The genetic basis of symbiosis in lichen *Usnea hakonensis*

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Summary

Symbiosis has been involved in many of evolutionary innovations in life's history, and considered as one of the most important interactions between organisms. Lichens are symbiotic associations of fungi, alga, and bacteria. As symbiotic entities, they are adapted to diverse environments, often where water supply is transient and nutrition is scarce. They are able to inhabit extreme environments such as polar regions, desert, and high altitudes. The principle of symbiotic interaction within a lichen is those between the fungus, mycobiont, and the photosynthetic partner, photobiont. Symbiotic association induce dramatic shifts in morphology and metabolism of both symbionts. The most prominent is the morphogenesis of symbiosis-specific structures (thalli), some of which are the most complex vegetative structures in the fungal kingdom. Inside a thallus, the photobiont benefits by the protection from environmental stresses while the mycobiont benefits by photosynthetic products supplied by the photobiont. However, genetic basis that function such symbiotic interaction between the mycobiont and the photobiont has not been revealed.

When I started the PhD. candidature, or even nowadays, characterization of genetic mechanisms in lichen symbiosis is a challenging topic with only few previous studies. Although high-throughput sequencing techniques have enabled to investigate genomes and transcriptomes of non-model organisms, those techniques have not been applied to investigate dual interaction between the mycobiont and the photobiont in a lichen.

In this thesis, I examined genetic mechanisms that regulate symbiotic interaction between a lichen-forming fungus and its photosynthetic partner by using *Usnea hakonensis*-*Trebouxia* sp. system. The symbiotic association of *U. hakonensis* and *Trebouxia* sp. can be resynthesized *in vitro* from isolated cultures of the fungus and the alga. To identify differentially expressed genes between the symbiotic and non-symbiotic states of this lichen symbiosis, I compared the transcriptomes of resynthesized thalli and isolated cultures of the mycobiont/photobiont by using high-throughput RNA sequencing techniques (RNA-seq). For an accurate comparison of the gene expression, I first sequenced and annotated the genomes of *U. hakonensis* and *Trebouxia* sp, which were both unavailable.

In Chapter 2, I performed the whole genome sequencing of the photobiont *Trebouxia* sp. The predicted size of the algal genome is 69 Mb, which is comparable to other *Trebouxia* algae. Unexpectedly, the genome sequencing revealed association of a novel Alphaproteobacterium, *Sphingomonas* sp. 'TZW2008' with the alga. This bacterium had not been visually identified during the 8 years of culturing in laboratory conditions, but the fluorescence *in situ* hybridization confirmed the localization of bacterial cells on algal cells. The bacterial growth was synchronized with the algal growth, and its dependency on algal photosynthetic products was identified. Although our study presented the bacterial benefit from the symbiotic interaction with the alga in terms of carbon source, algal benefit from the interaction remained unclear. However, closely related bacteria were also detected in *U. hakonensis* thalli collected in the field, indicated that this bacterium might also be the essential participant of the lichen symbiosis.

In Chapter 3, the genome of the mycobiont, *U. hakonensis* was determined. The fungal genome was annotated by using RNA-seq reads derived from the transcriptome of the isolated fungal strain. The genome was found to be compact, showing close proximity of neighbouring genes. When conventional genome annotation methods were used, few reads derived from the mRNAs with read-through sequences connected the neighbouring genes, resulted in mis-annotation. Therefore, I developed a pipeline that includes extra steps to eliminate those reads with read-through sequences. The genes in the annotated genome were classified into gene families according to the sequence similarity. Structure of the gene families were then compared among fungi with different lifestyles (lichen-forming, free-living, and parasitic fungi). The results indicated that some of the transporter gene families were contracted in the lichen-forming and the parasitic fungi, whereas three gene families were only identified in the lichen-forming fungi, and predicted to be lichen-specific. The common genomic features identified in the lichen-forming fungi may reflect long-term symbiotic association with algae.

In Chapter 4, finally, I examined the differential gene expression between the symbiotic and non-symbiotic states of the *U. hakonensis*-*Trebouxia* sp. system. The sequenced genomes of the mycobiont and the photobiont, separately discussed in Chapter 2 and 3, were re-annotated using RNA-seq reads derived from the transcriptomes of resynthesized thalli and isolated cultures. Re-annotation provided us with new genes, some of which may represent symbiosis-specific gene expression. More than half of the identified differentially expressed genes had no similar sequences in the public database, indicating

the possibility that the lichen obtained novel genes for symbiosis. On the other hand, genes common to other organisms were indicated to play important roles in symbiosis. As expected, transfer of photosynthetic products from the photobiont to the mycobiont was indicated from the results. Moreover, transfer of nutrients, such as nitrogen, phosphorus, and carbon dioxide, from the mycobiont to the photobiont was also indicated. In the *U. hakonensis*-*Trebouxia* sp. system, it appears that the bidirectional transfer of nutrients is the foundation of its symbiotic association. However, many of the up-regulated genes in the symbiotic state still awaits discovery of their functional roles in symbiosis.

In this thesis, I identified the genes that change expression before and after the *in vitro* symbiosis of lichen *U. hakonensis*. Functions of the genes up-regulated in the symbiotic state were predicted in terms of symbiotic interaction between the mycobiont and the photobiont. In the process, the genomes of the *U. hakonensis* and *Trebouxia* sp. were sequenced and annotated. A novel lichen-associated bacterium was detected and its roles in symbiotic association were predicted. Further investigation into expression of the identified genes in symbiosis of *U. hakonensis* in nature would lead us to a deeper understanding of genetic mechanisms underlying lichen symbiosis.

Contents

Chapter 1

General Introduction

The importance of symbioses in history of life and ecosystems has been increasingly perceived by modern society. It is now widely accepted that eukaryotes are originated from ancient symbiosis (Martin *et al.*, 2015), and the colonization of land by plants was accomplished with symbiotic fungi (Delaux *et al.*, 2015). Recently effects of microbiota on human health have been focused, reminding us that we *per se* are at the midst of symbiotic interaction (Cho & Blaser, 2012; Hooper *et al.*, 2012; Thaiss *et al.*, 2016). The concept of "symbiosis" was first coined by researchers studying lichens in late 19th century (Honegger, 2000; Trappe, 2005). In 1878, at a general meeting of German naturalists, Heinrich Anton de Bary, a professor of the University of Strassburg, proposed a term "symbiosis' to describe "associations of organisms of different species"(Oulhen *et al.*, 2016). The introduction of the term unified then distinguished associations between phylogenetically distinct organisms into a single concept and enabled researchers to perceive complex and variable interactions in nature in wider view.

1.1 Categories of Symbiosis

There are three main terms to categorize symbiotic associations: parasitism, commensalism, and mutualism. A symbiotic association in which one partner benefits and the other harmed by the association is called parasitism. An association in which both partners benefit by is called mutualism. An association in which one benefits by and the other neither harmed nor benefited is called commensalism.

Parasitism

Among the three terms, parasitism has the longest history. It was introduced in English in early 17th century from the Greek parasitos "(person) eating at another's table". Just like the word, in parasitic symbiosis, a symbiont, the parasite, gains nutrition from the other, the host, and in return causes disadvantage or harm to it. Accordingly, all viruses are parasites (if they are considered as living organisms) requiring host cells for

reproduction and in many cases (but not always) causing disease. Viruses associate with all forms of life ranging from prokaryotes to eukaryotes. They share six common stages upon infection. First, a virus binds to specific receptors on the surface of the host cell (attachment/adsorption). Then it enters the cell by endocytosis or direct fusion of the viral and host membranes (penetration). After the entry, protein coats of the virus are broken down releasing the viral genome (uncoating). Some viruses such as bacteriophages inject only their genomes into the host cell and leave their protein coats outside. Once getting into the host cell, the viral genome is replicated and viral genes are expressed for protein synthesis by the ribosomes of the host cell (replication). The extent of which viruses depend on the host cell for replication varies among types of viruses. In general, DNA viruses (viruses with DNA genomes) are more dependent requiring host cell enzymes for replication. Viral genomes and proteins gather at a certain place of the cell forming new virus particles (assembly). Matured virus particles are released from the host cell, often resulting in the death of the cell (release). During their reproductive processes, viruses interfere with normal activities of the host cells and in many cases cause disease in their hosts. Ebola virus, responsible for the repeating outbreaks of Ebola virus disease in Africa, is one of the most acute examples of viral symbiotic associations. However, not all viruses give severe damage to their hosts. Some viruses establish long-lasting symbioses and such persistent infections are mostly asymptomatic. Likewise, in host-parasite interactions between organisms from various phylogenetic groups, the extent of "harm" to the hosts ranges from lethal to barely detectable. Therefore more specific terms, which I would not refer to in this place, are used to describe complex and wide-ranged interactions between the parasite and the host.

Mutualism

On the contrary to parasitism, mutualism is a symbiotic association which both symbionts benefit from. The benefit is primarily nutrition but it can also be protection from environmental stress or reproductive success. Many mutualistic symbioses with

nutritional benefit include autotrophic organisms such as autotrophic bacteria, algae, and plants as a symbiotic partner. Of those, symbiosis between plants and mycorrhizal fungi is one of the best-studied associations due to its importance in terrestrial ecosystems. Mycorrhizal fungi collect nutrients, namely phosphorus and nitrogen, from the surrounding soil and provide them to the associated plant roots. In return, the fungi receive carbohydrates that are photosynthetically fixed by the plant. In general, mycorrhizal symbiosis enhances plant productivity in nutrient-poor soil by serving the plant with phosphorous and nitrogen, the vital nutrients for plant growth. In estimate, more than 80% of all plant species form mycorrhizal symbioses (Heijden *et al.*, 2015). Considering the role of plants as producers, mycorrhizal symbioses are probably the most important mutualistic symbioses on earth. Sometimes symbionts gain multiple benefits from the symbiosis. In mutualistic associations between clownfishes and sea anemones, the clownfish is protected from predators by living within poisonous tentacles of the sea anemone and at the same time, provided with food that are fragments of the prey and wastes from the anemone (Litsios *et al.*, 2012). In cases of microorganisms associated with insects or animals, microbes obtain suitable habitats as well as nutrients from their hosts (Leser & Mølbak, 2009). However, the benefit symbionts receive from the symbiotic life are difficult to assess and are not fully understood in most associations.

Commensalism

Due to our limited knowledge of symbioses, commensalism is probably the most difficult to assess compared to the other two. In commensal symbiosis, a symbiont (commensal) receives benefit from the association while the other (host) is neither advantaged nor disadvantaged. Mites on insects, barnacles on whales, remoras, and some microbes living in animal guts are considered as examples of commensals. However, it is still in question whether these organisms are truly neutral to their hosts. Commensal symbioses can sometimes turn into mutualistic or parasitic under certain circumstances. Some remoras present mutualistic character by feeding on ectoparasites of their hosts,

and *Candida albicans*, a harmless commensal living in the human gut, can cause an infection following the downfall of the host immune system (Mayer *et al.*, 2013).

Parasitism, mutualism, and commensalism are not the categories representing "fixed" associations between organisms but rather representing "state" of associations that are changeable from one to another. In nature, symbiotic associations are kept upon subtle balance of environmental, developmental, and genetic factors. Changes in those factors may result in different outcomes. For example, viruses can stay latent in the host cell under certain circumstances (Traylen *et al.*, 2011) or can be more dangerous in one species (or individual) than another (Mandl *et al.*, 2015). Likewise, mycorrhizal associations can be parasitic under phosphorus or nitrogen rich condition or for few weeks after the germination (Johnson *et al.*, 1997; Litsios *et al.*, 2012; Nouri *et al.*, 2014). Although how symbiotic association develops is not fully understood, contribution of coevolution, reciprocal evolutionary change that occurs between the symbionts, has been indicated in the studies such as: hosts and parasites (Decaestecker *et al.*, 2007; Ebert, 2008; Morran *et al.*, 2011), and flowering plants and pollinators (Machado *et al.*, 2005).

Classification of symbiotic associations

Symbiosis can be further classified into endosymbiosis or ectosymbiosis depending on the location of a symbiont. If a symbiont resides inside the cell of the other, the symbiosis is called endosymbiosis, and it is called ectosymbiosis if a symbiont resides outside the cell of the other. According to this definition, microorganisms living in animal guts are ectosymbionts. Symbionts that are completely dependent on the symbiotic association and cannot live without it are called obligate symbionts. If symbionts can also live in free-living condition they are called facultative symbionts. Many parasites especially viruses are the obligate symbionts, which cannot survive outside the host cells.

1.2 Lichens

A number of examples tell us that symbiosis is an important lifestyle for the organisms moreover, concerned profoundly with their evolutionary innovations. For lichens, symbiosis also acted as breakthroughs in their ecological obstacles. Lichens are symbiotic association of fungi (mycobionts) and photosynthetic partners (photobionts), which can be either algae or cyanobacteria or sometimes both. They can be found practically in all terrestrial environments from tropical to polar regions, places where neither mycobionts nor photobionts can survive in separate. Fossil records suggest that emergence of lichens goes back to 400-600 million years ago and as being one of the first colonizers of the land (Heckman *et al.*, 2001; Honegger, 2008). Symbiotic lives of lichens are considered to be mutualistic. The mycobiont nutritionally benefits from photosynthetic products fixed by the photobiont, whereas the photobiont gains suitable environment, protected from strong light, temperature extremes, and drought. Both symbionts, especially fungi, experience remarkable transformation by the association.

Mycobiont

Approximately 20% of fungal species are lichenized. Most of the lichen-forming fungi belong to Ascomyceta, account for approximately 40% of the phylum. Less than 0.5% of lichen-forming fungi belong to Basidiomycota, account for approximately 0.3% of the phylum. Some orders of Ascomycetes (Acarosporales, Gyalectales, Pertusariales, Trichotheliales, and Lichinales) exclusively consist of lichen-forming fungi, but other orders of Ascomycetes and the orders of Basidiomycetes consist of fungi with diverse nutritional strategies (lichens, saprophytes, pathogens, mycorrhizas, etc.) (James *et al.*, 2006; Miadlikowska *et al.*, 2006). Molecular phylogenies indicate that lichenization occurred independently in several fungal lineages (Gargas *et al.*, 1995; Lutzoni *et al.*, 2001). Moreover, Lutzoni *et al*. (2001) demonstrated that some of the extant non-lichenized fungi are derived from lichen-forming ancestors, implying that lichenization can be transient. In nature, lichen-forming fungi are obligate symbionts, almost exclusively found in the symbiotic state. But some species can be cultured independently

in a laboratory under appropriate condition, proving that they are physiologically facultative symbionts. Therefore, the lichen symbiosis is probably not an ultimate goal for fungi but rather an advantageous option. Although lichens are symbiotic entities comprised of the mycobiont and the photobiont, their scientific names are conventionally referred to the name of the mycobiont.

Photobiont

Photosynthetic partners of lichens cover both prokaryotic and eukaryotic world. Almost 40 genera of cyanobacteria and algae have been reported as photobionts. Some 85% of the mycobionts are associated with green algae, whereas 10% are with cyanobacteria and 3-4% are with both green algae and cyanobacteria (Honegger, 2008). Photobionts are considered as facultative symbionts since free-living populations have been found in nature. However, views on *Trebouxia*, one of the most common lichen photobionts, are controversial whether their free-living cells are abundant or rare in nature (Ahmadjian, 1988; Bubrick *et al.*, 1984; Sanders & Lücking, 2002; Sanders, 2005). Ahmadjian argued that *Trebouxia* algae are obligate symbiotic green algae, highly coevolved with their fungal partners and can no longer live by themselves (Ahmadjian, 1988). So far, there is no clear evidence to confirm his view. However, it is known that the mycobiont can be selective in its choice of the photobiont (Beck *et al.*, 2002; Fedrowitz *et al.*, 2011; Piercey-Normore & DePriest, 2001; Piercey-Normore, 2006; Rikkinen *et al.*, 2002; Wirtz *et al.*, 2003). The extent of fungal selectivity ranges from low to high possibly affected by environmental conditions. In some lichen species, individual thalli were found to associate with multiple strains of photobionts (Muggia *et al.*, 2014; Piercey-Normore, 2006). Such low selectivity of mycobionts for photobionts is hypothesized as adaptation to changing environmental conditions, since retaining diverse photobionts might give the mycobiont a margin for optimizing the symbiotic associations in variable conditions (such as temperature, humidity, and illumination). Low selectivity is also reported in lichens growing in harsh environments where choice of photobionts is limited (Romeike

et al., 2002; Wirtz *et al.*, 2003). Environmental conditions might affect mycobiont-photobiont interactions at larger scale. Lichen communities growing in similar habitats were reported to share photobionts in phylogenetically close relations (Peksa & Škaloud, 2011; Rikkinen *et al.*, 2002). In such cases, choice of photobionts seems to correlate more with environmental factors than phylogenetic characterizations. Extensive studies on lichen communities and environmental factors are definitely needed to understand selectivity, or further coevolution, between mycobionts and photobionts.

Symbiotic phenotypes

Symbiotic associations of lichens induce dramatic shifts in morphology and metabolism of both symbionts. When a mycobiont starts association with a compatible photobiont, it produces a species-specific symbiotic morphology called "thallus". The basic structure of a lichen thallus comprises photobiont cells enveloped by fungal hyphae. In general, thalli are classified into three main groups that are: crustose, foliose, and fruticose types. While majority of lichens produce morphologically simple thalli without internal stratification, foliose and fruticose lichens develop structures organized in layers such as, upper cortex, photobiont layer, medulla, and lower cortex. Leaf-like thalli of foliose lichens and hair-like, strap-shaped or shrubby thalli of fruticose lichens are the most complex vegetative structures in the fungal kingdom. Stratified structures of lichen thalli are exclusively made up of polymorphic hyphae of the mycobionts, whereas the photobionts, which are usually placed inside the thalli, do not directly contribute to the morphology. However, they too experience morphological changes during symbiotic associations. Reduced filamentous forms, modified cell size, and converted chloroplast structure have been observed (Friedl & Büdel, 2008; Peksa & Skaloud, 2008).

The principal of metabolic interaction between the mycobiont and the photobiont in lichen symbioses is the transfer of photosynthetic products. In lichens with green algal photobionts, sugar alcohols such as ribitol, erythritol, and sorbitol are released, whereas glucose is released from cyanobacterial photobionts. Once photosynthetic products are

taken up by the mycobiont, they are quickly converted to polyols (such as mannitol and arabitol) for fungal use (Palmqvist *et al.*, 2008). Mechanisms behind the carbohydrates transfer haven't been elucidated yet. However, Wang *et al*. (2014) identified several sugar transporter genes in the genome of lichen-forming fungus, *Endocarpon pusillum*. The transcription levels of some sugar transporters were up-regulated during the symbiosis under the laboratory condition. Furthermore, they confirmed that the secretion of carbohydrates from the photobiont cells occurred only when the photobiont was co-cultured with the mycobiont. Their result suggests that the carbohydrates transfer is a character specific to the symbiotic association. Besides fixing carbon, cyanobacterial photobionts fix atmospheric nitrogen and provide it to the mycobiont as well (Nash III, 2008). Studies have shown that cyanolichens (lichens that associate with cyanobacteria) contribute to the nitrogen cycling in various ecosystems. Nutrition transfer between the symbionts is anticipated to be bidirectional: inorganic nutrients such as nitrogen, phosphorous, and magnesium are considered as candidates of the transfer from the mycobiont to the photobiont. Amongst, nitrogen transfer is the best examined. Nitrogen is the essential nutrient for life as a component of proteins and nucleic acids and its availability is reported to affect growth of lichen symbionts (Makkonen *et al.*, 2007; Palmqvist & Dahlman, 2006). Recently McDonald *et al*. (2012) analysed nucleotide sequences of ammonium transporter/ammonia permease (AMTP) across 191 genomes representing main lineages of life, and found that lichen-forming fungi, belonging to leotiomyceta, preferentially retained AMTP genes similar to that of land plants, whereas those were practically absent from the non-lichen fungal genomes. In a further study, which surveyed distribution of the gene within several lineages of lichen-forming fungi, correlation between the retention of the plant-like AMTP genes in lichens and their availability of nitrogen was indicated (McDonald *et al.*, 2013). Lichens without internal (cyanobacterial photobionts) or external (nitrogen-rich habitats) sources of nitrogen retained plant-like AMTP genes. Also, analysis on the genome of a lichen-forming fungus, *Endocarpon pusillum* revealed that gene families of nitrogen transporters have been

expanded over evolutionary time of the fungus (Wang *et al.*, 2014). Although Wang *et al*. detected up-regulation of nitrogen transporters during the symbiotic association, there has been no direct evidence for nitrogen transfer from the mycobiont to the photobiont. In fact, Beck and Mayr (2012) suggested that the amount of nitrogen transferred from the mycobiont to the green algal photobionts may be very limited and algae seem to have mycobiont-independent mechanisms for nitrogen uptake.

In this manner, our knowledge is still far from understanding complex and diverse characters of lichen symbiosis. Indeed, the very basic question on their symbiotic association remains unanswered. Are they mutualistic or parasitic?

