mol/L NaC1, 100 mmol/L Tris, 20 mmol/L EDTA, 1% (*m*/*v*) polyvinyl polypyrrolidone), and sonicated in a Branson 5510 sonicator bath (Branson Ultrasonic Corp., New York, USA). The extracts were heated at 70 °C and further extracted in 24:1 (*v*/*v*) chloroform : isoamyl alcohol followed by ethanol precipitation and resuspension in TE buffer (10 mmol/L Tris, 1 mmol/L EDTA). A sample of *Sterocaulon alpinum*, collected in August 2011 (Hljóðaklettar, Iceland, lat 65.950, long –16.533), was used as an inland lichen control. Extracted DNA was PCR amplified in an MJR PTC-200 thermocycler (MJ Research Inc.)

Table 1. Colony types as determined by eye and under the dissection microscope.

Type	Colour	Sheen[a]	Convexity[a]	Other features	Representative strains
A	Black, white fizz on top	–	+	Mouldy appearance; hard to the touch	UA-AR0101, UA-AR0102, UA-AR0116
B	Buff	+	0		UA-AR0105, UA-AR0106, UA-AR0107, UA-AR0115, UA-AR0224, UA-AR0230
C	Bright yellow	0/+	0	Veins visible under the dissection microscope	UA-AR0104, UA-AR0202, UA-AR0238, UA-AR0305, UA-AR0413, UA-AR0416, UA-AR0419, UA-AR0433, UA-AR0436, UA-AR0502, UA-AR0504, UA-AR0511, UA-AR0516
D	Orange	++	0/+	Small colonies; veins sometimes visible	UA-AR0110, UA-AR0113, UA-AR0132, UA-AR0218, UA-AR0220, UA-AR0301, UA-AR0303, UA-AR0316, UA-AR0335, UA-AR0336
E	White (light buff)	+	–/0		UA-AR0109, UA-AR0121, UA-AR0125, UA-AR0204, UA-AR0206, UA-AR0208, UA-AR0215, UA-AR0222, UA-AR0232, UA-AR0302, UA-AR0307, UA-AR0313, UA-AR0315, UA-AR0410, UA-AR0422, UA-AR0432, UA-AR0435, UA-AR0505
F	Buff	–	–	Filiform margin; very large colonies; surface motility	UA-AR0117
G	Bright yellow	+	+/++		UA-AR0127, UA-AR0328, UA-AR0503
H	Creamy yellow	+	0		UA-AR0103, UA-AR0119, UA-AR0322, UA-AR0414, UA-AR0424
I	Pink	++	0		UA-AR0120, UA-AR0213, UA-AR0320, UA-AR0418
J	Red or dark orange	–/0	++/+++	Irregular, lumpy form	UA-AR0131, UA-AR0219, UA-AR0227, UA-AR0309, UA-AR0430
K	Colorless	++/+++	–/0		UA-AR0124, UA-AR0217, UA-AR0225, UA-AR0235, UA-AR0241, UA-AR0329, UA-AR0427, UA-AR0428
L	Bright red	+	+	Veins visible under the dissection microscope	UA-AR0129, UA-AR0216, UA-AR0221, UA-AR0229
M	White	–/0	+++	Rough, crystalline appearance	UA-AR0311, UA-AR0412
O	Pastel yellow	0/+	0		UA-AR0114, UA-AR0134, UA-AR0226, UA-AR0325, UA-AR0420
P	White	0/+	–	Undulate to lobate colony margin; surface motility	UA-AR0245, UA-AR0310, UA-AR0506
R	Dark yellow	–	0/+		UA-AR0423
S	Light yellow	–/0	–/0	Undulate to lobate colony margin; surface motility	UA-AR0201, UA-AR0501

[a]+, ++, +++, indicates that the indicated property is more pronounced than normally observed for *Escherichia coli* under standard growth conditions; –, less pronounced; 0, about equally pronounced.

using the primer pair 515F-GC (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGGCCAGCAGCCGCGGTAA-′3) and 806R (5′-GGACTACCAGGGTAT-CTAAT-′3) (McIntosh et al. 2008) at a final concentration of 1.0 μmol/L in a total volume of 25 μL of PCR mixture, containing approximately 10 ng of purified DNA and 0.3 μL of *Taq* polymerase (New England Biolabs, Ipswich, Massachusetts). The PCR reaction was as follows: initial denaturation was at 94 °C for 5 min, followed by 30 cycles of 50 s at 94 °C, 1 min at 56 °C, and 1 min at 72 °C, and a final extension step at 72 °C for 5 min. DGGE analysis was performed as described by Muyzer et al. (1993) in Dcode Universal Mutation Detection System (BioRad Laboratories, München, Germany). PCR products were loaded onto a 40%–60% denaturing gradient of urea–formamide in a 8% polyacrylamide gel and electrophoresed at 60 °C under 60 V for 14 h. The gel was subsequently stained in SYBR Gold Nucleic Acid Stain (Invitrogen, California, USA) and imaged under UV light using the Syngene InGenius LHR gel documentation system (Synoptics Ltd., Cambridge, UK).

Results

Bacterial isolation

Culturable bacteria on MA plates were enumerated by colony count after 5, 7, 9, 12, and 18 days of incubation at 15 °C (Fig. 1). The viable count in all samples increased throughout the incubation time, except for the knotted kelp control sample (AR05) whose viable count remained steady after 9 days of incubation. Therefore, the lichen-associated microbiota was found to be in large part composed of slowly growing bacteria, whereas the bulk of the knotted kelp-associated microbiota grew rapidly on MA at 15 °C. The final count varied among the samples from 6.0×10^4 to 3.5×10^8 CFU/g, with *L. helicopis* yielding significantly (by Student's *t* test, $p < 0.05$) lower colony counts than the other lichens. However, the peroxide-treated *L. helicopis* sample was found to contain a substantial count of slowly growing microbiota that appeared after 18 days of incubation.

Table 2. The most common colony types and their relative abundance in lichen samples after 18 days of incubation on marine agar plates at 15 °C.

Sample[a]	Dominant colony	2nd colony type[b]	3rd colony type[b]	Other colony types
AR01	D (92%)	G (2%)	E (2%)	A, B, C
AR02	L (49%)	D (44%)	E (5%)	B, C, I
AR03	D (73%)	E (9%)	L (9%)	B, C, K
AR04	C (63%)	B (13%)	E (8%)	R, L, K, D, O
AR05	B (68%)	C (26%)	E (6%)	S, K

[a]AR01, *Lecanora helicopis;* AR02, *Verrucaria ceuthocarpa;* AR03, *Hydropunctaria maura;* AR04, *Caloplaca verruculifera;* and AR05, *Ascophyllum nodosum.*

[b]For a description of colony types, see Table 1. Percentages are the mean values determined from colony counts of at least 2 countable plates or, where necessary, estimated by plate section count.

The peroxide treatment was not sufficient to sterilize the outer surface of the lichens, as determined by touch-plating on MA agar (data not shown), indicating that in spite of the rigorous washing and peroxide treatment, some of the isolates are likely to be associated with the lichen surface rather than endolichenic.

A total of 93 isolates from the seashore lichens listed in the section Sampling were subcultured and deposited into the University of Akureyri culture collection where they are stored in

Table 3. Identity of cultured strains as revealed by partial 16S rRNA gene sequencing.[a]

Strain	Colony type[b]	Source lichen	GenBank acc. No.	Most similar Megablast refseq_rna hit[c] (acc. No., aligned sequence length, % identity)	Class
UA-AR0101	A	*Lecanora helicopis*	JX874140	*Streptomyces microflavus* (NR_043854, 925, 99)	*Actinobacteria*
UA-AR0104	C	*L. helicopis*	JX874173	*Salinibacterium amurskyense* (NR_041932, 922, 99)	*Actinobacteria*
UA-AR0105	B	*L. helicopis*	JX874141	*Agrococcus baldri* (NR_041543, 954, 99)	*Actinobacteria*
UA-AR0109	E	*L. helicopis*	JX874142	*Salinibacterium amurskyense* (NR_041932, 625, 99)	*Actinobacteria*
UA-AR0110	D	*L. helicopis*	JX874174	*Psychrobacter faecalis* (NR_028966, 930, 99)	*Gammaproteobacteria*
UA-AR0113	D	*L. helicopis*	JX874175	*Psychrobacter faecalis* (NR_028966, 955, 99)	*Gammaproteobacteria*
UA-AR0114	O	*L. helicopis*	KF914714	*Micrococcus antarcticus* (NR_025285, 995, 99)	*Actinobacteria*
UA-AR0116	A	*L. helicopis*	JX874169	*Streptomyces microflavus* (NR_043854, 742, 99)	*Actinobacteria*
UA-AR0117	F	*L. helicopis*	JX874176	*Staphylococcus cohnii* (NR_036902, 924, 98)	*Bacilli*
UA-AR0119	H	*L. helicopis*	JX874143	*Agrococcus jenensis* (NR_026275, 627, 99)	*Actinobacteria*
UA-AR0120	I	*L. helicopis*	JX874144	*Salinicoccus roseus* (NR_026311, 346, 98)	*Bacilli*
UA-AR0125	E	*L. helicopis*	JX874145	*Bacillus asahii* (NR_040793, 306, 95)	*Bacilli*
UA-AR0127	G	*L. helicopis*	JX874146	*Eudoraea adriatica* (NR_042628, 626, **86**)[d]	*Flavobacteria*
UA-AR0129	L	*L. helicopis*	JX874147	*Hymenobacter actinosclerus* (NR_026470, 671, **90**)[e]	*Cytophagia*
UA-AR0201	S	*Verrucaria ceuthocarpa*	JX874148	*Bacillus murimartini* (NR_042084, 342, 99)	*Bacilli*
UA-AR0209	E	*V. ceuthocarpa*	JX874177	*Psychrobacter maritimus* (NR_027225, 961, 99)	*Gammaproteobacteria*
UA-AR0213	I	*V. ceuthocarpa*	KF914715	*Rhodothermus marinus* (NR_074728, 475, **88**)	*Bacteroidetes* Incertae sedis
UA-AR0216	L	*V. ceuthocarpa*	JX874178	*Sphingopyxis baekryungensis* (NR_043014, 983, 99)	*Alphaproteobacteria*
UA-AR0222	E	*V. ceuthocarpa*	JX874189	*Psychrobacter maritimus* (NR_027225, 697, 97)	*Gammaproteobacteria*
UA-AR0224	B	*V. ceuthocarpa*	JX874179	*Loktanella salsilacus* (NR_025539, 840, 99)	*Alphaproteobacteria*
UA-AR0226	O	*V. ceuthocarpa*	JX874149	*Micrococcus antarcticus* (NR_025285, 830, 99)	*Actinobacteria*
UA-AR0227	J	*V. ceuthocarpa*	KF914713	*Psychrobacter maritimus* (NR_027225, 856, 97)	*Gammaproteobacteria*
UA-AR0230	B	*V. ceuthocarpa*	JX874180	*Loktanella salsilacus* (NR_025539, 962, 99)	*Alphaproteobacteria*
UA-AR0235	K	*V. ceuthocarpa*	JX874150	*Micrococcus luteus* (NR_037113, 924, 99)	*Actinobacteria*
UA-AR0238	C	*V. ceuthocarpa*	KF914712	*Altererythrobacter luteolus* (NR_043151, 1278, 95)	*Alphaproteobacteria*
UA-AR0245	P	*V. ceuthocarpa*	JX874181	*Bacillus safensis* (NR_041794, 758, 99)	*Bacilli*
UA-AR0301	D	*Hydropunctaria maura*	JX874151	*Lewinella marina* (NR_041594, 984, **94**)[f]	*Sphingobacteria*
UA-AR0302	E	*H. maura*	JX874182	*Bacillus safensis* (NR_041794, 956, 99)	*Bacilli*
UA-AR0305	C	*H. maura*	JX874152	*Psychrobacter namhaensis* (NR_043141, 843, 99)	*Gammaproteobacteria*
UA-AR0307	E	*H. maura*	JX874168	*Pseudoalteromonas paragorgicola* (NR_042076, 949, 98)	*Gammaproteobacteria*
UA-AR0309	J	*H. maura*	JX874153	*Jannaschia helgolandensis* (NR_028976, 870, 99)	*Alphaproteobacteria*
UA-AR0310	P	*H. maura*	JX874154	*Bacillus aerius* (NR_042338, 463, 99)	*Bacilli*
UA-AR0311	M	*H. maura*	JX874155	*Jannaschia pohangensis* (NR_043910, 760, 98)	*Alphaproteobacteria*
UA-AR0315	E	*H. maura*	JX874156	*Bacillus idriensis* (NR_043268, 419, 100)	*Bacilli*
UA-AR0316	D	*H. maura*	JX874157	*Altererythrobacter luteolus* (NR_043151, 508, 97)	*Alphaproteobacteria*
UA-AR0320	I	*H. maura*	JX874158	*Jannaschia donghaensis* (NR_044162, 978, 97)	*Alphaproteobacteria*
UA-AR0322	H	*H. maura*	JX874183	*Micrococcus flavus* (NR_043881, 582, 97)	*Actinobacteria*
UA-AR0325	O	*H. maura*	JX874170	*Aurantimonas manganoxydans* (U53824, 654, 95)	*Alphaproteobacteria*
UA-AR0336	D	*H. maura*	KF914711	*Lewinella marina* (NR_041594.1, 1334, 94)	*Sphingobacteria*
UA-AR0412	M	*Caloplaca verruculifera*	JX874159	*Jannaschia pohangensis* (NR_043910, 840, 98)	*Alphaproteobacteria*
UA-AR0413	C	*C. verruculifera*	JX874160	*Sphingopyxis marina* (NR_043954, 615, 98)	*Alphaproteobacteria*
UA-AR0414	H	*C. verruculifera*	JX874161	*Sphingomonas desiccabilis* (NR_042372, 335, 97)	*Alphaproteobacteria*
UA-AR0416	C	*C. verruculifera*	JX874184	*Aurantimonas coralicida* (NR_042319, 696, 97)	*Alphaproteobacteria*
UA-AR0419	C	*C. verruculifera*	JX874185	*Aurantimonas kwangyangensis* (NR_044195, 784, 96)	*Alphaproteobacteria*
UA-AR0420	O	*C. verruculifera*	JX874186	*Micrococcus luteus* (NR_037113, 952, 99)	*Actinobacteria*
UA-AR0422	E	*C. verruculifera*	JX874162	*Psychrobacter frigidicola* (NR_042222, 816, 98)	*Gammaproteobacteria*
UA-AR0423	R	*C. verruculifera*	JX874163	*Aquimarina intermedia* (NR_042444, 814, **92**)[f]	*Flavobacteria*
UA-AR0424	H	*C. verruculifera*	JX874187	*Sphingomonas suberifaciens* (NR_043394, 1020, 95)	*Alphaproteobacteria*
UA-AR0430	J	*C. verruculifera*	JX874188	*Lewinella antartica* (NR_044281, 1021, **91**)	*Sphingobacteria*
UA-AR0433	C	*C. verruculifera*	JX874171	*Micrococcus flavus* (NR_043881, 722, 99)	*Actinobacteria*
UA-AR0436	C	*C. verruculifera*	JX874172	*Micrococcus flavus* (NR_043881, 655, 98)	*Actinobacteria*
UA-AR0501	S	*Ascophyllum nodosum*	JX874164	*Cellulophaga baltica* (NR_025286, 707, 99)	*Flavobacteria*
UA-AR0503	G	*A. nodosum*	JX874165	*Maribacter aquivivus* (NR_025750, 943, 98)	*Flavobacteria*
UA-AR0504	C	*A. nodosum*	JX874166	*Krokinobacter diaphorus* (NR_041274, 616, 100)	*Flavobacteria*
UA-AR0505	E	*A. nodosum*	JX874167	*Pseudoalteromonas paragorgicola* (NR_025654, 396, 99)	*Gammaproteobacteria*

[a]DNA was extracted from pure cultures using the UltraPure DNA isolation kit (MoBio). An approximately 1450 nt fraction of the 16S rRNA gene was amplified from primers 27F and 1492R using standard PCR conditions. PCR products were purified using the NucleoSpin Extract II kit (Macherey–Nagel) and partially sequenced from the 27F primer binding site on an Applied Biosystems 3130XL DNA analyzer.

[b]For a description of colony types, see Table 1.

[c]Sequences were aligned against the refseq_rna database at NCBI (http://blast.ncbi.nlm.nih.gov/) using the Megablast algorithm. The hit producing the highest alignment score was selected in each case.

[d]When BLASTed against the nr database, highest similarity, 94%, was observed against sequence GQ167328, described as uncultured clone LVBR10aH12.

[e]When BLASTed against the nr database, 92% similarity was observed against sequence FR682734, *Hymenobacter* sp. strain R-36591.

[f]When BLASTed against the nr database, results were identical (same bacterial species, same identity percentage).

Fig. 1. Enumeration of the culturable microbiota following incubation on marine agar at 15 °C. Mean values and standard deviation by colony counts from duplicate plates are shown. The bacteria were extracted from *Lecanora helicopis* (AR01), *Verrucaria ceuthocarpa* (AR02), *Hydropunctaria maura* (AR03), *Caloplaca verruculifera* (AR04), and *Ascophyllum nodosum* (AR05).

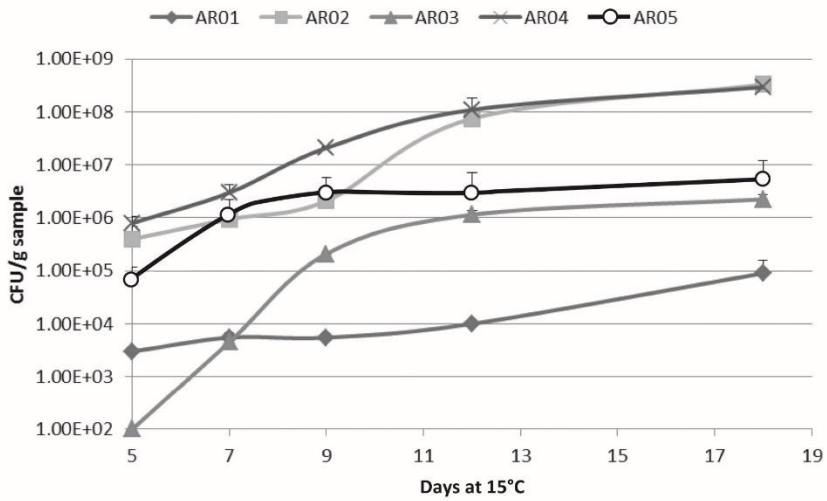
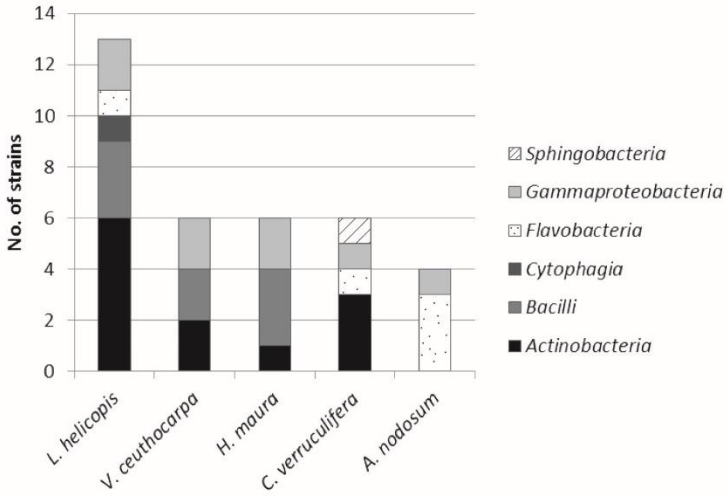


Fig. 2. Taxonomic diversity of selected strains.



28% (*v*/*v*) glycerol at −80 °C. Re-culturability from frozen stocks has been confirmed for all isolates.

Colonial diversity of the cultured microbiota

The diversity of the cultured microbiota was assessed both by colony morphology and by 16S rRNA gene sequencing (see section Taxonomy below). The isolation plates were examined by eye, as well as under the dissection microscope, and colony types were defined (Table 1). A clear difference in colony morphology was observed in the bacterial plate counts between the different lichen species. Most of the colony types were rare, with one or a few representatives per plate; the samples being strongly dominated by one or a few colony types (Table 2). In general, the seashore lichen-associated, MA-culturable microbiota appears to be characterized by a strong presence of bacteria forming colony types D, G, and L. However, the individual lichen species appear to harbor populations distinct from each other, with *L. helicopis* and *H. maura* harboring populations strongly dominated by colony type D, whereas the *V. ceuthocarpa*-associated community is codominated by bacteria forming colony type L and the *C. verruculifera*-associated microbiota is dominated by colony type C.

