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Microbiome change by symbiotic invasion in lichens

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Running title: Microbiome change by symbiotic invasion in lichens

Summary

Lichens are obligate symbioses between fungi and green algae or cyanobacteria. Most lichens resynthesize their symbiotic thalli from propagules, but some develop within the structures of already existing lichen symbioses. *Diploschistes muscorum* starts as a parasite infecting the lichen *Cladonia symphycarpa*, and gradually develops an independent *Diploschistes* lichen thallus. Here we studied how this process influences lichen associated microbiomes and photobionts by sampling four transitional stages, at sites in Sweden and Germany, and characterizing their microbial communities using high-throughput 16S rRNA genes and photobiont-specific ITS rDNA sequencing, and fluorescence *in situ* hybridization. A gradual microbiome shift occurred during the transition but fractions of *Cladonia*-associated bacteria were retained during the process of symbiotic reorganization. Consistent changes observed across sites included a notable decrease in relative abundance of Alphaproteobacteria with a concomitant increase in Betaproteobacteria. Armatimonadia, Spartobacteria, and Acidobacteria also decreased during the infection of *Cladonia* by *Diploschistes*. The lichens differed in photobiont specificity. *Cladonia symphycarpa* was associated with the same algal species at all sites but *Diploschistes muscorum* had a flexible strategy with different photobiont combinations at each site. This symbiotic invasion system suggests that partners can be reorganized and selected for maintaining potential roles rather than depending on particular species.

Introduction

Lichens are obligate symbioses between fungi and green algae or cyanobacteria, resulting in the production of a joint phenotype unique among microbial symbioses, the lichen thallus. Within the protective association of the lichen thallus, the fungal and algal components have been able to extend their range of ecological niches considerably and sustain much more exposed and stressful habitats compared with their free-living, non-symbiotic relatives. While many lichens represent classic examples of mutualistic symbioses, it has been suggested that lichens are better viewed as an analogy to human crop domestication, where fungi cultivate the algal constituent (Lücking *et al.*, 2009). A number of reviews on lichens as symbiotic systems, their component diversity, physiology, metabolism, contributions to ecosystems and environmental roles, can be found in Nash (2008).

In addition to the fungal symbiont, many species of other fungi can inhabit the lichen thalli and interact with their hosts in different ways, ranging from commensalism to parasitism. More than 1800 species of lichenicolous (lichen-inhabiting) fungi are known to science (Lawrey and Diederich, 2003), and rather than representing a single phylogenetic group, they are scattered in many unrelated fungal lineages. Many lichenicolous fungi are closely related to lichens, and were also suggested as a first stage of de-lichenization towards the evolution of other fungal lifestyles (Lutzoni *et al.*, 2001). In reality, many lichenicolous fungi have the same type of relationship with their host algae as the host lichen fungus itself, and may better be viewed as lichens, irrespective of if they form an independent thallus within or on the surface of the host thallus (Rambold and Triebel, 1992). Lichenicolous lichens, which establish their own mutualistic thallus, grow within the limits of their host's structures, sometimes forming rather small areas of infection. For other species, parasitism is only a transient stage of early development. Such parasites require infecting other lichens before continuing as independent mutualistic symbioses. The host lichen is often destroyed and the

lichenicolous fungus takes advantage of the algae available in the host thallus. This established photobiont can be directly accessed without wasting energy to capture and grow a new algal population. Thus lichenicolous behaviour can provide a starting benefit for parasitic lichens, as in *Rimularia insularis* where maintenance of the host's algae was observed by de los Rios *et al.* (2002). This, however, is not always the case. One example is *Diploschistes muscorum*, a common species in lichen-dominated biological soil crusts which infects lichens of the genus *Cladonia* (Fig. 1). Friedl (1987) found that *Diploschistes* starts as a lichenicolous fungus, invading the *Cladonia* thallus and utilizing the alga at an intermediate stage. Then *Diploschistes* replaces the *Cladonia* photobiont with a more suitable alga, together with which it forms an independent free-living *Diploschistes* thallus. The process in which horizontal transfer of algal partners occurs among fungal lineages was referred to as algal switching (Piercey-Normore and DePriest 2001). The photobiont switch in *D. muscorum* may indicate that the mycobiont has a moderate selectivity level, as it can establish symbiosis with two different algal species. And while several studies have examined established photobiont communities in *Diploschistes* and host *Cladonia* thalli (Bačkor *et al.* 2010, Škaloud and Peksa 2010, Škaloud *et al.* 2015), to the best of our knowledge, none have investigated the photobiont switching process with the *Diploschistes* take-over of *Cladonia*.

