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Bacterial communities in Antarctic lichens

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Abstract: To date, many studies surveying the bacterial communities in lichen thalli from diverse geographical areas have shown that Alphaproteobacteria is the predominant bacterial class in most lichens. In this study, bacterial communities in several Antarctic lichens with different growth form and substrates were analysed. The bacterial community composition in fruticose and foliose lichens, *Cladonia*, *Umbilicaria* and *Usnea*, and crustose lichens, *Buelia granulosa*, *Amandinea coniops* and *Ochrolechia parella*, from King George Island was analysed by pyrosequencing of bacterial 16S rRNA genes. Results showed that Proteobacteria, Acidobacteria, Actinobacteria and Bacteroidetes were predominant phyla. The predominant bacterial class in most of the samples was Alphaproteobacteria. *Acetobacteriaceae* of the order *Rhodospiralles* in Alphaproteobacteria was the most abundant bacterial family in Antarctic lichens. The LAR1 lineage of the order *Rhizobiales*, a putative N-fixer which has been frequently observed in lichens from temperate areas, was detected only from a few samples at low frequency. It is expected that other bacterial taxa are working as N-fixers in Antarctic lichens. From the PCoA analysis of the Fast UniFrac distance matrix, it was proposed that the microbial community structures in Antarctic lichens were affected by host species, growth form and substrates.

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Key words: *Cladonia*, lichen-associated bacteria, *Umbilicaria*, *Usnea*

Introduction

Lichen is a symbiotic organism usually comprised of a fungus (mycobiont) and a green algae or cyanobacterial partner (photobiont), although multiple algal genotypes in a thallus have been reported from several lichen species (Guzow-Krzeminska 2006, Ohmura *et al.* 2006, Piercey-Normore 2006, Grube & Muggia 2010, Casano *et al.* 2011, Park *et al.* 2015). They are abundant and often dominant life form in Antarctic terrestrial ecosystems (Øvstedal & Lewis Smith 2001).

Lichens contain many bacterial inhabitants as well as lichenized fungi and algae. Bacterial communities in the lichen thalli have been researched by cultural approaches, fluorescence *in situ* hybridization (FISH) staining, molecular fingerprinting methods and recently by next-generation sequencing approaches (Scott 1956, González *et al.* 2005, Cardinale *et al.* 2006, 2008, Liba *et al.* 2006, Grube *et al.* 2009, Bates *et al.* 2011, Hodkinson *et al.* 2012, Lee *et al.* 2014). Predominance of Alphaproteobacteria has been observed in many of the bacterial community studies (Cardinale *et al.* 2008, Grube *et al.* 2009, Hodkinson *et al.* 2012) and it has also been shown that lichen-associated bacteria form highly structured, biofilm-like assemblages on fungal surfaces (Grube *et al.* 2009). Physiological tests of cultivable bacteria have suggested involvement of associated bacteria in nutrient cycling via nitrogen fixation, lytic activities, hormone production and phosphate mobilization (Grube *et al.* 2009). A phylogenetic lineage called a lichen-associated *Rhizobiales* lineage (LAR1), a putative nitrogen fixer, has been consistently abundant in lichens from temperate climate zones and Alaska (Hodkinson & Lutzoni 2009, Bates *et al.* 2011). Antagonistic activity against other microorganisms has been another suggested role of endolichenic bacteria (Grube *et al.* 2009). Therefore, the microbial communities found in lichen thalli are considered to be normal flora and contribute to the lichen holotypes by providing lytic activities, complementing the lichen budget and producing bioactive substances (Grube *et al.* 2009).

Grube *et al.* (2009) demonstrated through FISH and SSCP fingerprinting techniques that the composition of bacterial communities in lichens are host-specific; these results are supported by subsequent studies using pyrosequencing (Bates *et al.* 2011). Other analyses also revealed effects of substrate (Cardinale *et al.* 2012b), age of the thallus (Mushegian *et al.* 2011, Cardinale *et al.* 2012b), photobiont type (Hodkinson *et al.* 2012) and geography (Cardinale *et al.* 2012a, Hodkinson *et al.* 2012) on the bacterial microbiome composition.