Taxonomy

A total of 55 isolates were selected for identification on the basis of sampling source and colony morphology to obtain an overview of the diversity of bacteria in the culture collection. The isolates were identified by partial 16S rRNA gene sequence analysis (Table 3). All sequences matched with entries in GenBank with similarities

Fig. 3. Phylogenetic trees of 16S rRNA gene sequences for (A) *Alphaproteobacteria* and (B) *Bacteroidetes*. Strains were selected on the basis of an initial BLAST search against the GenBank refseq_rna database. The sequences were multiply aligned using ClustalW and a phylogenetic tree constructed using the neighbor-joining method. Bootstrap values (1000 iterations) are shown.

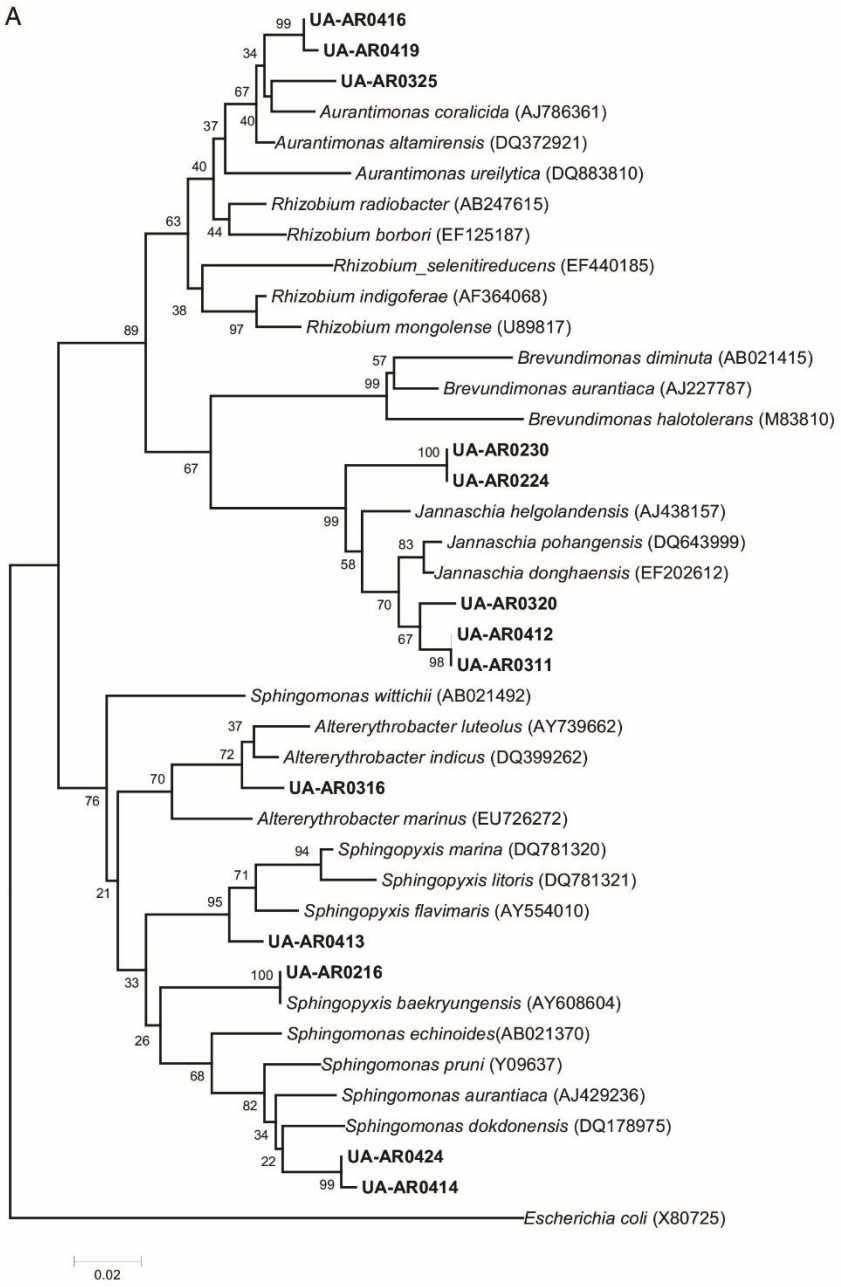
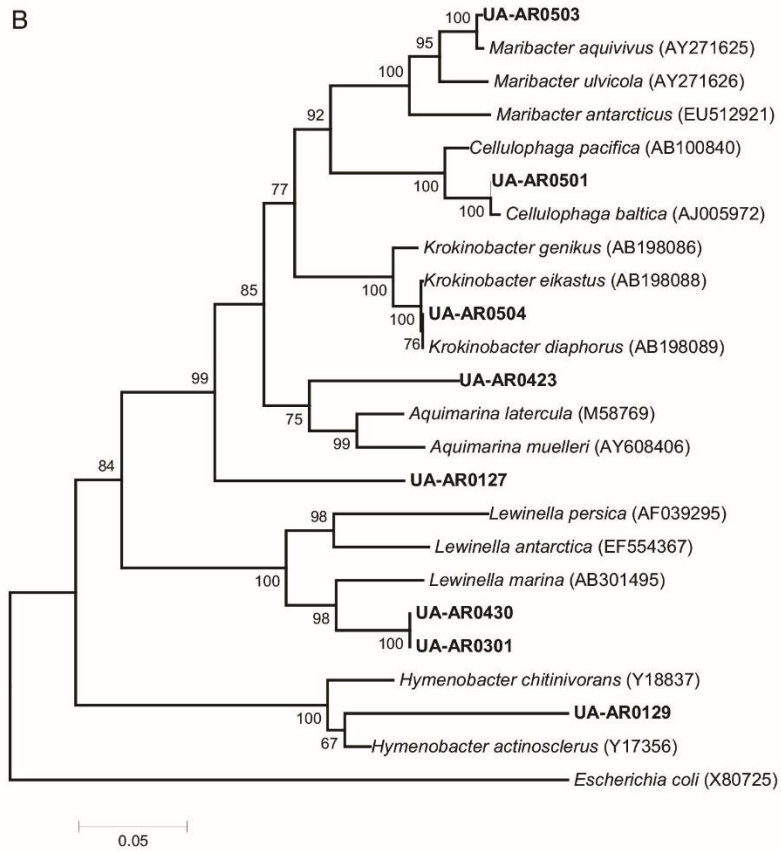


Fig. 3 (*concluded*).



ranging from 86% to 100%. The collection was found to contain members of 7 classes: *Alphaproteobacteria*, *Bacilli*, *Actinobacteria*, *Flavobacteria*, *Cytophagia*, *Sphingobacteria*, and *Gammaproteobacteria*. The taxonomic composition of the isolates was found to be specific for each lichen species and reflects the heterogeneity in colony types as discussed above, although the strain identities do not exhaustively cover the culture collection (Fig. 2). Phylogenetic trees of 16S rRNA gene sequences are shown in Figs. 3A and 3B.

DGGE-based fingerprinting

To obtain a culturing-independent assessment of endothallic microbial diversity, an approximately 290 nt section of the 16S rRNA gene (nt 515–806 by *E. coli* numbering) was amplified by PCR and loaded onto DGGE gels. A typical gel is shown in Fig. 4. The gel supports our hypothesis that the lichen-associated bacteria are specifically associated with their lichen hosts. While there are bands that appear in most or all lichen samples, most of the DGGE bands are either unique to a single lichen species or present in only a few.

Functional and biodegradative properties

A total of 27 isolates were screened for production of several extracellular enzymes at 15 °C. Isolates were selected on the basis of colony morphology and taxonomy, where available. Properties tested were inorganic phosphate solubilization, nitrogen fixation; and degradation of several glycans, protein, and the dye Azure B, indicative of potential lignin biodegradation (Table 4). Of the screened isolates, 52% displayed proteolytic activity, 41% glucanolytic, and 41% amylolytic; however, none of them showed chitinolytic activity. In addition, 41% showed phosphate-solubilizing activity whereas degradation of the dye Azure B was observed for only 11% of the tested isolates. Nitrogen fixation was observed for 22% of isolates.

Discussion

Diversity of the cultured microbiota

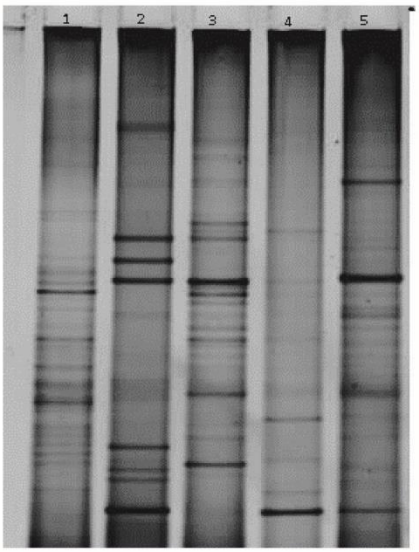
High numbers of bacteria in lichen thalli have been reported in the literature. Grube et al. (2009) reported that about 10^5 to 10^6 bacteria were observable by confocal laser scanning microscopy per cubic millimetres of lichen thalli (about 10^7 bacteria/g) in *Cladonia arbuscula* and *Umbilicaria cylindrica*. These figures are similar to the MA-culturable final viable counts presented in this study, indicating that the bulk of the lichen-associated bacteria are culturable on MA at 15 °C. This is somewhat surprising given the oft held assumption that the vast majority of environmental bacteria are poorly or not at all culturable (Shade et al. 2012) and indicates that our culture collection is likely to contain representatives of most of the numerically significant taxa present in the thalli of the sampled lichens. We expect there to be unculturable

Table 4. Biodegradative properties of selected strains.

Isolate	Origin	Phylogenetic analysis[a]	BBG[b]	Xylan[c]	Chitosan[c]	Casein[c]	Starch[c]	Azure B[d]	Phosphate[e]	NFA[f]
UA-AR0101	*Lecanora helicopis*	*Streptomyces microflavus*	+	–	–	–	+	–	–	–
UA-AR0103	*L. helicopis*	NA	–	–	–	–	+	–	–	–
UA-AR0104	*L. helicopis*	*Salinibacterium amurskyense*	–	–	–	–	–	–	–	–
UA-AR0105	*L. helicopis*	*Agrococcus baldri*	–	–	–	–	–	–	–	+
UA-AR0106	*L. helicopis*	NA	–	–	–	–	+	–	–	–
UA-AR0110	*L. helicopis*	*Psychrobacter faecalis*	–	–	–	–	+	–	–	–
UA-AR0113	*L. helicopis*	*Psychrobacter faecalis*	–	–	–	–	–	–	+	–
UA-AR0114	*L. helicopis*	NA	–	–	–	–	+	–	–	–
UA-AR0115	*L. helicopis*	NA	–	–	–	+	–	–	+	–
UA-AR0117	*L. helicopis*	*Staphylococcus cohnii*	–	–	–	–	–	–	–	–
UA-AR0125	*L. helicopis*	*Bacillus asahii*	+	+	–	+	–	–	–	–
UA-AR0134	*L. helicopis*	NA	–	–	–	(+)	–	–	(+)	–
UA-AR0201	*Verrucaria ceuthocarpa*	*Bacillus murimartini*	+	+	–	+	–	–	–	(+)
UA-AR0202	*V. ceuthocarpa*	NA	+	–	–	+	–	–	–	–
UA-AR0209	*V. ceuthocarpa*	*Psychrobacter maritimus*	+	+	–	+	–	+	+	+
UA-AR0226	*V. ceuthocarpa*	*Micrococcus antarcticus*	–	–	–	+	–	–	–	+
UA-AR0235	*V. ceuthocarpa*	*Micrococcus luteus*	+	+	–	+	–	–	–	+
UA-AR0302	*Hydropunctaria maura*	*Bacillus safensis*	+	+	–	+	–	+	(+)	–
UA-AR0305	*H. maura*	*Psychrobacter namhaensis*	+	–	–	+	–	–	+	–
UA-AR0310	*H. maura*	*Bacillus aerius*	+	–	–	+	+	–	+	–
UA-AR0315	*H. maura*	*Bacillus idriensis*	–	–	–	+	+	–	+	+
UA-AR0322	*H. maura*	*Micrococcus flavus*	–	–	–	–	–	–	+	+
UA-AR0413	*Caloplaca verruculifera*	*Sphingopyxis marina*	+	+	–	+	+	+	+	–
UA-AR0414	*C. verruculifera*	*Sphingomonas desiccabilis*	+	+	–	–	–	–	+	–
UA-AR0419	*C. verruculifera*	*Aurantimonas kwangyangensis*	–	–	–	–	+	–	–	–
UA-AR0420	*C. verruculifera*	*Micrococcus luteus*	–	–	–	+	+	–	–	–
UA-AR0424	*C. verruculifera*	*Sphingomonas suberifaciens*	–	–	–	–	+	–	–	–

Note: +, good degradation; (+), intermediate degradation; –, no degradation.
[a]NA, not analyzed.
[b]Barley β-glucan (BBG) performed on 0.5× nutrient agar plates supplemented with azurin cross-linked BBG (Megazyme).
[c]Soluble starch, casein, xylan, and chitosan hydrolysis tests performed using standard methods.
[d]Clearance zones on R2A agar supplemented with Azure B, indicative of lignin degradation.
[e]Clearance zones on NBIRP medium, indicative of inorganic phosphate mobilization.
[f]Clearance zones on nitrogen-free agar (NFA).

Fig. 4. Denaturing gradient gel electrophoresis gel showing the banding patterns of the inland fruticose lichen *Stereocaulon alpinum* (lane 1), and the 4 crustose seashore lichen samples of the present study (*Lecanora helicopis*, *Verrucaria ceuthocarpa*, *Hydropunctaria maura*, and *Caloplaca verruculifera* in lanes 2 to 5, respectively).



bacteria present as well, but they probably do not form a numerically dominant part of the total microbiota. The finding of Grube et al. (2009) that *Lecanora polytropa* contained significantly lower number of culturable bacteria (about 10^4/g) is also in agreement with our findings for *L. helicopis*.

Alphaproteobacteria

According to several recent molecular studies (Cardinale et al. 2008; Hodkinson and Lutzoni 2009; Bates et al. 2011; Bjelland et al. 2011), *Alphaproteobacteria* are expected to form a major part of the lichen-associated microbiotas. In the present study, 15 alphaproteobacterial isolates were identified. Interestingly, only 3 of them could be assigned to the *Rhizobiales*, postulated by Hodkinson and Lutzoni (2009) to contribute nitrogen fixation to the lichen symbiotic association. Other alphaproteobacterial isolates identified in the present study can be assigned to the *Sphingomonadales* and *Rhodobacterales*. The rhodobacterial isolates were found to be most similar to members of either the genus *Jannaschia*, strictly aerobic photoheterotrophs (Wagner-Döbler et al. 2003), often associated with marine and seashore environments (Wagner-Döbler et al. 2003; Adachi et al. 2004; Macián et al. 2005; Yoon et al. 2005; Kim et al. 2008*b*), or the genus *Loktanella*, strictly aerobic, moderately halotolerant chemoheterotrophs, often associated with marine environments (Trappen et al. 2004). While *Jannaschia* is well represented in our culture collection, it should be pointed out that while they are capable of photosynthetic growth, it is of an anoxygenic, heterotrophic nature and, thus, unlikely to contribute to the lichen symbiotic association as an auxiliary photobiont. The *Sphingomonadales* isolates were more diverse; each of the 3 isolates being most similar to members of separate genera, 2 within the *Sphingomonadaceae* family and 1 within the *Erythrobacteraceae* family. The sphingomonads are heterotrophs that are widely

distributed in nature, commonly found in soils and aqueous environments (Kersters et al. 2006), including marine and seashore environments (Cavicchioli et al. 1999, 2003; Fredrickson et al. 1999; Kim et al. 2008*a*; Notomista et al. 2011). *Sphingopyxis* has been noted to be particularly abundant in permanently cold marine waters (Ting et al. 2010). Many sphingomonads have been noted for their potential for oligotrophic growth (Cavicchioli et al. 2003) and the ability to degrade a variety of carbon sources, including aromatics (Fredrickson et al. 1999). The *Erythrobacteraceae* are represented by strain UA-AR0316, which shows greatest similarity to *Altererythrobacter luteolus*, a slightly halophilic marine heterotroph (Yoon et al. 2005; Kwon et al. 2007). Interestingly, while several alphaproteobacterial isolates were identified from each of the lichen species *V. ceuthocarpa*, *H. maura*, and *C. verruculifera*, none of the identified *L. helicopis* isolates were found to belong to the *Alphaproteobacteria*, reflecting the much lower yield of MA-culturable bacteria from this lichen.

Bacteroidetes

Bacteroidetes have not been prominent in the lichen-associated microbiota reported thus far. In the study of Bates et al. (2011), who used culture-independent methods to investigate the microbiotas of 4 species of saxicolous, foliose lichens, only 1 of the 26 most abundant lichen-associated phylotypes reported belonged to this phylum and was only found in 1 of the 4 lichens investigated (*Umbilicaria americana*). Bjelland et al. (2011), who studied the microbial communities of 4 crustose, saxicolous lichens and their rock substrata, found only 1 of the lichens, *H. maura*, to harbor *Bacteroidetes*. In the present study, however, 10 of the 55 identified isolates could be assigned to this phylum. Out of these, 3 were isolated from the knotted kelp control sample, the remaining 7 from 3 of the 4 studied lichens (Table 3). The *Bacteroidetes* isolates are quite diverse, representing 3 distinct classes, *Flavobacteria*, *Cytophagia*, and *Sphingobacteria*, and each isolate representing a different species. Intriguingly, each of the 5 lichen-associated isolates displays a low degree of similarity to all GenBank sequences. Thus, they are all likely to represent novel taxa. The *L. helicopis*-associated isolate UA-AR0129, representing the *Cytophagia*, is most similar to members of the genus *Hymenobacter*, red-pigmented, rod-shaped, obligate aerobes (Oren 2006) that have been found in disparate environments, including Antarctic soil (Hirsch et al. 1998), museum air (Buczolits et al. 2002), and irradiated pork (Collins et al. 2000). The *Sphingobacteria* are represented by the *H. maura* isolate UA-AR0301 and the *C. verruculifera* isolate UA-AR0430. Both isolates are most similar to members of the genus *Lewinella*, which comprises halophilic, chemoorganotrophic, carotenoid-pigmented marine bacteria (Khan et al. 2007). A common characteristic of *Sphingobacteria*, including *Lewinella*, is their ability to degrade biomacromolecules, particularly all kinds of polysaccharides, like agar, starch, cellulose, yeast cell-wall β-glucan, succinoglycan, pectin, alginate, and heparin (Reichenbach 2006). Also known for extensive degradative capabilities, the *Flavobacteria* are found in various habitats, including soil, freshwater, sea water, seashore environments, food and dairy products, various diseased animals, and more. They have been found to be very important in freshwater environments (Brümmer et al. 2000; Kirchman 2002) and in marine environments, particularly in the polar regions (Bowman et al. 1997; Ravenschlag et al. 2001). The *Flavobacteria* are represented by 2 lichen associates: the *L. helicopis*-associated isolate UA-AR0127 was found to be most similar to the newly described genus *Eudoraea* whose only member stems from Adriatic surface waters (Alain et al. 2008), and the *C. verruculifera*-associated isolate UA-AR0423, most similar to members of the genus *Aquimarina*, heterotrophic, aerobic, dark-yellow or brownish-colored, gliding bacteria producing flexirubin-type pigments that have been isolated from seawater samples (Nedashkovskaya et al. 2005) and a sea urchin from the Sea of Japan (Nedashkovskaya et al. 2006).

The role of associated microbiota in the symbiotic association

Although the role of the nonphototrophic, lichen-associated bacteria in the symbiotic association has remained poorly characterized, some important roles have been suggested. Among these are nitrogen fixation (Scott 1956; Cardinale et al. 2006; Liba et al. 2006), antifungal and antibacterial defenses of the lichen (González et al. 2005), and nutrient transfer from the rock surface to the fungal hyphae in saxicolous lichens (Banfield et al. 1999). Bacterial biofilms are also thought to play an important role in surface attachment of saxicolous lichens (de los Ríos et al. 2002). In the present study, selected isolates were screened for their biodegradation properties at 15 °C. The proportion of strains showing glucanolytic or phosphate-solubilizing activity is higher than reported with bacteria associated with *Cladonia arbuscula*, *Lecanora polytropa*, and *Umbilicaria cylindrica* (Grube et al. 2009). A large fraction of the inorganic phosphates present in nature is immobile and, therefore, unavailable to non-phosphate-mobilizing organisms (Nautiyal 1999). Several microorganisms have been found to be involved in the natural phosphorus cycle by releasing organic acids, which are responsible for mineral phosphate solubilization (Rodríguez and Fraga 1999). A relatively high percentage of phosphate-solubilizing bacteria was observed in present study (41%). We suggest that these lytic and phosphate-solubilizing bacteria are involved in nutrient transfer in the thallus and may enhance the mobilization of nutrients. Chitinolytic activity was detected for none of the tested strains in present study. Only 3 of the tested strains degraded the dye Azure B, suggesting that potential lignin degradation is not commonly found among bacteria associated with seashore lichens. Nitrogen fixation has been suggested to be among the most important role of lichen-associated microbiota (Cardinale et al. 2006; Liba et al. 2006; Grube et al. 2009). Interestingly, only 7 of the tested isolates in the present study were nitrogen fixing. However, environmental nitrogen limitation is unlikely to be a major issue in the seashore environment with typical seawater nitrate concentration of several micromoles per litre (Johnson and Petty 1983) perhaps obviating the need for a nitrogen-fixing symbiont.