Symbiotic relationships

Controlled parasitism is another interpretation of lichen symbiosis almost ever since their dual nature was first proclaimed by the Swiss botanist Simon Schwendener in 1867 (Honegger, 2000). When fungal hyphae envelope photobiont cells and penetrate their surface, specialized structure called "haustorium" are formed (Honegger, 1984). Haustoria are common features of parasitic fungi used for uptake of nutrition from the host cells, and because those in lichens are sometimes found associated with dead photobiont cells, they are considered as indication of parasitism. Another "parasitic" feature is fungal control on the population of photobionts (Honegger, 1993; Palmqvist, 2000). Mycobionts are speculated to regulate the population of photobionts in order to keep the nutritional balance between the symbionts in changing environment. Within thalli, photobionts reproduce only asexually (except for those belonging to the order Trentepohliales) often with reduced cell size or cell cycles compared to the cultured cells. Thus, photobionts are considered to be enslaved by the mycobionts, housed, maintained, and controlled within a thallus made of fungal hyphae.

Lichen-associated microorganisms

Non-phototrophic bacteria in lichen thalli were first reported in 1925 (Uphof, 1925).

Since then, a variety of bacteria have been found on the surface of and within thalli supposedly play supportive roles in lichen symbioses (Aschenbrenner *et al.*, 2016; Sigurbjörnsdóttir *et al.*, 2016). Functions such as nutrient supply, resistance against biotic and abiotic stress, support for photosynthesis and for growth of two eukaryotic partners, detoxification, and thallus degradation abilities have been indicated (Grube *et al.*, 2009; Grube *et al.*, 2015; Liba *et al.*, 2006; Sigurbjörnsdóttir *et al.*, 2015) which are assumed to enhance the persistence of lichens in harsh environment where they are often found. Importance of microorganisms, which are neither the mycobiont nor the photobiont, in lichen symbioses has been gradually revealed. Recently Spribille *et al*. (2016) identified a group of basidiomycete yeasts ubiquitously associated with Parmeliaceae lichens and indicated their involvement in construction of thalli, which have long been considered as products of a single fungus (mycobiont).

Resynthesis of lichen symbiosis and the objective of this study

One and a half centuries after Schwendener's finding of the dual nature of lichens, increasing evidence began to reveal that lichens are complex symbioses involving multiple organisms. However, the bulk of genetic mechanisms underlying their symbioses, even dual interaction between the mycobiont and the photobiont, still remains unknown. Resynthesis of symbiosis in culture has been the hindrance to molecular studies of lichen symbiosis. Even today, the symbiotic state can be resynthesized only in limited species. Within the limitation, previous studies indicated establishment of symbiotic relationships involves induction and suppression of gene expression in both fungal and algal partners (Armaleo & Miao, 1999; Trembley *et al.*, 2002). However, it took quite a while to identify each gene sequence involved in the development of symbiotic association until Joneson *et al*. (2011) used Suppressive Subtractive Hybridization (SSH) and quantitative PCR (qPCR). They identified the genes presenting up-regulated expression in the resynthesized *Cladonia grayi*, when compared with the non-symbiotic cultures of the mycobiont and the photobiont. Their study was first to indicate

communication between the symbionts in early lichen development. Later, Wang *et al*. (2014) combined high-throughput sequencing techniques with qPCR to identify differential expression of genes indicated to be "symbiosis-related". In their study, the genome of lichen-forming fungus, *Endocarpon pusillum*, was sequenced and characterized. The expansion and contraction of gene families in the fungal genome indicated the effect of lichenization. Interestingly, most of the sugar transporter gene families have been lost from the *E. pusillum* genome possibly reflecting the specialized carbohydrate source of lichen-forming fungi. Wang *et al*. suggested that during evolution, the fungus has lost the sugar transporter genes that were unnecessary for the transfer of algal photosynthetic products. By using qPCR, they verified up-regulated expression of sugar transporter genes and genes involved in sugar metabolism, as well as genes involved in the mycobiont-photobiont recognition, nitrogen transfer and metabolism, in the co-cultures of the mycobiont and the photobiont. However, the exploration of differentially expressed genes in the previous studies was limited by the qPCR method, which requires to specify the genes to be quantified. High-throughput RNA sequencing (RNA-seq) is a technique that can quantify expression of unidentified genes in transcriptome. In this thesis, I used RNA-seq to identify differential gene expression between the symbiotic and non-symbiotic states of lichen *Usnea hakonensis*, in an attempt to elucidate the genetic basis of lichen symbiosis. *U. hakonensis* (mycobiont) and *Trebouxia* sp. (photobiont) is an ideal system to investigate genetic mechanisms of lichen symbiosis. This system is capable of *in vitro* resynthesis of the symbiosis by using non-symbiotic isolated cultures of the mycobiont and the photobiont. In Chapter 2, and 3, the genomes of the mycobiont, *U. hakonensis* and the photobiont, *Trebouxia* sp. were determined. In Chapter 4, expression of each gene in the fungal/ algal genome was compared between the symbiotic and non-symbiotic states. Functions of the differentially expressed genes were predicted and their roles in the symbiotic association were discussed. Importantly, a considerable number of genes that showed up-regulated expression in the symbiotic state had no similar sequences in the public database,

indicating lichen-specific mechanisms. This thesis provides valuable information for the elucidation of distinct mechanisms underlying lichen symbiosis. Also, the methodology presented here would help future studies that investigate the genetic basis of symbiosis in other lichen species.

Chapter 2

Physical contact and carbon transfer between a lichen-forming *Trebouxia* alga and a novel *Alphaproteobacterium*

Introduction

Lichens are traditionally described as symbiotic organisms composed of a fungal partner (mycobiont) and photosynthetic partners (photobionts). The symbiotic interaction provides a mycobiont with carbon nutrition, and photobionts with a suitable habitat that protects them from abiotic and biotic stresses (Nash, 2008). In addition to the mycobiont and the photobiont, lichens are known to host various organisms on or within their symbiotic structures (thalli) and are sometimes considered to be self-contained ecosystems (Farrar, 1976; Grube *et al.*, 2009; Nash, 2008). Recent progress in molecular techniques elucidated supportive roles of these 'guests' in functioning lichen symbioses. By using high-throughput sequencing techniques Spribille *et al*. (2016) identified a group of basidiomycete yeasts that ubiquitously associate with Parmeliaceae (Lecanoromycetes) lichens, indicating that more species than expected could be fundamentally involved in the establishment of lichen ecosystems.

Non-phototrophic bacteria associated with lichens were first reported in 1925 by Uphof followed by studies that revealed the diversity of culturable lichen-associated bacteria and suggested their possible roles in lichen symbiosis (Aschenbrenner *et al.*, 2016; Sigurbjörnsdóttir *et al.*, 2016). However, culture-dependent studies could not describe the overall composition of bacterial communities due to limitations in culturing techniques, and left the bulk of lichen-associated bacteria unknown (Cardinale *et al.*, 2006). Nowadays, culture-independent techniques, especially high-throughput sequencing techniques, have revealed the diversity and abundance of lichen-associated bacteria (Bates *et al.*, 2011; Grube *et al.*, 2009; Grube *et al.*, 2015; Sigurbjörnsdóttir *et al.*, 2015). Lichens are hypothesised to have mechanisms that select bacterial species beneficial for their symbioses (Bates *et al.*, 2011; Cardinale *et al.*, 2008; Grube *et al.*, 2009; Grube *et al.*, 2015; Hodkinson *et al.*, 2012). Indeed, lichen-associated bacteria are inferred to have functions such as nutrient supply and recycling of resources, which would enhance the persistence of lichens, which are often found in nutrient-poor environments (Grube *et al.*, 2009; Grube *et al.*, 2015; Liba *et al.*, 2006; Sigurbjörnsdóttir *et al.*, 2015). Moreover,

recent metagenomic and proteomic studies suggested the contribution of bacteria to lichens survival under extreme and changing ecological conditions (Grube *et al.*, 2015; Printzen *et al.*, 2012).

The roles of bacteria in algal growth and survival are better understood in interactions between non-lichen-forming algae (Fuentes *et al.*, 2016; Ramanan *et al.*, 2016). Several studies reported that bacteria supply nutrients such as vitamin B_{12}, iron, nitrogen, phosphorus and carbon to algae often, but not always, in exchange for photosynthetic products (Amin *et al.*, 2009; Croft *et al.*, 2006; Kazamia *et al.*, 2012; Watanabe *et al.*, 2005). Such complementary functions are also predicted in interactions between lichen-associated non-phototrophic bacteria and the photobiont (Aschenbrenner *et al.*, 2016; Sigurbjörnsdóttir *et al.*, 2016). Although addition of lichen extracts to media can improve the culturability of lichen-associated bacteria (Biosca *et al.*, 2016), little is known about their special requirements for nutrition and physical contact with mycobionts and photobionts.

Here, we present the physical contact and carbon transfer between a *Trebouxia* alga and a novel Alphaproteobacterium. A strain of *Trebouxia* algae isolated from a fruticose lichen *Usnea hakonensis* has been cultured for 8 years. The whole genome sequencing of this *Trebouxia* strain revealed the presence of a cryptic bacterium that covers the surface of algal cells. Our experiments showed that the bacterial growth is dependent on an algal photosynthetic product, ribitol, which *Trebouxia* algae are known to release from cells abundantly in lichens.

Materials and methods

Algal and bacterial strains and growth conditions

The algal strain used in this study was provided by Dr. Yoshiaki Kon, Tokyo Metropolitan Hitotsubashi High School, Japan. It was isolated from *Usnea hakonensis* collected from Kanagawa Prefecture, Japan (35°26′N, 139°10′E) in 2008 by the method

described previously (Kon *et al.*, 1993). A strain of the alga (*Trebouxia* sp. 'TZW2008') associated with the cryptic bacterium (*Sphingomonas* sp. 'TZW2008') has been maintained for 8 years. Since 2012, the algal strain has been kept on 2% agar plates of autotrophic C medium [containing, 15 mg $Ca(NO_3)_2 \cdot 4H_2O$, 10 mg KNO_3, 5 mg ß-Na_2glycerophosphate$\cdot 5H_2O$, 4 mg $MgSO_4 \cdot 7H_2O$, 0.01 µg vitamin B_{12}, 0.01µg Biotin, 1 µg Thiamine HCL, 0.3 mL PIV metals (100 mg $Na_2EDTA \cdot 2H_2O$, 19.6 mg $FeCl_3 \cdot 6H_2O$, 3.6 mg $MnCl_2 \cdot 4H_2O$, 1.04 mg $ZnCl_2$, 0.4 mg $CoCl_2 \cdot 6H_2O$, 0.25 mg $Na_2MoO_4 \cdot 2H_2O$, in 100 mL solution), 50 mg Tris, per 100 mL of medium, pH 7.5] (Ichimura, 1971). In addition, the culture was grown on 3 % agar plates of heterotrophic medium MY [containing 2% (w/v) malt extract, 0.2% (w/v) yeast extract, pH 5.8] and checked for colonies of contaminant bacteria.

Genome DNA extraction, library construction, sequencing, and de novo assembly

Algal colonies were scraped off the agar plate, washed twice with liquid C medium and centrifuged at 7,000 g for 5 min to collect algal cells. The cells were kept at -20 °C until use. Genomic DNAs were extracted and concentrated using NucleoSpin Plant II (Macherey-Nagel, Düren, Germany) following the protocol using Buffer PL2. DNA libraries were constructed using TruSeq DNA PCR-Free Library Preparation Kit (Illumina, Inc., San Diego, CA) following the manufacturer's instructions. Short DNA sequences (paired-end 125 bp) were determined from the libraries by Illumina HiSeq2500 platform (short reads). After removal of the adaptor sequences and low-quality reads, the reads were assembled into scaffolds by using CLC genomic workbench (https://www.qiagenbioinformatics.com/) with a word size of 64.

Scaffold sorting by average coverage per site

The short reads were mapped to the assembled scaffolds and an average coverage per site was calculated for each scaffold by using CLC genomic workbench. All scaffolds larger than 2 kb were sorted into two groups by the average coverage of x2,000 (Table

2.1). Partial sequences of scaffolds were submitted to BLASTN search (Altschul *et al.*, 1990) against the NCBI database (nr/nt: https://www.ncbi.nlm.nih.gov).

Phylogenetic analysis of the alga and the bacterium

The internal transcribed spacer (ITS) rDNA sequence of *Trebouxia* sp. 'TZW2008' was amplified using AL1500bf (Helms *et al.*, 2001) and LR3 (Friedl & Rokitta, 1997) primers. Specific primers for the alga were designed to fill the gap left by the sequencing of the PCR products amplified with AL1500bf and LR3 (Trebo_kon_ITS in Table 2.2). PCR in 25 μL reactions was run using Takara ExTaq DNA polymerase (Takara Bio, Inc., Shiga, Japan) with the following PCR conditions: 30 cycles of denaturation for 10 sec at 98 °C, annealing for 30 sec at 58 °C, and extension for 1 min at 72 °C, and final extension at 72 °C for 5 min. The ITS rDNA sequence was aligned with the ITS rDNA sequences retrieved from Ohmura *et al*. (2006) and the NCBI database. The 16S rDNA sequence of *Sphingomonas* sp. 'TZW2008' was found on a bacterial contig. The sequence was aligned with bacterial 16S rDNA sequences selected from Bates *et al*. (2011) and those of *Sphingomonas* species additionally collected from the NCBI database. The phylogenetic trees were constructed in *MEGA* version 6 (Tamura *et al.*, 2013) using the p-distance algorithm and the neighbour-joining algorithm (Saitou & Nei, 1987), with 1,000 bootstrap replications.

Detection of Sphingomonas-like bacteria in field-collected lichens

We investigated field-collected lichens (*U. hakonensis*) to detect lichen-associated bacteria similar to *Sphingomonas* sp. 'TZW2008'. Lichens were sampled in 2013 at the same location as 2008 [Kanagawa Prefecture, Japan (35°26′N, 139°10′E)]. Total RNAs were extracted using RNeasy Plant mini kit (QIAGEN, Venlo, the Netherlands) and cDNAs were synthesized using SMARTer cDNA synthesis kit (Takara Bio, Inc., Shiga, Japan). We designed specific primers (Tr_bac in Table 2.2) to amplify the 16S rDNA sequence similar to that of *Sphingomonas* sp. 'TZW2008'. We used cDNAs as PCR

templates because cDNAs synthesized from total RNAs generally contain rDNA sequences. PCR in 25 μL reactions was run using Takara ExTaq DNA polymerase (Takara Bio, Inc., Shiga, Japan) with the following PCR conditions: 35 cycles of denaturation for 10 sec at 98 °C, annealing for 30 sec at 66 °C, and extension for 30 sec at 72 °C, and final extension at 72 °C for 5 min. A phylogenetic tree was constructed by the method described above using amplified sequences and sequences of *Sphingomonas* species used in the phylogenetic analysis of the bacteria.

Table 2.1: Overview of the algal and the bacterial genomes

	***Trebouxia* sp. 'TZW2008'**	***Sphingomonas* sp. 'TZW2008'**
Predicted genome size (Mb)	69	3.5
No. of scaffolds (> 2kb)	677	24
Average coverage	x371	x6,245
N50	223	273

Table 2.2: PCR primers used in this study

Primer	Forward (5'-3')	Reverse (5'-3')	Target
Tr_bac	AGGAATCCATCTCTGGAAGC	GCCGCAAGGTTAAAACTCAA	bacterial 16S rDNA
Sphingo-like	GTACAAGGCCTGGGAACGTA	TGATGAAGGCCTTAGGGTTG	bacterial 16S rDNA
Trebo_act1	CTCAATGGTGGAGCTAGATG	AGCAGAGCGTGAAATTGTCC	algal *actin* gene
Trebo_kon_ITS	CCTGTGGGAGGATTTCTCAA	AAGTTCAGCGGGTGTTCTTG	algal ITS rDNA

Detection of a novel bacterium by fluorescence in situ hybridization (FISH)

The bacterial 16S rDNA sequence was amplified by PCR using specific primers (Sphingo-like in Table 2.2) and genomic DNA of *Sphingomonas* sp. 'TZW2008' as a template. The PCR products were labelled with either Digoxigenin-11-dUTP (Roche, Basel, Switzerland) or DNP-11-dUTP (Perkin Elmer, Waltham, MA) by secondary PCR. The labeled rDNA sequences were collected by ethanol precipitation and resuspended in hybridization solution (50% formamide and 10% dextran sulphate in 2xSSC) for use as probes. For fluorescence *in situ* hybridization (FISH), algal colonies cultured with the bacterium on an agar plate were cut out with agar substrate and placed on a Poly-L-lysine-hydrobromide coated glass slide for 5 min until the colonies adhered to the slide surface. The samples were fixed with Carnoy solution (methanol: acetic acid = 3:1) for 30 min, dried for 30 min at room temperature, and baked at 70 °C for approximately 1 hour. FISH was carried out in a previously described manner (Kawamura *et al.*, 2012; Tanabe *et al.*, 2002) with slight modifications. The glass slide with the samples was denatured in 70% formamide in 2xSSC at 70 °C for 2.5 min, and dehydrated in a series of ice-cold ethanol (70%, 85%, and absolute) for 3 min each. The denatured probes were applied onto the glass slide and the slide was covered with a coverslip and sealed. Hybridization was performed in a moist chamber at 37 °C for 37 hours. The samples were washed twice in 2xSSC, three times in 0.1x SSC at 60 °C for 5 min each, and blocked with 5% BSA in 4xSSC with 0.2% Tween-20 for 30 min at 37 °C in the dark. Anti-Dinitrophenyl (DNP) rabbit antibody (Sigma, Kanagawa, Japan) and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) were diluted to 1:200 and used to detect DNP-labelled probes. Monoclonal anti-Digoxigenin mouse antibody (Sigma, Kanagawa, Japan) and Cy3-conjugated sheep anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were diluted to 1:200 and used to detect DIG-labelled probes. The samples were treated with RNase (Sigma, Kanagawa, Japan; 100 μg/ml) during a series of antibody detections to reduce the non-specific background signals. Nuclear DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, Kanagawa, Japan)

and TOPRO-3 (Molecular Probes, Inc., Eugene, OR) simultaneously, and the slides were mounted in Vectashield Antifade (Vector Laboratories, Inc., Burlingame, CA). Fluorescent images were observed using a Leica DM5000B epi-fluorescence microscope equipped with an Plan-Apochromat 63x/1.4 OIL objective lens and the filter set for DAPI, Green, and Cy3, captured by a Leica CW4000 system (Leica Microsystems, Hessen, Germany). Counterstained nuclei were coloured blue (DAPI), FISH signals were coloured green (DNP-labelled probes) and magenta (DIG-labelled probes), respectively. For 3D image analysis, confocal laser scanning microscope (LSM510META, Carl Zeiss Microscopy, Thuringia, Germany) equipped with a Plan-Apochromat 63×/1.4 Oil objective lens and argon (488 nm) and helium-neon (543/633 nm) lasers was used. Fluorescent images for each optical section were recorded in 200 nm z-intervals and collected sequentially in three separate RGB channels with Red (Cy3), Green (Alexa488), and Blue (Cy5; TOPRO-3), respectively. The image stacks were processed with microscope operating software (LSM5, Carl Zeiss Microscopy, Thuringia, Germany) and three-dimensional images were reconstructed using Amira 3.1.1 software (FEI, Hillsboro, OR).

Search for genes representing symbiotic interactions

To investigate interactions between *Trebouxia* sp. 'TZW2008' and *Sphingomonas* sp. 'TZW2008', genes reportedly involved in algae-bacteria symbiotic interactions were searched for. Sequences of the species closely related to *Trebouxia* sp. 'TZW2008' and *Sphingomonas* sp. 'TZW2008' were used as queries in the search. For green algae, we chose *Chlamydomonas reinhardtii*, *Volvox cateri* f. *nagariensis*, and *Coccomyxa subelliopsoidea*. For *Sphingomonas* bacteria, we chose *Sphingomonas taxi*, *Sphingomonas wittichii*, and *Sphingomonas* sp. 'WHSC-8'. Gene sequences were retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the NCBI database, which are listed in Appendix Table S2.1. BLASTX searches (Gish & States, 1993) were locally performed against the algal cDNA and bacterial genome sequences.

When the e-values of a result were larger than or equal to 1e-20, the gene was considered absent in the genome.

Effect of antibiotics on the alga and the bacterium

In an attempt to test the effect of antibiotics on algal and bacterial growth, three antibiotics were selected. Ampicillin, kanamycin, and chloramphenicol were added to the autotrophic C medium at final concentrations 50, 250, 500 and 1000 μg/mL for ampicillin, 50 μg/mL for kanamycin, and 32.5, 162.5, 325 and 650 μg/mL for chloramphenicol. The strain of alga associated with the bacterium was inoculated onto media containing the antibiotics at each concentration. Three experimental replicates were conducted. The cultures were collected 1, 7, and 14 days after inoculation. At each sampling point, algal growth was measured by absorption at 680 nm normalized at 750 nm using a spectrophotometer UV-1800 (Shimadzu Kyoto, Japan). DNA was extracted from the samples and stored at -20 °C for use in quantitative PCR (qPCR) analysis.