Studies on the microbial community have usually been conducted for the lichens from temperate and subtropical areas (Cardinale *et al.* 2008, Grube *et al.* 2009,

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Bates *et al.* 2011, Hodkinson *et al.* 2012). Microbial communities in polar lichens have attracted the interest of microbial ecologists, but there are limited studies on Alphaproteobacteria using culturing, fingerprinting, pyrosequencing and clone library techniques (Selbmann *et al.* 2010, Printzen *et al.* 2012, Lee *et al.* 2014, Sigurbjörnsdóttir *et al.* 2014, 2015). Data on the bacterial community structure in Antarctic lichens will provide a basis for understanding the ecological roles of bacterial microbiomes in adaptation to extreme environments.

In the current study, we investigated bacterial diversity in Antarctic lichens through bacterial 16S rRNA genes using the 454 pyrosequencing method. To test the effects of growth form and substrate, fruticose, foliose and crustose lichens inhabiting mosses and rocks were included for comparative analyses. To our knowledge, this is the first report of bacterial communities in Antarctic lichens determined using high-throughput sequencing methods. Along with the previous study of algal and fungal communities in Antarctic lichens (Park *et al.* 2015), this data will provide a basis for understanding the ecological roles of the bacterial microbiomes in adaptations to specific environmental conditions.

Materials and methods

Lichen samples

Twelve lichen samples that were used for bacterial community analyses were collected from Barton and Weaver peninsulas on King George Island, Antarctica (Table I). The detailed information for locality, identification and phylogeny is described in Park *et al.* (2015). DNA was isolated using a Wizard® Genomic DNA Purification kit (Promega, Madison, WI) and purified by a CTAB method as described in Park *et al.* (2015).

Pyrosequencing, sequence processing and taxonomic assignment

Bacterial 16S rRNA gene sequences were determined by the 454 GS-FLX sequencing technique using primer sets including adapter sequences (Table S1 will be found at http://dx.doi.org/10.1017/S0954102016000286). Sequences were read only with reverse primers. Sequencing templates were prepared by pooling three independent PCR amplification products by 25 cycle reactions to reduce PCR biases.

Bacterial 16S rRNA gene sequences were pre-processed with PyroTrimmer software (Oh *et al.* 2012). The pre-processing procedures included trimming of barcode, linker and primer sequences, sorting of sequences for each sample based on barcode and primer sequence information, trimming of low quality bases at the 3' ends and filtering out sequences with low average quality

Table I. Summary of sequencing results.

Sample	CL1 *Cladonia borealis*	CL2 *Cladonia borealis*	CL3 *Cladonia gracilis*	UM1 *Umbilicaria antarctica*	UM2 *Umbilicaria antarctica*	UM3 *Umbilicaria antarctica*	US1 *Usnea aurantiaco-atra*	US2 *Usnea aurantiaco-atra*	US3 *Usnea aurantiaco-atra*	CR1 *Buellia granulosa*	CR2 *Amandinea coniops*	CR3 *Ochrolechia parella*
Growth forms	Fruticose	Fruticose	Fruticose	Foliose	Foliose	Foliose	Fruticose	Fruticose	Fruticose	Crustose	Crustose	Crustose
Substrate	Moss	Moss	Moss	Rock	Rock	Rock	Rock	Rock	Moss	Rock	Rock	Rock
Sequence reads												
Bacterial	353	550	2141	355	1361	1883	295	31	2889	683	1076	262
	(15.9%)	(36.4%)	(25.5%)	(6.6%)	(37.3%)	(66.8%)	(9.6%)	(0.3%)	(55.0%)	(46.7%)	(66.8%)	(15.9%)
Plastid	1873	961	6241	5047	2285	2819	3066	9458	2365	780	535	1644
	(84.1%)	(63.6%)	(74.5%)	(93.4%)	(62.7%)	(33.2%)	(90.4%)	(99.7%)	(45.0%)	(53.3%)	(33.2%)	(84.1%)
Total	2226	1511	8382	5402	3646	2819	3066	9489	5254	1463	1611	1644