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II

Analysis of the *Peltigera membranacea* metagenome indicates that lichen-associated bacteria are involved in phosphate solubilization

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Although lichens are generally described as mutualistic symbioses of fungi and photosynthetic partners, they also harbour a diverse non-phototrophic microbiota, which is now regarded as a significant part of the symbiosis. However, the role of the non-phototrophic microbiota within the lichen is still poorly known, although possible functions have been suggested, including phosphate solubilization and various lytic activities. In the present study we focus on the bacterial biota associated with the foliose lichen *Peltigera membranacea*. To address our hypotheses on possible roles of the non-phototrophic microbiota, we used a metagenomic approach. A DNA library of bacterial sequence contigs was constructed from the lichen thallus material and the bacterial microbiota DNA sequence was analysed in terms of phylogenetic diversity and functional gene composition. Analysis of about 30 000 such bacterial contigs from the *P. membranacea* metagenome revealed significant representation of several genes involved in phosphate solubilization and biopolymer degradation.

INTRODUCTION

Lichens are generally characterized as the symbiotic association of a fungal partner (mycobiont), green algae and/or cyanobacteria (Nash, 2008), and together the lichen symbiotic partners form a thallus, which may be able to tolerate and sustain growth in hostile and dynamic environments where they could not survive alone (Bjelland *et al.*, 2011). Lichens generally have high tolerance to ecological extremes in temperature, low nutrient and/or water availability and UV light intensities and can therefore be found in a wide range of habitats, often where few other organisms survive (Beckett *et al.*, 2008; Boddy *et al.*, 2010). Although generally described as the symbiosis of a fungus and a photosynthetic partner, recent studies have revealed a high diversity and abundance of non-phototrophic bacteria present in lichen thalli (Cardinale *et al.*, 2006, 2008; Grube *et al.*, 2009; Hodkinson & Lutzoni, 2009; Bjelland *et al.*, 2011; Mushegian *et al.*, 2011). The presence of this non-phototrophic microbiota has long been known (Henkel & Yuzhakova, 1936; Henkel & Plotnikova, 1973), and members are now suggested to be involved in several important roles within the symbiosis, including iron and phosphate mobilization, hormone production, nitrogen fixation and several lytic activities (Liba *et al.*, 2006; Grube *et al.*, 2009). Since many lichens can thrive on extremely nutrient-poor substrates, it has also been suggested that the non-phototrophic microbiota plays an important role in the lichen thalli by facilitating supply of crucial nutrients (González *et al.*, 2005; Cardinale *et al.*, 2006; Liba *et al.*, 2006). Phosphorus (P) is a primary element involved in all major metabolic pathways (Khan *et al.*, 2010; Zhao *et al.*, 2014; Sharma *et al.*, 2013). In nature, fully oxidized P occurs as phosphate, and the majority is present in insoluble form that cannot be directly utilized by organisms (Richardson *et al.*, 2001). P must therefore be released from organic and inorganic compounds and it is well known that phosphate-solubilizing bacteria can make it available to plants (Chhabra *et al.*, 2013; Richardson *et al.*, 2001; Sashidhar & Podile, 2010; Zhao *et al.*, 2014; Sharma *et al.*, 2013). These pathways are complex and several key enzymes are involved, including alkaline and acid phosphatases, phytases and phosphonatases (Rodriguez *et al.*, 2006; Sharma *et al.*, 2013). A variety of bacteria are known to convert bound phosphorus to a soluble form and the genera *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* have been reported as the most significant

Abbreviation: COG, cluster of orthologous groups.

The GenBank/EMBL/DDBJ accession numbers for the bacterial contigs obtained from the *Peltigera membranacea* thallus material are KP422466–KP452497.

phosphate-solubilizing bacteria (Sturz & Nowak, 2000; Sudhakar *et al.*, 2000). *Proteobacteria*, particularly *Alphaproteobacteria*, form the largest part of the non-phototrophic bacteria in many lichens (Cardinale *et al.*, 2006, 2008; González *et al.*, 2005; Grube *et al.*, 2009; Hodkinson & Lutzoni, 2009; Liba *et al.*, 2006; Muggia *et al.*, 2013; Schneider *et al.*, 2011). Many of the proteobacterial genera listed above are commonly found in lichens, and recent studies have shown that one of the potential roles of the associated microbiota could well be phosphate solubilization (Liba *et al.*, 2006; Grube *et al.*, 2009; Sigurbjörnsdóttir *et al.*, 2014). Thus we hypothesize in the present study that phosphate solubilization is an important trait of the lichen-associated non-phototrophic microbiota.

Several previous studies have characterized lichen-associated microbiota with culture-based experiments (Cardinale *et al.*, 2006; González *et al.*, 2005; Grube *et al.*, 2009; Henkel & Plotnikova, 1973; Liba *et al.*, 2006). However, the vast majority of micro-organisms found in natural habitats remain unculturable in the laboratory (Amann *et al.*, 1995), underscoring the relevance of culture-independent approaches. By using metagenomics, the functional gene composition of microbial communities can be assessed, yielding a more extensive description than phylogenetic methods (Thomas *et al.*, 2012). The first study of lichen-associated bacteria based on metagenomic methods was conducted by Cardinale *et al.* (2006), where a great diversity of bacteria was shown to be present in the lichen thalli sampled.

The present study focuses on the non-phototrophic microbiota associated with the foliose lichen *Peltigera membranacea*, commonly found in Icelandic vegetation. *P. membranacea* is a cyanolichen, with *Nostoc* cyanobacteria as photobiont (Miadlikowska & Lutzoni, 2004). A metagenomic library was constructed from a collection of lichen thalli and the bacterial microbiota were analysed in terms of phylogenetic diversity and functional gene composition. Genes encoding phosphatases as well as other enzymes were identified in order to predict possible roles of the associated non-phototrophic microbiota of the lichen.

METHODS

Lichen samples, DNA extraction, sequencing and assembly. *P. membranacea* (accession nos: XBB013, Biology Laboratory, University of Iceland; LA-31632, Icelandic Institute of Natural History) was collected on 21 September 2008 at Keldnagil, Reykjavik, Iceland within a 12 m span (coordinates 64° 64.7′ N, 21° 46.6′ W). At this site *P. membranacea* thalli are fairly large (mostly 4–8 cm wide and more than 12 cm long), loosely attached to a substrate consisting mainly of the mosses *Hylocomium splendens* and *Pleurozium schreiberi*, intermingled with *Empetrum nigrum*, *Vaccinium uliginosum* and *Betula nana*, and contain very little soil material. The sample consisted of 8–10 cm long thalli from approximately 20 different individuals collected into a clean aseptic plastic box. The thalli were cleaned under a stereomicroscope until no epiphytes or extraneous material could be seen [analysis of the DNA extracted yielded a low level (<1 %) of non-*Peltigera* eukaryotic sequences] and extensively washed with distilled water before DNA extraction as previously described (Sinnemann *et al.*, 2000). Dried lichen (1.2 g) was placed in a mortar and cooled with liquid nitrogen before thorough grinding. The powder was incubated at 65 °C for 20 min. with 15 ml lysis buffer (10 mM Tris/HCl pH 8.0, 50 mM EDTA, 100 mM NaCl, 1 % SDS and 40 ng RNase A), with occasional gentle mixing. The lysis mixture was divided between 2 ml tubes and centrifuged for 4 min at 20 000 ***g***. Supernatant (1 ml) was transferred to a new 2 ml tube and 428 µl 10.5 M ammonium acetate was added. The mixture was kept on ice for 20 min and centrifuged for 4 min at 20 000 ***g***. Then, 1250 µl supernatant was transferred to a new 2 ml tube, 800 µl 2-propanol was added and the mixture was centrifuged for 4 min at 20 000 ***g***. The supernatant was carefully removed, and 1 ml TE buffer (10 mM Tris/HCl pH 8.0 and 1 mM EDTA) was added. The tubes were incubated at 50 °C for 20–60 min with occasional agitation until the pellet was dissolved. The tubes were centrifuged briefly and the supernatant was transferred to a new tube if a pellet was visible. An equal volume of phenol/choloroform/isoamyl alcohol (25 : 24 : 1) was added, and the tubes were vortexed briefly and centrifuged for 4 min at 20 000 ***g***. The aqueous phase was transferred to a fresh 2 ml tube without disturbing the interphase, an equal volume of saturated chloroform was added, and after brief vortexing the tubes were centrifuged for 4 min at 20 000 ***g***. The aqueous phase was again transferred to a fresh tube without disturbing the interphase, 0.1 vol 3 M sodium acetate (pH 5) and 2 vol ethanol were added, and after brief vortexing the tubes were centrifuged for 4 min at 20 000 ***g***. The supernatant was discarded and the pellet washed with ~500 µl 70 % EtOH. After air-drying, 100 µl TE buffer was added and the pellet brought into solution at 50 °C for 2–5 min with gentle tapping. Approximately 2 mg high molecular weight (>50 kb) DNA was recovered from 4 g dried lichen thalli. This DNA was processed for sequencing at a commercial facility (Microsynth) via Roche 454 technology. Approximately 1.76 Gb of 454 data (~340 base mean read length) was obtained. The 454 reads were assembled into contiguous sequences (contigs) using the Newbler (Roche) software (Chevreux *et al.*, 1999).

Construction and screening of the metagenomic library. Contigs were sorted into three bins based on significant scores with tblastn (Altschul *et al.*, 1990) against protein databases of filamentous ascomycetes ($P<10^{-3}$) and of *Nostoc* ($P<10^{-6}$). The remaining contigs were mainly bacterial and were used for the construction of the metagenomic library used in this study. The non-*Nostoc* contigs were first assigned to taxonomic groups by Infernal and SeqMatch matching against the RDP database (Wang *et al.*, 2007). Based on the assignments, 15 reference genomes were selected for extraction of the non-*Nostoc* bacterial metagenome: *Acidiphilium cryptum* JF-5, *Acidobacterium capsulatum* ATCC 51196, *Akkermansia muciniphila* ATCC BAA-835, *Cytophaga hutchinsonii* ATCC 33406, *Frankia alni* ACN14a, *Methylobacterium chloromethanicum* CM4, *Nocardia farcinica* IFM10152, *Opitutus terrae* PB90_1, *Oxalobacter formigenes* HOxBLS uid32497, *Pedobacter heparinus* DSM 2366, *Phenylobacterium zucineum* HLK1, *Rubrobacter xylanophilus* DSM 9941, *Sinorhizobium meliloti* 1021, *Sorangium cellulosum* So_ce_56 and *Sphingomonas wittichii* RW1. The BLASTN algorithm was used to compare the non-*Nostoc* bacterial community with the 15 reference genomes and resulted in 150 573 contigs, composing the metagenomic library. Contigs that returned results with *E*-values below 10^{-3} from the BLASTN search were extracted from the library and imported into CLC Genomic Workbench (CLC Bio). The number of actual contigs used for further analysis in this study was 30 033. The BLASTX suite was run via CLC against the 'non-redundant protein sequence' database from NCBI for assembly-based functional analysis and an overview of the taxonomic profiles. Based on the BLASTX results, the contigs were mapped manually to metabolic pathways using KEGG orthology groups and Cluster of Orthologous Groups (COG) categories (Tatusov *et al.*, 2000). The bacterial contigs were deposited in GenBank under accession numbers KP422466–KP452497.

RESULTS AND DISCUSSION

Taxonomic diversity of total DNA from *P. membranacea*

In the present study, the non-*Nostoc* bacterial metagenome of the cyanolichen *P. membranacea* was studied. An initial extraction of the contigs generated using 454 technology based on 16S rRNA homology yielded 518 contigs, whereof 14 were determined to be of non-bacterial origin or of insufficient quality for classification. The remaining 504 contigs were classified into 64 operational taxonomic units, which, while not composing a fully rarefied collection (analysis not shown), were deemed likely to contain representatives of the most abundant taxa present. The majority of contigs (381) were found to belong to the *Cyanobacteria*, presumably mostly to the *Nostoc* photobiont, but the remaining 123 contigs were found to represent a population dominated by *Proteobacteria* (58 %), followed by *Bacteroidetes* (14 %), *Actinobacteria* (11 %) and *Verrucomicrobia* (8 %) (Fig. 1).

The proteobacterial population is strongly dominated by *Alphaproteobacteria* (70 %), followed by *Betaproteobacteria* (15 %), *Gammaproteobacteria* (8 %) and *Deltaproteobacteria* (6 %). This is in good accordance with several recent studies on lichen-associated bacteria, which have shown that the most common taxa in the growing parts of lichens belong to *Alphaproteobacteria* (Bates *et al.*, 2011; Bjelland *et al.*, 2011; Hodkinson *et al.*, 2012; Mushegian *et al.*, 2011; Grube *et al.*, 2012). Of the orders within *Alphaproteobacteria*, we found *Rhizobiales* to be dominant among the microbiota associated with *P. membranacea* (42 % of alphaproteobacterial sequences), although the *Rhodospirillales* (32 %), *Caulobacterales* (14 %) and *Sphingomonadales* (10 %) are also well represented. The betaproteobacterial fraction, on the other hand, appears quite homogeneous and is almost exclusively made up of members of the *Burkholderiales*. Hodkinson *et al.* (2012) reported that the *Rhizobiales* was generally dominant in the bacterial part within nine different lichens, including *Peltigera*, which also agrees well with our results. Our results are thus in general agreement with other recent studies (Cardinale *et al.*, 2006, 2008; González *et al.*, 2005; Grube *et al.*, 2009; Hodkinson & Lutzoni, 2009; Hodkinson *et al.*, 2012; Liba *et al.*, 2006; Muggia *et al.*, 2013; Schneider *et al.*, 2011).

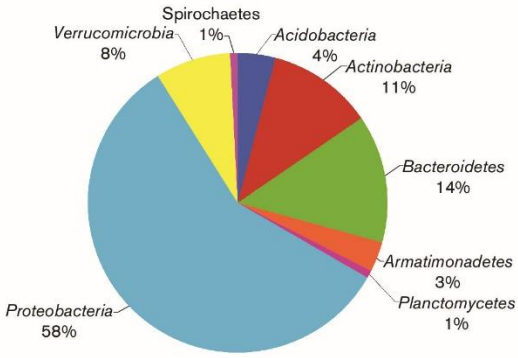

Fig. 1. Phylum-level composition of the bacterial microbiota as determined by Infernal and SeqMatch matching against the RDP database (http://rdp.cme.msu.edu/index.jsp). A total of 504 contigs, selected based on similarity to universal 16S rRNA primer sequences, were classified into 64 operational taxonomic units, likely to represent the most abundant taxa.

Based on these inital results, 15 reference genomes were selected for BLASTN binning into a putative non-*Nostoc* bacterial metagenome based on *E*-values below 10^{-3}. This resulted in a 95 Mb metagenome composed of 30 033 contigs, which were then compared with protein databases using the BLASTX algorithm. The taxonomic analysis, based on the highest similarity to known or predicted proteins and a low *E*-value from the BLASTX search, largely agreed with the previous 16S rRNA analysis, although agreement between the taxonomic identities of the BLASTN and BLASTX hits was in many cases poor. Of the coding sequences identified, the predominant fraction (86 %) of the total metagenome is from *Proteobacteria*, with *Actinobacteria* and *Bacteriodetes* less common (8 and 3 %, respectively). Only 1 % belong to *Firmicutes*, *Acidobacteria* and *Verrucomicrobia*, and other phyla are present at lower abundances (Fig. 2a). The extracted metagenome is thus expected to contain genes from all major groups present in the non-cyanobacterial bacteriome, with *Proteobacteria* being particularly well represented. The proteobacterial coding sequences identified are from the *Alphaproteobacteria* (55 %), *Betaproteobacteria* (31 %) and *Gammaproteobacteria* (13 %). Representatives from *Epsilon-*, *Delta-* and *Zetaproteobacteria* are few (Fig. 2b). Among the *Alphaproteobacteria*, *Rhizobiales* and *Sphingomonadales* are the most frequent orders. Within *Rhizobiales*, *Bradyrhizobiaceae* and *Methylobacteriaceae* are prominent, but members of *Beijerinckiaceae*, *Phyllobacteriaceae*, *Rhizobiaceae* and *Xanthobacteraceae* are also present in lower quantities. The majority of the *Sphingomonadales* belong to *Sphingomonadaceae*. Genes presumed to be from members of the order *Burkholderiales* are dominant among the taxon *Betaproteobacteria*, the majority belonging to the family *Comamonadacea*, and this agrees well with a recent metagenomic study on the associated microbiota of lichens (Grube *et al.*, 2014).

Functional gene composition and key enzymes

Functionalities encoded in the bacterial contigs were mapped to KEGG orthologue groups and COG categories. As can be seen from Fig. 3, nearly half of the sequences (14 362 contigs) could be mapped to metabolic categories. A total of about 10 000 contigs were classified as enzymes participating in cellular processes and signalling or information storage and processing. The remaining contigs (6275) were poorly characterized.

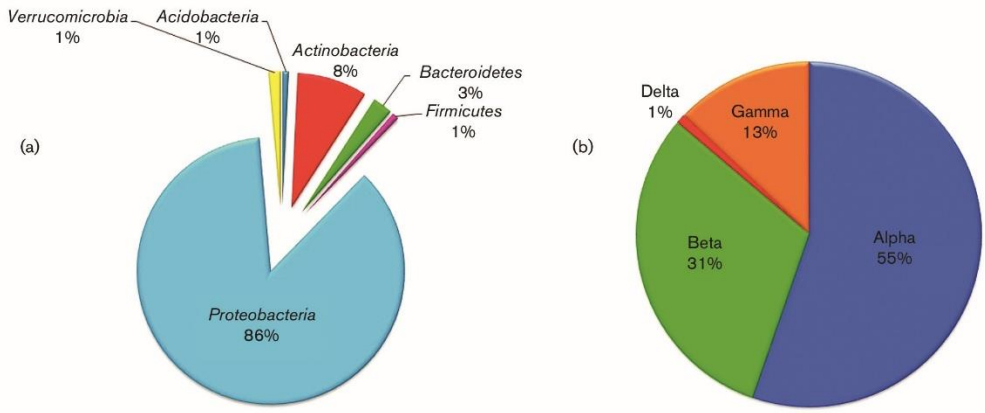

Fig. 2. Taxonomic composition of the non-*Nostoc* bacterial component of the metagenome. A total of 30 033 contigs, with *E*-values below 10^{-3}, from a BLASTN search against 15 selected reference genomes, were assigned to phyla (a) and the proteobacterial fraction (24 034 contigs) to classes (b). The analysis is based on the highest similarity with known or predicted proteins from a BLASTX search against the 'non-redundant protein sequence' database from NCBI via CLC Genomic Workbench.

Genes contributing to phosphate solubilization. Several strains isolated from various lichen thalli have been reported as phosphate-solubilizing (Liba *et al.*, 2006; Grube *et al.*, 2009, 2014; Sigurbjörnsdóttir *et al.*, 2014). Given that lichens often thrive under extreme ecological conditions, we hypothesized that phosphate solubilization could be an important role of the lichen-associated non-phototrophic microbiota. About 1335 contigs suggesting the presence of enzymes involved in phosphate metabolism were found in our dataset. Phosphatases that participate in the release of inorganic P were particularly prominent (588 contigs), but organic, alkaline and transport phosphatases are also present, although in lower numbers. We estimate there are more than 20 gene copies encoding alkaline phosphatase per ~5 Mb genome equivalent of the non-phototrophic bacterial metagenome. In a recent metagenomic study

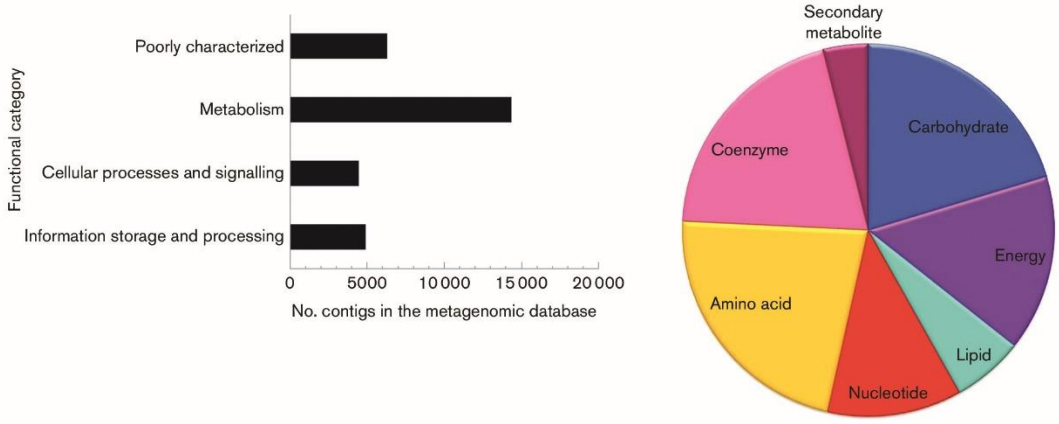

Fig. 3. Functional classification of the non-*Nostoc* bacterial metagenome. Based on BLASTX results, the bacterial contigs were assigned to major COG categories (bar chart) and metabolic functions (pie chart). A total of 14 362 contigs were mapped to metabolic categories.