To estimate the relative copy number of the bacterial genome to the algal genome, the copy number of algal actin gene and the bacterial 16S rRNA gene in the DNA extracted from the collected cultures were quantified by qPCR. Plasmid DNA was prepared as standard samples for quantification of each gene by using specific primers for the algal actin gene (Trebo_act1 in Table 2.2) and the bacterial 16S rRNA gene (Tr_bac in Table 2.2). Partial sequences of the algal actin gene and the bacterial 16S rRNA gene were amplified and each PCR product was cloned into pMD20 vector (Takara Bio, Inc., Shiga, Japan) using Ligation Kit version 2 (Takara Bio, Inc., Shiga, Japan). qPCR reactions were performed with Thermal Cycler Dice TP800 (Takara Bio, Inc., Shiga, Japan) using the program of an initial denaturing step at 95 °C for 30 sec, followed by 40 cycles of denaturing at 95 °C for 5 sec and extension at 55 °C for 1 min. Two types of reaction mixture containing either the algal or the bacterial primer set were prepared with SYBR® Premix Ex Taq™ II (Takara Bio, Inc., Shiga, Japan). For every qPCR reaction, a series of diluted plasmid DNA (10 pg, 1 pg, 0.1 pg, 10 fg, and 1 fg, per μL) was prepared to

construct a standard curve for the algal and bacterial genes. All the qPCR reactions were run with three replicates. The copy number of the algal and the bacterial gene in each sample DNA was quantified by using the respective standard curves. The relative copy number of the bacterial genome to the algal genome in each sample DNA was estimated from these gene copy numbers.

Bacterial growth on medium supplemented with various carbohydrates

To identify the carbon source of the bacterium associated with the alga, 2% ribitol, glucose, mannitol, or sucrose solution (w/v) was spread over the surface of the heterotrophic MY media. The strain of alga associated with the bacterium was inoculated onto each medium with a platinum loop. The 16S rDNA sequences of bacterial colonies that grew on the ribitol-supplemented medium were amplified by PCR using Tr_bac primers (see the section 'Detection of *Sphingomonas*-like bacteria in field collected lichens' for the PCR condition. The condition was modified to 25 cycles with the annealing temperature 58 °C). Bacterial colonies were carefully sampled by using micro glass tubes, avoiding any contamination by algal colonies.

Algal and bacterial growth on autotrophic medium

Autotrophic C media inoculated with the strain of alga associated with the bacterium were cultured under a 12h/12h light-dark cycle and in constant darkness. Three experimental replicates were conducted for each condition, collected one, three, six, and twelve days after the inoculation. At each sampling point, 3 mL of liquid C medium was added to a medium and algal cells were scraped off the medium with a spreader. A 2 mL aliquot was collected for measurement of algal growth using a spectrophotometer UV-1800 (Shimadzu, Kyoto, Japan) and DNA was extracted for the qPCR analysis (described in the above sections). Algal and bacterial growth was estimated from the copy number of algal actin gene and the bacterial 16S rRNA genes, respectively.

Data deposition

The DDBJ accession numbers for the genomic sequences of *Trebouxia* sp. 'TZW2008' are BDIU01000001-BDIU01000677, for the genomic sequences of *Sphingomonas* sp. 'TZW2008' are BDJB01000001-BDJB01000024, and the 16S rDNA sequences of *Usnea hakonensis* are LC197944 (Uh_seq1) and LC197945 (Uh_seq2).

Results

Culture of a green alga isolated from a lichen contained a novel bacterium

A green alga belonging to the genus *Trebouxia* (hereafter called *Trebouxia* sp. 'TZW2008') was isolated from a *Usnea hakonensis* in 2008 by Dr. Yoshiaki Kon and has been kept as a strain for 8 years. No bacterial contamination was observed during the culturing. Phylogenetic analysis using the sequences of ITS rDNA showed that *Trebouxia* sp. 'TZW2008' was closely related to *Trebouxia corticola* (Figure 2.2a), which is a common symbiont of lichens (Nash, 2008).

Genomic DNA was isolated from cultured *Trebouxia* sp. 'TZW2008' and sequenced by Illumina Hiseq2500 platform. The 260 million reads (125 bp in length) were *de novo* assembled into 1,997 scaffolds. Seven hundred and one scaffolds remained after the removal of scaffolds smaller than 2 kb.

We mapped the reads on the scaffolds and calculated the average number of reads mapped to each site of a scaffold and found that the scaffolds are distinguishable into two groups: a high coverage group (the average coverage per site ≥ x2,000) and a low coverage group (< x2,000). When the scaffolds are plotted with the length against the count of mapped reads, they are clearly separated into high- and low-coverage groups (Figure 2.1). The BLASTN search against the NCBI nucleotide database (https://blastncbi.nlm.nih.gov) showed that several large scaffolds in the high-coverage group were homologous to the genomic sequence of the gram-negative bacterium *Sphingomonas taxi* (CP009571.1), whereas large scaffolds in the low-coverage group

were homologous to sequences of algal species belonging to Chlorophyta.

The BLASTN search of all the scaffolds in the high-coverage group revealed that these were homologous to algal chloroplastic, mitochondrial, rDNA sequences (shown as triangles in Figure 2.1), and *Sphingomonas* sequences (squares). In consequence, 24 out of the 50 scaffolds were defined as bacterial sequences. The phylogenetic analysis of 16S rDNA sequences, found on a bacterial scaffold also showed that the bacterium belonged to the genus *Sphingomonas* (Figure 2.2b). This suggested the presence of a *Sphingomonas* species (hereafter called *Sphingomonas* sp. 'TZW2008') in the algal culture.

Genome size predicted from the sum of the scaffolds was 69 Mb for *Trebouxia* sp. 'TZW2008' and 3.5 Mb for *Sphingomonas* sp. 'TZW2008', which are comparable to closely related species: *Trebouxia gelatinosa* (57 Mb; https://www.ncbi.nlm.nih.gov/genome/?term=Trebouxia), *Trebouxia decolorans* (55 Mb) (Beck *et al.*, 2015), *S.* taxi (4 Mb; https://www.ncbi.nlm.nih.gov/genome/?term=Sphingomonas+taxi), *S.* wittichii (6 Mb; https://www.ncbi.nlm.nih.gov/genome/?term=Sphingomonas+wittichii), *Sphingomonas* sp. 'WHSC-8' (5 Mb; https://www.ncbi.nlm.nih.gov/genome/?term=Sphingomonas+WHSC-8). Average coverage for the predicted genome and N50 value are x371 and 223 kb for the alga, and x6245 and 273 kb for the bacterium (Table 2.1).

Novel bacterium interacts with the lichen-forming alga through carbon transfer

We cultured the algal strain on the heterotrophic MY medium for 1 month in order to promote growth of the cryptic bacterium associated with the alga. However, no visible bacterial colonies were observed (Figure 2.3a). Therefore we performed FISH, targeting the 16S rDNA sequence of the bacterium. FISH not only confirmed its existence but also revealed the localization of bacterial cells on algal cells (Figure 2.3b and 2.3c). Moreover, 3D analysis clearly visualized bacterial cells covering the surface of algal cells. A large number of bacterial cells (red and green signals) were detected even in deep positions

between algal cells (Figure 2.3d). These observations suggested that *Sphingomonas* sp. 'TZW2008' is unculturable without the alga. The quantification of the algal and bacterial growth on the autotrophic C medium under photosynthesis-activated and -inhibited conditions revealed that the alga and bacterium grew in a synchronized manner (Figure 2.4). They grew dynamically only when algal photosynthesis was active (after day 6), compared to photosynthesis-inhibited condition (t-test, alga: $p = 0.047$; bacterium: 0.025), indicating that both the alga but and the bacterium are dependent on the algal photosynthetic product for growth.

To clarify the carbon source of *Sphingomonas* sp. 'TZW2008', the alga with the bacterium was cultured on the heterotrophic MY medium supplemented with carbohydrates (ribitol, glucose, mannitol, or sucrose) previously identified in lichen thalli (Aubert *et al.*, 2007; Richardson & Smith, 1968b). As shown in Figure 2.5(a-c), bacterial colonies distant from algal colonies were observed on the ribitol-, glucose-, mannitol-supplemented media. We amplified and sequenced the 16S rRNA gene from these colonies, and verified that *Sphingomonas* sp. 'TZW2008' formed the colonies. *Trebouxia* photobionts are known to produce ribitol as a photosynthetic product (Richardson & Smith, 1968a). Previous studies indicated that it is the main carbohydrate transferred from *Trebouxia* photobionts to mycobionts (Lines *et al.*, 1989; Richardson & Smith, 1968b) and abundantly exists in lichen thalli (Aubert *et al.*, 2007). Within lichen thalli, ribitol is indicated to be metabolized by a fungal partner via the pentose phosphate pathway (PPP) (Lewis & Smith, 1967; Lines *et al.*, 1989). Our gene search identified bacterial genes similar to those required in the proposed ribitol metabolism via the PPP, and inferred the ability of *Sphingomonas* sp. 'TZW200' to utilize ribitol (Table 2.3, Figure 2.6). Along with ribitol, glucose is a well-documented photosynthetic product that is transferred from a photobiont to a mycobiont in lichen thalli (Drew & Smith, 1967; Hill, 1972; Tysiaczny & Kershaw, 1979). The gene search confirmed that the bacterial genome retains all the genes required in the glycolysis and Entner-Doudoroff pathway. Once taken up by the mycobiont, both ribitol and glucose are converted to mannitol that is abundantly

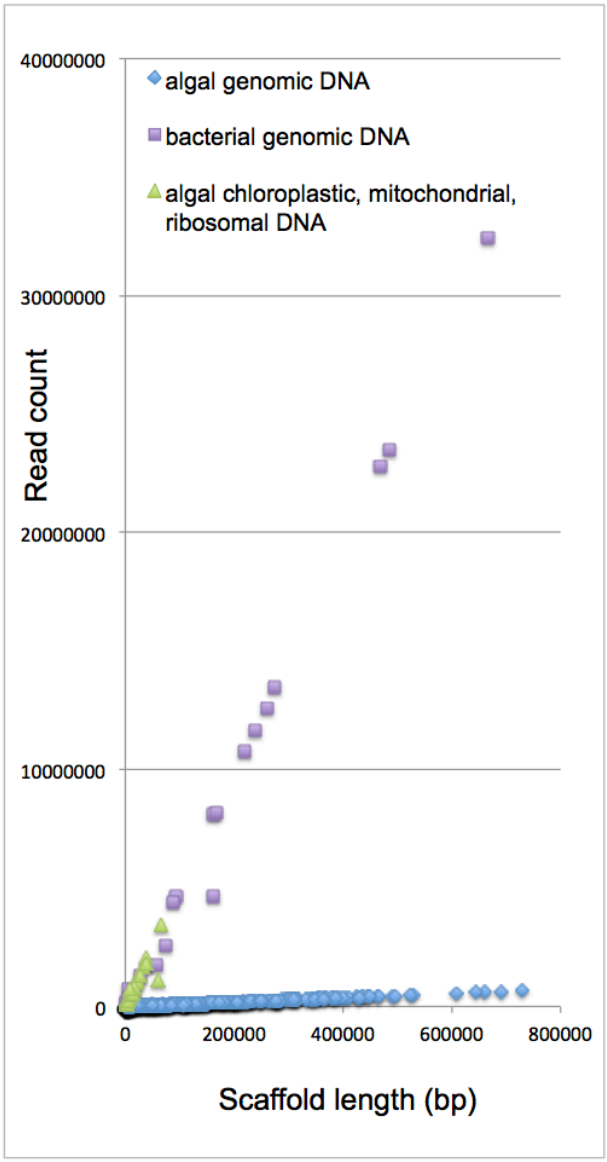


Figure 2.1: Genome sequencing of the alga isolated from the lichen *Usnea hakonensis* The plotting of a number of reads mapped on a scaffold against the length of a scaffold. Scaffolds in the high coverage group are homologous to bacterial sequences (shown in squares) or chloroplastic, mitochondrial, and rDNA sequences of algal species (triangles). Scaffolds in the lower coverage group are homologous to algal species (diamonds).

a

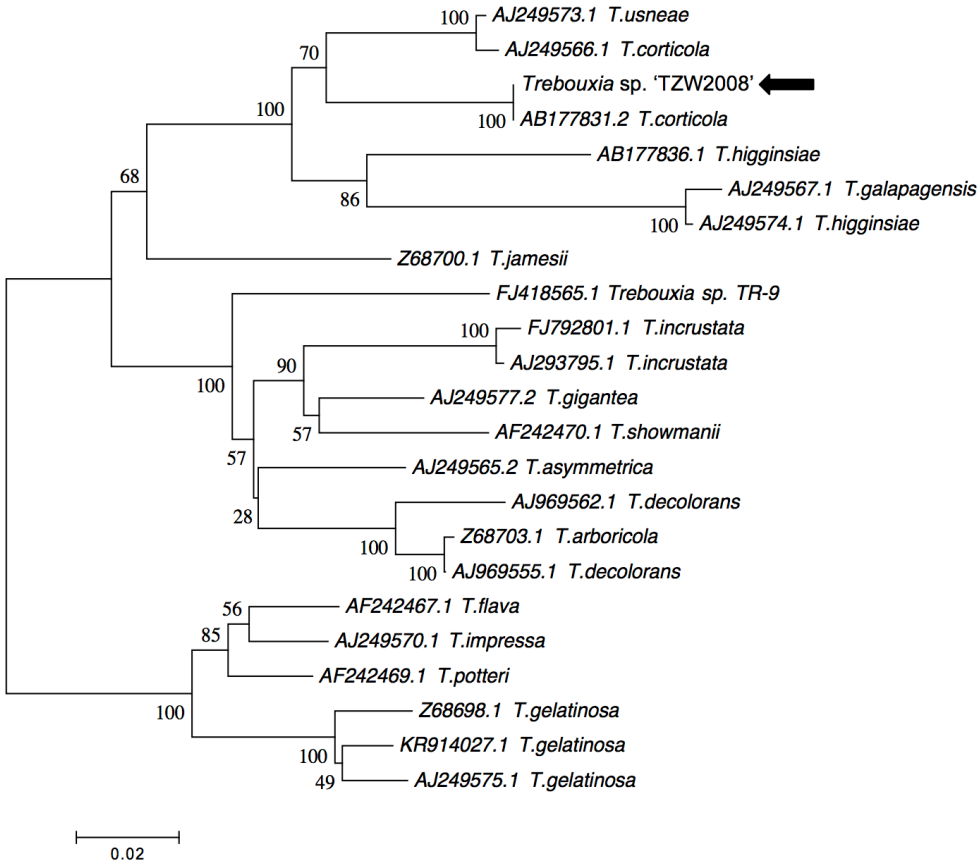
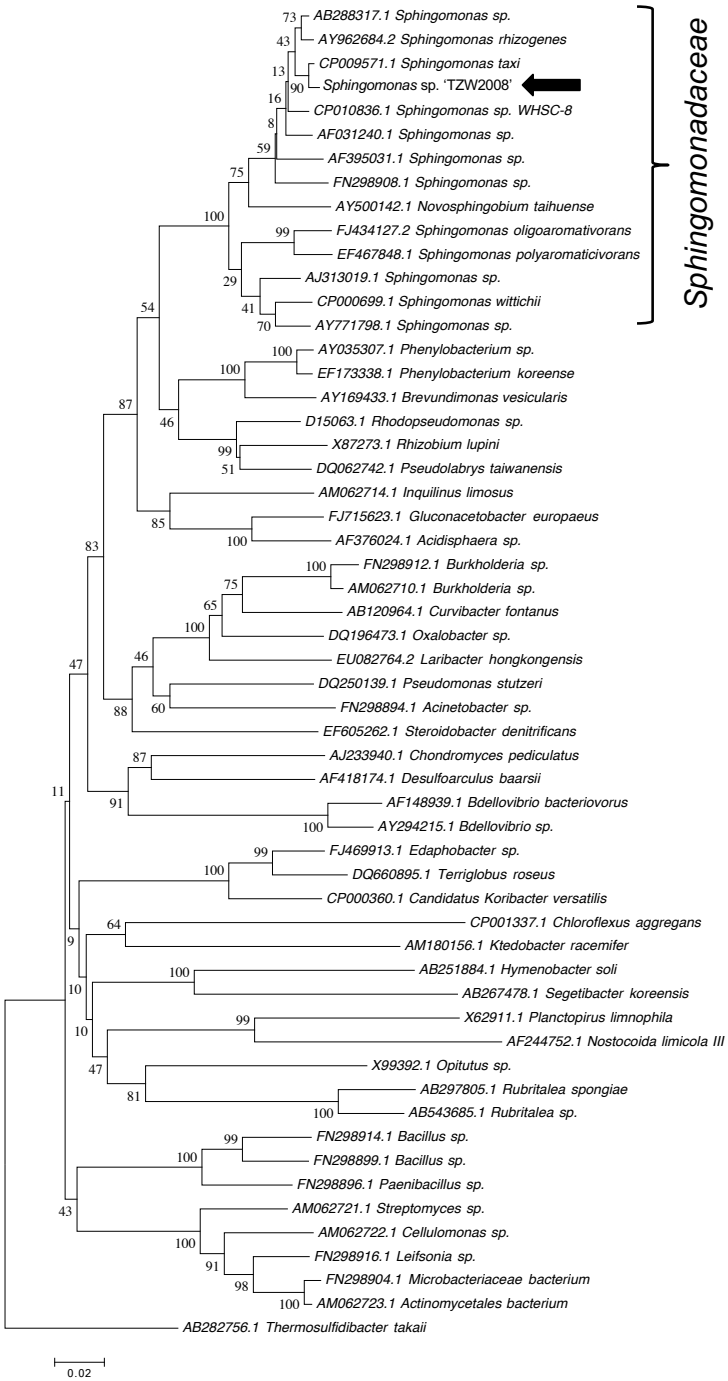


Figure 2.2: Phylogenetic relationships of (a) the alga and (b) the bacterium based on ITS rDNA and 16S rDNA sequence, respectively. Scale bars represent 0.02 substitutions per site. The arrows show the position of *Trebouxia* sp. 'TZW2008' in (a) and *Sphingomona*s sp. 'TZW2008' in (b).

Figure 2.2: (continues)

b



contained in lichen thalli (Aubert *et al.*, 2007; Richardson & Smith, 1968a). Although no gene similar to mannitol dehydrogenase gene (required in the mannitol degradation) was found in the bacterial genome, we identified a gene similar to that of sorbitol dehydrogenase. This enzyme is reported to oxidize a number of sugar alcohols including mannitol (Negm & Loescher, 1979). Therefore, the bacterium might use this enzyme to degrade mannitol rather than mannitol dehydrogenase. On the other hand, no bacterial colonies were observed on the sucrose-supplemented medium (Figure 2.5d). Consistent with this, two genes encoding enzymes that metabolize sucrose were not identified in the bacterial genome. These results indicate the inability of *Sphingomonas* sp. 'TZW2008' to use sucrose as its carbon source.

The results of FISH and the culturing experiments indicate that, during its growth, the alga is tightly associated with bacterial cells as if wearing them around its cell wall, and provides ribitol to them. The gene search suggested that the bacterium does not have genes of enzymes that function in the digestion of cellulose, one of the major components of the algal cell wall. Therefore *Sphingomonas* sp. 'TZW2008' is likely to obtain extracellular ribitol released by *Trebouxia* sp. 'TZW2008'.

Bacterial roles in the interaction between the novel bacterium and the lichen-forming alga

To examine whether the association of the bacterium and the alga is symbiotic or not, we searched for genes associated with algae-bacteria symbiotic interactions in the genome of the bacterium. The average coverage of the algal and the bacterial genomes sequenced in this study was x371 and x6,245, respectively, and sequences were well assembled (Table 2.1). Therefore, we consider that it is less likely to overlook genes by misassembly. Nitrogen fixation, phosphate solubilization, and vitamin B_{12} synthesis are well-studied bacterial roles in mutualistic interaction with algae. First, the ability of *Sphingomonas* sp. 'TZW2008' in nitrogen fixation was investigated. *Sphingomonas* species used as query sequences retain several enzymatic genes involved in nitrogen fixation. However, no similar sequence to those genes were found in the bacterial

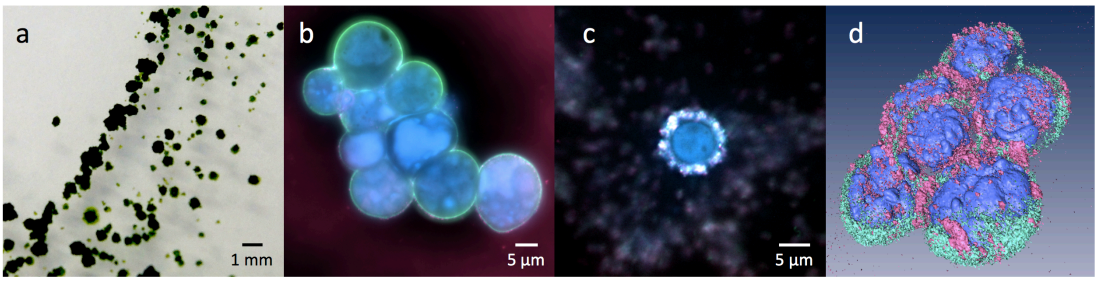


Figure 2.3: Localization of a novel bacterium in culture. (a) Algal colonies on the heterotrophic MY medium. A scale bar represents 1 mm. The FISH images showing the localization of bacterial cells represented by 16S rDNAs (red or green) around (b) aggregated algal cells (blue) and (c) a single algal cell. Bacterial cells are visualized in white where red and green fluorescence are merged on the bacterial nuclei (blue). (d) The 3D image was reconstructed by using Amira 3.1.1 software from the image stacks scanned by the confocal microscope LSM510meta. Algal colonies are coloured in blue, which are covered intensely by bacterial cells that are coloured in red or green.