Bacterial groups, such as Alphaproteobacteria and Acidobacteria, have been consistently recovered from lichen thalli (Cardinale *et al.*, 2008; Grube *et al.*, 2012; Muggia *et al.*, 2013), and evidence is rapidly accumulating that these microbes may generally play integral roles in the lichen symbiosis (Grube *et al.*, 2009, 2015; Hodkinson and Lutzoni, 2009; Bates *et al.*, 2011; Printzen *et al.*, 2012; Erlacher *et al.*, 2015). Other complex community interactions between symbiotic microbes and eukaryotes are of course well-known, for example, among marine sponges or within animal guts (Webster, 2014). Microbial spatial localization patterns in multispecies associations may further reflect conditions as substrate

concentration gradients, or biotic interactions as microbial coaggregation or resource competition (Daims and Wagner, 2011). Little is known, however, about community and spatial changes that occur in microbiomes during lichen-to-lichen parasitization-takeover events. Considering that previous studies suggest lichens maintain species-specific microbial communities (Grube *et al.*, 2009; Bates *et al.*, 2011), it is reasonable to expect marked microbiome shifts as one lichen species infects, and eventually overcomes, another. In order to address this knowledge gap, we investigated lichen microbiome dynamics as the parasitic lichen *Diploschistes muscorum* takes over *Cladonia symphycarpa* using high-throughput 16S rRNA gene and photobiont-specific ITS rDNA sequencing to track bacterial and algal transitions during the infection process, and employed fluorescence in situ hybridization (FISH) to localize bacteria in the *Cladonia* and *Diploschistes* lichen thalli.

Results

Four stages in the *Cladonia-Diploschistes* transition were sampled with three replicates each on the three study sites (Gynge and Dröstorp in Öland, Sweden, and Gössenheim in Bavaria, Germany): A) *Cladonia* with no visible infection, B) early infection stage defined by the first visible *Diploschistes* thallus, C) late-stage infection with parts of the *Cladonia* thallus still identifiable, and D) final stage with only a fully developed *Diploschistes* thallus (Fig. 1A-D).

Richness, diversity and taxonomic composition of bacterial communities

We tested for differences in bacterial richness (number of operational taxonomic units, OTUs, at 97 % similarity level) and evenness across infection stages. There were no differences in number of bacterial OTUs when rarefied to 1010 sequences per stage (two sample *t*-test, $p > 0.05$), and stages did not differ in estimated richness (Chao 1 index, two sample *t*-test, $p > 0.05$), and there was no general trend in evenness or phylogenetic diversity between stages

(Shannon index and Faith's Phylogenetic Diversity, two sample *t*-test, $p > 0.05$ for both). The 16S rDNA amplicon survey revealed (Fig. 2A) the dominance of Proteobacteria (57.2 %, percentages here and elsewhere are averages across all samples), Bacteroidetes (12.1 %), Acidobacteria (11.0 %), Actinobacteria (5.8 %) and Verrucomicrobia (3.7 %) in the system. Several other phyla, including Chloroflexi, Planctomycetes, Armatimonadetes and Cyanobacteria, with relative abundances less than 4 % of the 16S rRNA reads, were present, possibly (at least Cyanobacteria) as casual epiphytes. At class level Alphaproteobacteria (37.1 %), Betaproteobacteria (17.1 %), Saprospirae (6.2 %), Actinobacteria (4.7 %) and Acidobacteria (4.4 %) were main contributors. Alphaproteobacteria were primarily represented by the orders Rhodospirillales (19.0 %; Acetobacteraceae, Rhodospirillaceae) and Rhizobiales (7.9 %; Methylobacteriaceae, Beijerinckiaceae, Bradyrhizobiaceae, Methylocystaceae), and Betaproteobacteria by Burkholderiales (16.7 %; Burkholderiaceae, Oxalobacteraceae). Among the Saprospirae, Saprospirales (6.2 %) and Cytophagales (3.4 %) were predominant. Abundant and frequently occurring members across stages were identified with the two-parameter model by Li *et al.* (2013) taking into account taxonomic abundance and ubiquity for each taxon. Eight members at family level were identified at the ubiquity cut-off of 80 % and abundance cut-off of 1 % (Fig. 2B).