(Grube *et al.*, 2014) on the associated microbiota of the lung lichen *Lobaria pulmonaria*, phosphatases were also well represented, supporting our primary hypothesis.

The process of mineral phosphate solubilization (MPS) is complex and involves several gene products (Chhabra *et al.*, 2013; Rodríguez *et al.*, 2006; Zhao *et al.*, 2013; Apel *et al.*, 2007). Acidification is a key step in the dissolution of many poorly soluble mineral phosphates, and hence, as proposed by Goldstein (1996), the direct oxidation of glucose to gluconic acid is a key step in some of the major mechanisms for MPS in Gram-negative bacteria, which are suggested to be more efficient at MPS than Gram-positive bacteria owing to secretion of organic acids from sugar metabolism (Sashidhar & Podile, 2009). The pathway is mediated by a membrane-bound glucose dehydrogenase and requires pyrroloquinoline quinone (PQQ) as a cofactor (Goldstein, 1995). Bacteria belonging to the genera *Achromobacter*, *Agrobacterium*, *Bacillus*, *Enterobacter*, *Erwinia*, *Escherichia*, *Flavobacterium*, *Mycobacterium*, *Pseudomonas* and *Serratia* have been found to be efficient in phosphate solubilization (Goldstein, 2001). Genes encoding PQQ-associated proteins were present in 27 contigs in our dataset. Thereof, PQQ-dependent dehydrogenases were encoded in 13 contigs. The remaining 14 contigs contained members of the PQQ biosynthesis operon: *pqqB* (two contigs), *pqqC* (five contigs), *pqqD* (three contigs) and *pqqE* (four contigs). All PQQ-associated quinoproteins and PQQ biosynthesis proteins found in the metagenome belong to Gram-negative *Proteobacteria*, showing greatest similarity to the corresponding genes from members of the *Rhizobiaceae*, *Sphingomonadaceae*, *Burkholderiaceae*, *Xanthomonadaceae* and *Myxococcaceae*. PQQ-associated proteins have previously been proposed to be among required factors in mineral phosphate metabolism. Thus, finding these genes among key members of the *Peltigera*-associated microbiome further supports our hypothesis that phosphate solubilization by the associate members plays an important role in the lichen symbiosis. Further supporting that conclusion is the comparatively large number of contigs encoding phosphatases (Fig. 4). Not surprisingly, the majority of phosphatase-encoding contigs in our dataset belong to *Proteobacteria*, particularly *Alphaproteobacteria*.

Other enzymes of interest. Sequences encoding proteins responsible for both central and degradative carbohydrate metabolism and transport are well represented, as expected. Furthermore, sequences assigned to proteins involved in energy production, amino acid metabolism and transport of nucleotides, coenzymes and lipids are also well represented. Although many of the enzymes are included in central metabolism, and thus expected in the dataset, the presence of others supports previous findings of amino acid release of the non-phototrophic microbiota of lichens (Schneider *et al.*, 2011; Liba *et al.*, 2006) and that the bacterial microbiota assists in re-allocating resources within the lichen (Schneider *et al.*, 2011).

Further classification of the metagenome into the major COG gene ontology categories can be seen in Fig. 5. A relatively large portion (19.8 %) of the metagenome remained poorly characterized, as BLASTX search resulted in hypothetical proteins with unknown functions. General function could only be predicted for a third of these poorly characterized entities and included many ABC transporter-like proteins that could not be assigned to a particular pathway. BLASTX searches resulted in no significant similarity for 12 % of the putative proteins (Fig. 5).

Recent functional and metagenomics studies have indicated that the bacterial microbiota of lichens possesses a

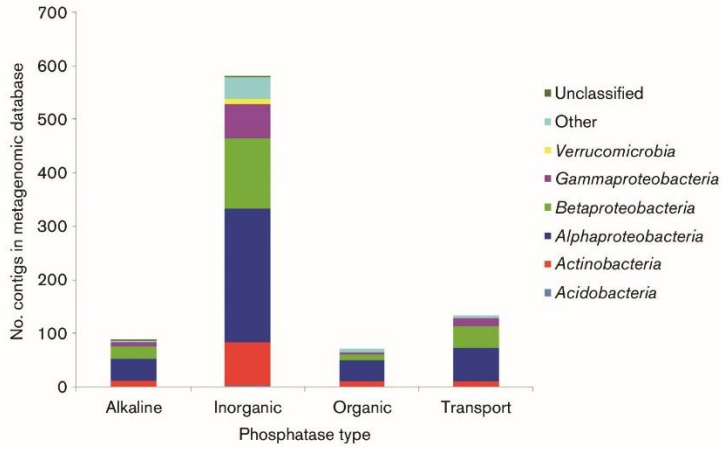

Fig. 4. Taxonomic distribution of 1335 contigs containing phosphatase genes as determined by BLASTX against the NCBI nr database.

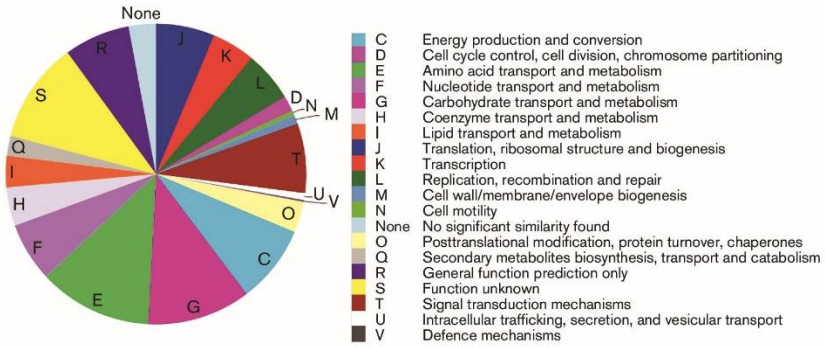

Fig. 5. Functional classification of the non-*Nostoc* bacterial metagenome. The 30 033 bacterial contigs were assigned to COGs, based on BLASTX results.

wide range of lytic activities: chitinolysis, proteolysis and glucanolysis (Cardinale *et al.*, 2006; Grube *et al.*, 2009, 2014; Sigurbjörnsdóttir *et al.*, 2014). Given the cellulose-rich environment of *P. membranacea*, a terricolous lichen that grows in close proximity to mosses and other vegetation, cellulolysis may be considered likely to be of particular importance for the *P. membranacea* holobiont. Cellulase systems are known and have been studied for several lichens, including *Peltigera canina* (de los Ríos *et al.*, 1997), a lichen closely related to *P. membranacea* used in our study. Thus, we screened our metagenomic dataset for cellulases, yielding several hits (172 contigs). Glycoside hydrolase (GH) gene fragments, especially GH family 5, were particularly abundant. Endocellulases (endoglucanases) belonging to GH family 5 break the internal bonds and disrupt the crystalline structure of cellulose. Although lichens generally obtain their carbon from the photosynthetic partner (Palmqvist, 2000), it has been hypothesized that cellulases found within the lichen are used for saprophytic activity, which could be beneficial for the symbiosis, e.g. when lichens are covered by snow (Beckett *et al.*, 2013). The presence of many cellulases within our dataset suggests that cellulases might help to exploit the substrate when lichens pursue facultative heterotrophy using the substrate matter under snow during long and dark winters and thus supports the previous finding of Beckett *et al.* (2013). However, cellulases might also help to degrade ageing cells in older parts of the thallus, together with proteases, which are indeed frequently found in our metagenomics dataset (369 contigs).

Enzymes participating in secondary metabolism were found in 654 contigs in our dataset. Among interesting enzymes found are carboxymethylenebutenolidase (EC 3.1.1.45) and phenol 2-monooxygenase (EC 1.14.13.7). Both these enzymes participate in gamma-hexachlorocyclohexane (HCH) degradation. HCH, also known as lindane, is widely used as an insecticide but has now been banned or restricted because of its toxicity and persistence in the environment (Cao *et al.*, 2013). The presence of these enzymes, and other enzymes related to secondary metabolites, in the metagenomic dataset might suggest that the associated microbiota somehow takes part in a defence mechanism within the symbiosis.

CONCLUSIONS

In the present study we have focused on the substantial non-phototrophic microbiota of the lichen *P. membranacea*, and hypothesized on their possible roles. We used metagenomic methods to elucidate the potential functional roles of endolichenic bacteria and hypothesized that phosphate solubilization is among the most important traits. A phylogenetic analysis of bacterial contigs from the metagenomic database revealed that the major part consists of *Proteobacteria*, with *Alphaproteobacteria* being particularly abundant. This supports previously published data on endolichenic bacteria, although metagenomic methods have not, to our knowledge, previously been used to identify the associated microbiota of *P. membranacea*. A search for genes contributing to phosphate solubilization within the dataset yielded many positive hits (1335 of 30 033 contigs), mainly belonging to *Alphaproteobacteria*, further supporting our hypothesis of the importance of phosphate solubilization within the symbiosis.

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III

Culturable phosphate-mobilizing, biosurfactant-producing and biodegradative bacteria associated with a sub-Arctic, terricolous lichen, *Peltigera membranacea*

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Abstract

By definition, lichens are the symbiotic association of fungi and a photosynthetic partner. However, non-phototrophic bacteria are also present and are thought to comprise an essential part of the lichen symbiosis, although their roles in the symbiosis are still poorly understood. In the present study, we isolated and characterized 108 non-phototrophic bacterial lichen-associates from the thalli of the terricolous lichen *Peltigera membranacea*. Among biodegradative and other nutrient-scavenging properties studied among selected isolates were phosphate-mobilization, biosurfactant production and degradation of napthalene and several biopolymers, suggesting organic and inorganic nutrient scavenging as roles for bacteria in the lichen symbiotic association. Identification by partial 16S rRNA gene sequencing revealed that the isolates comprised 18 genera within the *Proteobacteria*, *Actinobacteria*, *Bacteroidetes,* and *Firmicutes*, many with high similarities with bacteria typically associated with the plant and rhizosphere environments, suggesting that plants may be important sources of terricolous lichen-associated bacteria, or vice versa.

Keywords: *Peltigera membranacea*, lichen-associates, biosurfactant producing, phosphate-mobilizing, biodegradative bacteria

Introduction

Lichens are usually defined as the symbiotic association between fungi and photosynthetic algal or cyanobacterial partners (Nash, 2008a). Together, the partners form a long-lived and resilient structure, the thallus, which makes it possible for both symbiotic partners to live under extreme environmental conditions and withstand extreme fluctuations in, for example temperature or availability of nutrients and/or water (Bjelland et al., 2011; Cardinale, Puglia, & Grube, 2006; Cardinale *et al.*, 2012; Grube *et al.*, 2009).

Lichens harbour non-phototrophic microbiota which are now increasingly regarded as significant players of the symbiosis (Cardinale, Steinova, et al., 2012). Overall, the taxonomic bacterial diversity has been relatively well established in number of studies, using culture dependent or culture independent methods (Aschenbrenner *et al.*, 2014; Bates *et al.*, 2011; Bjelland *et al.*, 2011; Cardinale, Puglia & Grube, 2006; Grube *et al.*, 2015, 2009; Lee *et al.*, 2014; Sigurbjornsdottir *et al.*, 2014). Although the microbial community composition is considered to be dependent on the lichen species (Bates et al., 2011), the thallus age (Cardinale, Steinova, et al., 2012), the type of photosynthetic symbiotic partner (Hodkinson *et al.*, 2012), habitat (González *et al.*, 2005) and the

secondary metabolites produced by the symbiotic fungi (Bjelland et al., 2011), a number of studies suggest that *Proteobacteria* generally dominate the microbacteria (Cardinale, Puglia & Grube, 2006; Cardinale *et al.*, 2008; González *et al.*, 2005; Grube *et al*., 2009; Hodkinson *et al*., 2012; Hodkinson & Lutzoni, 2009; Liba *et al*., 2006; Muggia *et al*., 2013; Schneider *et al*., 2011).

The functional roles of lichen-associated bacteria are still largely unknown although several putative roles have been suggested, such as nitrogen fixation, defence against pathogens, nutrient scavenging, phosphate solubilization, and growth-promoting effects (Grube and Berg, 2009; Grube *et al*., 2015; Sigurbjornsdottir *et al.*, 2014).

About 90 species of foliose lichens belong to the genus of *Peltigera* (Xavier *et al.*, 2012) with a *Nostoc* cyanobacteria as the photobiont (Miadlikowska and Lutzoni, 2004). Metagenomic studies on *Peltigera* lichens have led to better understanding of the distribution and possible contribution of archaea and bacteria to the symbiosis in the lichen thallus. Analysis on the *P. membranacea* metagenome has shown *Proteobacteria* to be the predominant phylum of prokaryotes in this lichen, with *Actinobacteria, Bacteriodetes*, and *Verrucomicrobia* also present in substantial amounts (Sigurbjornsdottir, Andresson and

Vilhelmsson, 2015). Various roles of the non-phototrophic microbiota in the symbiotic association have been suggested, such as glucan degradation and phosphate solubilisation (Grube *et al*., 2013; Sigurbjornsdottir, Andresson and Vilhelmsson, 2015).

In the present study, we address further the potential nutrient-scavenging roles of cultured bacterial isolates, associated with *P. membranacea* from a sub-Arctic heathland habitat, focusing in particular on biodegradative activity and metabolic profiling of taxa identified by 16S rDNA sequencing, suggesting their potential functional roles within the symbiotic association.

2 Materials and methods

2.1 Sampling and strain isolation

Peltigera membranacea lichen thalli were sampled in August 2012, April 2013, and April 2014 at Þingmúli, eastern Iceland (65°2'16.66''N, 14°37'5, 82''W). Samples were washed with sterile water and surface sterilized by immersion in 3.0% H_2O_2 for 5 min to remove epithallic bacteria. The thalli samples were ground in Butterfields buffer, using a sterile mortar and a pestle. Serial dilutions in Butterfields Buffer were performed and 100 µL plated on nine types of media. The media used were: Tryptic Soy Agar (TSA, Difco™), Actinomycete Isolation Agar

(AIA, Difco™), Brain Heart Infusion Agar (BHI, Fluka), Reasoner's 2A agar (R2A, Difco™) and *Peltigera* Extract Agar (PEA, 200 mL *Peltigera* extract [20 g *P. membranacea* thallus, 2.0 g NaCl, 200 mL distilled water, boiled for 40 minutes, filtered through a coffee filter, diluted to 400 mL with distilled water, autoclaved], 0.4 g Na_2HPO_4, 0.4 g KNO_3, 0.4 g $MgSO_4 \cdot 7H_2O$, 6.0 g Bacto Agar, diluted to 400 mL with distilled water, heated until dissolved, pH adjusted to 7.0, autoclaved), a selective medium for isolation of inorganic phosphate-mobilizing bacteria (NBRIP medium (Nautiyal, 1999)), and differential media indicating cellulase (CLA), xylanase (XA) or amylase (STA) activity (Plate Count Agar (PCA, Difco) supplemented with 0.1% (w/v) azurine cross-linked xylan or cellulose (Megazyme, Wicklow, Ireland), or 0.2% soluble starch, respectively).

Plates were incubated in the dark at 4°C, 15°C and 22°C until no new colonies appeared (up to 4 weeks).

Colonies were isolated and purified using morphological characteristics and streaked onto respective media. Stocks of purified isolates were prepared by suspending a loopful of growth in 1.0 mL 28% (v/v) glycerol and are stored at -80°C in the University of Akureyri culture collection.

2.2 Plate Wash PCR (PW-PCR)

A modified Plate Wash PCR (PW-PCR) method from Stevenson *et al.* (2004) was used prior to isolating pure cultures. One plate of each isolation medium type was sacrificed. Plates were flooded with Bead Solution, obtained from the UltraClean® Microbial DNA Isolation Kit (MoBio, 2010) in order to dissolve colonies completely. The UltraClean® Microbial DNA Isolation Kit was used for extraction of total DNA from the colony solution, which was then PCR amplified in an MJR PTC-200 thermocycler (MJ Research Inc., MA, USA) using group specific primer pairs (see Table 1 for details). The PCR reaction was performed as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 51°C for 45 s, 72°C for 30 s; final extension was performed at 72°C for 7 min.

Bearing the results from the PW-PCR in mind, selected colonies were then picked from all media types and streaked onto fresh media in order to isolate representatives from each taxonomic group observed with PW-PCR.

2.3 Screening of *Peltigera*-associated bacteria for bio surfactant activity

Detection of bio surfactant activity was carried out for 95 isolates (DG and MAS isolates) using a drop collapse assay (Jain *et al.*, 1991); 50 µL

of distilled water was pipetted on fixated parafilm and a sterile tooth pick used to collect the test isolates. The test isolates were suspended in the water droplet and checked for drop collapse. Water was used as a negative control and a culture of Pseudomonas fluorescens as a positive control.

2.4 Functional assays with *P. membranacea*-associated isolates

Isolates were subjected to functional assays based on different growth media. Degradative assays were performed on 1.5% agar plates as follows. Plates were incubated for up to 7 days at 22°C. Casein degradation was assayed by the appearance of clear halos on 50% (v/v) skim milk in NA. *B. subtilis* DSM and sphingomonad AR0413 were applied as positive and negative controls, respectively. Luxuriant growth and clear halos on NBRIP medium (Nautiyal, 1999) and nitrogen free agar (NFA) was taken to indicate possible ability to mobilize inorganic phosphates and nitrogen fixation. *Rhodococcus* sp. KA1105 and *Bacillus* sp. KA0514 were employed as positive and negative controls for both tests, respectively. Glycanolytic activity was tested against barley beta-glucan, birch xylan and cellulose in half-strength Tryptic Soy Agar (Becton Dickinson), supplemented with the appropriate 0.2% (w/v) AZCL- cross-linked polymers (Megazyme, Wicklow, Ireland).

Appearance of blue halos was indicative of degradation. *E. coli* DSM 1103 was used as a negative control. Positive controls were *Saccharophagus degradans* DSM 17024, *Bacillus* sp. KA0902 and *Cellulophaga chitinilytica* DSM 17922 for betaglucan, xylan and cellulose, respectively.

To screen for chitinase activity, R2A broth supplemented with colloidal chitin and bromocresol purple, was used. A color change from yellow to dark purple was taken as indicative of chitin degradation (Agrawal and Kotasthane, 2012).

Selected isolates were screened for naphthalene degradation. Half strengthened TSB medium, supplemented with naphthalene crystals was used. Solution of Fast Blue B (Sigma Aldrich) was added to the cultures and a color change to pink/purple indicated naphthalene degradation. Chitin and naphthalene degradation tests were performed in 2.0 mL Eppendorf tubes and a loop full of the appropriate isolate added to the medium. The tubes were incubated at 22°C for up to 7 days.

Furthermore, selected representatives from each taxonomic class were applied to three different API systems (bioMérieux, Marcy-l'Etoile, France) for further screening. The API systems used were API 20E, API zym and API 50CH. A loop full of each isolate was suspended in sterile

dH20 (for API 20E and API zym) and CHB/E medium (for API 50CH) and inoculated to each strip according to the manufacturer´s protocol. Changes in colour (depending on each system) were noted and strips read when at least three positive tests on each strip were noted.

2.4 Phylogenetic analysis

For DNA extraction from a single colony of pure cultures, the Ultra Clean® Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) was used according to the manufacturer's instructions. The 16S rRNA gene was PCR amplified in an MJR PTC-200 thermocycler (MJ Research Inc., MA, USA) using the universal primer pair 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'- GGT TAC CTT GTT ACG ACT T-3') at a final concentration of 1.0 µM in a total volume of 25 µL of PCR mixture. The PCR reaction was carried out as follows: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 51°C for 45 s, 72°C for 30 s; final extension was performed at 72°C for 7 min. Amplicons were separated by agarose gel electrophoresis (1.3%, 25 min at 110V) in TAE buffer stained with SybrSafe and visualized under UV. The amplicons were purified with the Nucleospin Extract II (Macherey-Nagel GmbH & Co.KG, Düren, Germany) kit according to the manufacturer´s protocol. Partial

sequencing of the purified PCR products was performed with a BigDye terminator kit and run on Applied Biosystems 3130XL DNA analyzer (Applied Biosystems, Foster City, USA) at Macrogen Europe, Amsterdam, the Netherlands. The same primers as used for PCR amplification were used for the sequencing. The obtained 16S rDNA sequences were subjected to multiple alignments at the Ribosomal Database Project (RDP) (Cole et al., 2009). Reference sequences were selected from the database and *Escherichia coli* provided as the outgroup sequence. The evolutionary history was inferred using MEGA6 program with neighbor-joining method (Tamura et al., 2011), based on the distance matrix generated according to the Tamura-Nei model. The confidence level of the tree topology was evaluated by bootstrap analysis using 10,000 sequence replications.