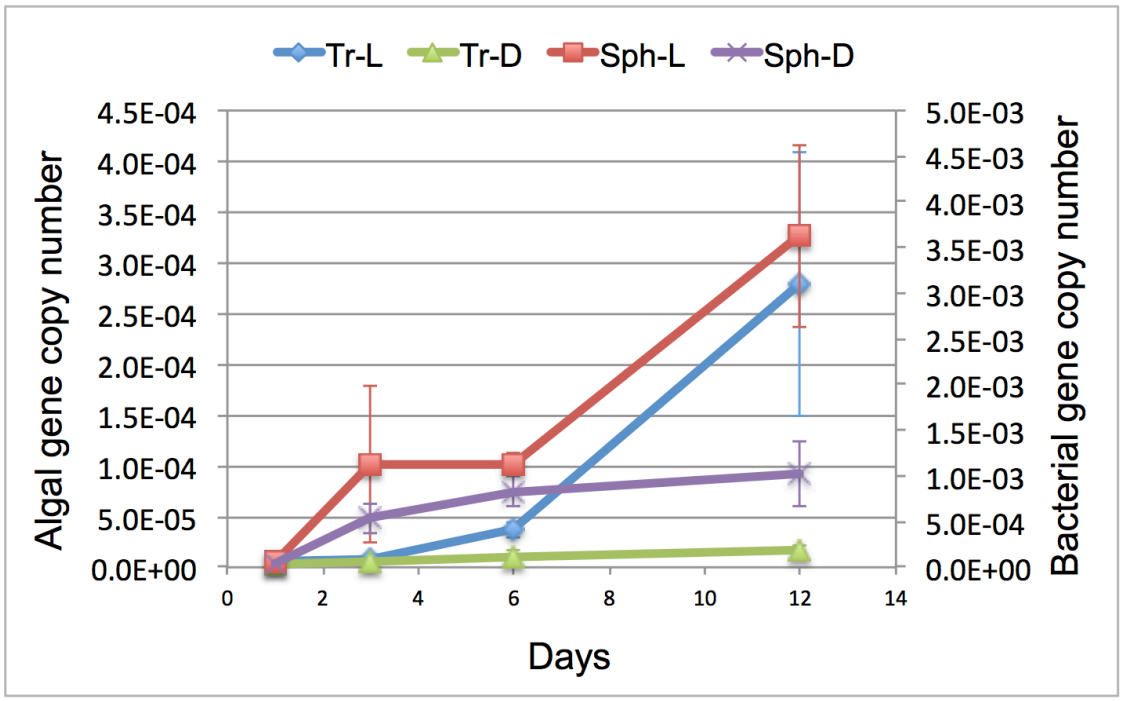


Figure 2.4: The algal and bacterial growth on the autotrophic medium. The strain of alga associated with the bacterium was cultured on the autotrophic C medium under photosynthesis activated and inhibited conditions that were 12h/12h light-dark cycle (indicated as Tr-L and Sph-L for the alga and the bacterium, respectively) and in the constant dark (indicated as Tr-D and Sph-D). The left and the right y axes represent the growth of the alga and the bacterium respectively, estimated by the quantification of the algal and the bacterial gene copy numbers by qPCR. The x axis shows days passed after the inoculation. The error bars represent the standard deviation of three biological replicates.

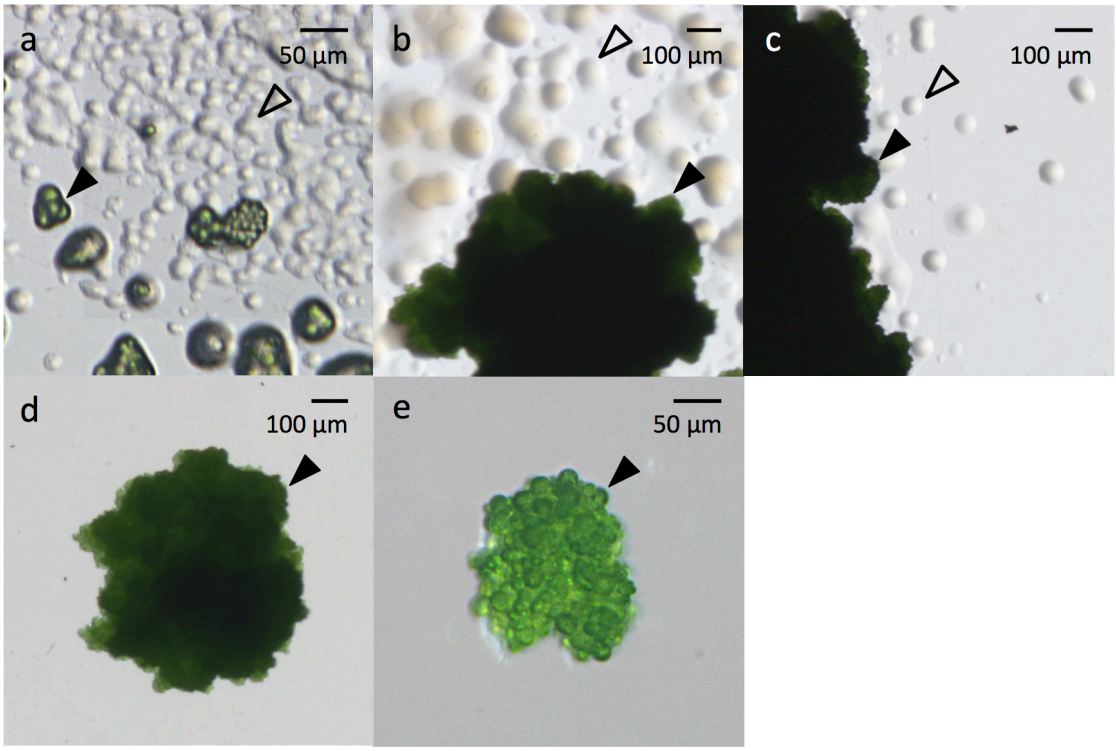


Figure 2.5: The carbon sources of the novel bacterium. The strain of alga associated with the bacterium grown on the heterotrophic MY medium supplemented with ribitol (a), glucose (b), mannitol (c), and sucrose (d). The open and filled arrowheads show a bacterial colony and an algal colony, respectively. (e) The same strain grown on the heterotrophic MY medium without carbohydrate supplementation. Scale bars represent 50 or 100 µm in each photo.

Table 2.3: Genes mapped onto the Pentose Phosphate Pathway in KEGG

Annotation	*EC number	Scaffold no.	**e-value
6-phosphogluconate dehydrogenase	1.1.1.44	3	8.0E-168
glucose 1-dehydrogenase	1.1.1.47	7	1.0E-31
glucose-6-phosphate 1-dehydrogenase	1.1.1.49	7	0.0
quinoprotein glucose dehydrogenase	1.1.5.2	***-	-
transketolase	2.2.1.1	3	0.0
transaldolase	2.2.1.2	3	4.0E-168
2-dehydro-3-deoxygluconokinase	2.7.1.45	-	-
ribose-phosphate pyrophosphokinase	2.7.6.1	3	7.0E-176
gluconolactonase	3.1.1.17	-	-
6-phosphogluconolactonase	3.1.1.31	7	8.0E-114
fructose-1,6-bisphosphatase II	3.1.3.11	13	3.0E-174
fructose-bisphosphate aldolase	4.1.2.13	-	-
2-dehydro-3-deoxyphosphogluconate aldolase	4.1.2.14	7	3.0E-96
xylulose-5-phosphate	4.1.2.9	7	-
phosphogluconate dehydratase	4.2.1.12	7	0.0
ribulose-phosphate 3-epimerase	5.1.3.1	1	2.0E-114
ribose 5-phosphate isomerase A	5.3.1.6	3	3.0E-45
ribose 5-phosphate isomerase B	5.3.1.6	9	2.0E-62
glucose-6-phosphate isomerase	5.3.1.9	31	0.0
phosphoglucomutase	5.4.2.2	12	0.0

*Enzyme commission number

** Blastx results with e-value < 1e-20 are considered significant

***Not found

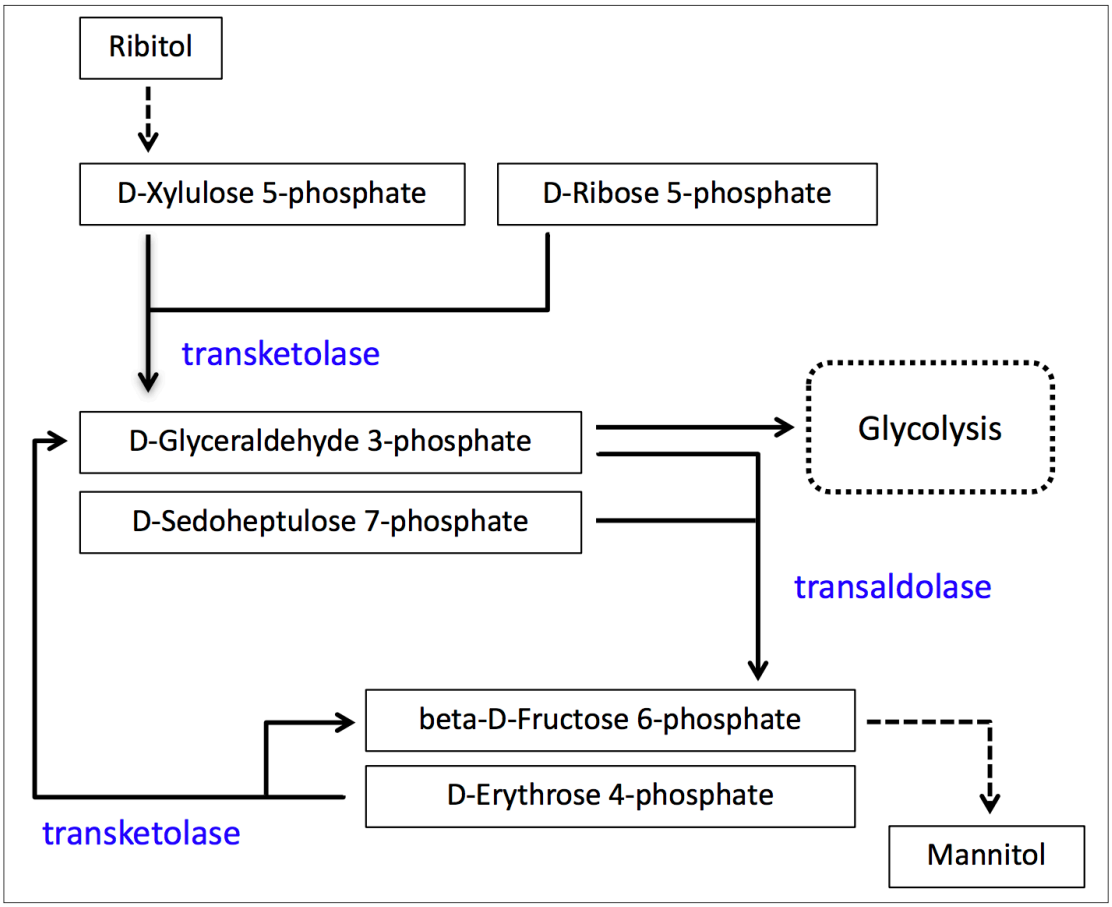


Figure 2.6: The metabolism of ribitol via the PPP. The proposed pathway metabolizing ribitol into mannitol in lichens. The enzymes necessary for the reactions are shown in blue. Sequences homologous to the genes encoding the two enzymes were found in the bacterial genome. Dashed lines represent uninvestigated input and output pathways of the PPP.

genome. *Sphingomonas* sp. 'TZW2008' formed colonies distant from the alga on the MY medium supplemented with carbon sources. Therefore *Sphingomonas* sp. 'TZW2008' can obtain nitrogen from MY medium containing various nitrogen sources. According to our investigation, nitrate and nitrite reductase genes were found to be missing from the genome of *Sphingomonas* sp. 'TZW2008', indicating that neither nitrate nor nitrite could be the source of nitrogen for the bacterium. The strain of alga has been subcultured on the autotrophic C medium in our laboratory since 2012. This medium contains nitrate as the sole nitrogen source. Therefore, during subculture in our laboratory, *Sphingomonas* sp. 'TZW2008' is likely to have relied on the alga for its nitrogen source. Second, phosphate solubilization ability was examined. Although the genetic mechanism of mineral phosphate solubilization activity is not fully understood, biosynthesis of gluconic acid, carried out by the enzyme glucose dehydrogenase (GCD), is considered as a key step in many gram-negative bacteria (Chhabra *et al.*, 2013; Sashidhar & Podile, 2010). In the genome of *Sphingomonas* sp. 'TZW2008' we identified a gene similar to that encoding pyrroloquinoline quinone (PQQ), the cofactor of GCD, but the gene encoding GCD was not found. In the genome of *Sphingomonas taxi*, GCD gene is encoded in the region where genes involved in phosphate regulation activity located. *Sphingomonas* sp. 'TZW2008' also retains all those regulatory genes but lacks the GCD gene. Finally, genes encoding enzymes involved in vitamin B_{12} biosynthesis were also not identified. Therefore, we predict that *Sphingomonas* sp. 'TZW2008' could not have supplied phosphate and vitamin B_{12} to the alga.

Previous studies reported the release of amino acids by lichen-associated bacteria (Liba *et al.*, 2006; Schneider *et al.*, 2011; Sigurbjörnsdóttir *et al.*, 2015). The constant supply of amino acids by a symbiotic partner can cause loss of genes involved in the biosynthesis of the supplied amino acids (Moran & Degnan, 2006; Shigenobu *et al.*, 2000). Therefore, we searched the genome of the alga for genes required in the biosynthesis of the 20 proteinogenic amino acids. We also searched the genome of the bacterium in case there was amino acid supply from the alga to the bacterium. However, both alga and bacterium

retained the genes in the biosynthetic pathways and may have been able to synthesize the amino acids *per se*.

Tight association between the novel bacterium and the lichen-forming alga

To eliminate the bacterium from the algal cells, the strain of alga associated with the bacterium was grown on autotrophic C media with antibiotics: ampicillin, kanamycin, and chloramphenicol. As shown in Figure 2.7(a), kanamycin at the final concentration of 50 μg/mL almost halted algal growth 7 days post-inoculation, whereas the other antibiotics had less effect even at higher concentrations (Figure 2.7b). The low growth rate of the alga on the kanamycin-supplemented medium may have been due to the inhibitory effect of kanamycin on algal chloroplasts (Bashir & Cho, 2016; Bourque *et al.*, 1976; Mentewab *et al.*, 2014).

The effect of each antibiotic on bacterial growth was observed by quantification of the 16S rRNA gene using qPCR (Figure 2.7c). The relative copy number of the bacterial gene to the algal gene was used as an indicator of remaining bacterial cells. Although the alga showed variation in growth rate between the three antibiotics at the concentrations 50 μg/mL for ampicillin and kanamycin, and 32.5 μg/mL for chloramphenicol, the relative copy number reached the same value as that of the control after 14 days. Following antibiotic treatment, the culture was transferred to a fresh autotrophic medium without antibiotics. The survival of the bacterium was confirmed by amplification of the 16S rDNA sequence by PCR. The higher concentrations of ampicillin and chloramphenicol succeeded in reducing the relative copy number to almost one-tenth of that in the control after 14 days (Figure 2.7d, t-test, $p < 0.05$), but failed to eliminate bacterial cells completely. These results indicated that *Sphingomonas* sp. ‘TZW2008’ is resistant to antibiotics and could not be easily separated from the alga.

Bacterial 16S rDNA sequences highly similar to that of *Sphingomonas* sp. ‘TZW2008’ were amplified from lichens collected at the same location in a different year, and formed a monophyletic group with *Sphingomonas* sp. ‘TZW2008’ (Figure 2.8). This suggested

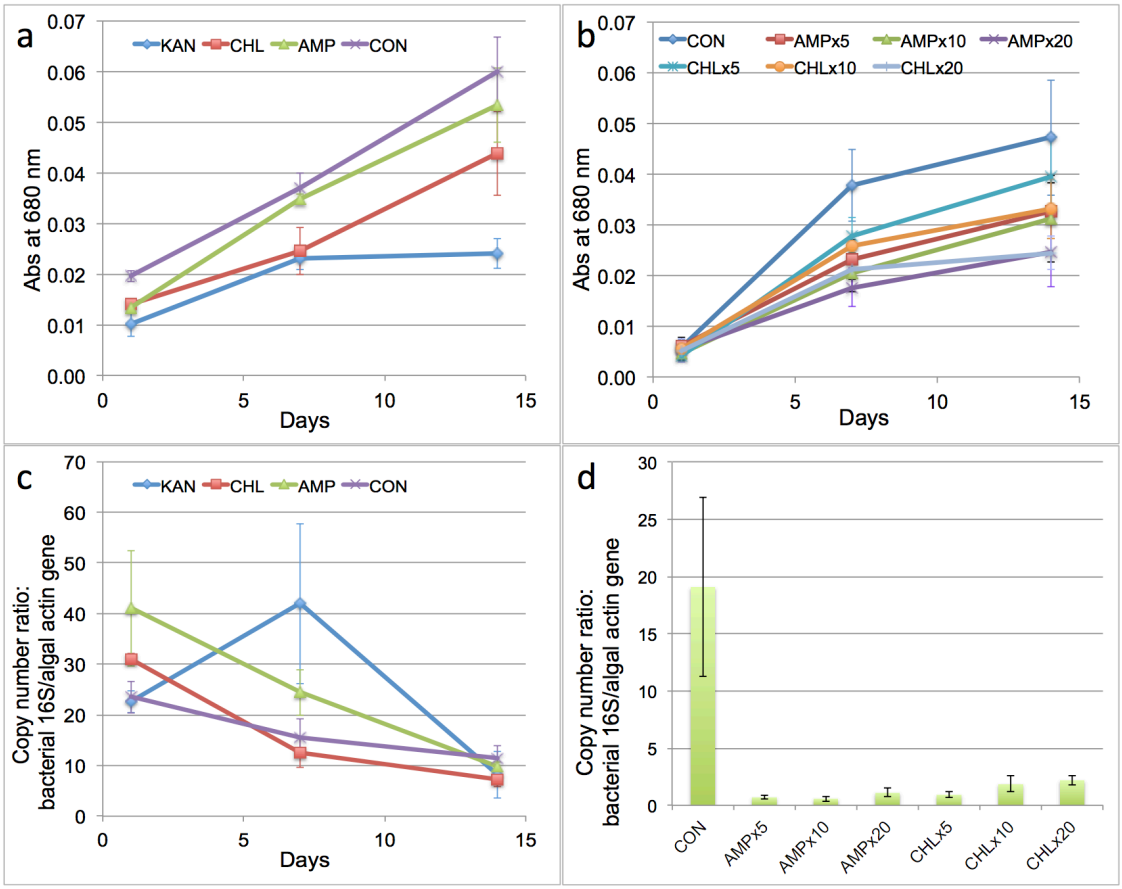


Figure 2.7: Effect of antibiotics on the bacterial growth. Growth of the alga and the bacterium on the autotrophic C medium with antibiotics. Effects of the three antibiotics on algal growth were tested at concentrations: (a) 50 µg/mL for ampicillin (AMP) and kanamycin (KAN), and 32.5 µg/mL for chloramphenicol (CHL); (b) five (x5), ten (x10), and twenty (x20) times higher than the initial concentrations (50 and 32.5 µg/mL) for ampicillin and chloramphenicol. CON indicates the algal growth on media without antibiotics. The x and the y axes represent days passed after the inoculation and absorption at 680 nm, respectively. Relative copy numbers of the bacterial genome to that of the algal genome were estimated at: (c) one, seven, fourteen days after the inoculation onto ampicillin (50 µg/mL), kanamycin (50 µg/mL), and chloramphenicol (32.5 µg/mL) supplemented media; (d) fourteen days after the inoculation onto ampicillin (x5, x10, x20 indicate concentrations 250, 500, 1000µg/mL) and chloramphenicol (x5, x10, x20 indicate 162.5, 325, 650 µg/mL) supplemented media. The x axes represent days passed after the inoculation (c) and the concentrations of the antibiotics (d), and the y axes represent relative copy number ratio, respectively. The error bars represent the standard deviation of three biological replicates.

that *Sphingomonas* sp. 'TZW2008' is a lichen-associated bacterium tightly associated with a lichen-forming alga.