Beta diversity patterns determined using NMDS

The samples of the four infection stages formed no clear clusters based on the Bray–Curtis dissimilarity metric but clustered separately (Fig. S1) when community differences were measured using unweighted and weighted UniFrac distance metrics. Although samples of stage B (initial infection) overlapped with *Cladonia* samples, while samples of stage C (progressed infection) overlapped with *Diploschistes* samples, ANOSIM test showed no significance for sample type (stage A-D; ANOSIM analyses of unweighted UniFrac distances

$R = 0.11$, $p > 0.05$, ANOSIM of weighted UniFrac $R = 0.15$, $p > 0.05$). The ordination of bacterial community structure data revealed that the main structuring factor was sampling site (ANOSIM analyses of unweighted UniFrac distances $R = 0.43$, $p = 0.007$, ANOSIM of weighted UniFrac $R = 0.35$, $p = 0.035$). As no consistent differences between the four stages were observed in the general bacterial community structure, we assessed the common and unique OTUs (Fig. 3A) and changes in abundance of bacterial taxa in the process of *Diploschistes* infection. Of the 1295 observed OTUs, only 9.1 % were present in all four infection stages (Fig. 3A). The main changes at higher levels included incremental increases and decreases in the process of infection and distinct maxima at particular infection stages. Armatimonadia (1.7 % in stage A to 0.5 % in stage D), Spartobacteria (6.1 % in stage A to 0.8 % in stage D), Acidobacteria (6.4 % in stage A to 1.3 % in stage D) and Alphaproteobacteria (52.3 % in stage A to 18.2 % in stage D) were more abundant at all sites; whereas Betaproteobacteria increased gradually in relative abundance in the process of infection. Orders with similar patterns at more than one location are shown in Fig. 3B. Although taxa at class and order level showed similar dynamics at all sites, their relative abundance within the bacterial communities was variable. We used the LEfSe tool to identify 16 bacterial taxa at order level and 21 at family level where relative abundance changes differed significantly between stages. The *Diploschistes* samples of all sites differed from the other infection stages in their higher abundances of Burkholderiales and Clostridiales (Fig. 3C).

Fluorescence in situ hybridization and confocal laser scanning microscopy

As the relative abundance of Alphaproteobacteria and Betaproteobacteria changed in the transition, taxonomic group-specific oligonucleotide probes were applied in fluorescence *in situ* hybridization (FISH). Consistent with this finding was the observation of Alphaproteobacteria-specific signals with 16S rRNA – FISH in *Cladonia* and

Betaproteobacteria-specific signals in *Diploschistes* (Fig. 4). Bacteria were visible at the upper cortex and the lower surface of *C. symphylicarpa* (Fig. 4A-D), but no bacteria were attached to the algal layer or medulla. In *Diploschistes* (Fig. 4E), FISH-CLSM revealed a low over-all density of bacteria (confirmed by light microscopy) suggesting a lower abundance compared with *Cladonia*.

Photobionts present in Cladonia and Diploschistes

All *Cladonia symphylicarpa* samples contained a single photobiont (*Asterochloris*) at all three sites. *Diploschistes muscorum*, however, had different photobionts at each site. At one site *Diploschistes* was associated with the same *Asterochloris* species as the *Cladonia*. At another site the *Diploschistes* samples were all associated with a *Trebouxia* only, possibly following a similar development and photobiont replacement as described by Friedl (1987). The most complicated mycobiont-photobiont relationship was found at the third site, where *Diploschistes* was associated with at least three different algae. All samples had the *Trebouxia* photobiont but, in addition, one sample still contained the initial *Asterochloris* present in *C. symphylicarpa*, and two samples had *Dictyoichloropsis symbiontica* as a second photobiont. The photobiont topologies resulting from the Maximum Likelihood analyses (Figs. S2-S4) were more resolved than from the Bayesian analyses, whereas the Bayesian trees were better supported. The topologies recovered were in agreement.

Discussion

To the best of our knowledge, this is the first study to report on bacterial community transitions that occur when one lichen species is parasitically invaded by another. The bacterial community of *Cladonia symphylicarpa* is replaced by a different community when *Diploschistes muscorum* infects and eventually replaces the *Cladonia* thallus. This transition

is gradual, with younger infections having a bacterial composition resembling the uninfected *Cladonia* community dominated by Alphaproteobacteria (Fig. 2A, 3B, 4). More advanced infections are more similar to the *Diploschistes* bacterial community even if these still retain fractions of *Cladonia*-associated bacteria. One recent study investigated effects of a fungal infection on the bacterial communities in lichens, *Rhagadostoma lichenicola* on *Solorina crocea* in the Eastern Alps (Grube *et al.*, 2012). Similar to other non-lichenized lichenicolous fungi, this species merely develops unstratified hyphal structures inside the host. Grube *et al.* (2012) found that the microbiome composition at phylum level remained largely the same, irrespective of the infection status of the host. Differences were only found at bacterial strain level. In contrast, in the present study we see clear differences at higher taxonomic levels, which correlate with the formation of a genuine symbiotic phenotype by the infecting fungus.