3 Results

3.1 Identification of isolates

Thalli of the foliose lichen *Peltigera membranace* were sampled in August 2012, April 2013 and April 2014 at the sampling site indicated in section 2.1. Same media types and culture conditions were used for the first two isolation procedures whereas more selective media were used in the third one. Colonies were picked from the isolation plates and

streaked onto fresh medium for purification. A total of 110 bacterial isolates were sub-cultured and deposited into the Universtity of Akureyri culture collection where they are stored in 28% (v/v) glycerol at -80°C. Re-culturability from frozen stocks has been confirmed for all isolates.

The diversity of isolates was further assessed by 16S rDNA gene sequencing. DNA was extracted, 16S rDNA amplified and sequenced from 85 isolates, selected on the basis of colony morphology and/or biodegradative properties. Sequences (average length 950 nucleotides) were analysed by MegaBLAST against the 16S ribosomal RNA sequences database and classified by the RDP Classifier at a confidence of 70% or better. The sequenced isolates were assigned to 4 bacterial phyla (*Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*). Isolates belonging to *Proteobacteria* (55%) were the most abundant, followed by *Bacteroidetes* (27%), *Actinobacteria* (10%) and *Firmicutes* (9%). Forty-eight isolates were assigned to the genera *Burkholderia* (8), *Enterobacter* (1), *Erwinia* (1), *Janthinobacterium* (1), *Kluyvera* (1), *Luteibacter* (3), *Pseudomonas* (27), *Sphingomonas* (5) and *Variovorax* (1) of *Proteobacteria*. There were 21 isolates from the phylum *Bacteroidetes*, represented by *Dyadobacter* (7), *Mucilaginibacter* (10) and *Pedobacter* (4). Nine isolates belonged to the phylum *Actinobacteria*, represented by M*icrobacterium* (1) and *Rhodococcus*

(7). One isolate from each of the genera *Macrococcus* and *Paenibacillus* of *Firmicutes* were obtained as well as 2 from *Bacillus* and 4 from *Sporocarcina*. The overall similarity of the isolates to known type strains ranged from 95-100% (see table S1 for details). Interestingly, eight isolates with ≤ 97% similarity to 16S rRNA gene sequences of known strains belonged to the genera *Erwinia*, *Mucilaginibacter*, and *Burkholderia* and may represent novel species. Sequences are accessible at GenBank KM658456-KM658497.

A phylogenetic tree of 16S rRNA gene sequences retrieved from isolates belonging to *Dyadobacter* was constructed and is shown in Fig. 1.

Different media were used for the isolation, including commercial nutrient media, such as TSA, media for slow growing bacteria, such as R2A and more specialized media to screen for specific enzymatic activity, such as cellulase, xylanase, and amylase activity and phosphate mobilization. From these media types, six isolates formed clearing halos on xylanase agar and six on NBRIP medium (phosphate mobilization). Although eight isolates were purified from cellulose agar, none of them were taken to have clear cellulase activity as no halos were formed on the agar.

3.2 PW-PCR and targeted isolation

The results from the Plate Wash PCR assay are shown in Fig. 2.

The modified method by Stevenson *et al*. (2004) was used to screen all media types for different taxonomic groups present. Different primer pairs were used and as seen from Fig. 2, clear bands at 1500 bp, yielded from the universal primer pair 27F/1492R, are observed from all media types. Bands are also detected at 550 and 650 bp, suggesting that *Alpha*- and *Betaproteobacteria* are present on all plates. *Actinobacteria* were found on BHI, AIA and one of the two PEA plates at 1250 bp. Additionally, a band specific for *Acidobacteria*, was observed on the same PEA plate at 1300 bp.

3.3 Bio surfactant activity

A total of 95 isolates were tested for bio surfactant activity, using a simple drop collapse test (Table 2). Of the tested isolates, 47 were positive, thereof 3 with very strong bio surfactant activity as compared to the positive control used. Details regarding taxonomic diversity of the tested isolates are shown in table S2.

3.4 Enzymatic activity

Isolates were screened for their extracellular enzymatic activity at 22°C. Properties tested were inorganic phosphate solubilisation, nitrogen

fixation, degradation of several glycans, protease activity and naphthalene degradation (Table 2).

Proteolytic activity, judged by a clearance halo on skimmed milk agar, was observed for 38% of the tested isolates, affiliated with *Microbacterium, Rhodococcus, Pedobacter, Bacillus, Macrococcus, Burkholderia, Janthinobacterium, Pseudomonas* and several unidentified isolates. Glucanolytic activity (beta-glucanase, cellulose and xylanase) was observed for 16, 12 and 9%, respectively. About 35% of the isolates had chitinolytic activity, the majority assigned to the genus *Pseudomonas*. Solubilization of inorganic phosphate was common, nearly half (45%) of the tested isolates are capable of phosphate solubilization. About one-third (31%) of the tested isolates formed clear halos on nitrogen free agar (NFA), suggesting they can fix nitrogen from the atmosphere. Naphthalene degradation was rare, only eight of the tested isolates, belonging to *Muciliganibacter, Pedobacter, Burkholderia, Pseudomonas* and a few unidentified isolates were positive. See table S2 for details of degradation tests.

For further characterization, selected isolates were applied to three different API systems (BioMerieux) according to the manufacter‘s directions. Isolates were selected on the basis of their taxonomy where

at least one representative from each class (API ZYM) or genus (API 20E and API CH50) was chosen.

A majority of the selected isolates were found to be beta-galactosidase and arginine dihydrolase producing, whereas none of the tested isolates produced lysine or ornithine decarboxylases or produced H_2S from sodium thiosulfate (Table 3). Glucose was fermented by all isolates except four (belonging to *Pedobacter, Bacillus, Sporocarcina* and *Janthinobacterium*), but inositol, D-sorbitol, L-rhamnose and D-sucrose was not utilized by any of the tested isolates. Nitrate was reduced to nitrite by six isolates. Of the total of 12 isolates applied to API ZYM, 7 gave a positive reaction for alkaline phosphatase (Table 4) and all of them, except two, also for acid phosphatase. Isolate MAS002 (belonging to *Dyadobacter*) gave positive reaction to only two tests, esterase C4 and N-acetyl-β-glucosiminidase. All the other tested isolates were postive for most of the tests included in API zym.

The growth of the selected isolates on API CH50 strips is summarized in Table 5. Two isolates, MAS022 and DG135 were only positive on three or fewer tests. The carbon assimilation pattern was, as expected, different between species. Nineteen carbon sources were assimilated by none of

the tested isolates (erythritol, L-sorbose, sorbitol, inulin, xylitol, L-arabitol, gluconate, 2-keto-gluconate, and 5-keto-gluconate).

4 Discussion

Although it has been suggested that the composition of the associated microbiota of lichens are somewhat dependent on the on lichen type (Bates et al., 2011), thallus age (Cardinale, Steinova, et al., 2012), type of photosynthetic symbiotic partner (Hodkinson et al., 2012), habitat (González et al., 2005) and the secondary metabolites produced by the symbiotic fungi (Bjelland et al., 2011), previously published studies have suggested that *Proteobacteria* generally dominate the microbacteria (Cardinale, Puglia and Grube, 2006; Cardinale *et al.*, 2008; González *et al.*, 2005; Grube *et al.*, 2009; Hodkinson *et al.*, 2012; Hodkinson and Lutzoni, 2009; Liba *et al.*, 2006; Muggia *et al.*, 2013; Schneider *et al.*, 2011). The *P. membranacea*-associated microbiota, as determined by metagenomic analysis, has been found to be dominated by members of the alphaproteobacterial orders *Rhizobiales*, *Sphingomonadales*, *Caulobacterales*, and *Rhodospirillales*, and the betaproteobacterial order *Bukholderiales* (Sigurbjornsdottir, Andresson and Vilhelmsson, 2015), not all of which are readily cultured by the methods used in the present study, where 110 bacterial strains were isolated from the thalli of *P.*

membranacea sampled from a scrub heath environment in eastern Iceland in August 2012, April 2013 and April 2014. We therefore did not expect our culturing efforts to yield a collection representative of the taxonomic composition of the microbiota, although some of the dominant taxa, such as *Sphingomonas* and *Burkholderia*, are represented, but focussed instead on obtaining a collection of isolates with certain biodegradative and other nutrient scavenging properties indicated by our previous study to be of importance to the *Peltigera*-associated microbiome (Sigurbjornsdottir, Andresson and Vilhelmsson, 2015). Incubation conditions and media types reflected this goal and included selective and differential media for isolation of phosphate-mobilizares, xylan degraders, cellulose degraders, and more. Prior to the isolation of pure culture, we used a modified method from Stevenson *et al.* (2004) to screen for taxonomic groups present on each media plate, thereby increasing the taxonomic diversity of our isolates.

Overall, the taxonomic composition of the isolates in the present study is dominated by *Proteobacteria* (54%), with representatives of *Bacteroidetes* (27%), *Actinobacteria* (10%) and *Firmicutes* (9%) also present, which is similar to other culture-based studies on lichen-associated bacteria. Surprisingly, only 5 isolates belong to *Alphaproteobacteria* and are all affiliated with the genus *Sphingomonas*.

Overall, the genus of *Sphingomonas* comprises plant-associated bacteria and some species are regarded as plant growthpromoting and thought to reduce plant diseases (H. Kim et al., 1998). In a previous study by Lee *et al.* (2014), *Alphaproteobacteria* and especially *Sphingomonas* are particularly abundant although alphaproteobacterial groups are poorly represented in other recent studies on cultured lichen associates (Cardinale, Puglia and Grube, 2006; Printzen *et al.*, 2012; Selbmann *et al.*, 2010). MAS005, selected arbitrarily to represent the sphingomonads of the present study, possesses for example alkaline phosphatase activiy (Table 4) and mobilizes inorganic phosphate (Table S2). It also displays several biodegradative properties, such as gelatinase, xylanase and betaglucanase activity (Table 3, S2), suggesting secretion of biodegradative enzymes as a potentially important role of these comparatively abundant members of the *Peltigera*-associated microbiota.

It has become increasingly apparent in the past few years that lichens harbour a specific bacteriota distinct from that of the surrounding environment. Nevertheless, exchange of bacteria from lichen to its environment and vice versa is thought likely to occur (Vilhelmsson *et al.*, 2016). In the present study, the terricolous lichen *P. membranacea* was sampled from grass swards in densely vegetated scrub heath habitat

types in eastern Iceland and typically plant- and rhizosphere-associated bacteria were indeed found among the isolates, as demonstrated by the gammaproteobacterial isolates, most of which were identified as close relatives of plant-associated bacteria.

The gammaproteobacterial isolates fall into three families; the *Enterobacteriaceae*, the *Xanthomonadaceae*, and the *Pseudomonadaceae*. Although the three isolates assigned to the family *Enterobacteriaceae* (MAS010, MAS017, and MAS019) appear somewhat diverse, being assigned to three genera (*Kluyvera*, *Enterobacter* and *Erwinia*, respectively) they all appear quite similar to bacteria known for their close association with plants, such as endophytic, plant growth-promoting or phytopathogenic bacteria (Hardoim *et al*., 2013; Palacio-Bielsa *et al.*, 2012; Wenbo, Kelly and Glick, 2001), and are all chitinolytic (Table S2). *Enterobacter* MAS017 further possesses both acid and alkaline phosphatase activity and *Erwinia* MAS019 is lipolytic (Table 4). Three strains (MAS015, MAS020, and MAS041) were assigned to the xanthomonad genus *Luteibacter*, all isolated on differential media for cellulose or strach degradation. Members of this genus have been known to inhabit the endohyphal environment in fungi (Hoffman *et al.*, 2013), suggesting that these bacteria may be endosymbionts of the mycobiont. Members of this

genus have been isolated from lichens before (Cardinale, Puglia and Grube, 2006; Grube *et al.*, 2009), but no attempts were made at characterizing their activity or role in the lichen symbiosis in these studies. In the present study, *Luteibacter* MAS020 was found to produce an acid phosphatase, possess both trypsin-like and gelatinase-like proteolytic activity activity, amino acid amidase activities, and produce biosurfactants (Tables 3, 4, and S2). Among its carbon sources were several monosaccharides, disaccharides and gycisides, such as glucose, xylose, galactose, fructose, lyxose, fucose, melibiose, inositol, *N*-acetylglucosamine, and esculin (Table 5), in good agreement with the published characterization of *L. rhizovicinus*, a species originally isolated from barley rhizosphere in Denmark (Johansen *et al.*, 2005). The association of *Luteibacter* with both the rhizosphere and the terricolous lichen *P. membranacea*, which in this study was isolated from dense grass swards, bring to mind the question of whether plant and lichen environments exchange bacterial symbionts to some extent (Vilhelmsson et al., 2016).

A large fraction of the gammaproteobacterial isolates were pseudomonads, perhaps not unexpectedly, as members of this genus are frequently isolated under similar conditions from various habitats in Iceland (Joelsson, Fridjonsdottir, and Vilhelmsson, 2013; Markusdottir

et al., 2013; Þorsteinsdóttir and Vilhelmsson, 2013) and have also been isolated from lichens (Selbmann et al., 2010). While it is not advisable to identify bacteria to higher resolution than the genus level by 16S rDNA analysis alone (Dahllöf, Baillie and Kjelleberg, 2000; Fox, Wisotzkey and Jurtshuk, 1992), we note that the isolates were found to be most similar to 6 commonly plant-associated species, namely *P. brassicacearum, P. fluorescens, P. frederiksbergensis, P. helmanticensis, P. tolaasii,* and *P. veronii*.

Seven isolates were assigned to the genus of *Burkholderia*, being most similar to the phytopathogenic species *B. glathei,* as well as to *B. sordidicola* and *B. terrestris* with similarities ranging from 97-99% to other known species. At least two of the eight *Burkholderia* isolates are capable of degrading naphthalene (Table S2), indicating polyaromatics degradation as a possible survival factor within the lichen environment. As judged by the partial metagenome of *P. membranacea*, *Variovorax* members are quite abundant (Sigurbjornsdottir, Andresson and Vilhelmsson, 2015). Based on a Blastn search, one isolate, MEA010, belongs to the genus *Variovorax* and is 99% similar to *V. paradoxus*. To date, *Variovorax* spp. have not been isolated from lichens before, although members of this genus is quite commonly found in soil and as plant associated bacteria (Han et al., 2011).

Previous studies on various lichen species have suggested the *Actinobacteria* as frequent members of the associated biota (Aschenbrenner *et al*., 2014; Bjelland *et al*., 2011; Grube *et al.,* 2012; Parrot *et al*., 2015; Selbmann *et al*., 2010). In our study, a total of 9 isolates belong to *Actinobacteria*, all assigned to the genera *Microbacterium* and *Rhodococcus*.

Members of the *Bacteroidete*s have been isolated from lichen thalli although they have not been prominent in the associated biota reported thus far (Bates et al., 2011; Lee et al., 2014; Sigurbjornsdottir et al., 2014). In the present study, isolates were affiliated with genera *Dyadobacter, Mucilaginibacter* and *Pedobacter* of the *Bacteroidetes* phylum. The genus *Dyadobacter* comprises 12 species isolated from various environmental samples but members have not, to our knowledge, been isolated from lichens thus far. The isolates, affiliated with *Dyadobacter*, in our study were most similar to the species *D. hamtensis* (6) and *D. psychrophilius* with similarities ranging from 97-99%. As seen from the phylogenetic tree, represented in Fig. 1, the *Dyadobacter* isolates in our study are distinguishable from *Dyadobacter* isolated from other sources as well as from other bacteria, isolated from lichen thalli and belong to *Bacteroidetes*. Thus, we suggest that the lichen thalli of *P. membranacea* harbour bacterial associates not found elsewhere. Isolates

in our study, belonging to the genus *Mucilaginibacter*, were a total of 10 and could be assigned to the following species: *M. dorajii* (1), *M. gynuensis* (5), and *M. rigui* (4).

We screened the isolates for production of several extracellular enzymes at 22°C. About one-third of the tested isolates were able to grow on nitrogen free medium, indicating their ability to fix nitrogen from the atmosphere. Nitrogen fixation is well known among many lichen bacterial associates and the members provide nitrogen to the lichen symbiotic system. However, such bacteria are mainly important in lichens with non-nitrogen fixating cyanobacteria (M. Grube and Berg, 2009). Since *P. membranacea* has a *Nostoc* cyanobacteria as the photosynthetic partner (Xavier et al., 2012), it is not surprising that only a small part of the tested isolates were able to fix nitrogen.

As suggested in the previous paper published on the metagenome of *P. membranacea*, phosphate solubilisation is among the most important roles of lichen associates (Sigurbjornsdottir, Andresson and Vilhelmsson, 2015). Within the metagenome, many genes contributing to phosphate solubilisation were detected, including phosphatases participating in the release of inorganic phosphorus, as well as organic, alkaline and transport phosphatases. Corresponding to the previous

results, 45% of the tested isolates from *P. membranacea* formed clearing zones on NBRIP agar, underscoring the importance of solubilisation of phosphates by the lichen associates. Similar results were published by Grube *et al.* (2015) on the metagenome of the lung lichen *Lobaria pulmonaria*. Furthermore, many of the selected isolates applied to API zym strips were positive for both alkaline and acid phosphatases, agreeing well the previous results on phosphate mobilizing ability of lichen associated bacteria.

One of the suggested roles of lichen associated bacteria is that they secrete enzymes that are needed for degradation of older thallus parts, possibly providing nutrients for younger parts and thus maintaining the nutrient cycle within the lichen thallus (Cardinale, Puglia and Grube, 2006; Grube & Berg, 2009; Grube *et al.*, 2015; Sigurbjornsdottir *et al.*, 2014). Therefore, we screened our isolates for protease and glycanase activity at 22°C. Protease activity was common among the isolates. About 38% tested positive in casein degradation suggesting that the isolates secrete proteases, suggesting extracellular protein degradation as an important role. Cellulase, glycanase, and xylanase activity was less common. Halos on media, supplemented with the appropriate polymer, were observed for 12%, 16%, and 9%, respectively, of the tested isolates. These results are, however, in good accordance with other recent

cultivation-based studies as well as metagenomics studies (Grube *et al*., 2015; Schneider *et al*., 2011; Sigurbjornsdottir, Andresson and Vilhelmsson, 2015).

The use of surfactants in various industry applications has increased enormously over the past decades (Marchant and Banat, 2012). Biosurfactants could possibly replace chemical surfactants and are currently widely studied. Among the most promising biosurfactants at the present time are rhamnolipids produced by *Pseudomonas* (Chrzanowski et al., 2012). We used a simple drop collapse test to screen selected isolates for possible bio surfactant activity. Of the 95 tested isolates, half (47) were positive, including three isolates with very strong activity as compared to the positive control we used. In addition, eight out of the 95 tested isolates could degrade naphthalene, suggesting that degradation of polyaromatics may be among the survival strategies of *Peltigera*-associated bacteria, albeit not a particularly common one. Thus, we suggest that non-phototrophic bacteria not only are ecologically important in the lichen symbiosis but also that they secrete enzymes of biotechnological commercial interest.

In the present study we isolated bacterial strains from the thalli of *P. membranacea*, yielding a taxonomically and functionally diverse

collection of isolates. This is the first study of the culturable bacteria from *P. membranacea* and is in good accordance with previously published results on the metagenome of this lichen, both in terms of taxonomy and enzyme activity. We find the number, identities and activities of taxa more commonly associated with plants noteworthy and possibly indicative of a more promiscuous interkingdom exchange of bacteria between plants and this terricolous lichen than previously thought. Overall, the bacteria in the present study possess a myriad of biodegradative activities, supporting their roles as nutrient scavengers in the lichen symbiotic association.

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Table 3. Results of API 20E on selected isolates.