Discussion

It is now widely recognized that diverse and abundant bacteria are associated with lichens (Aschenbrenner *et al.*, 2016). In the last decade, the specificity of lichen-associated bacteria and their contribution to lichen symbiosis have been suggested. Bates *et al*. (2011) reported that bacterial community composition in lichens is distinct from that of surrounding soils. The community composition of lichen-associated bacteria is suggested to be specific to mycobiont species (Bates *et al.*, 2011; Grube *et al.*, 2009), whereas Hodkinson *et al*. (2012) inferred photobiont type (green algae or cyanobacteria) is the more significant factor that shapes the community. Large geographical scale, substrate type, lichen secondary metabolites, lichen growth type, and age of thallus are also factors likely to affect the community composition (Bates *et al.*, 2011; Cardinale *et al.*, 2008; Cardinale *et al.*, 2012; Grube *et al.*, 2009; Grube *et al.*, 2015; Hodkinson *et al.*, 2012). Although there is no clear answer to what shapes bacterial communities, they are assumed to reflect requirements in lichen symbioses (Aschenbrenner *et al.*, 2016). Physiological assays on isolated strains of lichen-associated bacteria confirmed their functions in nitrogen fixation, phosphate solubilization, amino acid excretion, lytic activities, and antagonistic activities (Cernava *et al.*, 2015; Grube *et al.*, 2009; Grube *et al.*, 2015; Liba *et al.*, 2006). Metagenomic and proteomic data also inferred their diverse functions in vitamin and hormone biosynthesis, detoxification, and resistance against biotic and abiotic stress (Grube *et al.*, 2015). These bacterial functions are assumed to sustain the integrity and robustness of lichens (Cardinale *et al.*, 2008; Grube *et al.*, 2009; Grube *et al.*, 2015). Therefore, lichen symbiosis is now considered to be a complex symbiotic assembly involving multiple species rather than a dual interaction between the mycobiont and the photobiont.

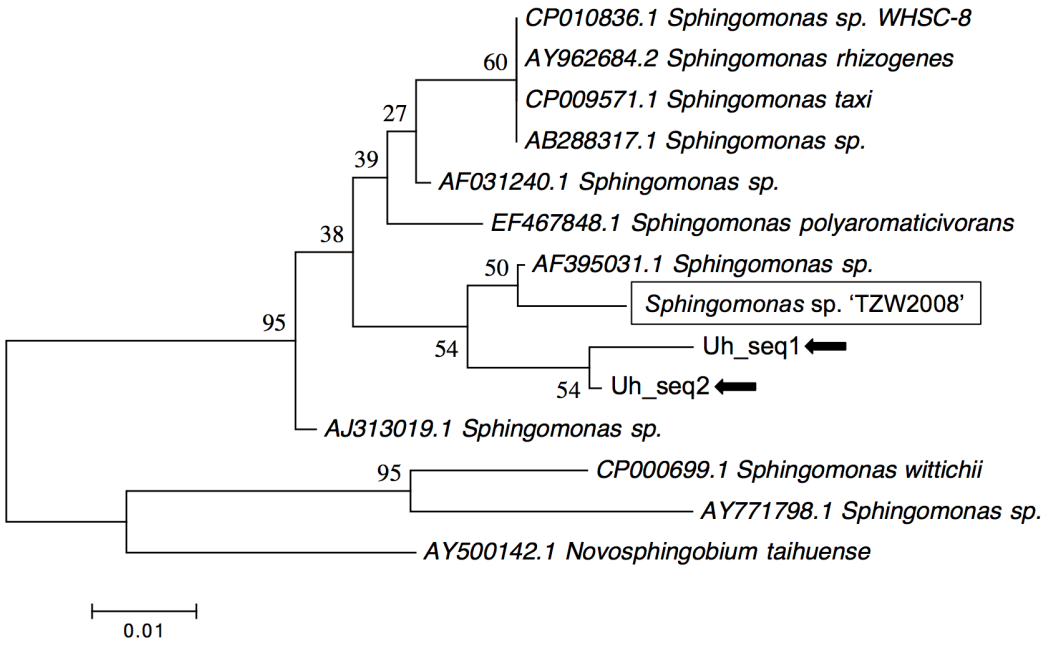


Figure 2.8: A phylogenetic tree of 16S rDNA partial sequences similar to that of *Sphingomonas* sp. 'TZW2008' amplified from lichen samples (*Usnea hakonensis*). Scale bars represent 0.01 substitutions per site. The arrows show position of the sequences isolated from *U. hakonensis*.

In this study, we sequenced the genome of a lichen-forming green alga *Trebouxia* sp. 'TZW2008' as well as the genome of an Alphaproteobacterium *Sphingomonas* sp. 'TZW2008'. Our genome search and experiments provided strong evidence for the specific interaction between *Trebouxia* sp. 'TZW2008' and *Sphingomonas* sp. 'TZW2008'. *Sphingomonas* sp. 'TZW2008' is strictly localized on algal cell walls and its growth is dependent on photosynthetic product of the alga. The bacterium was able to use ribitol, which is known to be produced by and released from *Trebouxia* algae (Richardson & Smith, 1968b), as a carbon source. Closely related bacteria were identified in lichen samples collected in the field, and therefore we concluded that this bacterium is less likely to have become associated with algal cells after its isolation from the lichen, but rather associated with the lichen. Alphaproteobacteria have been reported as the dominant taxon in bacterial communities of several lichen species (Bates *et al.*, 2011; Cardinale *et al.*, 2008; Grube *et al.*, 2009; Grube *et al.*, 2015; Hodkinson *et al.*, 2012; Schneider *et al.*, 2011; Sigurbjörnsdóttir *et al.*, 2015). Since Alphaproteobacteria were hardly detected in culture-dependent studies, most of them are suspected to be unculturable, highly specific to lichen symbioses (Cardinale *et al.*, 2008). Previous studies identified several carbohydrates in lichen thalli such as glucose, sucrose, trehalose, ribitol, arabitol, mannitol, and also some sugar phosphates (Aubert *et al.*, 2007; Drew & Smith, 1967; Richardson & Smith, 1968b). Among those, ribitol, glucose, and mannitol are the major carbohydrates responsible for the carbon flow within lichens, are abundant in thalli (Aubert *et al.*, 2007; Drew & Smith, 1967; Eisenreich *et al.*, 2011). The preference of *Sphingomonas* sp. 'TZW2008' for ribitol, glucose, and mannitol in our experiments might represent the characteristic of *Sphingomonas* sp. 'TZW2008' as an endolichenic bacterium specialized in environments within lichen thalli. Lichen thalli are known to deposit various extracellular secondary metabolites many of which are reported to have antibiotic effects (Kosanić & Ranković, 2015; Nash, 2008). The fungal partner of *Trebouxia* sp. 'TZW2008', *U. hakonensis,* is known to produce a secondary metabolites called usnic acid (Kon *et al.*, 1990), well known for its antibiotic effect (Kosanić &

Ranković, 2015). Therefore, the resistance shown by *Sphingomonas* sp. 'TZW2008' towards the three types of antibiotic may possibly correlate with endolichenic environment that contains antibacterial secondary metabolites. The presence of closely related *Sphingomonas* bacteria in other *U. hakonensis* samples suggested that this group of bacteria might be involved in symbiotic association of *U. hakonensis*.

Our study clearly shows that the bacterium benefits from symbiotic interaction with the alga in terms of carbon source. On the other hand, the benefit for the alga, in return for the nutritional support given to the bacterium, remains unclear. In mutualistic interactions between algae and bacteria reported in previous studies, the latter often support algae in terms of nitrogen fixation, phosphate solubilization, and vitamin B_{12} supply (Fuentes *et al.*, 2016; Ramanan *et al.*, 2016). However, according to our gene searches we predict that *Sphingomonas* sp. 'TZW2008' may not have genes directly related to these functions and the benefit of the interaction seems unidirectional. It is worthy of note that *Trebouxia* sp. 'TZW2008' and *Sphingomonas* sp. 'TZW2008' are derived from a lichen where interactions with a mycobiont and/or other lichen-associated bacteria are expected. Taking this into account, the benefit might not be for the alga *per se* but for the lichen symbiosis as a unit. Considering its preference for ribitol, glucose, and mannitol, *Sphingomonas* sp. 'TZW2008' may colonize thalli and play a supportive role in carbohydrate turnover in lichens. Previous studies showed that external carbohydrates, identical to photosynthetic products of the photobiont, inhibit the carbon transfer from the photobiont to the mycobiont in lichen thalli (Drew & Smith, 1967; Hill & Smith, 1972; Richardson & Smith, 1968b). Therefore, if accumulation of excess ribitol within thalli interferes with carbon transfer between the photobiont and the mycobiont, one potential role of *Sphingomonas* sp. 'TZW2008' is to utilize excess ribitol within the thalli, which would support the transfer of ribitol from the alga to the fungus. Support for nutrient cycling can be a great benefit for lichens that often grow widely in nutrient-poor habitats.

The close physical contact between *Trebouxia* sp. 'TZW2008' and *Sphingomonas* sp. 'TZW2008' shown in this study allows us to speculate that the latter could be vertically transferred with lichen propagules (symbiotic propagules of the mycobiont and the photobiont) like the bacterial starter community proposed by Aschenbrenner *et al*. (2014). Since inherited lichen-associated bacteria are suggested to influence the establishment of lichen symbiosis in a new environment (Aschenbrenner *et al.*, 2016), it might be advantageous for *Trebouxia* sp. 'TZW2008' to 'wear' this *Sphingomonas* sp. 'TZW2008' even outside lichen thalli to establish a new symbiotic ecosystem. However, further experiments on bacterial metabolic products and their effects on algal and fungal metabolism, as well as localization of the bacterium in lichen thalli, are required to elucidate roles of *Sphingomonas* sp. 'TZW2008' in lichen symbiosis. Such experiments will help to further elucidate the sophisticated mechanism of symbiosis in lichens.

Chapter 3

Lichen-specific genetic features indicated by characterization of gene families in lichen-forming fungus

Introduction

Lichens are symbiotic associations of a heterotrophic fungal partner, an autotrophic photosynthetic partner (algae/cyanobacteria), and supportive microorganisms (Aschenbrenner *et al.*, 2016; Honegger, 2008; Spribille *et al.*, 2016). They are considered as one of the most successful ecological units, for their broad range of habitats and persistence in extreme environmental conditions where water and nutrition is scarce. More than 40 % of the described ascomycetes, one-fifth of all fungal species, are known to be lichen-forming, indicating that lichenization has been a prevailing strategy in fugal evolution. Recent progress in molecular techniques, especially that in high-throughput sequencing, has enabled sequencing of genomes and transcriptomes of non-model organisms including lichens. So far, more than 10 species of lichen-forming fungi and algae have been subjected to genome or transcriptome sequencing (Beck *et al.*, 2015; Carniel *et al.*, 2016; Dal Grande *et al.*, 2017; Junttila & Rudd, 2012; McDonald *et al.*, 2013; Wang *et al.*, 2014; Wang *et al.*, 2015). A number of fungal or algal genes with or without known function have been reported as candidate genes that characterize lichen symbioses. However, in most cases those genes were identified by either gene prediction or *de novo* transcriptome assembly, without a comprehensive study on genome and transcriptome. Therefore, until now transcription of genes “on site” have not been discussed in lichens. Previous studies have reported importance of using actual transcriptome data for genome annotation (Singh *et al.*, 2017). High-throughput RNA sequencing (RNA-seq) is a powerful method in sequencing transcriptome at massive scale. It has contributed to detect novel transcripts, new exons, untranslated regions in genomes (Nagalakshmi *et al.*, 2008; Wang *et al.*, 2010; Zhang *et al.*, 2010), and has identified a number of non-coding RNAs, which are recently described to have considerable functions in numerous biological processes (Fatica & Bozzoni, 2014; Quinn & Chang, 2016).

Here, we present the first comprehensive study on genome and transcriptome of a lichen-forming fungus *Usnea hakonensis*. Genomic DNA and RNA extracted from axenic

culture of the fungus were sequenced by using Illumina Hiseq2500 platform. The transcriptome-based genome annotation revealed close proximity of genes in the fungal genome and identified considerable number of non-coding RNAs. Gain and loss of genes in certain gene families of lichen-forming fungi related to their symbiotic lifestyle has been previously indicated (McDonald *et al.*, 2013; Wang *et al.*, 2014). Some of the gene families we identified in the present study also seem to have lichen-specific characteristics when compared with other fungi that are lichen-forming, free-living or parasitic.

Materials and Methods

Strain and growth condition

The fungal strain used in this study was provided by Dr. Yoshiaki Kon, Tokyo Metropolitan Hitotsubashi High School, Japan. It was isolated from *Usnea hakonenesis* collected from Kanagawa Prefecture, Japan (35°26′N, 139°10′E) in 2008 by the method described previously (Kon *et al.*, 1993). The strain of the fungus has been maintained for eight years on slant MY media (composed of 2% (w/v) agar, 2% (w/v) malt extract, 0.2% (w/v) yeast extract and pH adjusted to 5.8).

Genome DNA extraction, library construction, sequencing and de novo assembly

Genomic DNAs were extracted and concentrated using NucleoSpin Plant II (Macherey-Nagel, Düren, Germany) following the protocol for fungi. DNA libraries were constructed using TruSeq DNA PCR-Free Library Preparation Kit (Illumina, Inc., San Diego, CA) following the manufacturer's instructions. Short DNA sequences (paired-end 125 bp) were determined from the libraries by Illumina HiSeq2500 platform (short reads). After removal of the adaptor sequences and low-quality reads, the reads were assembled into scaffolds by using CLC genomic workbench (https://www.qiagenbioinformatics.com/) with a word size of 64.

RNA extraction, library construction and sequencing

Total RNAs were extracted from three biological replicates using RNeasy mini kit (QIAGEN, Venlo, the Netherlands) following the protocol for filamentous fungi. RNA libraries were constructed using NEBNext Poly(A) mRNA Magnetic Isolation Module and NEBNext Ultra RNA Library Prep Kit for Illumina (New England Bio Labs, Ipswich, MA) following the manufacturer's instructions. Short cDNA sequences (paired-end 125 bp) were determined from the libraries by Illumina Hiseq2500 platform.

Gene annotation and identification of gene families

The sequenced reads were mapped to the assembled genome by using TopHat v2.1.0 (Kim *et al.*, 2013; Trapnell *et al.*, 2009). Reads mapped to the bases with coverage < 30 were eliminated from the total mapped reads by using SAMtools v1.3.1 (Li *et al.*, 2009) and BEDTools v2.26.0 (Quinlan & Hall, 2010). The remaining reads were re-mapped to the genome. The re-mapped reads from the three replicates were merged by using SAMtools and genes were annotated on the genome by using Cufflinks v2.1.1 (Trapnell *et al.*, 2010). These processes eliminated reads that connected neighbouring genes as shown in Figure 3.1. We used Augustus (Stanke *et al.*, 2004; Stanke & Morgenstern, 2005) to predict coding sequences in the annotated genes using the annotated information of *Aspergillus fumigatus* as a reference. The coding sequences were converted to protein sequences. For functional prediction, the protein sequences were subjected to a BLASTP search against the NCBI non-redundant protein sequences database (nr). For gene family identification, the protein sequences were used as queries and a database. Genes reciprocally hit by this search were grouped into the same gene family. The same cut-off value (e-value < 1e-39) were used in both searches.

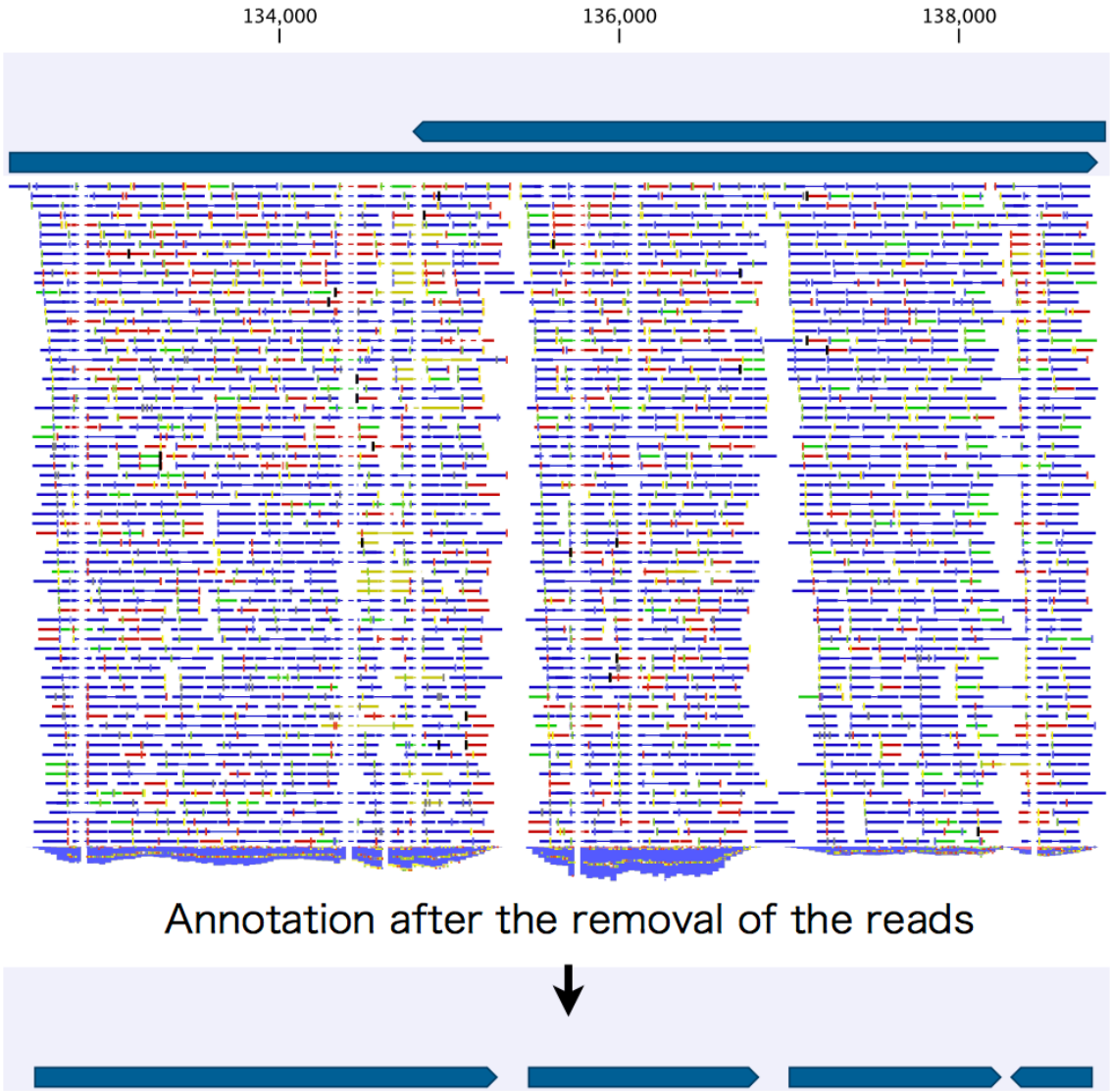


Figure 3.1: Genome annotation using RNA-seq reads. Each short line in the figure represents the read mapped to the genome. A small number of reads with read-through sequences connect neighbouring genes causing annotation of fused or overlapped genes (as shown by the arrows in the upper raw). Removal of the reads mapped to the low coverage regions (< 30 reads per base) cuts the connections and restores consistency between the annotation and the mapping (as shown in the lower raw).

Usnea hakonensis gene families in other fungal genomes

Other fungal genomes were searched for the identified gene families. Protein sequences were downloaded from five fungal genomes available at NCBI, *Aspergillus fumigatus* (GCA_000002655.1: https://www.ncbi.nlm.nih.gov/assembly/GCA_000002655.1), *A. oryzae* (GCA_000269785.2: https://www.ncbi.nlm.nih.gov/assembly/GCA_000269785.2), *Candida albicans* (GCF_000182965.3: https://www.ncbi.nlm.nih.gov/assembly/GCA_000182965.3), *Endocarpon pusillum* (GCF_000464535.1: https://www.ncbi.nlm.nih.gov/assembly/GCA_000464535), and *Umbilicaria pustulata* (GCA_900169345.1: https://www.ncbi.nlm.nih.gov/assembly/GCA_900169345.1). Each downloaded dataset was formatted as a BLASTP database. Twenty gene families (≥ 10 genes were assigned in *U. hakonensis*) were selected. For each gene family, assigned *U. hakonensis* coding sequences were converted to protein sequences, and used as queries in BLASTP searches against each of the five fungal databases. However, in a search using *U. hakonensis* sequences as queries, some genes in the database that should be assigned to the same gene family could be overlooked for low sequence similarities, due to the genetic distance between *U. hakonensis* and the targeted fungal species. Therefore, we performed an additional BLASTP search using protein sequences in the database, found homologous to the *U. hakonensis* sequences in the first search, as queries. The genes hit by these searches were grouped together to form a gene family in the targeted fungal species. In all the searches results with e-value < 1e-39 were considered significant.