The *Diploschistes* infection causes successive breakdown of the structural integrity of *Cladonia* and degradation of its hyphae. It is, however, hardly possible to quantify the extent of breakdown of individual hyphae and as we never found an enrichment of generalist decomposers, we interpret this thallus replacement and takeover as a process without a phase of unspecific bacterial decomposition. Instead a distinct but gradual shift in the bacterial community was observed when one lichen replaces another through parasitism. This observation corroborates previous indications of host specific composition of lichen-associated microbiota (Grube *et al.*, 2009; Bates *et al.*, 2011). Moreover, the changes here occur in similar habitats with similar microclimate, and also proceed similarly when comparing samples originating from geographically distant sites. The results also suggest that the more homogeneous bacterial community in the attacked symbiosis is replaced with more varied microbiota in the parasite, as differences are found across the sampling sites. The higher variation of the bacterial communities in the parasite is paralleled by its broader compatibility with different algae. The most obvious change in the transition is that the

215 Alphaproteobacteria dominating the *Cladonia* community decrease, at all sites.
216 Betaproteobacteria of the Burkholderiales increase substantially when *Diploschistes* takes
217 over, at two of the three sites. This increase is partly due to Oxalobacteraceae, a group known
218 to occur in lichens (Lang *et al.*, 2007). As members of this group are involved in oxalate
219 degradation, this increase could be correlated with the substantial production of Ca-oxalate in
220 *Diploschistes* thalli that is apparent from our own observations of microscopic sections. The
221 decline of Alphaproteobacteria was due mostly to the loss of Rhodospirillales,
222 Caulobacteriales and Sphingomonadales, while notably the relative abundance of lichen-
223 associated Rhizobiales (LAR1), a clade of bacteria so far known only from lichens
224 (Hodkinson and Lutzoni, 2009; Bates *et al.*, 2011) did not change significantly in the course
225 of infection. Changes were not consistent across all sites, an increase in Actinobacteria was
226 observed at two sites (2.3 % to 6.7 % at Dröstorp and 1.6% to 10.7 % at Gössenheim). While
227 the Alphaproteobacteria did significantly decrease in samples from the Dröstorp site, the
228 corresponding increase in Betaproteobacteria was not as evident, instead by the final stage
229 there was a significant increase in the abundance of Chloroflexi (13.1 % of all sample
230 sequences on average), a group with low representation (2.0 %) at other sites for all stages.

231 The coexistence and interactions of bacteria and fungi have been observed in several
232 environments and comprise polymicrobial communities to symbiotic associations of fungal
233 hyphae and bacterial cells, for example mycorrhiza helpers. Bacteria aid in mycorrhiza
234 formation and functioning due to nutrient mobilization, nitrogen fixation and protection from
235 pathogens (Frey-Klett *et al.*, 2007) and communities associated with mycorrhiza are specific
236 for the plant host (de Boer *et al.*, 2005; Frey-Klett and Garbaye, 2005; Frey-Klett *et al.*,
237 2011). Lichen-associated bacteria contribute to similar beneficial functions for the host
238 (Grube *et al.*, 2009, 2015; Erlacher *et al.*, 2015) and communities contributing functions to
239 maintenance of symbiotic integrity are apparently adjusted to the fungal determined

architecture. Here, we found a number of taxonomic groups with nitrogen-fixing members, including Rhodospirillales and Rhizobiales, indicating that bacterial associates may contribute to lichen nitrogen requirements, as was suggested previously (Hodkinson and Lutzoni, 2009; Bates *et al.*, 2011). Interestingly, the observed decline of Acetobacteriaceae in the Alphaproteobacteria was paralleled by a relative increase of *Burkholderia*, some of which are also able to fix nitrogen.