	Phylogenetic analysis / strain ID																
	Actinobacteria			*Bacteriodetes*		*Firmicutes*				*Alpha-proteobact.*	*Beta-proteobact.*		*Gammaproteobact.*				
	Microbacterium MAS059	*Rhodococcus* DG114	*Dyadobacter* MAS002	*Mucilaginibacter* MAS022	*Pedobacter* MAS018	*Bacillus* DG131	*Macrococcus* DG124	*Paenibacillus* DG135	*Sporosarcina* DG121	*Sphingomonas MAS005*	*Burkholderia* MAS009	*Janthinobacterium* MAS001	*Erwinia* MAS019	*Enterobacter* MAS017	*Kluyvera* MAS010	*Luteibacter* MAS020	*Pseudomonas* MAS054
Hydrolysis of:																	
ONPG	+	+	-	+	+	+	-	+	+	-	-	-	+	+	+	-	-
Arginine dihydrolase	-	-	+	-	+	+	-	-	+	+	+	+	+	+	-	-	+
Lysine decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	-	-	+	+	-	-	-	-	-	+	+	+	-	-	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	+
Tryptophan deaminase	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-

Indole production	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Acetoin production	+	-	-	+	+	+	-	-	-	-	+	-	+	+	+	-	+
Gelatinase activity	+	+	+	-	+	+	+	+	+	+	+	-	-	-	-	+	+
Fermentation/ oxidation:																	
D-glucose	+	+	+	+	-	-	+	+	-	+	+	-	+	+	+	+	+
D-mannitol	+	-	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-melibiose	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	+
Amygdalin	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-
L-arabinose	+	+	+	-	-	-	-	-	-	+	+	-	+	+	+	-	+
NO_3 reduction	+	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-
NO_2 reduction	-	-	+	-	+	+	-	-	-	-	-	-	-	+	+	-	+

Table 4. Results of API ZYM tests on selected isolates.

	Phylogenetic analysis / strain ID											
	Actinobacteria	*Bacteroidetes*			*Firmicutes*		*Alpha-proteobact.*	*Beta-proteobact.*	*Gammaproteobact.*			
Enzyme	*Microbacterium* MAS059	*Dyadobacter* MAS002	*Mucilaginibacter* MAS022	*Pedobacter* MAS018	*Bacillus* DG131	*Sporosarcina* DG121	*Sphingomonas* MAS005	*Burkholderia* MAS009	*Erwinia* MAS019	*Enterobacter* MAS017	*Luteibacter* MAS020	*Pseudomonas* MAS054
Alkaline phosphatase	+	-	+	+	-	-	+	+	-	+	-	+
Acid phosphatase	+	-	+	-	+	-	-	-	+	+	+	+
Naphthol phosphatase	+	+	+	+	+	-	+	+	+	+	-	+
Esterase (C 4)	+	+	-	+	+	+	+	+	-	+	-	+
Esterase Lipase (C 8)	+	-	-	+	+	+	+	+	+	+	-	+
Lipase (C 14)	-	-	+	+	-	+	-	-	+	-	-	-
Leucinearylamidase	+	-	+	+	+	-	+	+	-	-	+	-
Valinearylamidase	+	-	+	-	+	-	-	+	-	-	+	-
Cysteine arylamidase	+	-	+	-	+	+	-	+	-	-	+	-
Trypsin	+	-	+	-	+	-	-	+	+	-	+	-
α-chymotrypsin	+	-	+	-	+	-	+	+	+	-	-	-
α-galactosidase	-	-	-	-	-	+	+	-	-	+	-	+
β-galactosidase	-	-	-	-	+	+	-	-	+	+	-	+
β-glucuronidase	+	-	-	-	-	-	+	-	+	+	-	+
α-glucosidase	+	-	-	-	-	-	-	-	+	+	-	+

β-glucosidase	+	-	-	-	+	+	-	-	+	+	-	+
N-acetyl-β-glucosiminidase	-	-	+	+	+	+	+	+	+	-	+	-
α-mannosidase	+	-	-	+	-	-	-	-	+	-	-	-
α-fucosidase	+	-	-	-	-	+	-	-	+	-	-	-

Table 5. Results of API 50CH on selected isolates.

	Phylogenetic analysis / strain ID																
	Actinobacteria		*Bacteriodetes*			*Firmicutes*				*Alpha proteo bact.*	*Betaproteobact.*			*Gammaproteobact.*			
	Microbacterium MAS059	*Rhodococcus* DG114	*Dyadobacter* MAS002	*Mucilaginibacter* MAS022	*Pedobacter* MAS018	*Bacillus* DG131	*Macrococcus* DG124	*Paenibacillus* DG135	*Sporosarcina* DG121	*Sphingomonas MAS005*	*Burkholderia* MAS009	*Janthinobacterium* MAS001	*Erwinia* MAS019	*Enterobacter* MAS017	*Kluyvera* MAS010	*Luteibacter* MAS020	*Pseudomonas* MAS054
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-arabinose	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+
L-arabinose	+	+	+	-	+	+	-	-	-	+	-	-	+	+	+	+	+
Ribose	+	+	+	-	-	+	-	-	+	+	+	-	+	+	+	-	+
D-xylose	+	+	+	-	+	+	-	-	-	+	+	-	-	-	-	+	+
L-xylose	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+
Adonitol	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+
β-Methyl-D-xyloside	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	-	+	+	-	-	-	-	+	-	+	+	+	+	+
D-Glucose	+	+	+	-	+	+	+	-	+	-	-	+	+	+	+	+	+
D-fructose	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+	+	+
D-mannose	+	+	+	-	+	+	-	-	+	-	+	+	+	+	+	-	+
L-sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Rhamnose	-	-	+	-	-	-	-	-	-	-	+	-	-	+	+	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Mannitol	+	+	+	-	-	-	+	-	-	-	-	+	+	+	+	-	-
Sorbitol	-		-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
α-Methyl-D-mannoside	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
α-Methyl-D-glucoside	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
N-acetyl-glucosamine	-	+	+	+	-	-	-	-	+	-	-	-	+	+	+	+	-
Amygdalin	-	+	-	-	+	+	-	-	+	-	-	-	-	-	+	-	-
Arbutin	+	+	+	-	-	+	-	-	+	-	-	-	+	+	+	-	-
Esculin	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
Salicin	-	+	+	-	-	-	-	-	+	-	-	-	+	+	+	-	-
Cellobiose	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Maltose	+	+	-	-	+	-	+	-	-	-	-	-	-	-	+	-	+
Lactose	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+
Melibiose	+	+	+	-	+	-	-	-	-	-	+	-	-	-	+	+	+
Sucrose	+	+	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-
Trehalose	+	+	+	-	+	+	-	-	+	-	-	-	+	+	+	-	+
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
D-Raffinose	+	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-
Starch	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Glycogen	-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B-Gentobiose	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Turanose	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
D-Lyxose	+	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	+

D-Tagatose	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	
D-Fucose	-	-	+	+	-	-	-	-	-	+	+	-	-	-	+	+	+	
L-Fucose	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	
D-Arabitol	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
2-keto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
5-keto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Table S1. Identity of isolates as revealed by partial 16S rRNA gene sequencing.

Taxon	Strain #	Isolation media	Seq. length	Most similar BLAST hit (16S db)	Max seq. ID (%)	Query Cov'ge (%)
Actinobacteria						
	MAS059	XA	1362	*Microbacterium oxydans*	99	100
	DG126	AIA	716	*Rhodococcus erythropolis*	99	99
	DG127	AIA	941	*R. erythropolis*	99	100
	DG129	TSA	953	*R. erythropolis*	99	99
	MAS037	STA	836	*R. erythropolis*	100	99
	MAS053	XA	876	*R. erythropolis*	99	100
	MAS060	XA	798	*R. erythropolis*	99	100
	DG114	PEA	583	*R. erythropolis*	95	99
Bacteroidetes						
	MAS002	R2A	719	*Dyadobacter hamtensis*	97	91
	MAS013	CLA	981	*D. hamtensis*	99	99
	MAS025	CLA	1071	*D. hamtensis*	99	100
	MAS031	R2A	1176	*D. hamtensis*	98	100
	MAS034	R2A	1329	*D. hamtensis*	98	100
	MAS040	STA	394	*D. hamtensis*	97	100
	MAS045	NBRIP	249	*D. psychrophilus*	99	100
	MAS003	R2A	1073	*Mucilaginibacter dorajii*	97	100
	MAS004	R2A	1099	*M. gynuensis*	99	100
	MAS006	R2A	965	*M. gynuensis*	96	100
	MAS028	R2A	1102	*M. gynuensis*	99	100
	MAS030	R2A	554	*M. gynuensis*	97	99
	MAS039	STA	1103	*M. gynuensis*	99	100
	MAS022	STA	813	*M. rigui*	99	100
	MAS024	STA	816	*M. rigui*	98	100
	MAS032	R2A	1018	*M. rigui*	99	100
	MAS061	XA	818	*M. rigui*	98	100
	MAS035	R2A	819	*Pedobacter borealis*	98	100
	MAS018	CLA	816	*P. roseus*	99	100
	MAS029	R2A	1352	*P. roseus*	99	100
	MAS011	CLA	1056	*P. soli*	98	100
Firmicutes						
	DG131	TSA	287	*Bacillus pumilus*	99	100
	DG133	TSA	1199	*B. safensis*	99	98
	DG124	BHI	992	*Macrococcus bovicus*	99	100
	DG135	TSA	687	*Paenibacillus xylanexedens*	99	100
	DG118	TSA	1424	*Sporocarcina aquimarina*	98	100

	DG125	BHI	983	*S. aquimarina*	99	100
	DG121	BHI	1022	*S. psychrophila*	98	99
	DG122	BHI	1450	*S. psychrophila*	98	99
Alphaproteobact.						
	MAS005	R2A	1027	*Sphingomonas faeni*	98	99
	MAS012	CLA	891	*S. faeni*	98	100
	MAS033	R2A	1240	*S. faeni*	99	99
	MAS036	R2A	1323	*S. faeni*	99	99
	MAS057	XA	1250	*S. faeni*	99	100
Betaproteobact.						
	MAS008	R2A	974	*Burkholderia glathei*	98	100
	MAS056	XA	1388	*B. sordidicola*	99	100
	MAS062	XA	1395	*B. sordidicola*	98	100
	MAS058	XA	1482	*B. sordidicola*		
	MEA006	TSA	1010	*B. sordidicola*	98	100
	MAS009	R2A	1351	*B. terrestris*	98	100
	MAS014	CLA	1362	*B. terrestris*	98	100
	MAS021	STA	1070	*B. terrestris*	97	99
	MAS001	R2A	1338	*Janthinobacterium agaricidamnosum*	98	100
	MEA010	AIA	714	*Variovorax paradoxus*	98	100
Gammaproteobact.						
	MAS017	CLA	554	*Enterobacter cancerogenus*	99	100
	MAS019	STA	509	*Erwinia persicina*	95	96
	MAS010	R2A	999	*Kluyvera intermedia*	98	100
	MAS015	CLA	1363	*Luteibacter rhizovicinus*	99	100
	MAS020	STA	1072	*L. rhizovicinus*	99	100
	MAS041	CLA	968	*L. rhizovicinus*	99	100
	DG102	TSA	1037	*Pseudomonas brassicacearum*	99	100
	DG107	TSA	1052	*P. brassicacearum*	99	100
	MEA002	TSA	959	*P. brassicacearum*	99	100
	MAS044	NBRIP	1081	*P. brassicacearum*	99	100
	DG105	TSA	971	*P. fluorescens*	98	100
	DG106	TSA	1014	*P. fluorescens*	96	100
	DG109	BHI	787	*P. fluorescens*	98	100
	DG116	AIA	960	*P. fluorescens*	99	99
	DG117	TSA		*P. fluorescens*		
	DG119	BHI	544	*P. fluorescens*	99	100
	MAS043	NBRIP	830	*P. fluorescens*	99	100
	MAS046	NBRIP	1038	*P. fluorescens*	99	100
	MAS047	NBRIP	1048	*P. fluorescens*	99	100

MAS054	XA	1434	*P. fluorescens*	99	100
MAS007	R2A	968	*P. fluorescens*	99	99
MAS027	R2A	1035	*P. fluorescens*	99	99
DG104	TSA	1348	*P. frederiksbergensis*	99	100
DG108	TSA	974	*P. frederiksbergensis*	99	100
DG113	PEA	520	*P. frederiksbergensis*	99	98
DG120	BHI	1372	*P. frederiksbergensis*	99	100
DG128	TSA	599	*P. frederiksbergensis*	99	100
DG112	AIA	630	*P. frederiksbergensis*	99	100
DG110	BHI	604	*P. helmanticensis*	98	99
DG123	BHI	513	*P. salomonii*	99	100
DG130	TSA	677	*P. salomonii*	99	99
MAS051	XA	1368	*P. tolaasii*	99	100
MAS048	NBRIP	1195	*P. veronii*	99	100

Table S2. Taxonomy and biodegradatvie results of P. membranacea-associated isolates

			Substrate or activity tested								
Taxon		Isolate ID	Barley betaglucane	CM-cellulose	Casein	Birchwood xylan	Chitin	N-fixation	Phosphate mobilization	Naphthalene degradation	Bio surfactant activity
Actinobacteria											
	Microbacterium	MAS059	-	-	+	-	-	-	-	-	-
	Rhodococcus	DG114	++	+	+	++	-	-	-	-	-
		DG126	-	-	-	-	-	(+)	-	-	-
		DG127	-	-	-	-	-	(+)	-	-	-
		DG129	-	-	-	-	-	+	-	-	-
		MAS053	-	-	-	-	-	+	+	-	-
		MAS060	-	-	-	-	-	+	+	-	+
		MAS037	-	-	-	-	+	+	+	-	+
Bacteroidetes											
	Dyadobacter	MAS002	-	-	-	-	-	+	-	-	+
		MAS013	-	-	-	-	-	-	-	-	-
		MAS025	-	-	-	-	-	-	-	-	+
		MAS031	-	-	-	-	+	-	-	-	-
		MAS034	+	-	-	-	-	-	-	-	-
		MAS040	-	-	-	-	-	-	-	-	-
		MAS045	-	-	-	-	+	-	+	-	-
	Mucilaginibacter	MAS003	-	-	-	-	-	+	+	-	-
		MAS004	-	-	-	-	+	-	-	-	-
		MAS006	-	-	-	-	-	+	-	-	-
		MAS028	-	-	-	-	-	-	-	-	-
		MAS030	+	+	-	-	-	+	-	-	-
		MAS039	-	-	-	-	-	-	-	-	-
		MAS022	-	-	-	-	-	-	-	+	+
		MAS024	+	-	-	-	-	-	-	-	+
		MAS032	-	-	-	-	-	-	-	-	+
		MAS061	+	-	-	-	-	+	-	-	+
	Pedobacter	MAS035	-	+	+	-	-	+	+	-	+
		MAS018	-	-	-	-	+	+	-	+	+
		MAS029	+	-	-	-	-	+	+	-	+
		MAS011	+	-	-	-	-	-	-	-	+
Firmicutes											
	Bacillus	DG131	+	-	+	+	-	-	-	-	-
		DG133	+	-	+	+	-	-	-	-	-

	Macrococcus	DG124	-	-	+	-	-	-	-	-	-
	Paenibacillus	DG135	+	+	-	+	-	-	+	-	-
	Sporocarcina	DG118	-	-	-	-	-	-	-	-	-
		DG122	-	-	-	-	-	-	-	-	-
		DG121	-	-	-	-	-	-	+	-	+
		DG125	-	-	-	-	-	-	-	-	+
Proteobacteria											
	Burkholderia	MAS008	-	+	-	-	+	+	+	-	-
		MAS056	-	-	-	-	-	+	-	-	-
		MAS058	-	-	-	+	-	-	-	+++	+
		MAS062	-	-	-	-	-	-	-	-	+
		MEA002	-	-	+	-		+	+		
		MAS009	-	-	-	-	-	-	-	+	-
		MAS014	-	-	-	-	-	-	-	-	+
		MAS021	-	-	-	-	-	-	-	-	+
	Enterobacter	MAS017	-	-	-	-	+++	+	-	-	+
	Erwinia	MAS019	-	-	-	-	+++	-	+	-	-
	Janthinobacterium	MAS001	-	-	+	-	-	-	+	-	-
	Kluyvera	MAS010	-	+	-	-	+	+	+	-	-
	Luteibacter	MAS015	-	-	-	-	+	-	-	-	+
		MAS020	-	-	-	-	-	-	-	-	+
		MAS041	-	-	-	-	-	+	-	-	+
	Pseudomonas	DG102	-	-	+	-	-	(+)	+	-	-
		DG107	-	-	+	-	+	-	+	-	-
		MAS044	+	-	-	-	-	-	-	-	-
		DG117	-	-	++	-	+	-	+	-	+
		MAS007	-	+	+	-	++	-	+	+	+++
		MAS027	+	+	+	-	++	+	+	-	+
		MAS043	-	+	+	-	-	-	+	-	-
		MAS046	-	-	+	-	-	+	+	-	-
		MAS047	-	-	-	-	-	+	+	-	+
		MAS054	-	+	+	-	+	+	+	-	++++
		DG104	-	-	+	-	-	-	+	-	+
		DG108	-	-	+	-	+	-	+	-	-
		DG120	-	-	+	-	+	-	+	-	+
		DG110	-	-	+	-	+	-	+	-	+
		DG105	-	-	++	-	+	-	-	-	+
		DG106	-	-	++		+	-	-	-	+
		DG109	-	-	++	-	+	-	+	-	+
		DG112	-	-	(+)	-	-	-	-	-	-
		DG113	-	-	+	-	-	-	+	-	-
		DG116	-	-	++	-	+	-	-	-	+
		DG119	-	-	++	-	+	-	+	+	+
		DG123	-	-	++	-	+	-	+	-	+

	DG128	-	-	+	-	+	(+)	+	-	-
	DG130	-	-	++	-	+	-	+	-	+
	MAS051	-	-	-	-	-	-	-	-	-
	MAS048	-	-	-	-	-	-	-	-	-
Sphingomonas	MAS005	+	-	-	+	-	-	+	-	-
	MAS012	-	-	-	+	-	-	-	-	+
	MAS033	-	-	-	-	-	+	-	-	+
	MAS036	-	-	-	-	+	+	+	-	-
	MAS057	-	-	-	+	-	-	-	-	-
Variovorax	MEA010	+	-	-	-	-	-	-	-	
Unidentified	DG101	-	-	+	-	-	-	+	+	-
	DG103	-	-	+	-	+	-	+	-	+
	DG115	-	-	++	-	+	-	+	-	+
	DG132	-	-	++	-	+	-	+	-	+
	DG134	-	-	++	-	+	-	+	-	+
	MAS016	-	-	-	-	-	+	-	-	-
	MAS023	-	-	-	-	-	+	+	-	-
	MAS026	-	-	-	-	+	+	+	-	-
	MAS038	-	-	-	-	-	-	-	-	-
	MAS042	-	+	+	-	++	-	+	+	+
	MAS049				-					
	MAS050	-	-	+	-	++	+	+	-	+++
	MAS052	-	-	-	+	-	-	+	-	+
	MAS055	-	+	+	-	++	-	+	-	+
	MEA003	-	-	-	-	-	-	-	-	
	MEA004									
	MEA005	-	-	-	-		-	-		
	MEA006	+	-	+	-		-	+		
	MEA007	++	-	-	-		-	-		
	MEA008									
	MEA009	-	-	-	-	-	-	-	-	
	MEA011									
	MEA012	-	-	-	-		-	-		
	MEA013	-	-	-	-		-	-		

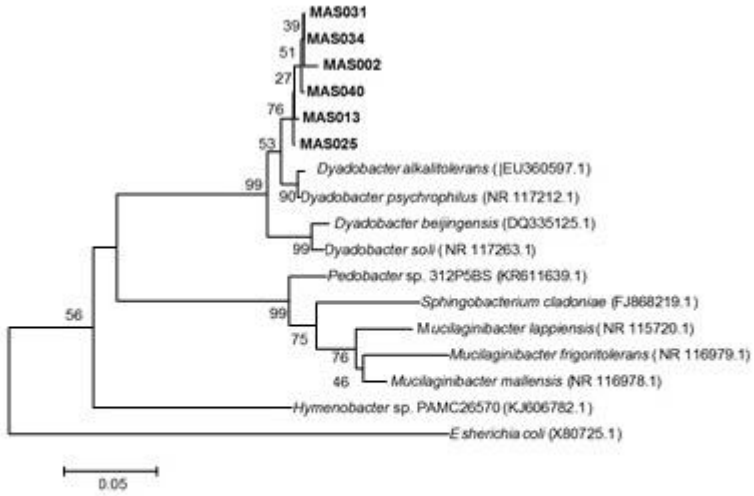

Figure 1. Evolutionary relationships of Dyadobacter isolated from P. membranacea and selected Bacteroidetes isolates from lichens. The optimal tree with the sum of branch length = 0.86716656 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches. All positions containing gaps and missing data were eliminated. A total of 381 positions were in the final dataset.