Amandinea coniops (Wahlenb.) M. Choisy ex Scheid. & H. Mayrhofer, *Buellia granulosa* (Darb.) C.W. Dodge, *Cladonia boreadis* S. Stenroos, *Cladonia gracilis* (L.) Wild., *Ochrolechia parella* (L.) A. Massal, *Umbilicaria antarctica* Frey & I.M. Lamb, *Usnea aurantico-atra* (Jacq.) Bory.

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scores and short sequences. The 3' ends of sequences with low quality values were trimmed when average quality scores for a 5 bp window size were lower than 20. Sequences with ambiguous nucleotides or shorter than 180 bp were discarded. Chimeric reads were detected using USEARCH (Edgar 2010) with the UCHIME *de novo* option (Edgar *et al.* 2011) and excluded for the downstream analysis. Bacterial 16S rRNA gene sequences of 12 lichen

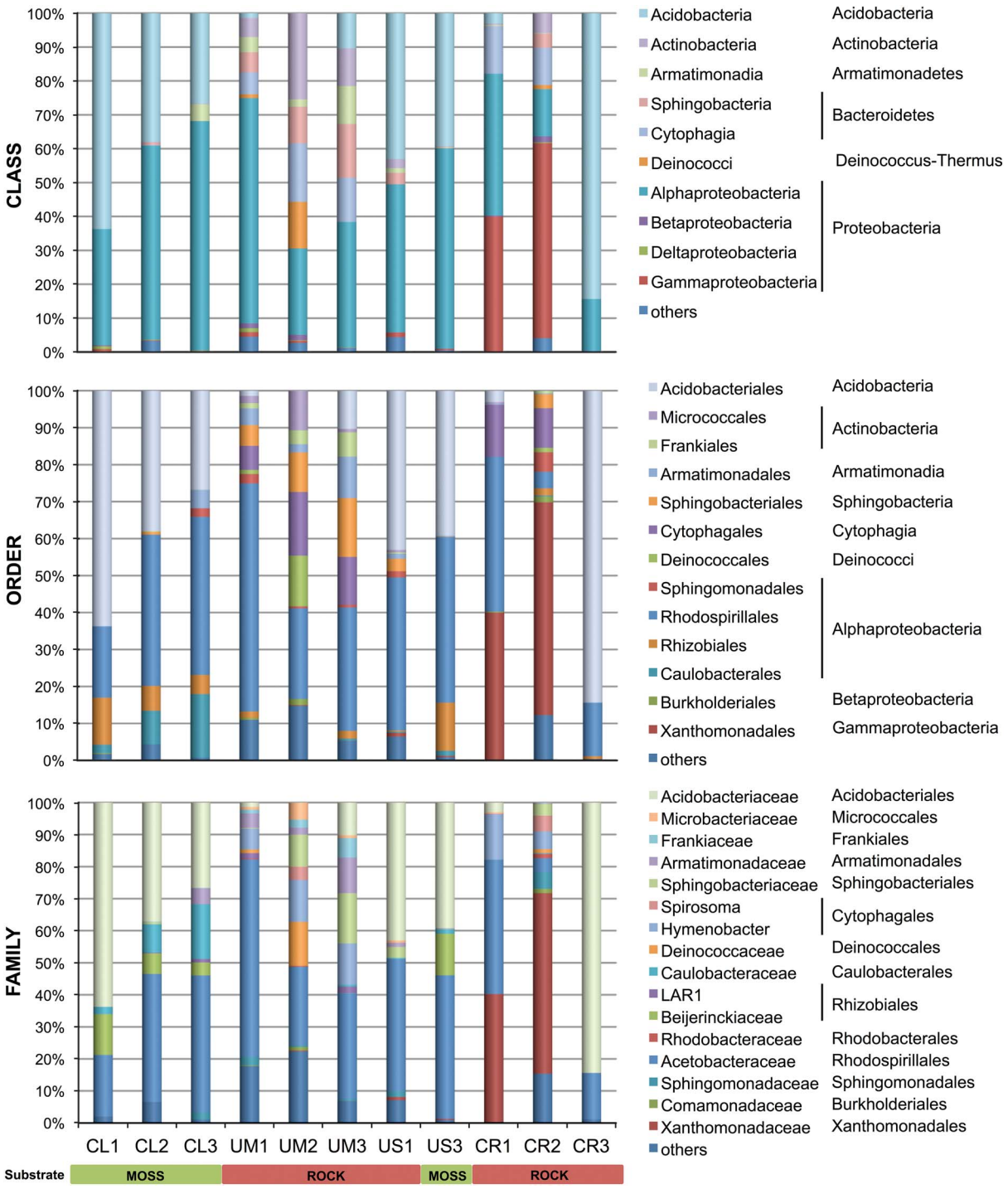


Fig. 1. Relative abundances of major families of 11 lichen samples. Sequences that were not included in major families were pooled to 'others'. Host lichen species are indicated by sample ID along the base of the figure. Substrates are indicated by the bar below the figure (green = moss, dark red = rock). Detailed taxonomic and abundance information for each OTU is presented in Table S2 found at http://dx.doi.org/10.1017/S0954102016000286.

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specimens were clustered altogether using CLUSTOM software (Hwang *et al.* 2013) with a 97% similarity cut-off. Taxonomic affiliation was determined using the EzTaxon-e database (Kim *et al.* 2012, www.ezbiocloud.net/eztaxon).

Fast UniFrac analysis