The two lichens studied clearly differ in physical parameters of their thallus structure, in particular the thallus surfaces differ in hydrophily. While the leafy scales of *Cladonia symphycarpa* have hydrophilic surfaces, where water droplets are easily percolating into the thallus, *Diploschistes* has hydrophobic surfaces, where droplets may persist much longer on the surface due to acetone-insoluble molecules (Lange *et al.*, 1997). We assume that these differences in host structure are major factors determining bacterial composition, as bacteria respond differently to water accessibility. A close association of bacteria (Rhizobiales) with the hydrophilic fungal cortex of *Lobaria* was found by Erlacher *et al.* (2015). In reindeer lichens, Cardinale *et al.* (2008) showed that the hydrophilic interior surfaces of the cylindric branches were inhabited by a bacterial biofilm, whereas the hydrophobic external surfaces of the thalli were hardly colonized. It is therefore likely that the surface hydrophobicity of *Diploschistes* impedes substantial bacterial aggregation here (Fig. 4E). The lichens further differ in their extracellularly deposited secondary chemistry: *D. muscorum* producing diploschistesic acid, sometimes with lecanoric acid, whereas *C. symphycarpa* contains atranorin and norstictic acids. Potential effects of secondary lichen metabolites on the growth on certain bacteria are known from culture assays (see review of Boustie and Grube, 2005). Thus, differences of thallus chemistry may also affect the composition of bacterial communities. It can also be argued whether bacteria enriched in the course of infection could

have both a detrimental effect on *Cladonia* and a beneficial effect on *Diploschistes*. The pending tests of such hypotheses would require thoughtful experimental approaches.

Our survey of algal photobionts over the course of the infection and eventual take-over of *Cladonia* by *Diploschistes* suggests a progression from a restricted photobiont maintenance in the host to a more generalist photobiont strategy by the parasitic lichen. All *Cladonia symphycarpa* samples contained a single photobiont at all three sites but *Diploschistes muscorum* has different algal partners at each site, changing algal partner during the transition at two sites but keeping the *Cladonia* photobiont at one, implying that an algal switch is not occurring at this site. This suggests that *D. muscorum* has a very flexible algal partner strategy. There is no specific photobiont which *D. muscorum* needs to wait for during the take-over of the host thallus, which is further supported by all these photobionts producing the same carbohydrate, ribitol (Honegger, 1991, 2012), and there is no indication that the optional algal switch influences the shift in bacterial community. Hodkinson *et al.*, (2012) suggested that the nature of the algal partner does influence the overall composition of the bacterial communities, but they compared green algal and cyanobacterial species, respectively, which often imply completely different thallus morphologies. The existence of multiple photobionts (identified as *Asterochloris irregularis* and *Trebouxia showmanii*) in *Diploschistes* has been demonstrated previously by Friedl (1987), and was later molecular studies (Bačkor *et al.*, 2010; Škaloud and Peksa, 2010; Škaloud *et al.*, 2015) have expanded the number of associated photobionts (*A. glomerata*, *A. lobophora*, and two undescribed *Asterochloris* species). In our study, all *Asterochloris* from *Diploschistes* and *Cladonia* samples group with the recently described *A. mediterranea* (Fig. S2), expanding the range of hosts of this photobiont, known only from *Cladonia* species (Moya *et al.*, 2015). Earlier studies have reported *Trebouxia showmanii* and *T. gigantea* as algal partners of *D. muscorum* (Friedl, 1987; Friedl and Gärtner, 1988). Our *Trebouxia* from *Diploschistes* represent the undescribed

Trebouxia “sp. URa3” (Fig. S3) which is here reported as algal partner of *Diploschistes* for the first time. Finally, *Dictyochloropsis* is reported from a *Diploschistes* species for the first time (Fig. S4). There are to our knowledge no other reports suggesting a similar generalistic photobiont strategy, but there are a couple of studies on lichenicolous lichens indicating at least a photobiont switch. *Chaenothecopsis consociata* at first associates with the host’s (*Chaenotheca chrysocephala*) *Trebouxia* photobiont but later replaces this with *Dictyochloropsis symbiontica* (Tschermak-Woess, 1980). In the *Rimularia insularis* infection of *Lecanora rupicola*, the *Rimularia* associates with the host’s photobiont (de los Rios *et al.*, 2002)

In our study, we found no evidence suggesting that the lichenicolous lichen *Diploschistes muscorum* is specific for a host lichen with a similarly structured microbiome. Instead, we observed a distinct shift of composition and pattern of variation in bacterial community and photobiont association in the transition from one lichen to the other. These results do not exclude that more similar communities may be observed in other cases of lichen parasitism involving unrelated lichens, with more similar thallus features shared by the host and its transforming parasite. The transitions seen in lichenicolous lichens suggest that these symbiotic systems integrate partners with a preference for maintaining functional roles, rather than particular species. Our study thus provides a promising first insight into the shifts observed during lichen parasitism, which represent a symbiotic invasion process with a complete reorganisation of all partners (fungi, algae, and bacteria), while the basic functionalities of the lichen symbiosis remain the same: a shape-providing fungal partner, carbohydrate-providing eukaryotic algae, and an associated bacterial community with multiple potential functions (Grube *et al.*, 2015). The particular functions of the bacteria, however, remain to be elucidated by experimental studies in this fascinating transition of bacterial communities.