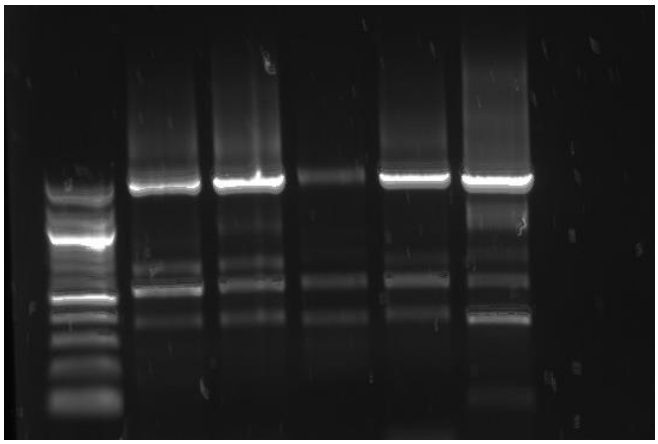

Figure 2. Results from the PW-PCR analysis. DNA was extracted from colonies on different media types. Wells represent samples (from left): 100 bp ladder, BHI, AIA, PEA, TSA and PEA media.

IV

Nutrient scavenging activity and antagonistic factors of non-photobiont lichen-associated bacteria: A review

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Abstract

Lichens are defined as the specific symbiotic structure comprising a fungus and a green alga and/or cyanobacterium. Up until recently, non-photobiont endothallic bacteria, while known to be present in large numbers, have generally been dismissed as functionally irrelevant cohabitants of the lichen thallus, or even environmental contaminants. Recent analyses of lichen metagenomes and innovative co-culture experiments have uncovered a functionally complex community that appears to contribute to a healthy lichen thallus in several ways. Lichen-associated bacteriomes are typically dominated by several lineages of *Proteobacteria*, some of which may be specific for lichen species. Recent work has implicated members of these lineages in several important ecophysiological roles. These include nutrient scavenging, including mobilization of iron and phosphate, nitrogen fixation, cellulase, xylanase and amylase activities, and oxidation of recalcitrant compounds, e.g. aromatics and aliphatics. Production of volatile organic compounds, conferring antibacterial and antifungal activity, has also been demonstrated for several lichen-associated isolates. In the present paper we review the nature of non-phototrophic endolichenic bacteria associated with lichens, and give insight into the current state of knowledge on their importance the lichen symbiotic association.

Keywords: lichen, bacteria, symbiosis, endothallic, microbiome

Introduction

Lichens

Lichens form fascinating symbiotic relationships, where two or three organisms associate into unique and easily recognizable structures, the vegetative thalli. The symbiosis yields a biotope that can be considered a small ecosystem and is usually composed of a fungal partner, the mycobiont, and one or more photosynthetic partners, the photobiont, which generally is either a green alga or a cyanobacterium (Nash, 2008a). Lichens can thrive in practically any terrestrial environment, they are among the earliest colonizers of severe habitats and recently exposed land (Gadd, 2007), and are often major components of widespread communities such as biocrusts (Sancho et al. 2014). Lichens are dominant in some parts of the planet's landscape and have been estimated to cover up to 8% of the total land surface (Ahmadjian 1995). Lichens are usually highly tolerant of harsh environmental conditions such as extremes in temperature, low water content, low nutrient availability and high UV light intensities. They are thus often found in environments where few other macroscopic organisms would thrive (Beckett et al. 2008), including extreme environments such as the Antarctic (Øvestal & Smith, 2001), the Arctic (Printzen, 2008), on mountain summits, as components of desert crusts (Büdel et al., 2009) and tropic regions (Morley & Gibson, 2004). Lichens are

perennial and slow-growing organisms that present a variety of colorful forms and maintain fairly uniform, long-lived morphologies, which can be maintained up to several thousand years. Despite their resistance to abiotic stresses in their dormant stage, lichens can be vulnerable to even slight changes in environmental conditions that can disrupt the integrity of the symbiotic association (Erlacher et al. 2015).

General description of the lichen symbiosis

Lichens represent a classic example of self-sustaining symbiotic interactions that today are recognized to be widespread in nature, and involving different kingdoms of life (Nash, 2008a). As a heterotrophic organism, the mycobiont requires carbohydrates, which are produced by the photobiont; sugar alcohols by most algal photobionts and glucose by cyanobionts (Nash, 2008a). The majority, or approximately 85% of lichens, contain green microalgae as photobionts. In addition to the principal photobiont, tripartite lichens are characterized by cyanobacteria associated within their thalli, usually in specific structures called cephalodia. In tripartite lichens, the cyanobacteria are apparently focused on supplying fixed nitrogen used by other members of the symbiosis (Rikkinen 2002). The nitrogen fixation has important effects on the lichen ecology, promoting colonization of special ecological niches, such as extremely oligotrophic habitats (Büdel and Scheidegger 2008).

Lichen growth and resources

Individual lichen growth is dependent on resource acquisition, followed by biosynthesis of cellular compounds, minus losses of thallus material through dispersal, fragmentation, grazing and necrosis (Palmqvist, 2000). Being poikilohydric organisms, unable to control their water status, environmental conditions have a large effect on lichen growth. However, when wet and metabolically active, lichens can convert incident light energy into new biomass just as efficiently as vascular plants. Successful lichen growth requires a balance between the carbon acquisition and mineral availability. Mineral nutrients, particularly nitrogen and phosphorous, are essential for thallus growth (Palmqvist et al. 2008).

About 40-50% of a lichen's dry mass is composed of carbon, which they mainly gain from photobiont photosynthesis (Green et al. 2008). The poikilohydric characteristic of lichens in general dominates their CO_2 exchange behavior. The rate of gas exchange dramatically changes in contrast to the thallus water content; when the water content is low, CO_2 diffuses but when hydrated, diffusion of CO_2 is blocked (Honegger 1991). The CO_2 exchange rates can vary among lichen species or lichen type and environmental conditions can also affect the exchange rates (Green et al. 2008). Nitrogen is involved in many processes for all symbionts and although it is an important

nutrient, fixed nitrogen can cause stress if supplied in excess (Johansson et al. 2010). The nitrogen acquisition mode can vary among lichen species, depending on the associated photobiont. Green algal lichens mostly depend on direct nitrogen deposition on the thallus surface whereas cyanobacterial lichens (either bi- or tripartite) carry out biological nitrogen fixation of atmospheric nitrogen (Dahlmann et al. 2004). Lichens can assimilate nitrogenous compounds as nitrate (NO_3^-), ammonium (NH_4^+) or organic N (Nash 2008b; Johansson et al. 2011). The effects of nitrogen limitation on lichen productivity have been relatively well studied (Palmqvist et al. 2008) but the effect of phosphorus limitation is not fully known. However, recent studies have e.g. shown that addition of phosphorus may increase growth rate, abundance and species richness of lichens, especially N_2-fixing species (McCune & Caldwell, 2009; Benner, Conroy, Lunch, Toyoda, & Vitousek, 2007; Benner & Vitousek, 2007). Only trace concentrations of phosphorus are present in the atmosphere as dust from soil erosion and rock weathering, and thus lichens have developed uptake pathways for their phosphate capture (Smith 1960; Farrar 1976).

Bacterial communities

Although lichens are generally defined only as bipartite or tripartite mycobiont-photobiont symbioses, the presence of other microorganisms in the lichen thalli has long been known. Following Johannes Uphof's early report

on “purple bacteria” in lichens, suggesting that non-cyanobacterial prokaryotes were present (Uphof 1925), a series of papers were published in the following years (Henkel and Plotnikova 1936; Iskina 1938; Panosyan and Nikogosyan 1966). Throughout most of the 20th century, however, the focus was primarily on the symbiotic components of lichens and their ability to produce secondary metabolites with biological activities (Huneck 1999; Muller 2001) and little was known about the endolichenic microbial communities, their function or diversity.

Bacterial diversity of the culturable biota

Some 10 years ago, the first modern-era study on the non-phototrophic lichen-associated microbiota was published (González et al. 2005). That study focused on *Actinobacteria*, isolated from 25 terrestrial lichen samples collected in Alaska, Hawaii and Reunion. The lichens sampled were not identified in the paper but were described as saxicolous (rock-dwelling) and arboricolous (tree-dwelling). A standard medium (YME agar) was used for actinobacterial cultivation from all lichen samples, resulting in a large number (337) of strains which were identified on the basis of DNA fingerprinting and fatty acid analysis. The majority of isolates belonged to the families *Micromonosporaceae* (142) and *Streptomycetaceae* (110), although *Pseudonocardiaceae* (30), *Nocardiaceae* (4), *Streptosporangiaceae* (2),

Thermomonosporaceae (7) and *Geodermatophilaceae* (1) were also detected. The isolates were further screened for the presence of genes involved in polyketide, polypeptide and isoprenoid biosynthesis, and antimicrobial activity against *Escherichia coli, Staphylococcus aures* and *Candida albicans*. At least one biosynthetic cluster was observed in a large part (over 60%) of the isolates and 27% showed antimicrobial activity against at least one of the targeted microorganisms.

The following year, two papers were published on lichen-associated bacterial communities (Cardinale et al. 2006; Liba et al. 2006). Cardinale and coworkers analyzed 11 different lichens, belonging to eight species and sampled at five different sites in Austria and France. The lichens sampled belonged to the genera *Cladonia, Pseudevernia, Hypogymnia* and *Roccella*. Bacteria from external and internal surfaces of the lichen thalli were isolated, using TY and sugar-rich/N-free medium, resulting in 34 morphologically distinct bacteria. Liba et al. (2006) sampled 5 foliose cyanolichens from rainforests in Brazil and isolated acetylene-reduction positive strains on nitrogen-free medium. The presence of *nifH* genes, involved in nitrogen fixation, was confirmed by dot-blot hybridization of genomic DNA and the microbiota was suggested to be involved in nitrogen fixation for the lichen symbiosis. The isolates (17) all belonged to different genera of *Gammaproteobacteria* and further analysis of

their functions showed them to be involved in phosphate solubilization, indole acetic acid (IAA) production and amino acid secretion.

Since these studies, a number similar publications have appeared. While many of them have targeted individual isolates or species, others have focused on special interests or the biotechnological potential of the microbiota. Overall, the taxonomic bacterial diversity has been fairly well established and in some studies, possible roles of the associated microbiota have been suggested (see Table 1 for overview).

Table ***Error! No sequence specified.****. A summary of culture dependent studies of lichen associates*

In the first study published on bacterial associates of Antarctic lichens, a number of psychrotolerant strains were revealed (Selbmann et al. 2010). Of the thirty bacterial isolates, a new species of *Deinococcus-Thermus* was reported and several other strains represented potential new taxa. The bacterial diversity of Antarctic and Arctic lichens was further studied by Kim et al. (2014) and Lee et al. (2014). Although no specific roles was suggested for the microbiota within the symbiosis, the strains isolated from the crustose lichen *Ochrolechia* sp. had antimicrobial and antioxidant activity (Kim et al. 2014). The isolates were found to belong to *Sphingomonas* and *Burkholderia*, genera abundantly

found in Antarctic and Arctic lichens (Lee et al. 2014). Lee et al. studied the microbiota of nine different lichens, recovering isolates affiliated with *Actinobacteria, Bacteroidetes, Deinococcus-Thermus, Firmicutes, Alpaproteobacteria, Betaproteobacteria* and *Gammaproteobacteria*. Although previous studies using cultivation methods had poor representation of *Alphaproteobacteria* (Cardinale et al. 2006; Selbmann et al. 2010), they were found to be predominant in Lee's study. The majority of the alphaproteobacterial isolates were affiliated with the genus *Sphingomonas*. In addition to *Sphingomonas*, members of the genera *Frondihabitans, Hymenobacter* and *Burkholderia* were isolated across lichen samples from both the Arctic and Antarctica and members of *Nakamurella, Streptomyces, Deinococcus, Paenibacillus, Aurantimonas, Methyloferula, Psycrobacter, Pseudomonas* and *Rhodanobacter* were recovered with lower frequency. Based on functional tests, the microbiota of the Arctic and Antarctic lichens were commonly found to have protease and lipase activity and were suggested to be involved in nutrient supply for the symbiosis as a whole. In a recent study on crustose lichens on the Northern Iceland seashore, at total of 93 bacterial strains were isolated from four different lichens. Based on 16S rDNA sequencing, the collection was found to contain members belonging to *Alphaproteobacteria, Bacilli, Actinobacteria, Flavobacteria, Cytophagia, Sphingobacteria* and *Gammaproteobacteria*. Selected isolates were further

tested for secretion of several extracellular enzymes and were found to be proteolytic, glucanolytic and amylolytic. Furthermore, a number of the tested isolates were phosphate-solubilizing and nitrogen fixing (Sigurbjörnsdóttir et al., 2014).

In summary, cultivation-based studies have revealed that the culturable non-phototrophic microbiota of lichens tends to be dominated by members of the phyla *Actinobacteria, Firmicutes* and *Proteobacteria*. Some studies also yielded members of *Deinococcus-Thermus, Bacteroidetes* and *Acidobacteria*. While some of the studies did not consider potential roles of the lichen associates, we can surmise that the culturable fraction of lichen-associated bacteriomes are involved in nitrogen fixation, phosphate solubiliszation, hormone production, nutrient supply via various lytic activities and that they play roles in defense mechanisms through antagonistic activities.

Bacterial diversity via culture-independent methods

While many studies have described the diversity of bacterial lichen-associates and their production of bioactive compounds (Davies et al. 2005; Yamamura et al. 2011), the culturable fraction is nevertheless believed to represent only a small part of the total bacterial biota in environmental samples (Amann et al. 1995). Several methods can be used to expose the unculturable bacterial

fraction, including microbial fingerprinting where PCR products yield analyzable banding patterns (Portillo et al. 2011). Different primers can be used, either universal or group specific, and the bands can further be excised from the gel matrix and characterized by sequencing. Such fingerprinting methods have been used in a number of studies, revealing the taxonomic diversity of the total bacterial biota of lichens (see Table 2 for overview). Although fingerprinting methods have in general many advantages, e.g. comparatively low cost, are not time consuming and banding patterns can directly be compared between samples, these methods generally lack the resolution required for thorough phylogenetic inference (Grube and Berg 2009). Instead, the analysis of 16S rDNA libraries, often supplanted by other conserved gene markers, is now commonly used to identify the total bacterial community in lichens (Grube et al. 2012; Aschenbrenner et al. 2014; Grube et al. 2015).

Table 3. A summary of culture independent studies of lichen associates

The location of bacteria in the symbiotic structure has been established in a number of studies, using fluorescent *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM). In the first published study where FISH-CLSM analysis of lichen-associated bacteria was performed, a protocol for cryosectioning of lichen fragments was optimized, and dense colonization of bacteria was mainly found on the internal thallus surfaces of *Cladonia arbuscula* (Cardinale et al. 2008). In the same study, group-specific FISH probes revealed the prominence of *Alphaproteobacteria* (>60% of all bacteria). *Actinobacteria* and *Betaproteobacteria* were also detected although at a much lower abundance. Few members of *Firmicutes* were found but *Gammaproteobacteria* were not detected. In this pioneering study, lichens were found to host more bacteria than previously thought and the method has since been used in several studies. Grube et al. (2009) studied the bacterial diversity of three lichen species, *C. arbuscula*, *Lecanora polytropa* and *Umbilicaria cylindrica*, collected from alpine environments. The number of bacteria per gram of *C. arbuscula* specimen were comparable to the previous study of Cardinale et al. (2008) but lower numbers were obtained for *L. polytropa* and *U. cylindrica*, 7×10^5 and 9×10^5 mm^{-3} of lichen volume, respectively. Using FISH group-specific probes, *Alphaproteobacteria* were estimated to form 45-75% of the total

bacterial biota but other groups much less. In this work, bacterial fingerprints from the three lichen species were compared for universal bacterial and group-specific fingerprints (*Alphaproteobacteria, Pseudomonas* and *Burkholderia*) and the bacterial biota found to be species-specific (Grube et al. 2009).

Interestingly, relatively few studies thus far have focused on the non-phototrophic bacteria of crustose lichens. In one of the few published studies, samples of *Hydropunctaria maura, Ophioparma ventosa, Pertusaria corallina* and *Rhizocarpon geographicum* were analysed (Bjelland et al. 2011). In this study, DGGE and clone library sequencing were used to determine the composition and abundance of bacteria. The classes *Acidobacteria*, *Proteobacteria* (*Alpha-* and *Betaproteobacteria*) and *Chloroflexi* dominated the bacterial biota in *O. ventosa, P. corallina* and *R. geographicum* although *O. ventosa* was slightly different and more often associated with *Beta-* and *Gammaproteobacteria.* In terms of abundance, the samples in this study had similar numbers of bacteria per cell of lichen thallus as previously reported by Cardinale et al. (2008) and Grube et al. (2009). *H. maura,* however, had a higher number, 1.8×10^{11} g^{-1} and the bacterial community reflected the marine influence, not detected in the other three lichen samples. Moreover, *Archaea* were, for the first time, shown to be associated with lichens.

Many studies have used culture independent methods to elucidate the functional role of the lichen-associates. In Grube's recent work (Grube et al. 2015) the functional gene composition of the lung lichen *Lobaria pulmonaria* was revealed as well as the taxonomic structure. *Proteobacteria* were found to be dominant, with *Alphaproteobacteria* the prominent taxon, but members of *Beta-*, *Gamma-* and *Deltaproteobacteria* were also present. Greatest homology to members of the taxa *Actinobacteria, Acidobacteria* and *Bacteriodetes* were found in 2, 2 and 1% of the total bacterial contigs, respectively. When the lichen metagenome was subjected to functional analyses with SEED and KEGG, most contigs were found to be involved in primary metabolism such as carbohydrate, energy, lipid, nucleotide and amino acid metabolism, glycan biosynthesis and genetic information processing. When the metagenome was further screened for selected functions genes involved in e.g. nutrient scavenging, resistance against biotic and abiotic stress factors, hormone production and various lytic activities were detected. In another recent study, the bacterial microbiota associated with *Peltigera membranacea* was analyzed in terms of taxonomic structure and functional gene composition. *Proteobacteria* composed the majority of the bacterial microbiota but *Acidobacteria, Actinobacteria, Bacteroidetes* and *Verrucomicrobia* were also detected. Genes involved in phosphate

scavenging were commonly found in the dataset, as were enzymes involved in various lytic activities (Sigurbjörnsdóttir et al., 2015).

Functional roles of lichen associates

Nitrogen is one of the essential elements lichens need for growth. In cyanolichens, the photobiont can gain nitrogen from the atmosphere but nitrogen fixation has been suggested to be one of the important roles of the non-cyanobacterial prokaryotes of lichens. Nitrogen is abundant in Nature, however, it is not always available in form which organisms can utilize. Atmospheric nitrogen must thus be fixed to usable form, in an energy-intensive transformation mediated by nitrogenase enzymes (Shridhar 2012). Nitrogen fixing bacteria, often referred to as diazotrophs, might deliver nitrogen suitable for the symbiotic partners of lichens (Kneip et al. 2007) a role of special importance in non-cyanobacterial lichens and in the case of nitrogen limiting conditions (Grube and Berg 2009).

Phosphorus (P) is another essential macronutrient for plants and lichens and is involved in all major metabolic pathways (Hayat et al. 2010; Sharma et al. 2013; Zhao et al. 2014). In soil, P exists in two forms, as organic and inorganic phosphates, and needs to be converted to soluble form for plants (Richardson et al. 2001). Phosphate solubilizing bacteria

(PSB) have the ability to release P and make it available to plants (Richardson et al. 2001; Sashidhar and Podile 2010; Sharma et al. 2013; Chhabra et al. 2013; Zhao et al. 2014). The pathways involved in phosphate solubilization are complex and several key enzymes are needed, including alkaline and acid phosphatases, phytases and phosphonatases (Rodríguez et al. 2006; Sharma et al. 2013). Organic acids, especially gluconic acid, are essential for acidification, a key step in the dissolution of many poorly soluble mineral phosphates. Goldstein (1995) proposed the direct oxidation of glucose to gluconic acid (GA), and in some cases 2-ketogluconic acid, to be a key step of the mineral phosphate solubilization (MPS) in some Gram-negative bacteria. Membrane-bound glucose dehydrogenase mediates the pathway and requires pyrroloquinolline quinone (PQQ) as a cofactor (Goldstein 1995). Bacteria of various genera have been reported to be efficient phosphate solubilizers, e.g. members of *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aerobacter, Flavobacterium* and *Erwinia*. The bacteria can solubilize mineral phosphate (inorganic compounds), including tricalcium phosphate, hydroxyl apatite and rock phosphate (Rodríguez and Fraga 1999; Rodríguez et al. 2006).