Principal co-ordinates analysis (PCoA) was performed for all bacterial OTUs and alphaproteobacterial OTUs based on distance matrices that were generated by Fast UniFrac (Hamady *et al.* 2009). Weighted Fast UniFrac analysis was conducted with log-transformed abundance data (percent abundance + 1) and a phylogenetic tree produced by neighbour-joining analysis based on the distance matrix calculated by pairwise sequence alignment using ClustalX (Thompson *et al.* 2002).

Results

Sequencing of 16S rRNA gene amplicons produced 1511 to 9489 sequence reads depending on the sample after the multiple quality check procedures (Table I). The conserved primers used in the current study allowed amplification of the plastid rRNA gene as well as the bacterial 16S rRNA gene. The proportion of the bacterial sequence reads was highly variable among samples, with a range of 0.3–66.8% (Table I).

Clustering of bacterial sequences altogether resulted in 382 bacterial OTUs from 12 lichen samples. A taxonomic summary of lichen-associated bacterial communities (Fig. 1 and Table S2 found at http://dx.doi.org/10.1017/S0954102016000286) showed that Proteobacteria were typically dominant (54.7%). However, two samples, *Cladonia borealis* (CL1) and *Ochrolechia parella* (CR3), were dominated by Acidobacteria (22.9%). Other major phyla included Bacteroidetes (10.9%), Actinobacteria (6.1%), Armatimonadetes (3.4%) and Deinococcus-Thermus (1.7%). Minor phyla (0.3%) included Chloroflexi, Cyanobacteria, Firmicutes, Gemmatimonadetes, Planctomycetes, Verrucomicrobia and MATCR.