Methods

Sampling

Samples were collected September 9th 2012 at the Swedish Soil Crust International (SCIN) site on Öland (Gynge Alvar, at 56°32'31.7''N, 16°28'42.2''E), April 7th 2013 at the German SCIN site ("Ruine Homburg" at Gössenheim, Bavaria, Germany, 50°01'34.8''N, 9°47'56.1''E), and June 6th 2013 at an additional site on Öland (Dröstorpe, at 56°35'29.7''N, 16°35'01.1''E). All sites are on limestone bedrock with a thin gravel layer on top of the bedrock, and the vegetation is composed of a colourful lichen-dominated biological soil crust (BSC) including *Cladonia* spp., *Diploschistes muscorum*, *Thamnolia vermicularis*, *Squammarina* spp., *Fulgensia* spp., *Psora decipiens*, *Toninia* spp., and cyanobacteria (Fig. S5). Here, the dominating *Cladonia* species in the BSC is *Cladonia symphylicarpa* and it is possible to follow the transition from *C. symphylicarpa* to *D. muscorum* (Fig. 1A-D) at all sites. A background to the SCIN study with descriptions of the SCIN sites is given by Büdel *et al.* (2014). Four transition stages were sampled with three replicates on each site. The sampling was simplified by the fact that both *Cladonia* and *Diploschistes* grew in distinct, well-delimited clumps or colonies, here treated as individual samples. Sampling in the field was conducted carefully avoiding contamination, and samples were kept on dry ice until arrival at the laboratory, and kept at -70 °C until processing. In the laboratory, debris and soil remnants were carefully removed before DNA extraction.

DNA extraction

The samples for this study were processed at two different occasions; the samples from Gynge in 2012 and the samples from Dröstorpe and Gössenheim in 2013. Ca.0.25g of every sample was put into sterile and pyrogen free tubes, lowered into liquid N₂, and ground with a

sterile, cooled pestle. DNA extraction was done with the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) following the manufacturer's protocol. An incubation step at 70° C for 10 min was included, followed by 2 × 30 sec of bead beating. Each extraction round included a negative control to test for contamination in the kit buffers.

16S PCR amplification and amplicon sequencing to assess bacterial community structure

The V4 hypervariable region of the 16S ribosomal RNA gene was amplified using GS FLX 454 one way read barcoded fusion primers (Lib-L kit, Primer A, Primer B, Roche 454 Life Science, Branford, CT, USA) with the template specific sequences F515 and R806 (Caporaso *et al.*, 2011a). PCR triplicates were run using illustra Hot Start Mix RTG beads (GE Healthcare, Little Chalfont, Bucks, UK) following the manufacturer's protocol with 0.4µM of each primer and 1µL of DNA extract. The PCR triplicates were pooled and purified with the QiaQuick Gel Extraction kit (Qiagen, Venlo, Netherlands). After DNA quantification with QuantiFluor dsDNA Dye (Promega, Madison, WI, USA) the samples were pooled equimolar and were run on a 2100 Bioanalyzer (Agilent, Waldbronn, Germany) for quality assessment and quantification. Emulsion PCR of pooled samples was performed with the GS Junior Titanium emPCR Kit (Lib-L) (Roche 454 Life Science, Branford, CT, USA) as per the manufacturer's instructions. Sequencing was run on a GS Junior instrument using GS Junior Titanium Sequencing Kit and PicoTiterPlate Kit (Roche Diagnostics, Basel, Switzerland).

Processing of amplicon sequence data

Raw sequence data were pre-processed using QIIME v. 1.8.0. (Caporaso *et al.*, 2011b). Sequences were denoised (Reeder and Knight, 2010), quality-trimmed and chimeric reads detected with ChimeraSlayer. Sequences were aligned using PyNAST (Caporaso *et al.*, 2010). For OTU-binning the Mothur clustering method (Schloss *et al.*, 2009) was utilized. Sequences