In many of the recent functional and metagenomic studies, one of the suggested traits of the bacterial microbiota of lichens is their wide range of lytic activities; chitinolysis, proteolysis and glucanolysis (Grube et al., 2015, 2009; Lee et al., 2014; Schneider et al., 2011). These extracellular enzymes are relatively abundant among lichen associates and are suggested to be involved in nutrient mobilization within the lichen symbiosis and contribute to the hydrolysis of organic compounds. The mycobiont comprises the major part of a lichen thallus and as fungal cells are mixtures of polysaccharides and proteins, bacterial strains with lytic activity are likely to degrade older parts of the thallus where antibacterial compounds are no longer present. Many foliose and fruticose lichens grow in a cellulose-rich environment and often in close proximity to mosses and other vegetation, thus cellulolysis might be particularly important for many lichen symbioses. Albeit lichens generally obtain their carbon from the photosynthetic partner (Palmqvist et al. 2008) it has been suggested that cellulase systems, found within lichens, are used for saprophytic activity which could be beneficial for the symbiosis, e.g. when lichens are covered by snow (Beckett, Zavarzina, & Liers, 2013).

Plant growth-promoting rhizobacteria (PGPR) can influence plant growth by synthesizing and exporting phytohormones which may act as regulators in plant growth and development (Hayat et al. 2010). One of the

physiologically most active phytohormones in plants is indole-3-acetic acid (IAA) and it is produced by several lichen associates (Liba et al. 2006; Grube et al. 2009). In plants, production of IAA is known to stimulate root elongation and long-term (e.g. cell division and differentiation) responses (Spaepen et al. 2007; Hayat et al. 2010). IAA has been shown to effect fungal growth (Gryndler et al. 1998) and cell division in unicellular algae (Lau et al. 2009). Therefore, IAA producing bacteria have been suggested to alter morphogenetic processes of lichen symbioses and influence both the mycobiont and algal partners (Grube and Berg 2009).

Bacteria of the phylum *Actinobacteria* are known for their antimicrobial properties and biosynthetic potential (Arul Jose and Jebakumar 2013) and have commonly been cultured from lichen species. It has thus been suggested that such activities are involved in the defense mechanism of lichens (Grube et al. 2009; Grube et al. 2015; Cernava et al. 2015b).

Although the presence of other microorganisms within the lichen thallus has long been recognized, the first conclusive studies were only published in the last decade. Culturing and culture independent methods have now been used to elucidate the taxonomic structure and functional gene composition of lichen associates. In most lichens studied thus far, *Proteobacteria* are most abundant although members from many other

classes have also been found, including *Archaea*. Studies now indicate that lichen associates have various roles within the lichen symbiosis, including nitrogen fixation, phosphate solubilization and nutrient mobilization through various lytic activities.

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Lichen species	Lichen habitat	Taxonomic diversity of the isolated microbiota	Roles of microbiota within the symbiosis/potential biotechnological applications	Reference
Unidentified	Terrestrial	*Actinobacteria*	None suggested/ Bioactive	González et al., 2005
Cladonia digitata, C. rangiferina, C. coniocracea, C. pyxidata, C. coccifera, Pseudevernia furfuraceae, Hypogymnia physodes, Rocella phycopis, R. fuciformis	Terrestrial	*Firmicutes, Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria*	N-fixing/ Diazotrophic bacteria	Cardinale et al., 2006
Canoparmelia caroliniana, C. crozalsiana, C. texana, Parmotrema sanctiangeli, P. tinctorum	Cyanolichens from rain forest in Brazil	*Gammaproteobacteria*	N-fixing, phosphate solubilization, IAA production, nutrition contribution via amino acid release	Liba et al., 2006
Cladonia arbuscula, Lecanora polytropa, Umbilicaria cylindrica	Saxicolous above tree line in Austria	*Alphaproteobacteria, Actinobacteria, Firmicutes, Betaproteobacteria, Gammaproteobacteria*	N-fixing, proteolysis, chitionlysis, glucanolysis, phosphate solubilization, IAA production, antagonistic	Grube et al., 2009
Acarospora flavocordia, Lecanora fuscobrunnea, Lecidea cancriformis, Rhyzocarpon sp., *Umbilicaria decussata, Usnea antarctica, Xanthoria elegans*	Antartic	*Actinobacteria, Gammaproteobacteria, Deinococcus-Thermus, Firmicutes*	None suggested	Selbmann et al., 2010

Cladonia sp. *Cladonia rangifernia*, *Sphaerophorus globose*	Epigeal bog in N-Russia (Karelia and Arctic tundra)	*Actinobacteria*, *Acidobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*	None suggested	Pankratov, 2012
Ochrolechia sp.	Arctic region	*Alphaproteobacteria*, *Betaproteobacteria*	None suggested/ antibacterial and antioxidant activity	Kim et al., 2014
Usnea sp., *Cladonia borealis*, *Psoroma* sp., *Stereocaulon* sp., *Umbilicaria* sp., *Cetraria* sp., *Cladonia* sp., *Ochrolechia* sp.	Antarctic and Arctic regions	*Actinobacteria*, *Bacteroidetes*, *Deinococcus-Thermus*, *Firmicutes*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*	Nutrient supply (proteolysis and lipase activity)	Lee et al., 2014
Lecanora helicopis, *Verrucaria ceuthocarpa*, *Hydropunctaria maura*, *Caloplaca verruculifera*	Seashore lichens, Northern Iceland	*Alphaproteobacteria*, *Bacilli*, *Actinobacteria*, *Flavobacteria*, *Cytophagia*, *Sphingobacteria*, *Gammaproteobacteria*	Proteolysis, glucanolysis, amylolysis, phosphate solubilisation, nitrogen fixation	Sigurbjörnsdóttir et al., 2014
Lobaria pulmonaria	Maple trees in the Alps		Nutrient supply phosphate solubilization, antagonistic activity	Grube et al., 2015
Lobaria pulmonaria	Three different locations in Austria	*Betaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria*, *Firmicutes*	Antagonistic activity	Cernava et al., 2015a
Lobaria pulmonaria	Three different locations in Austria		Volatile organic compound production, spermidine production, hydrogen cyanide production	Cernava et al., 2015b

Lichen species	Lichen habitat	Technique(s) applied	Taxonomic diversity of the associated microbiota	Potential activity of the associated microbiota	Reference
Cladonia digitata, C. rangiferina, C. coniocraea, C. pyxidata, C. coccifera, Pseudevernia furfuracea, Hypogymnia physodes, Rocella phycopsis, R. fuciformis		ITS fingerprinting, sequencing of bands	*Betaproteobacteria, Gammaproteobacteria*, Actinobacteria	N-fixing	Cardinale et al., 2006
Cladonia arbuscula	Mountain ridge in Styria, Austria	FISH, SSCP	*Alphaproteobacteria,* Actinobacteria, *Betaproteobacteria*	None suggested	Cardinale et al., 2008
Cladonia cristatella, C. cryptochlorophaea, C. sobolescens, C. peziziformis, C. subtenuis, Flavoparmelia caperata, Parmotrema perforatum, Peltigera phyllidiosa	Virginia and N-Carolina, USA	Sanger sequencing (direct of PCR products using universal primers)	*Alphaproteobacteria,* Acidobacteria, *Gammaproteobacteria*	None suggested	Hodkinson & Lutzoni, 2009
Cladonia arbuscula, Lecanora polytropa, Umbilicaria cylindrica	Saxicolous above tree line in Styria, Austria	FISH, SSCP	*Alphaproteobacteria,* Actinobacteria, *Betaproteobacteria*	High abundance of *nifH* genes, diazotrophic bacteria	Grube et al., 2009

Hydropunctaria maura, Ophioparma ventosa, Pertusaria corallina, Rhizocarpon geographicum	Saxicolous lichens from SW-Norway	DGGE, clone libraries	*Alphaproteobacteria, Betaproteobacteria,* Actinobacteria, Acidobacteria, Firmicutes, Chloroflexi, Bacteroidetes	None suggested	Bjelland et al., 2011
Xanthoparmelia plittii, X. somloensis	Foliose lichens sampled in Massachusetts, USA	Pyrosequencing of 16S rRNAs	*Alphaproteobacteria,* Acidobacteria, Bacteroidetes, *Gammaproteobacteria, Deltaproteobacteria*	None suggested	Mushegian et al., 2011
Lobaria pulmonaria	Foliose lichen, arboicolous	FISH, pyrosequencing	*Alpaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria,* Bacteroidetes, Actinobacteria, Firmicutes, Verrucomicrobia	Nutrient availability (various lytic activity), resource reallocation (amino acid release)	Schneider et al., 2011
Parmelia sulcate, Rhizoplaca chrysoleuca, Umbilicaria americana, U. phaea	Foliose lichens from granite rock outcrops, Colarado, USA	Pyrosequencing of 16S rRNAs	*Alphaproteobacteria,* Acidobacteria, *Gammaproteobacteria*, Firmicutes, Verrucomicrobia, Planctomycetes, Actinobacteria, *Betaproteobacteria,* Bacteroidetes, *Deltaproteobacteria*	N-fixing, phosphate solubilisation	Bates et al., 2011
Lobaria pulmonaria	Styria, Austria	FISH, SSCP	*Alphaproteobacteria*	N-fixing	Cardinale et al., 2012a

Cetraria islandica, Lobaria pulmonaria, Lecanora polytropa, Cladonia arbuscula, Umbilicaria cylindrical, Cladonia coccifera	Different parts of thalli sampled (e.g. old, whole, young), Styria, Austria	FISH, SSCP	*Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria,* Actinobacteria	None suggested	Cardinale et al., 2012
Solorina crocea	Styria, Austria	Pyrosequencing of 16S rRNAs	Acidobacteria, Proteobacteria, Planctomycetes, Actinobacteria	N-fixing	Grube et al., 2012
Cladonia sp., *Flavocetraria* sp., *Ophioparma* sp., *Umbilicaria* sp., *Usnea* sp., *Dictyonema* sp., *Leptogium* sp., *Peltigera* sp., *Sticta* sp.	Tropical or arctic latitudes	Pyrosequencing of 16S rRNAs	*Alphaproteobacteria,* Acidobacteria, *Betaproteobacteria, Gammaproteobacteria,* Actinobacteria, Verrucomicrobia, Planctomycetes, Bacteroidetes, Deltaproteobacteria, Firmicutes	None suggested	Hodkinson et al., 2012
Cetraria aculeata	Collected from different places; Antarctica, Spain, Germany and Iceland	PCR with group specific primers, clone libraries	*Alphaproteobacteria*	None suggested	Printzen et al., 2012
Arthrocaphis citronella, Baeomyces placophyllus, B. rufus, Icmadophila ericetorum, Psora decipiens, Trapeliopsis granulosa	Styria, Austria	FISH	*Alphaproteobacteria,* Acidobacteria	None suggested	Muggia et al., 2013

Lobaria pulmonaria	Maple trees in the Alps	Pyrosequencing of 16S rRNAs	*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Epsilonbacteria*, Actinobacteria, Acidobacteria, Bacteroidetes	Nutrient supply, pathogen defence, abiotic stress resistance, detoxification of metabolites, lytic activity	Grube et al. 2015
Lobaria pulmonaria	Styria, Austria	SSCP, pyrosequencing of 16S rRNAs, FISH	*Alphaproteobacteria*, Bacteroidetes, Verrucomicrobia, *Deltaproteobacteria*, Actinobacteria, *Betaproteobacteria*, *Gammaproteobacteria*, Acidobacteria, Planctomycetes	None suggested	Aschenbrenner et al., 2014
Lobaria pulmonaria	Three different locations in Austria	Pyrosequencing of 16S rRNAs	*Betaproteobacteria*, *Gammaproteobacteria*, Actinobacteria, Firmicutes	Antagonistic activity	Cernava et al., 2015a
Lobaria pulmonaria	Two mountain forests	Pyrosequencing of 16S rRNAs, FISH	*Alphaproteobacteria*	N-fixing, auxin and vitamin production, stress protection	Erlacher et al., 2015
Peltigera membranacea	Sub-Arctic heathland, Iceland	Shotgun pyrosequencing, rRNA analysis	*Alphaproteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Betaproteobacteria*, *Verrucomicrobia*, *Gammaproteobacteria*, *Deltaproteobacteria*	Phosphate solubilization, nutrient availability (cellulolytic, glucanolytic), defence mechanism within the symbiosis.	Sigurbjörnsdóttir et al., 2015

5 Appendix

Are lichens potential natural reservoirs for plant pathogens?

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Most ecological studies on plant pathogenic bacteria have focused, perhaps understandably, on the agricultural environment. Interest is increasing however, in the occurrence of plant pathogenic bacteria in

habitats outside of agriculture. Seminal work by Morris et al. (2008), for instance, has shown that *Pseudomonas syringae* occupies a wide range of niches linked with the water cycle, including alpine lakes, streams and snow. Moreover, it is becoming clear that traits that are linked to adaptation to biotic and abiotic stress in the nonagricultural environment can have a secondary function as virulence factors in plants (Morris et al., 2009). Indeed, adaptation to non-host environments has been suggested to have played a nontrivial role in the evolution of *Pseudomonas syringae* phytopathogenicity and to affect its epidemic potential (Bartoli et al., 2015).

A reservoir sensu Haydon *et al*. is defined as ”one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population“ (Haydon et al., 2002). While in some cases, plant pathogens are known to survive and persist in plant debris left on the soil surface, in and on seeds, in soil, in association with perennial hosts, in water, on or inside insects, or even on inorganic objects such as machinery and packaging material, for the most part, the natural reservoirs of most plant pathogens remain unidentified.

Lichens are symbiotic associations of fungi, algae and bacteria that are present in most environments, often quite prominently so. Many lichen species are found in close association with plants, growing for example on tree trunks or on grassland or heath scrub soils, albeit rarely to an apparent detrimental effect to plant health. Nevertheless, recent metagenomic, amplicon-based, and culture-based investigations have indicated that some plant pathogens, or their very close relatives, are present in lichen-associated microbiomes, in some cases in significant numbers (Cardinale et al., 2008; Sigurbjörnsdóttir et al., 2014). Should we perhaps regard these unassuming and generally welcome members of the environmental vegetation as potential reservoirs for established or emerging plant diseases?

According to the traditional textbook definition, lichens are bipartite symbiotic associations of a mycobiont (usually an Ascomycete or a Basidiomycete) and a photobiont (a green alga or a cyanobacterium). The mycobiont provides the structural bulk of the lichen thallus, while the photobiont subsists in colonies or layers sheltered beneath fungal peripheral layers. In many species, either or both partners produce secondary metabolites with antibacterial activity, thus providing a somewhat selective environment in which complex bacterial communities

thrive, some in biofilm-like surface communities (Grube et al., 2015) and others endothallically, below the surface of extracellular polysaccharides (Cardinale et al., 2008; Grube et al., 2009). Thus, lichens are hosts to large populations of bacteria whose identity and role in the lichen symbiotic association has been gradually emerging in recent years (Hodkinson and Lutzoni, 2009; Sigurbjörnsdóttir et al., 2015). Although there is considerable interspecies and inter-study variability, it has become clear that most lichens harbour a sizeable, complex, Proteobacteria-dominated microbiota, with dominant families typically comprising the Rhizobiaceae, Methylobacteraceae, Sphingomonadaceae, Acetobacteraceae, Rhodospirillaceae, Comamonadaceae and Burkholderiaceae (Aschenbrenner et al., 2014; Sigurbjörnsdóttir et al., 2015). Typically, taxa more commonly associated with phytopathogens, such as members of the Xanthomonadaceae, Pseudomonadaceae, and Enterobacteriaceae are also present, albeit in lower numbers. While many of the bacteria present in lichen thalli have doubtless never been cultured, members of many or even most of the dominant lichen-associated taxa have been isolated and grown in pure culture (Cardinale et al., 2006; Sigurbjörnsdóttir et al., 2014). Among the reported bacterial isolates from lichens, phylogenetic analysis has revealed several close relatives of known plant pathogens, including *Pseudomonas syringae* (Liba et al.,

2006) and *Burkholderia glathei* (Cardinale et al., 2006). Plant pathogenicity has, however, not been established, or indeed experimentally addressed, for these or any other lichen-associated isolates to our knowledge. Among bacteria indicated by metagenomic or amplicon-based analysis to be present in lichen-associated microbiomes that have not been cultured to our knowledge are a few plant pathogens. For example, both functional genes and 16S rDNA from *Xanthomonas* and its close relative *Xylella* were observed in the membranous dog lichen (*Peltigera membranacea*) metagenome (Sigurbjörnsdóttir et al., 2015), but have not as yet been cultured from that species. The finding of *Xanthomonas*-like organisms in lichens is interesting because *Xanthomonas* spp. are known to survive poorly when unprotected by host tissues. Further culturing efforts are thus called for.

Our recent analysis of a partial shotgun metagenome of the microbiome associated with *P. membranacea* (Sigurbjörnsdóttir et al., 2015), yielded a number of multiple hits on several genes involved in lichen secondary metabolite resistance, inorganic phosphate mobilization, biopolymer degradation and several other potentially important functions in thallus colonization and symbiosis, as well as several genes likely to play a role in plant pathogenicity. Among plant virulence genes found in the

metagenome are several homologs of type III and type IV secretion systems from diverse bacteria, including xanthomonads, burkholderiae, sphingomonads, and acidobacters. However, it should be borne in mind that some genes that BLAST searches identify as virulence factors might just as well be involved in mutualistic interactions. As so much of the previous work has been done on pathogenic relations, the BLAST results are expected to be biased and underestimate positive interactions. Indeed, bacteria closely related to known plant growth-promoting bacteria have been observed in a number of studies on lichen-associated bacteria (Cernava et al., 2015; Hodkinson and Lutzoni, 2009; Sigurbjörnsdóttir et al., 2015). Detailed data mining of completed and ongoing shotgun metagenome sequencing data is likely to yield a more complete picture of the plant pathogenic potential of members of the lichen-associated microbiome. A curated lichen-associated metagenome database would facilitate such work.

Given the size and complexity of both the endothallic and epithallic bacterial communities, as well as the restrictive environment, one might surmise a considerable scope for evolutionary acquisition of plant pathogenicity traits to be present. Adaptation to the fungal/algal environment is likely to select for traits useful for plant cohabitation, and indeed, production of phytohormones and volatile organics has been

observed in several lineages of lichen-associated bacteria (Aschenbrenner et al., 2014; Cernava et al., 2015). Further, the high abundance of associated bacteria yields a significant gene pool that may be available for lateral genetic exchange, possibly facilitating virulence gene acquisition. The lichen-associated genetic mobilome has as yet barely been studied at all. Only a few genomes of lichen-associated bacteria have thus far been published, all sphingomonads. As in other sphingomonads, lichen-associated Sphingomonadaceae strains appear hosts to several prophages and other mobile elements (Aylward et al., 2013), suggesting high capacity for lateral genetic exchange. To assess the potential of the lichen-associated microbiome for interspecific exchange of genetic material, we call for a thorough and systematic push for whole genome sequencing of lichen-associated bacteria, obtaining several genomes from each major lineage of lichen-associated taxa, as well as a more extensive database of shotgun metagenomic data.

The potential for microbial population exchange between lichens and plants should also not be discounted offhand. Both arboricolous and several terricolous lichens are found in quite intimate proximity to plants and vertical transmission of bacterial populations on lichen propagules has been observed for the arboricolous lung lichen, *Lobaria pulmonaria*

(Aschenbrenner et al., 2014). Whether, and if so, to what extent, interkingdom exchange of bacterial symbionts occurs between lichens and plants has to our knowledge not been investigated.

While the idea of lichens as potential plant pathogen reservoirs is in many ways attractive, we should hasten to point out that there are striking dissimilarities between plants and lichens as microbial habitats, calling for some notable differences in selection pressures and adaptation mechanisms of plant-associated versus lichen-associated bacteria. For instance, temporal variation in humidity can be expected to be much more dramatic in the lichen environment, with periodic droughts occurring frequently within the lichen thallus. This environmental variability calls for an environmental adaptability not necessarily seen in plant pathogens, which normally are r-strategic (that is, rely on fast growth and rapid substrate exploitation for selective advantage). Survival experiments of plant pathogens in lichen thalli, such as spraying and subsequent monitoring of GFP-tagged plant pathogens onto lichens in a field setting are therefore needed to assess the survivability of plant pathogens in the lichen environment. Conversely, lichen-associated bacteria closely related to known plant pathogens may of course be in fact adapted lichen symbiovars with no plant pathogenic traits at all and thus need to be tested for their growth in plant models.

In summary, we feel that lichens ought to be considered as potential non-host reservoirs for phytopathogenic bacteria, including *P. syringae*, *B. glathei* and xanthomonads which have been repeatedly observed through molecular or, in some cases, culture-based, observations to be present in significant numbers in or on lichen thalli. Much remains to be done, however, before the role of lichens in these pathogens' ecology and evolution can be reliably elucidated. Among tasks to be tackled are systematic data mining of extant and emerging metagenomes for genes encoding virulence factors, identification and quantification of prophages and other mobile genetic elements within lichen-associated microbiomes, assessment of population exchange potential between lichens and plants, and rigorous characterization of potentially plant pathogenic isolates.

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