For g6463, g3106, and g5573 families whose functions were not predicted by the BLASTX search, we searched the Pfam database (Pfam 31.0: http://pfam.xfam.org) for matching entries with e-value cut-off < 1e-39.

Phylogenetic analysis

The 18S ribosomal DNA sequence of *U. hakonensis* was aligned with fungal 18S rDNA sequences selected from James *et al*. (2006). The phylogenetic tree was constructed in

MEGA version 7 (Kumar *et al.*, 2016) using the p-distance algorithm and the neighbour-joining algorithm (Saitou & Nei, 1987) with 500 bootstrap replications.

Results

De novo assembly and annotation of the fungal genome

A lichen *Usnea hakonensis* was collected in 2008 by Dr. Yoshiaki Kon in Tanzawa Japan. The fungus was isolated from a lichen, apart from its algal partner, and maintained as independent culture in the laboratory (Figure 3.2). Genomic DNA and total RNA were extracted from cultured *U. hakonensis* and sequenced by Illumina Hiseq 2500 platform. The 227 million reads (125 bp in length) were *de novo* assembled into 2,971 scaffolds. Eight hundred and seventy-nine scaffolds remained after the removal of scaffolds smaller than 2 kb. The genome size predicted from the sum of the scaffolds (< 2 kb) is 41.2 Mb. The average coverage and N50 value are x506 and 166 kb respectively (Table 3.1).

RNA sequencing yielded 146 million reads, which were then used to annotate the genome. The fungal genome was annotated with 13,695 genes in total. Among those genes, 7,054 coding sequences were predicted in 6,875 sequences (50.2 %) by Augustus, while none were predicted in 6,820 (49.8 %).

Identification of gene families

The BLASTP search was locally performed against the NCBI nr database. Out of the 7,054 coding sequences, 2,465 (35 % of the total) were similar to proteins with known functions, 2,937 (42 %) were similar to those with unknown functions, and 1,652 (23 %) showed no significant similarity to any known protein sequences in the database (e-value < 1e-39) (Figure 3.3).

To identify gene families within the transcriptome of *U. hakonensis*, the BALSTP search was also performed against the own protein sequences. Among the 7,054 protein-coding genes, 5,609 were defined as a single copy genes which had no significant similarity with

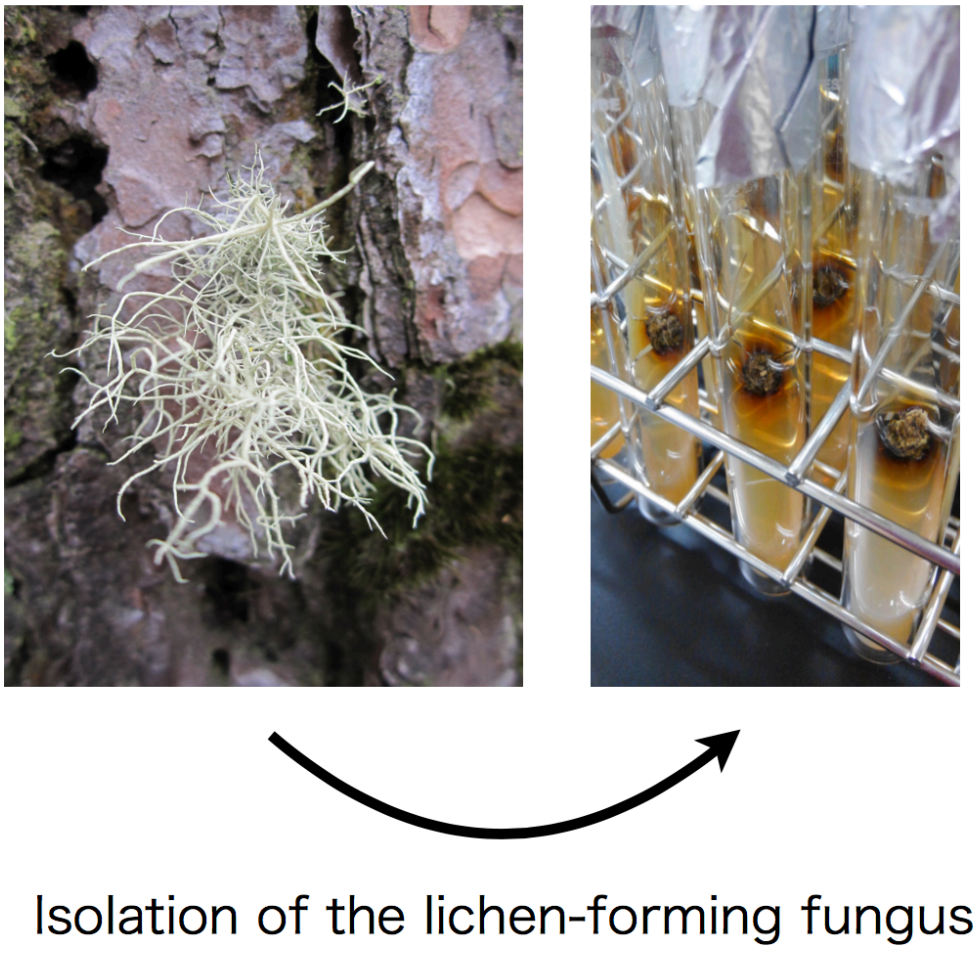


Figure 3.2: *Usnea hakonensis*. The left shows a thallus of *Usnea hakonensis* in the field. The right shows the strain of the mycobiont isolated from a natural thallus.

Table 3.1: Overview of genome sequencing and annotation.

Scaffolds > 2kb	
Predicted genome size (Mb)	41.2
No. of scaffolds	879
Average coverage	x506
N50 (kb)	166
No. of transcribed genes	13695
Transcribed region (Mb)	21.3
Transcribed region (%)	51.7
No. of predicted genes	7054

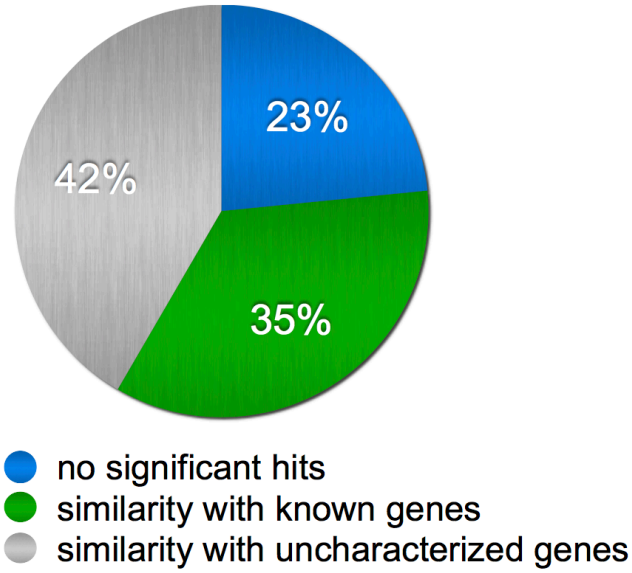


Figure 3.3: Result of the BLASTP search of the predicted coding sequences against the NCBI nr database. The sequences which had no similar sequences in the database with significance (e-value <1e-39) are shown in blue. The sequences matched with protein sequences of known and uncharacterized function are shown in green and grey, respectively.

other genes in the transcriptome with e-value < 1e-39. The remaining 1,445 genes were sorted into 432 gene families. The numbers of genes in one gene family ranged from two to 24 (Table 3.2). For the 20 families that consist of more than 10 genes, we examined their functions and presence in other fungal genomes (Table 3.3).

Comparative genome analysis of the gene families

Genomes of two lichen-forming fungi (*Endocarpon pusillum* and *Umbilicaria pustulata*), two free-living fungi (*Aspergillus fumigatus* and *A. oryzae*), and one parasitic fungus (*Candida albicans*) were searched for the gene families identified in the *U. hakonensis* transcriptome. We first performed a BLASTP search against the target fungal genomes by using sequences of *U. hakonensis* as queries. Then, performed second BLASTP search by using homologous protein sequences found in the target fungal genomes as queries, to avoid underestimation of the number of genes in each family due to the genetic distance from *U. hakonensis* (Figure 3.4).

All the 20 gene families of *U. hakonensis* were also present in genomes of examined five fungi. Despite differences in genome size, the number of predicted protein-coding genes (Table 3.3), and phylogenetic distance from *U. hakonensis*, numbers of sequences assigned to some gene families (TIP49: g5457, Helicase: g2986, Serine/threonine kinase: g3390, Mitogen-activated Protein Kinase (MAPK): g1309, and Helicase: g5166) are almost constant throughout the six fungi. Predicted function of the largest gene family in *U. hakonensis*, was Major facilitator superfamily (MFS) transporter. In total, five out of the 20 gene families were annotated as transporter families (three MFS transporter families: g162, g6745, g5383, one sugar transporter family: g754, and one amino acid transporter family: g2490). The number of genes assigned to these transporter families are tend to be large in the free-living *Aspergillus* fungi, and small in the parasitic *Candida albicans* (Figure 3.5). Three protein families (lichen-specific A, B, and C: g6463, g3106, g5573) with unknown functions were exclusively identified in the genomes of lichen-forming fungi (Figure 3.5).

Table 3.2: Number of single genes and multi-gene families identified in the transcriptome of the fungus.

single genes	5609
Multigene families	
No. of genes in a family	No. of gene families
2	253
3	78
4	36
5	17
6	15
7	8
8	4
9	1
10	2
11	3
12	3
13	1
14	4
15	1
17	2
19	2
20	1
24	1

Table 3.3: Size of the five fungal genomes and the numbers of genes and proteins predicted in each genome.

Fungal species	Size (Mb)	Gene	Protein
Endocarpon pusillum Z07020	37.1	9238	9238
Umbilicaria pustulata	39.2	8268	8268
Aspergillus fumigatus Af293	29.4	9916	9630
Aspergillus oryzae 3.042	36.6	11639	11397
Candida albicans SC5314	14.3	6263	6030

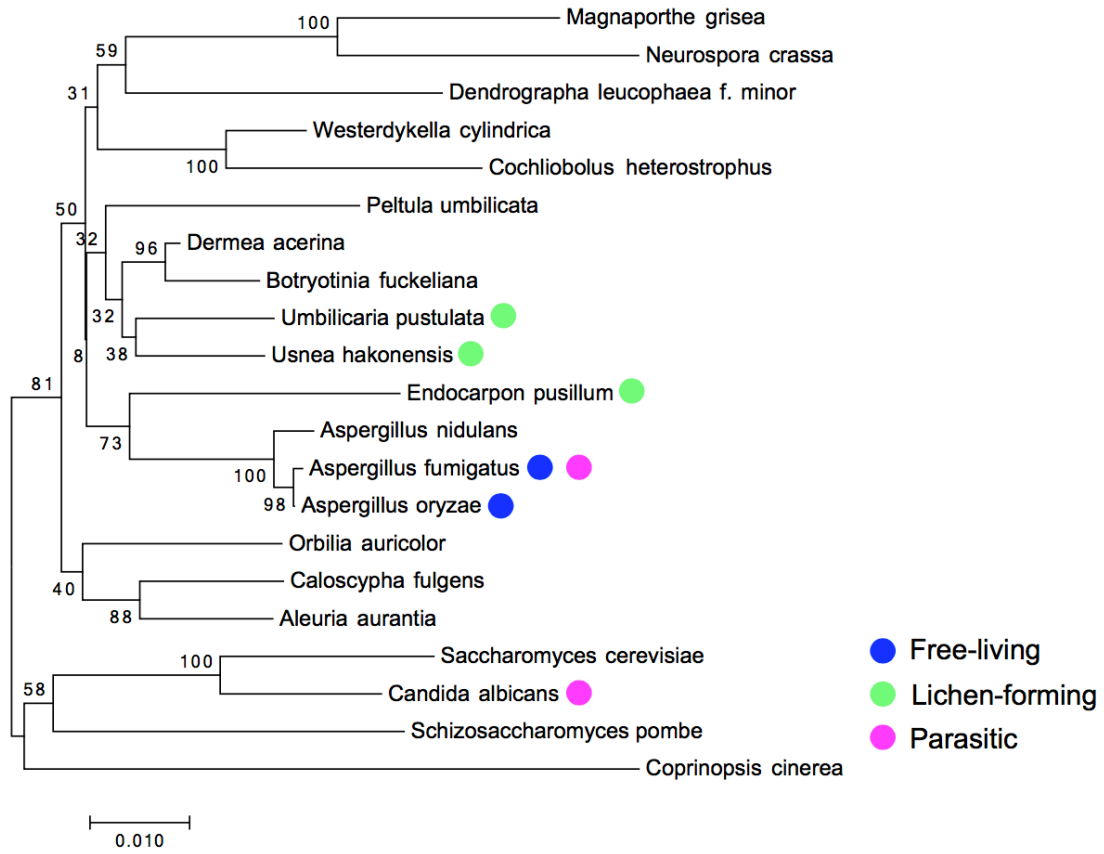


Figure 3.4: Phylogenetic tree of 18S rDNA partial sequences of the fungi. The six fungal species used in the comparative genome analysis of this study are marked by the circles. The colours of the circles represent the life-style of each fungus. The scale bar represents 0.01 substitutions per site.

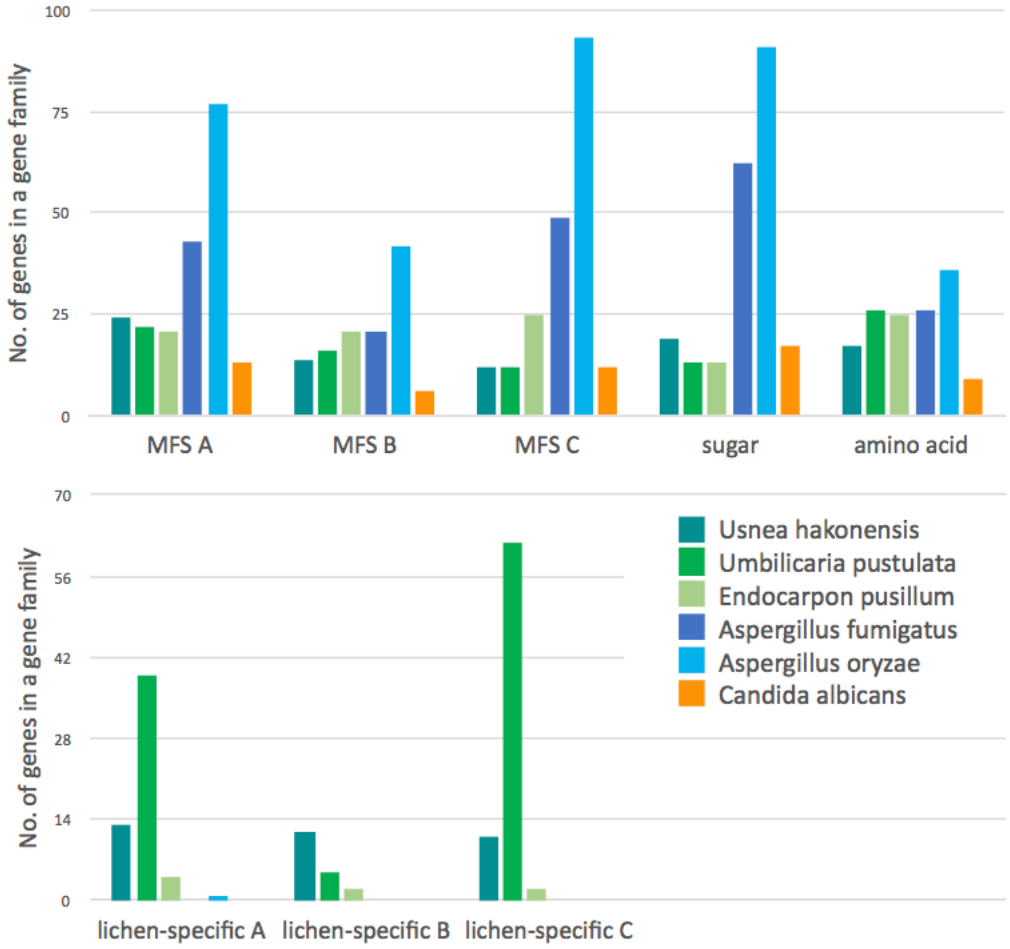


Figure 3.5: Number of genes assigned to the five transporter and the three lichen-specific families. The transcriptome of *U. hakonensis* and the genomes of the five fungal species were searched for.

Gene families annotated as, Polyketide synthase (PKS): g772, Cytochrome p450: g3602, Glucose-methanol-choline (GMC) oxidoreductase: g789, Kinesin: g5873, and O-methyltransferase: g395 were not identified in the genome of *Candida albicans* (Table 3.4).

Discussion

This is the first study that has sequenced and annotated the genome of a lichen-forming fungus in a genus *Usnea* and investigated its genetic features. The predicted genome size and the number of protein-coding genes of *Usnea hakonensis* are 41.2 kb and 7,054, respectively. The results are comparable to that of other lichen-forming fungi *Endocarpon pusillum*, (37.5 Mb in size annotated with 9,285 genes (Wang *et al.*, 2014), and *Umbilicaria pustulata*, 39.2 Mb in size annotated with 8,268 genes (Dal Grande *et al.*, 2017). However, the genes we identified in the present study represent those that had been expressed in independently cultured *Usnea hakonensis*. We expect more genes to be encoded in the genome, since previous studies have reported genes specifically expressed during lichen symbioses (Joneson *et al.*, 2011; Trembley *et al.*, 2002; Wang *et al.*, 2014).

The mapping of the RNA-seq reads to the assembled genome revealed that almost half of the transcribed genes were without predictable coding sequences. They are likely to be non-coding RNAs that have been reported to engage in numerous biological processes (Fatica & Bozzoni, 2014; Quinn & Chang, 2016). As the functional importance of non-coding RNAs are increasingly notified, the proper identification of transcribed regions becomes more essential. However, identification of transcribed regions was problematic in our annotation process. The mapping process revealed that genes are densely located in the genome of *U. hakonensis*, a characteristic which have been reported in compacted eukaryotic genomes (Pombert *et al.*, 2014; Rolfe *et al.*, 2016; Williams *et al.*, 2005). A small number of reads derived from mRNAs with read-through sequences at the 3' ends connected neighboring genes and caused prediction of excessive overlapped or large

Table 3.4: Number of genes in each gene family, found in the transcriptome of *U. hakonensis* and in the genomes of the five fungal species.

		lichen-forming		free-living		parasitic	
gene family	Predicted function	*U. hakonensis*	*E. pusillum*	*U. pustulata*	*A. fumigatus*	*A. oryzae*	*C. albicans*
g162	MFS transporter A	24	21	22	43	77	13
g6745	MFS transporter B	14	21	16	21	42	6
g5383	MFS transporter C	12	25	12	49	93	12
g754	sugar transporter	19	13	13	62	91	17
g2490	amino acid transporter	17	25	26	26	36	9
g6463	lichen-specific A	13	4	39	0	1	0
g3106	lichen-specific B	12	2	5	0	0	0
g5573	lichen-specific C	11	2	62	0	0	0
g5457	TIP49	20	19	19	19	19	19
g2986	helicase	17	22	23	22	23	23
g3390	serine/threonine kinase	15	21	19	21	29	23
g1309	MAPK	12	11	12	13	15	14
g5166	helicase	10	14	15	15	15	13
g827	aldehyde dehydrogenase	10	11	9	26	26	8
g3113	casein kinase	14	3	3	2	3	3
g772	polyketide synthase	19	24	18	19	29	0
g3602	cytochrome P450	14	13	12	13	42	0
g789	GMC oxidoreductase	14	9	6	8	22	0
g5873	kinesin	14	49	125	11	24	0
g395	o-methyltransferase	11	2	4	4	15	0

fused-genes (Figure 3.1). Therefore, we eliminated mapped reads at low coverage regions to avoid fusion of the genes (as described in the Materials and Methods section). Our genome annotation pipeline shown in Figure 3.6 may be useful in analyses of other compact genomes in order to identify *bona fide* transcribed genes.