were classified using BLAST. To calculate UniFrac sample distances (Lozupone and Knight, 2005), Maximum Likelihood phylogeny was inferred with FastTree (Price *et al.*, 2009). For QIIME downstream analyses chloroplast or mitochondria sequences were excluded, and three samples per stage were pooled and rarefied to 1,010 sequences per sample. Megan (MEtaGenome ANalyzer) was used for taxonomic comparison of samples (Huson *et al.*, 2007). A two-sample *t*-test for alpha diversity comparison between infection stages was applied. The shared taxa (core members) across infection stages were identified using a two-parameter model and visualized with a ubiquity vs abundance plot (taxonomic ubiquity cut-off = 0.8, abundance = 0.01). “Abundance” is defined as the proportion of a taxon in a sample and “ubiquity” describes the proportion of samples in which a taxon is detected (Li *et al.*, 2013). For identification of taxa with similar occurrence patterns, a hierarchical cluster analysis was performed (Shade *et al.*, 2013). Non-metric Multidimensional Scaling (NMDS) was used to compare sample distances and visualize clustering for both Bray–Curtis dissimilarity (OTU-based metric), and weighted and unweighted UniFrac (phylogenetic metrics) distance matrices, using the R phyloseq package (McMurdie and Holmes, 2013; R Core Team, 2013). Additional data are given in the Supporting Information.

Photobiont identification

ITS rDNA region of photobionts in transition stages A and D was amplified with primers ITS1.T and ITS4.T (Kroken and Taylor, 2000), PCR settings initial denaturation at 95° C for 5 min followed by 35 cycles of at 95° C 30 sec, annealing at 56° C 30 sec and extension at 72° C 1 min with a final extension of 8 min 72° C. The Gynge samples in stage D resulted in PCR products of two different lengths. The PCR samples were gel-cleaned with Sigma GenElute Agarose Spin Columns (Sigma-Aldrich Inc., St Louis, USA), and reamplified. The PCR products were purified using Exonuclease I and FastAP™ Thermosensitive Alkaline

Phosphatase (Thermo Fisher Scientific). For sequencing reactions the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) was used, following the manufacturer's protocol, and fragments were separated on an ABI3130xl Genetic Analyzer (Applied Biosystems). BLAST searches indicated that the 21 newly generated sequences belonged to three green algal genera; *Asterochloris*, *Trebouxia*, and *Dictyochloropsis* (Tab. S1). Three alignments were produced and a summary is present in Tab. S1. For the *Asterochloris* alignment, sequences representing lineages in Škaloud and Peksa (2010), Peksa and Škaloud (2011), and Řídká *et al.* (2014) were selected, together with three sequences of *Asterochloris mediterranea*. The *Trebouxia* alignment included a broad selection of taxa from O'Brien (2014). The *Dictyochloropsis* alignment was based on the "Dictyochloropsis clade 2" from Dal Grande *et al.* (2014), as this included both free-living and lichenized algae. The alignments were aligned manually in AliView 1.09 (Larsson, 2014), ambiguous regions were delimited manually and excluded. Evolutionary models were estimated separately for the three ITS partitions (ITS1, 5.8S, and ITS2), using the Bayesian Information Criterion in jMODELTEST (Posada, 2008). ML searches were run using GARLI 2.0 (Zwickl, 2006), stopping after 5,000 generations if no improvement of the Ln likelihood ≤ 0.01 was detected, with a maximum of 500,000 generations. Support was assessed with 1,000 replicates of ML bootstrapping under the same search models. Bayesian analyses were conducted in MrBayes 3.2.1 (Ronquist *et al.*, 2012). Ten million Markov chain Monte Carlo generations were sampled every 500th step and the first 25 % were discarded as burn-in. We ran two parallel runs each with four chains and pooled the samples after burn-in. Mixing and convergence was confirmed through the statistics provided by the program.

Fluorescence in situ hybridization and confocal laser scanning microscopy

As our knowledge of microbial spatial localization in lichen thalli is limited, fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes is a very useful technique to identify and visualize microbes and their occurrence in microhabitats without the need of culturing, as it preserves the three-dimensional structure of the sample, and relates spatial arrangement of organisms to substrate gradients (Amann *et al.*, 2001). For details on sample fixation, embedding, sectioning and hybridization protocol see Maier *et al.* (2014). The oligonucleotide probes applied for hybridization were: 16S rRNA targeted 5'-end-labeled Cy3 (indocarbocyanine) probe EUB338 mix (an equimolar combination of EUB388, EUB338II and EUB338III [Daims *et al.*, 1999]), Cy5 (indodicarbocyanine) labelled ALF968 (Loy *et al.* 2007), Betaproteobacteria – specific FITC labelled Bet42a (Manz *et al.*, 1992) and 5'-end-labelled Cy5 probe Burkho (Hogardt *et al.*, 2000). The negative controls non-EUB338 were 5'-end-labeled with Cy3, Cy5 and FITC fluorescent dye (Wallner *et al.*, 1993). Details on the probes are given in the Tab. S2, and are available at probeBase (Loy *et al.*, 2007). The FISH assay specificity was confirmed by double-hybridizations of group-specific probes with the general probes EUB. Thallus sections were observed with a Leica TCS SPE (Leica Microsystem, Heidelberg, Germany) and bacteria recorded with Leica ACS APO 40x OIL CS (1.15 NA) and Leica ACS APO 63x OIL CS (1.30 NA) objective lenses. The excitation settings were 488 nm, 532 nm, and 635 nm. The wavelength ranges set to detect the emission signal of the fluorochromes were fluorochrome FITC at 500–550 nm, Cy3 at 550–600 nm, and Cy5 at 650–700 nm. Optical sections were taken every 1.0 μ m and stacked.