The proportion of each phylum was highly variable depending on the sample. *Cladonia* and *Usnea* contained Alphaproteobacteria and Acidobacteria as major bacterial groups, and Armatimonadetes, Actinobacteria, Bacteroidetes, Gammaproteobacteria, Deltaproteobacteria and Betaproteobacteria as minor groups. In contrast, *Umbilicaria* contained Alphaproteobacteria, Acidobacteria, Bacteroidetes, Actinobacteria and Armatimonadetes as major bacterial groups with variation depending on the sample. Acidobacteria, Deinococcus-Thermus, Betaproteobacteria, Gammaproteobacteria and Deltaproteobacteria constituted the minor groups in *Umbilicaria*. The three crustose lichen samples showed highly variable microbial community structures. *Buellia granulosa* (CR1) and *Amandinea coniops* (CR2) contained Alphaproteobacteria, Gammaproteobacteria and Bacteroidetes as major groups. In contrast, most of the bacterial communities of *O. parella* (CR3) were occupied by Acidobacteria and Alphaproteobacteria. Betaproteobacteria, Deltaproteobacteria, Cyanobacteria, Chloroflexi, Firmicutes, Plantomycetes, Verrucomicrobia and SM2F11 were rarely recognized in the crustose samples.

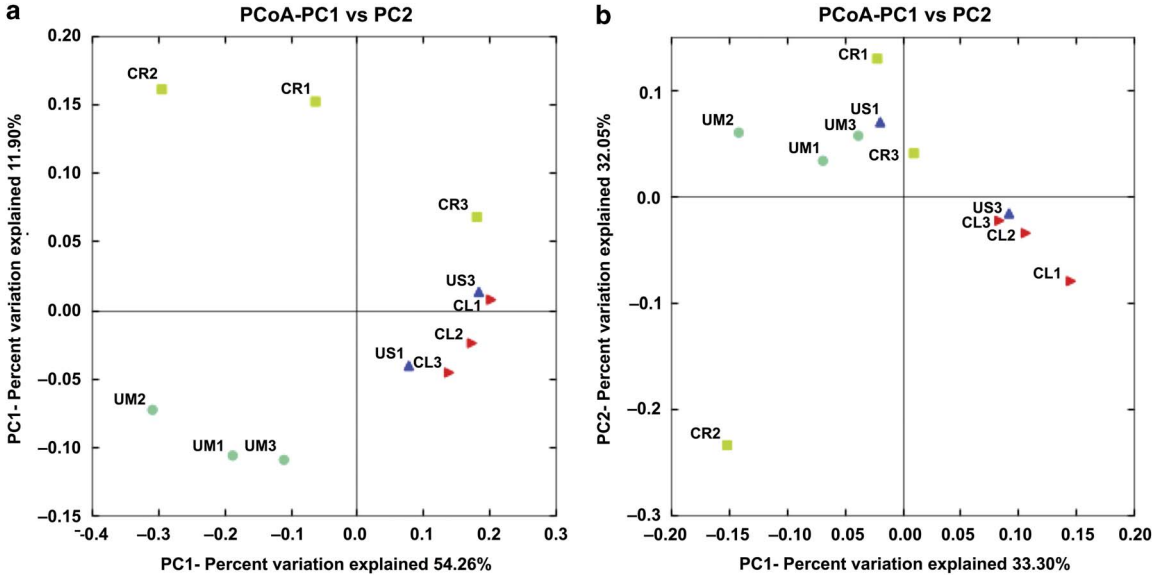


Fig. 2a. Principal co-ordinates analysis (PCoA) of all lichen-associated bacterial OTUs and **b.** alphaproteobacterial OTUs based on distance matrices generated by Fast UniFrac analyses.