The effect of long-term symbiotic association is often reflected in the genomes of symbionts. Gain or loss of genes occurs corresponding to the necessity in symbiotic life, sometimes resulted in expansion or contraction of gene families (Martin *et al.*, 2008; Martin *et al.*, 2010; Moran, 2007; Pombert *et al.*, 2014; Wang *et al.*, 2014). To examine the effect of lichenization on the genome of *U. hakonensis*, we classified the identified protein-coding genes into families according to the sequence similarity. We considered that families with large set of transcribed genes (≥ 10 genes) should represent active families in the *U. hakonensis* transcriptome, possibly representing characteristics of a lichen-forming fungus. To examine how symbiotic life style have affected those families, we searched genomes of five other fungal species for homologs of the genes of *U. hakonensis* assigned to the families. Two lichen-forming (*Endocarpon pusillum* and *Umbilicaria pustulata*), two free-living (*Aspergillus fumigatus* and *A. oryzae*), and one parasitic (*Candida albicans*), fungi were chosen for the analysis. Although g5457, g2986, g3390, g1309, and g5166 families were stable in size in all the six fungi, families of transporter genes showed a tendency to differ among fungal life styles. The number of genes assigned to the transporter gene families are tend to be large in free-living and small in parasitic fungi. This result is consistent with previous studies that have reported gene loss in the genomes of parasites resulted from increased dependency on host's metabolism (Katinka *et al.*, 2001; Zhou *et al.*, 2009). *Aspergillus fumigatus* is a saprophytic fungus, whose natural niche is the soil. However, it could also cause diseases in immunosuppressed hosts and thus considered to be a weak pathogen (Latgé, 1999). Therefore, reduced number of transporter genes in *A. fumigatus* compare to *A. oryzae* observed in this study may be a reflection of its parasitic life style.

MFS transporter (A and C) and sugar transporter families are reduced in the three lichen-

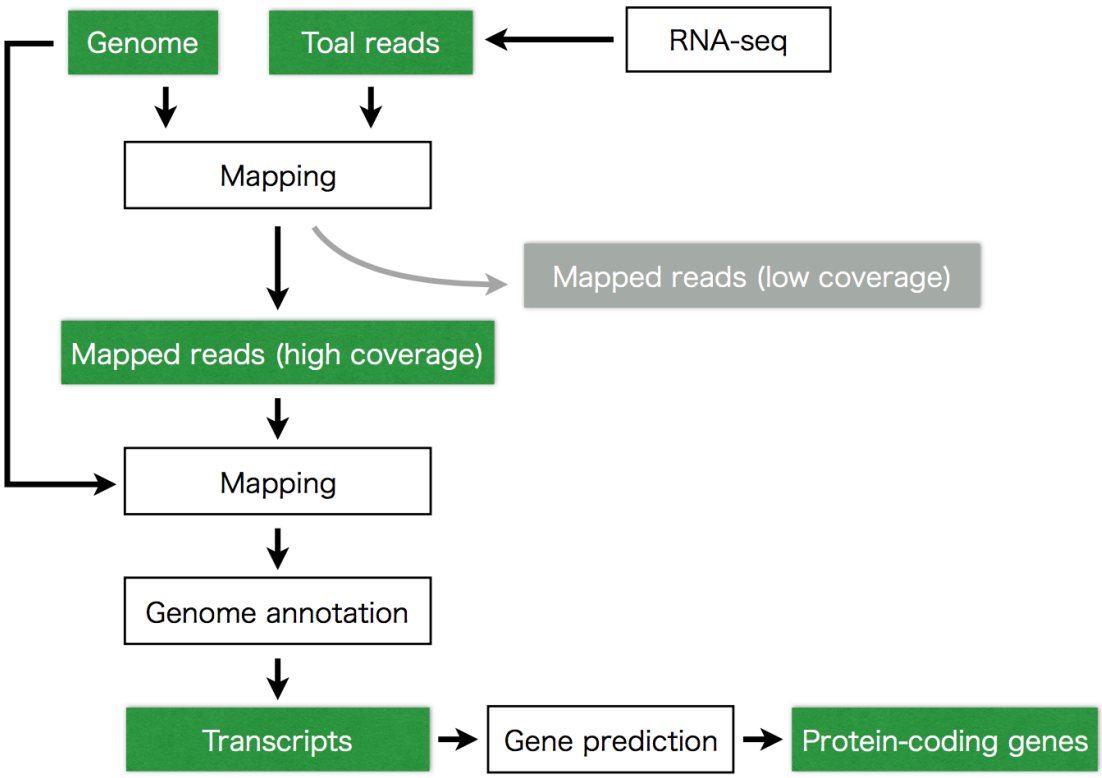


Figure 3.6: Genome annotation pipeline used in this study. Reads mapped to the low coverage regions (<30 reads per base) were removed before the annotation.

forming fungi compare to the free-living. Wang *et al*. (2014) reported the loss of sugar transporter genes in the genome of *E. pusillum* compare to 14 non lichen-forming fungi. They proposed that the reduction is a consequence of the lichenization, which supplies the fungus with specific carbohydrates produced by the algal partner and reduced fungal necessity to uptake other sugars from environment. Similar scenario could also be hypothesized from our results. Although we do not know the exact substrates for the remaining transporters, reduction of the transporter families may characterize specific transport activities between the fungus and the alga in lichens.

Three gene families were only identified in the genomes of the lichen-forming fungi. Protein-coding genes assigned to these families shared no significant similarities with genes of known function. Moreover, no matching domains were found in the Pfam database, indicating that these three gene families are likely to be novel families. The number of genes assigned to each of the three families varied among the examined lichen-forming fungi, which possibly represents species-specific character of these families.

The gene annotation pipeline we used in the present study has succeeded to identify actual transcribed genes in the genome of *U. hakonensis*. However, the cut-off value for the base coverage was too low for genes with extremely high expression, and a small number of fused genes were left. We used a gene prediction tool to separate those remained fused genes, but more fundamental solution should be needed in future to elucidate *bona fide* transcription in compact genomes. Although our method is far from perfection, our results presented several important genetic trends of lichen-forming fungus. The genomic and transcriptomic information provided in this study would be useful for future studies in screening genes or gene families involved in lichenization and to elucidate evolution of lichen symbioses.

Chapter 4

Bidirectional nutrients transfer between the mycobiont and the photobiont inferred by gene expression analysis of lichen *Usnea hakonensis*

Introduction

When the symbiotic nature of lichens was first recognized by Schwendener (Honegger, 2000) in 1867, lichens were described as associations of fungi and algae. Centuries after his finding, progress in molecular techniques has provided us with knowledge of diversity and functional roles of lichen-associated bacteria, leading us to consider that lichens are actually multi-species symbioses (Aschenbrenner *et al.*, 2016; Kono *et al.*, 2017). Meanwhile, only few studies have applied molecular techniques to investigate symbiotic interaction between a fungus, (mycobiont) and a photosynthetic partner (photobiont), the core association that sustains lichen symbiosis.

In lichen symbiosis, a mycobiont develops a specific structure called thallus, in which photobiont cells are extracellularly located. Establishment of symbiotic association is a multistep process accompanied by morphological and physiological alterations of both symbionts. Regulation of the process at the level of gene expression has been indicated more than a decade ago (Armaleo & Miao, 1999; Trembley *et al.*, 2002). However, it was not until recently that differential expression of genes between the symbiotic and non-symbiotic states were quantified and roles of differentially expressed genes in symbiosis have begun to be discussed (Joneson *et al.*, 2011; Wang *et al.*, 2014). Studies on gene regulation in lichen symbioses have been performed *in vitro*. Cultured isolates of the mycobiont and the photobiont, which represented the non-symbiotic state, were co-cultured to resynthesize lichen thalli, which represented the symbiotic state. The *Cladonia gray*i-*Asterochloris* sp. resynthesis system was used in Jonseon *et al*. (2011), from which they suggested that communication between the mycobiont and the photobiont begins before the direct contact. Wang *et al*. (2014) used the *Endocarpon pusillum*-*Diplosphaera chodatii* system to confirm the up-regulation of several fungal genes, presumably symbiosis-related, in the symbiotic state. Their results suggested that *in vitro* lichen resynthesis can be used as a model system to investigate genetic mechanisms underlying lichen symbiosis, even though resynthesis of matured thalli cannot be achieved in any lichen species to date.

In these previous studies, a quantitative PCR method was used to quantify differential gene expression. The target genes should be specified in this method, which limits the choice of genes in an analysis. Progress in high-throughput RNA sequencing (RNA-seq) technologies enabled global quantification of expressed genes in non-model organisms. Comparative analysis of transcriptomes using RNA-seq have been applied to various symbiotic interactions presenting its validity (Grote *et al.*, 2017; Rosenthal *et al.*, 2011; Tisserant *et al.*, 2013).

In the present study, we applied RNA-seq techniques to lichen *Usnea hakonensis* for the first time. The *U. hakonensis*-*Trebouxia* sp. system is another resynthesis system established by Kon *et al*. (1993). When the mycobiont, *U. hakonensis* and the photobiont, *Trebouxia* sp. are co-cultured, resynthesis-specific structure similar to natural thalli will be induced. At the time we started this study, neither genomes nor transcriptomes of *U. hakonensis* and *Trebouxia* sp. have been sequenced. To accomplish the final goal of the study, which was to create a catalogue of symbiosis-related genes, we first started from annotation of the genomes, and subsequently sequenced transcriptomes for comparative analysis of fungal and algal gene expression between the symbiotic and non-symbiotic states.

Materials and Methods

Sample preparation

All cultures were incubated under a 12h/12h light-dark cycle, approximately 20 μmol $m^{-2}s^{-1}$ illumination at 18℃. Isolated cultures of the alga were collected after one month of incubation following the method described in Chapter 2. Resynthesized thalli of *Usnea hakonensis* were provided by Dr. Yoshiaki Kon. Isolated cultures of *U. hakonensis* and *Trebouxia* sp. were co-cultured following the method described in Kon *et al*. (1993). After three months, resynthesized thalli, fibrils, were collected by using tweezers. The collected samples were stored in RNA-later at -80℃ until the RNA extraction. Three biological

replicates were prepared for each type of the sample.

RNA extraction, library construction, and sequencing

Total RNAs of the algal cultures and the resynthesized thalli were extracted using RNeasy mini kit (QIAGEN, Venlo, the Netherlands) following the protocol for plants. RNA libraries were constructed using NEBNext Poly(A) mRNA Magnetic Isolation Module and NEBNext Ultra RNA Library Prep Kit for Illumina (New England Bio Labs, Ipswich, MA) following the manufacturer's instructions. Short cDNA sequences (paired-end 125 bp) were determined from the libraries by Illumina Hiseq2500 platform (RNA-seq).

Genome annotation

The fungal and the algal genomes were annotated following the genome annotation pipeline described in Chpater 3. The RNA-seq reads from Chapter 3, derived from the transcriptome of the fungal isolates were also used in the annotation. The genomes were first annotated by using reads from the fungal/algal isolates. Then, re-annotated by using reads from the resynthesized thalli as well.

Differential expression analysis and functional prediction of the differentially expressed genes

The reads derived from the transcriptome of resynthesized thalli (symbiotic state) and the fungal and the algal isolates, were mapped to the annotated genomes by using CLC genomic workbench (https://www.qiagenbioinformatics.com). Expression value, RPKM (Reads Per Kilobase of exon model per Million mapped read) of each gene was calculated from the number of reads mapped to the gene. (Mortazavi *et al.*, 2008). The average RPKM of the three replicates were compared between the symbiotic and non-symbiotic states. Significance of the differential expression was confirmed by the exact test for two-group comparisons (Robinson & Smyth, 2008) with Bonferroni correction (p-value cut-

off of 0.05). Results of the statistical tests on the fungal and algal genes are shown in Appendix Table S4.1 and S4.2, respectively. Differentially expressed genes (DEGs) were subjected to BLASTX searches against the NCBI non-redundant protein sequences database (nr) with e-value cut-off of 1e-39.

Results

Annotation of the genomes

Thalli of a lichen *Usnea hakonensis* was resynthesized under laboratory condition by Dr. Yoshiaki Kon using isolated strains of *U. hakonensis* and *Trebouxia* sp. as previously described in Kon *et al.* (1990). Transcriptomes of resynthesized *U. hakonensis* thalli and isolated cultures of *Trebouxia* sp. were sequenced by using Illumina Hiseq2500 platform with three biological replicates, results of which are summarized in Table 4.1. The yielded reads and previously sequenced reads derived from the transcriptome of isolated fungal cultures, were firstly used to annotate *U. hakonensis* and *Trebouxia* sp. genomes following the genome annotation pipeline described in Chapter 3. To verify the differences in annotation with or without reads from resynthesized thalli, each genome was annotated twice by using two types of dataset, 1) reads derived from the isolated fungal or algal cultures, and 2) those combined with reads derived from the resynthesized thalli. The former identified 13,695 fungal and 12,235 algal genes on each genome, while the latter identified more genes, 24,023 and 19,041, respectively (Figure 4.1).

Differential gene expression between the symbiotic and non-symbiotic states

The reads were then used to evaluate the expression of genes for the comparative transcriptome analysis, by following the next three steps. First, the reads were mapped on the fungal or the algal annotated genomes. Second, the expression value (RPKM: Reads Per Kilobase of exon model per Million mapped reads) (Mortazavi *et al.*, 2008) for each annotated gene on the fungal and the algal genome was calculated. Third, the values were

compared between the resynthesized thalli (symbiotic state) and the isolated fungal/algal

Table 4.1: Yields of RNA-seq and results of mapping for the three replicates of each sample type

	Total number of reads	Number of reads mapped to fungal genome		Number of reads mapped to algal genome	
Resynthesized 1	119118914	67281614	(56.5%)	24239567	(20.3%)
Resynthesized 2	126331702	64856126	(51.3%)	37410193	(29.6%)
Resynthesized 3	121890284	61097919	(50.1%)	35775001	(29.4%)
Fungal isolates 1*	38253798	29881267	(78.1%)	-**	
Fungal isolates 2*	27698854	21607126	(78.0%)	-	
Fungal isolates 3*	28707094	22357966	(77.9%)	-	
Algal isolates 1	32719326	-		28860913	(88.2%)
Algal isolates 2	32711120	-		28831918	(88.1%)
Algal isolates 3	29800098	-		26322272	(88.3%)

* sequenced in our previous study.

** mapping was not performed.

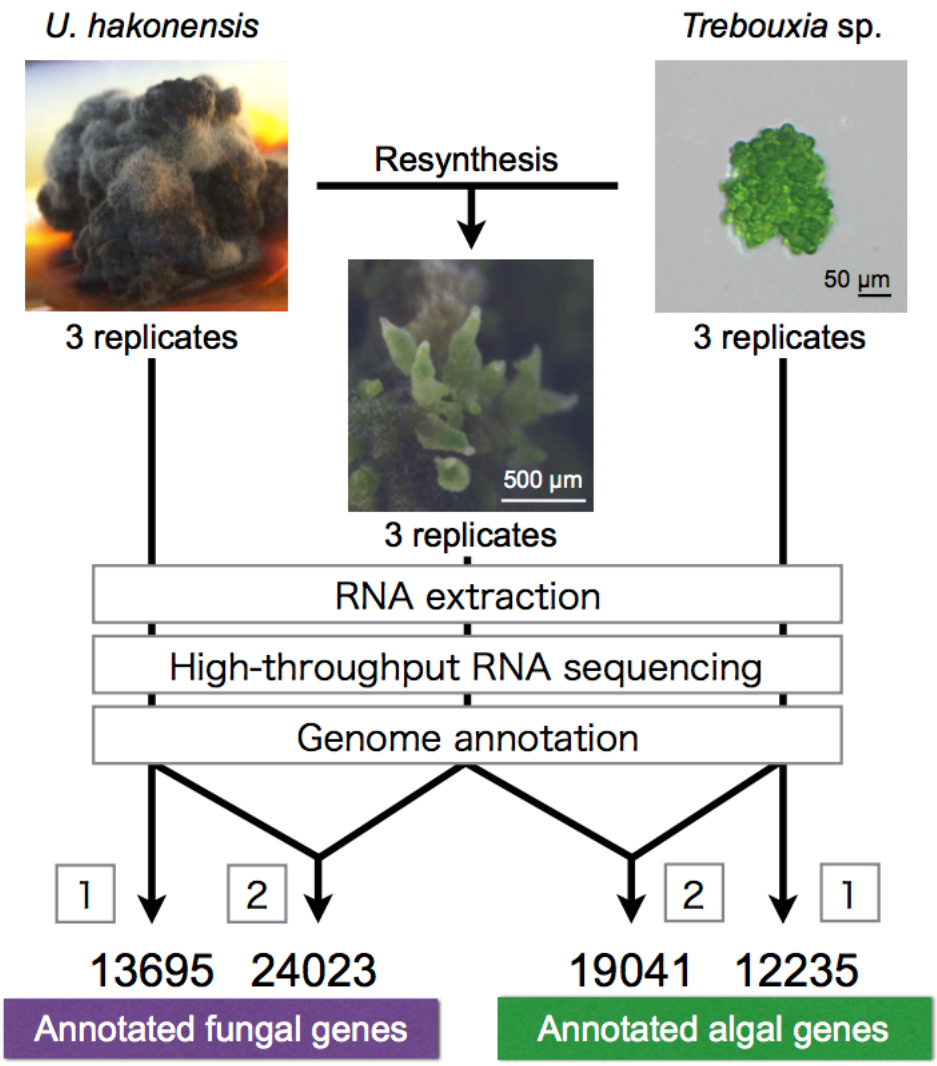


Figure 4.1: Process of genome annotation. Three biological replicates were prepared for each sample type. First, RNA-seq reads derived from the isolated cultures of the fungus/alga were solely used in the first annotation. Then, those were merged with the reads derived from the resynthesized thalli and used in the second annotation.

cultures (non-symbiotic state) (Figure 4.2). In the fungal genome, 474 protein-coding genes were identified to show significantly higher expression (up-regulated) in the symbiotic state than in the non-symbiotic state whereas 865 genes were up-regulated in the non-symbiotic state. In the algal genome, 412 and 816 genes were up-regulated in the symbiotic and non-symbiotic states, respectively.

The identified differentially expressed genes (DEGs) were subjected to BLASTX searches against the NCBI nr database. Out of the 474 and 865 fungal up-regulated genes in the symbiotic and non-symbiotic states, 155 (32.7%) and 509 (58.8%) genes had similar sequences in the database. Whereas only 13 (3.2%) and 56 (6.9%) genes among the 412 and 816 algal up-regulated genes in the symbiotic and non-symbiotic states, respectively, had similar sequences in the database. In both species, the proportions of the DEGs similar to genes with known function are larger in the non-symbiotic state than in the symbiotic state (Figure 4.3).

Functional prediction of the differentially expressed genes

We manually classified the DEGs, up-regulated in the symbiotic state according to their function predicted from the BLASTX searches. In total, 71 fungal and five algal genes were classified into 10 functional categories: Carbohydrate metabolism, Cytochrome P450, Detoxification, Fatty acids synthesis, Methylation, Phospholipid synthesis, Proteolysis, Secondary metabolism, Translation, and Transporter (Table 4.2). In the fungus, the most frequent category is Transporter (18 genes), followed by Carbohydrate metabolism (14), Secondary metabolism (8), and Cytochrome P450 and Proteolysis (7). The five algal DEGs were categorized into Translation (2), Fatty acid synthesis (1), Phospholipid synthesis (1), and Proteolysis (1).

Differential expression of the fungal gene families

In the previous chapter, we have identified the gene families within the transcriptome of isolated fungal cultures. To examine the effect of symbiotic association on the fungal

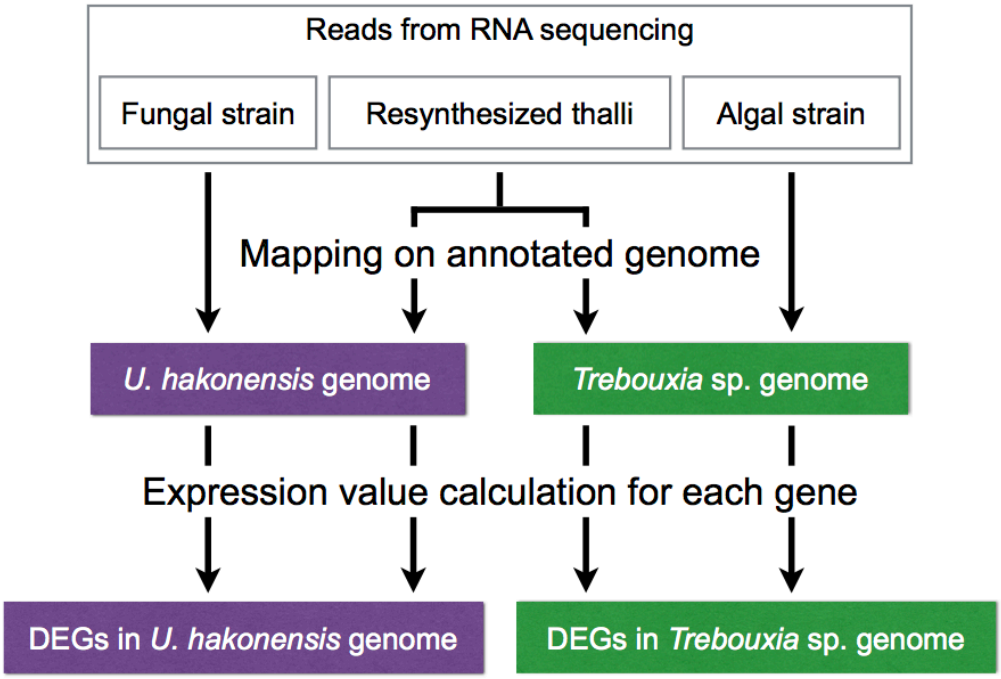


Figure 4.2: Process of differential expression analysis. Expression value (RPKM) was calculated for each gene, and compared between the symbiotic (resynthesized thalli) and the non-symbiotic state (isolated cultures).