Data deposition

454 FLX sff-flowgrams are deposited in the NCBI Sequence Read Archive (SRP056133).

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616

Figure legends

Fig. 1. A. *Cladonia symphycarpa*. B. Initial *Diploschistes* infection. C. Progressed *Diploschistes* infection, with *Cladonia* remnants visible. D. *Diploschistes muscorum*. Photos: A. E. Timdal, B-D. M. Westberg.

Fig 2. A. Bacterial community structure based on amplicon 16S rDNA data. Pie charts show the proportion of reads assigned to *Cladonia* (grey) and *Diploschistes* (blue) samples (97 % similarity threshold, rarefaction with a depth of 1,010 reads per sample). B. Ubiquity-Abundance plot (family-level) with cut-offs of ubiquity=0.8 abundance=0.01. Coloured lines represent taxa above cut-offs, non-core taxa are plotted in grey, two-parameter cut-off in red.

Fig. 3. A. Network showing the number of bacterial OTUs found only in *Cladonia*, *Diploschistes* or the infection stages as well as shared OTUs (based on amplicon 16S rRNA gene data; rarefaction with a depth of 1,010 reads per sample). B. Hierarchical clustering was used to determine taxa with similar occurrence patterns in the course of *Diploschistes*-infection. C. LEfSe results detect 16 bacterial orders with significant differential abundance. Symbols: A=*Cladonia*, B=initial infection, C=progressed infection, D=*Diploschistes*.

Fig. 4. Bacteria colonize thalli of *Cladonia* and *Diploschistes*, visualized using fluorescence *in situ* hybridization with confocal laser scanning microscopy (merged 3-colour images). A) and B1) Vertical section of an uninfected *Cladonia* squamule (50 μ m); Eubacteria (red) and Alphaproteobacteria (pink) in the cortex, thallus reflection (grey), algal autofluorescence (blue). B2) Enlargement of B1. C) Vertical section of an uninfected *Cladonia* squamule (50 μ m); Eubacteria (red) and Alphaproteobacteria (pink) in the lower surface, thallus reflection (grey). D) Vertical section of an uninfected *Cladonia* squamule (50 μ m); Eubacteria (red), *Burkholderia* spp. (pink) Betaproteobacteria (yellow) in the cortex, thallus reflection (grey), algal autofluorescence (blue). E) Vertical section of *D. muscorum* thallus (50 μ m); Eubacteria

641 (red), *Burkholderia* spp. (pink) Betaproteobacteria (yellow), thallus reflection (grey). al: algal
642 layer; co: cortex; me: medulla; ls: lower surface. Scale=20µm.
643

644 **Supporting information**

645

646 **Supplementary Methods.** *Processing of 454 sequence data.*

647 **Table S1.** Voucher information, cultured algal strain or herbarium/isolate number, and

648 Genbank accession numbers of the sequences used in this study. Newly produced sequences

649 are in bold.

650 **Table S2.** Oligonucleotide probes used for fluorescence *in situ* hybridization in this study.

651 **Fig. S1.** Ordination using Non-Metric Multidimensional Scaling (NMDS) derived from

652 unweighted and weighted UniFrac; based on amplicon 16S rRNA gene data, rarefaction with

653 a depth of 1,010 reads per sample.

654 **Fig. S2.** Unrooted most likely tree of *Asterochloris*.

655 **Fig. S3.** Unrooted most likely tree of *Trebouxia*.

656 **Fig. S4.** Unrooted most likely tree of *Dictyochloropsis*.

657 **Fig. S5.** Study site at Gynge alvar, Öland, Sweden. A typical lichen-dominated biological

658 soil-crust area on limestone-pavement. *Diploschistes* thalli are visible as greyish clumps

659 scattered over the area.