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The only bacterial class that was present as a major component in most of the samples was Alphaproteobacteria (13.9–67.6%). When Alphaproteobacteria was subdivided at order level, *Rhodospirillales* (76.4%), *Rhizobiales* (11.7%), *Caulobacteriales* (8.7%) and *Sphingomonadales* (2.5%) were the major orders observed. The dominant order, *Rhodospirillales*, was mostly occupied by *Acetobacteriaceae* (99.9% of *Rhodospirillales* sequences) and most of them (97.9%) could not be assigned to a known bacterial genus. The next major order in Alphaproteobacteria was *Rhizobiales*, but the frequency was highly variable depending on the sample. *Rhizobiales* was frequently found in the three *Cladonia* samples and the *Usnea* moss sample (US3). The most frequent OTUs in *Rhizobiales* belonged to *Beijerinckiaceae*, including the genera *Beijerinckia* and *Methylorosula*. The only three OTUs were included in LAR1 (7.8% of *Rhizobiales* sequences) which was defined by Hodkinson & Lutzoni (2009) and were found in one *C. gracilis* (CL3) and two *Umbilicaria* samples (UM1 and UM3).

Acidobacteria, the second major phylum, was abundant in the three *Cladonia* and *Usnea* samples, one *Umbilicaria* (UM3) sample and the *A. coniops* sample (CR2). Most of the Acidobacteria OTUs included *Acidobacteriaceae* (99.6% of acidobacterial sequences) and were classified as *Acidobacterium*, *Granulicella* and *Terriglobus.* Gammaproteobacteria was the major bacterial group in *B. granulosa* (CR1) and *A. coniops* (CR2) samples, and was mostly represented by major OTUs, *Frateuria aurantia* (ex Kondô & Ameyama) Swings *et al.* in CR1 and *Rhodanobacter panaciterrae* Wang *et al.* in CR2 (80.9% and 97.6% of Gammaproteobacteria, respectively). Bacteroidetes was the major phylum identified in the three *Umbilicaria* samples, and the CR1 and CR2 samples. *Sphingobacteriales* and *Cytophagales* were equally abundant in the three *Umbilicaria* and CR2 samples, but only *Cytophagales* was observed in CR1.

Based on a distance matrix calculated by weighted Fast UniFrac analysis for all bacterial OTUs, PCoA of 11 lichen samples, excluding US2 which contained a low frequency of bacterial sequences, resulted in close clustering of the same mycobiont genera (Fig. 2a). The three *Cladonia* samples and two *Usnea* samples with fruticose growth forms were closely related, and the three *Umbilicaria* samples were clustered together. In contrast, the three crustose samples of different mycobiont genera were not clustered together. When frequency data for alphaproteobacterial OTUs was used for PCoA analysis, the four moss inhabiting samples (CL1, CL2, CL3 and US3) were more closely related, and the three *Umbilicaria* samples, one *Usnea* sample (US1) and two crustose samples (CR1 and CR3) inhabiting rocks were closely related. One crustose sample (CR2) was not related to any of the other samples (Fig. 2b).

Discussion

Lichens have long been recognized as symbiotic organisms between two major components, mycobiont and photobiont. However, it has also been recognized through culturing approaches that lichens harbour diverse bacterial species as components of the lichen thalli (González *et al.* 2005, Cardinale *et al.* 2006, Liba *et al.* 2006, Selbmann *et al.* 2010, Lee *et al.* 2014). Recently FISH staining, DNA fingerprinting and high-throughput sequencing techniques have been applied to improve our understanding of the microbial community structures in lichen thalli (Cardinale *et al.* 2008, Grube *et al.* 2009, Bates *et al.* 2011, Hodkinson *et al.* 2012). Studies on microbiomes in the human and animal gut suggest that microbiomes in animals affect health and body condition of host animals (Turnbaugh *et al.* 2006, Warnecke *et al.* 2007). Similarly, microbiomes in lichens have ecological and physiological roles for all of the lichen thallus (Grube *et al.* 2009). Until now, most of the studies on microbial communities in lichen thalli have been conducted for lichens living in temperate climate zones (Grube *et al.* 2009, Hodkinson & Lutzoni 2009, Bates *et al.* 2011) and rarely in the cold environment of the Arctic (Sigurbjörnsdóttir *et al.* 2014, 2015). Therefore, studies on lichens living in Antarctica will provide data complementary to the previous studies on bacterial communities in lichen thalli from temperate and Arctic areas. Fungal and algal community analyses have been performed on the same lichen samples (Park *et al*. 2015). Therefore, data on bacterial community structure will provide a balanced view on the microbiome composition in Antarctic lichen thalli.

Studies on bacterial communities repeatedly reveal Alphaproteobacteria as one of the major bacterial groups in lichen thalli comprising up to 60–70% of the total bacterial communities (Cardinale *et al.* 2008, Grube *et al.* 2009, Bates *et al.* 2011, Hodkinson *et al.* 2012, Sigurbjörnsdóttir *et al.* 2015). This trend was continued in Antarctic lichens, although the first major bacterial group was Acidobacteria or Gammaproteobacteria in some cases (CL1, CR2 and CR3). Some crustose lichens that were in full contact with the substrate examined by Grube *et al.* (2009) and Hodkinson *et al.* (2012) were shown to be different from the macrolichens. It was revealed that composition of all bacterial OTUs was dependent on the mycobiont genus from the PCoA (Fig. 2a). It was also suggested that growth form may affect the microbial community by showing that fruticose lichens, *Cladonia* and *Usnea*, were grouped together in PCoA based on bacterial OTU frequency (Fig. 2a). Substrate is proposed as another putative factor for alphaproteobacterial communities as the PCoA indicated that the composition of Alphaproteobacteria was closely related in the four lichen samples from mosses and in most

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of the lichens from rocks (Fig. 2b). The work of Printzen *et al.* (2012) also suggested that the composition of lichen alphaproteobacterial communities was affected by environmental conditions. Therefore, host species, growth form and substrate might be putative factors that affect bacterial communities in lichen thalli. To understand the effects of growth form and habitat as a potential determinant of bacterial community composition more precisely, further studies with larger sample sizes are needed.

In previous studies of bacterial communities in lichen thalli, the LAR1 lineage of *Rhizobiales* has been recognized as an important symbiotic component that may provide fixed nitrogen to lichen ecosystems (Hodkinson & Lutzoni 2009, Bates *et al.* 2011). However, LAR1 lineage was recovered from only three samples at low frequency (CL3, US3 and UM1; 7.8% of *Rhizobiales*). Instead, the dominant order from Alphaproteobacteria was *Rhodospirillales* and mostly found as *Acetobacteriaceae*. It is well known that many species of *Acetobacteriaceae* are involved in nitrogen-fixing processes (Pedraza 2008, Saravanan *et al.* 2008). Although direct evidence was not obtained in the current study, it is proposed that *Acetobacteraceae* might contribute to the lichen ecosystem as nitrogen fixers. Other well-known nitrogen fixers that were observed in the Antarctic lichen samples from this study included *Beijerinckia*, *Bradyrhizobium*, *Burkholderia* and *Acinetobacter* (Rösch *et al.* 2002, Grube *et al.* 2009, Bates *et al.* 2011).

To date, many researchers have speculated that lichens may be specific microecosystems (Cardinale *et al.* 2008, Grube *et al.* 2009, Bates *et al.* 2011, Park *et al.* 2015) and have studied the roles of lichen microbiomes. We examined bacterial diversity in Antarctic lichens to improve our understanding of the bacterial community structure. Although this study was conducted with a limited number of lichen samples, the analysis raised several interesting issues, including the complex composition of bacterial OTUs and their dependency on host species, growth form and substrate.

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Author contributions

C.H.P. performed the experiments, analysed the data and wrote the manuscript. S.G.H. conceptually designed the work and wrote the manuscript. K.M.K. and O.S.K. analysed the data. G.J. revised the article.

Supplemental material

Two supplemental tables will be found at http://dx.doi.org/10.1017/S0954102016000286.

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