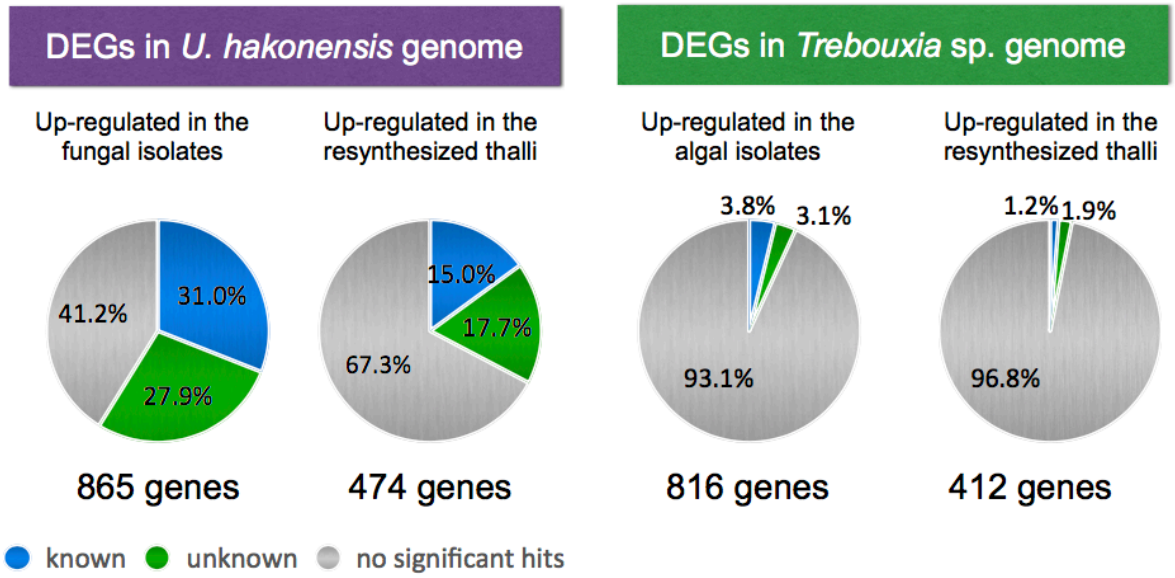


Figure 4.3: Differentially expressed genes (DEGs) identified in the fungal and the algal genomes. Homologous sequences were searched in the NCBI nr database by BLASTX searches with the e-value cut-off of 1e-39.

Table 4.2: Functional categories of fungal and algal DEGs, up-regulated in the resynthesized thalli

	up-regulated in resynthesized thalli	
	fungal	algal
carbohydrate metabolism	14	-*
cytochrome P450	7	-
detoxication	4	-
fatty acid synthesis	2	1
methylation	2	-
phospholipid synthesis	0	1
proteolysis	7	1
secondary metabolism	8	-
translation	-	2
transporter	18	-
uncategorized	9	-

* Not identified.

transcriptome, DEGs in the gene families were also examined. We chose 12 gene families large in size (≥ 10 family members), which could either be assigned to the above functional categories of DEGs or indicated to be lichen-specific. DEGs were identified in eight gene families, five of which contained both DEGs in the symbiotic and non-symbiotic states. The most prominent was the sugar transporter gene family, which more than half of the genes were differentially expressed. On the other hand, all of the genes assigned to the three lichen-specific gene families did not show differential expression (Figure 4.4).

Discussion

In nature, lichen-forming fungi are found exclusively associated with their algal partners, showing phenotypes specific to symbiosis. However in laboratory conditions, lichen symbiosis is rarely resynthesized from isolated cultures of the mycobiont and the photobiont, and never grows into natural lichen thalli. To date, resynthesis experiments are successful in limited species of lichens, and only few of them have been investigated using molecular techniques (Joneson *et al.*, 2011; Trembley *et al.*, 2002; Wang *et al.*, 2014).

When the isolates of *U. hakonensis* and *Trebouxia* sp. are co-cultured, they exhibited distinct structures, which are referred to as “fibrils”, consist of fungal hyphae and algal cells. The fibrils present morphological and chemical characters similar to thalli of *U. hakonensis* in nature, indicating that symbiotic interaction between the mycobiont and the photobiont were resynthesized. Therefore *U. hakonensis* is an ideal system to investigate genetic mechanisms of lichen symbiosis. By using this system, we aimed to identify differential expression of fungal and algal genes between resynthesized thalli and isolated cultures. In advance, we annotated the fungal and the algal genomes, which is valuable to raise accuracy of the comparative transcriptome analysis. In the previous chapters, the genomes of *U. hakonensis* and *Trebouxia* sp. were determined. Furthermore,

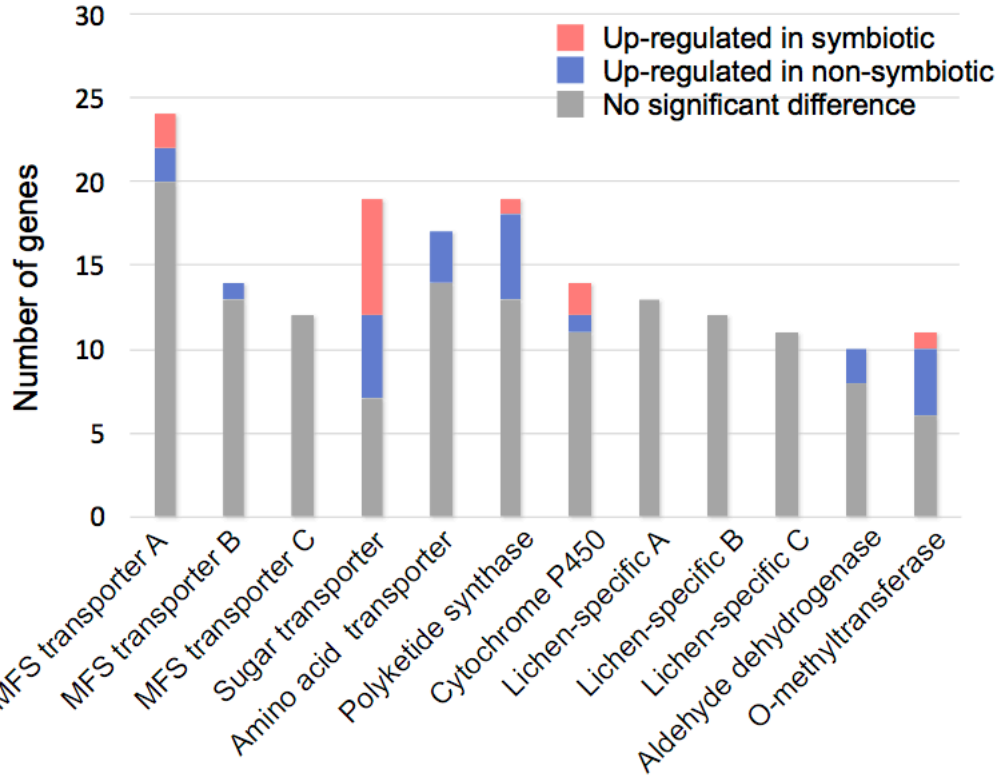


Figure 4.4: Number of DEGs in the gene families. The predicted functions of the gene families correspond to the functional categories of DEGs identified in this study.

the fungal genome was annotated by using RNA-seq reads derived from the transcriptome of isolated cultures of the mycobiont. In the present study, transcriptomes of resynthesized thalli and isolated cultures of the photobiont were sequenced. When we re-annotated the fungal genome using the RNA-seq reads derived from the transcriptome of resynthesized thalli and isolated cultures, considerable number of genes were newly annotated. This was also observed in the annotation of the algal genome (Figure 4.1). One of the possibilities for the cause of these altered annotations is increased number of reads used in the transcripts-based annotation process. However, previous studies indicated that expressions of certain genes are induced by symbiotic association (Joneson *et al.*, 2011; Trembley *et al.*, 2002; Wang *et al.*, 2014). Therefore we concluded that some, if not all, of the new annotations are due to the genes expression of which were induced by the symbiotic association.

Symbiotic association of the mycobiont and the photobiont is known to change expression of genes in both partners (Joneson *et al.*, 2011; Trembley *et al.*, 2002; Wang *et al.*, 2014). However, those genes have been partially reported and their functional roles in symbiosis remain largely unknown. The RNA-seq performed in this study identified differentially expressed genes (DEGs) between the symbiotic and non-symbiotic states of *U. hakonensis* in unprecedented amount. Results of the BLASTX searches revealed that more than half of the DEGs had no similar sequences in the database. The proportion of those genes with no similar sequences is larger in the alga than the fungus, and larger in the symbiotic state than the non-symbiotic state (Figure 4.3). The observed bias in the blast results between the fungus and the alga may primarily be explained by the deviation of registered species in the database. On the other hand, the bias observed within the species, between the symbiotic and non-symbiotic states, may indicate that genes up-regulated in the symbiotic state are more specialized in lichen-forming species that have not been characterized so far.

Among the annotated fungal genes that were up-regulated in the symbiotic state, transporter genes are the most abundant. Considering that association of the mycobiont

and the photobiont is the core event in resynthesized thalli, these genes are likely to function in import or export of molecules between the fungus and the alga.

Transport from the photobiont to the mycobiont

Two fungal genes, annotated as sugar transporter genes, were up-regulated. When the isolated cultures of the mycobiont and the photobiont were co-cultured, small projections made from fungal hyphae, referred to as fibrils, were formed on the surface of tissues. The fibrils enveloped algal cells while having no direct contact with the medium (Kon *et al.*, 1990). As the symbiotic state, we used those fibrils. Therefore we concluded that the source of carbohydrates for mycobiont was shifted by the symbiotic association, possibly from the medium to the photosynthetic products of the alga.

Fungal metabolism of the transported molecules

The up-regulation of some oxidoreductase genes and genes involved in carbohydrate metabolism may represent the catabolic process of imported sugars inside the mycobiont. Furthermore, subsequent use of carbohydrates in the synthesis of lichen-specific metabolites have been indicated (Elix & Stocker-Wörgötter, 2008). Lichen-forming fungi are known to produce a number of secondary metabolites, most of which are uniquely found in lichens. We identified several polyketide synthase genes (categorized as "Secondary metabolism") among the genes up-regulated in the symbiotic state. Polyketide synthases are enzymes known to function in the biosynthesis of several lichen-specific metabolites (Elix & Stocker-Wörgötter, 2008). We assume that these polyketide synthase genes and other genes related to metabolism (cytochrome P450 and fatty acids elongation genes) may be involved in lichen-specific metabolic activities.

Transport from the mycobiont to the photobiont

Nitrate and phosphate transporter genes were also identified as DEGs. Although little is known with evidence, nutrient flux from the mycobiont to the photobiont is considered

essential for lichen symbiosis. In lichen thalli, photobiont cells are enveloped by fungal hyphae, isolated from surrounding environment. Therefore, algae must receive nutrients necessary for their metabolic activities from the mycobiont. Nitrogen supply from the fungus to the alga was previously indicated by Wang *et al*. (2014). They identified up-regulation of a nitrate transporter gene in *Endocarpon pusillum* when it was co-cultured with the photobiont. They proposed that the mycobiont absorbs various nitrogen sources from the environment and convert them to forms which meet the requirement of the photobiont. In the present study, up-regulation of allantoate permease genes were also identified in the symbiotic state. In some legumes, allantoate is indicated as a transport molecule of nitrogen fixed by symbiotic bacteria (Raso *et al.*, 2007). Therefore, allantoate could also be the source of nitrogen that meets the requirement of *Trebouxia* sp. Transfer of phosphorus from the mycobiont to the photobiont was predicted for the first time.

Interestingly, higher expression of fungal carbonic anhydrase gene was identified in the symbiotic state. Carbonic anhydrase catalyzes the reversible conversion of bicarbonate ion and carbon dioxide. In plants, it is known to support photosynthesis by means of CO_2 transfer and fixation (Tiwari *et al.*, 2005). The up-regulation of this enzyme indicates that the fungus may support/regulate the algal photosynthesis through CO_2 supply.

Algal metabolism of the transported molecules

Carbon, nitrogen and phosphorous are essential elements for organisms. They are involved in major metabolic activities including the synthesis of proteins, lipids, and fatty acids. Therefore, the supply of these nutrients by the mycobiont could activate algal metabolism, which is implied by enhanced expression of algal genes encoding ribosomal protein, phosphomethylethanolamine N-methyltransferase, and 3-oxoacyl-ACP synthase in the symbiotic state.

Differential expression of the fungal gene families

We expanded our view from the expression of each gene to those of gene families.

Expressions of 12 fungal gene families described in Chapter 3 were investigated (Figure 4.4). While genes were steadily expressed in most of the gene families, sugar transporter gene family showed a distinctive expression pattern. More than half of the sugar transporter genes were differentially expressed, with almost equal number of genes that were up-regulated in either the symbiotic or non-symbiotic state. This result indicated that many of the genes in this family are involved in the altered transportation of sugars induced by the symbiotic association, possibly indicating the importance of this family in evolution of lichen symbiosis.

Surprisingly, none of the genes in the families inferred as lichen-specific changed expression between the symbiotic and non-symbiotic states. The absence of DEGs in the lichen-specific families may indicate their low relevance to symbiosis or possibly their limited expression during the development of symbiosis. Increased expression of genes only in a particular developmental stage of symbiosis has been previously reported (Joneson *et al.*, 2011; Wang *et al.*, 2014). Thus, the resynthesized thalli used in the experiment were possibly not in the stage where these gene families to function. More detailed analyses focused on developmental stages of lichen symbiosis are needed to elucidate roles of these gene families.

Observation of the differential expression at the level of gene families provided us with new information. Even when the function of a gene was not inferred by the database search, its function could sometimes be predicted from other genes assigned to the same gene family. More importantly, as we've seen in the case of sugar transporters, connecting differentially expressed genes in terms of gene families could lead us to deeper understanding of lichen symbiosis.

Conclusion

In the present study, we screened expression of genes in mass amount and identified differentially expressed genes between the symbiotic and non-symbiotic states of a lichen *U. hakonensis*. From our results, we hypothesize that essential nutrients are transferred

between the mycobiont and the photobiont, and metabolic activities of both symbionts are sustained by those nutrients (Figure 4.5). The bidirectional transfer of materials via transporter proteins indicated in this study may represent the mutualistic relationship between the mycobiont and the photobiont in lichen symbiosis. Our study provides a model for future researches which attempt to investigate genetic mechanisms underlying lichen symbiosis in detail. However, the results are limited to the resynthesized symbiosis of *U. hakonensi.* Studies on broader lichen species using resynthesized and natural lichen samples are needed for the full characterization of their genetic specificity.

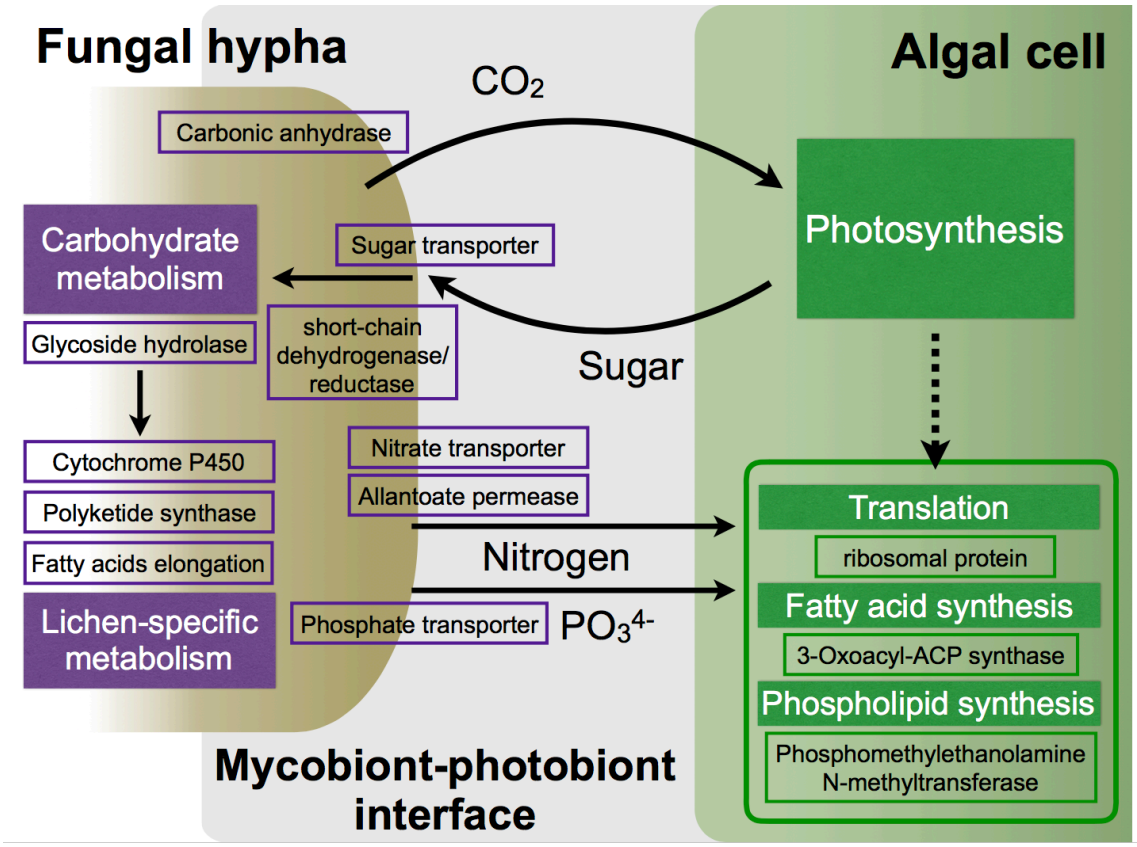


Figure 4.5: Predicted symbiotic interactions between the mycobiont and the photobiont. Bidirectional transfer between the symbionts are suggested by the up-regulation of various fungal transporter genes. Photosynthetic products are transferred from the photobiont to the mycobiont, which are subsequently used in the fungal metabolic activities possibly to produce lichen-specific substances. Nitrate, allantoate, and phosphate are transferred from the mycobiont to the photobiont to support various algal metabolic activities. The mycobiont may also release carbon dioxide at the mycobiont-photobiont interface to support/regulate the algal photosynthesis. The broken arrow represents a pathway without supportive DEGs from the present study.

Chapter 5

Conclusion and perspectives

Lichens can be readily found in our daily life. They often grow on soil, bark, rocks, and leaves in our gardens. However, even centuries after the finding of their symbiotic character, the true nature of lichen symbiosis still awaits exploration. It is apparent that symbiosis is the key factor for evolution of lichens. As symbiotic entities, they are adapted to extreme environments, which are characterized by frequent cycles of desiccation and rehydration, temperature extremes, high light intensities, and low nutrition availability. Recently studies using molecular techniques have begun to reveal supportive roles of lichen-associated bacteria in persistence of lichens in extreme environmental conditions. Nevertheless, the bulk of genetic mechanisms that regulate symbiosis remains unknown.

The aim of this thesis was to elucidate the genetic basis of lichen symbiosis. Although lichens are now considered as multi-species symbiosis, their foundation is the association between the mycobiont and the photobiont. Solving genetic mechanisms that regulate their interaction will lead us to deeper understanding of complex symbiotic association in lichens.

Usnea hakonensis-*Trebouxia* sp. is an ideal system to investigate genetic mechanisms of lichen symbiosis. The mycobiont *Usnea hakonensis* belongs to Lecanoromycetes, a fungal class which is almost exclusively consist of lichen-forming fungi. Likewise, the photobiont *Trebouxia* sp. belongs to one of the most frequent genera of photobionts. Thus, their genetic features are likely to be shared by broad lichen species. More importantly, this system is capable of *in vitro* resynthesis of the symbiosis by using isolated cultures of the mycobiont and the photobiont, amenable to molecular investigation.

Therefore, this system was used to identify differential gene expression before and after the symbiosis, each state represented by the isolated fungal/algal cultures and the resynthesized lichen thalli. For comprehensive quantification of gene expression, I used high-throughput sequencing techniques. For accurate comparison of the gene expression, genomes of the mycobiont and the photobiont annotated in fine quality were essential. Hence, in advance to transcriptome analysis, I preformed whole genome sequencing and transcriptome-based annotation of the fungal and the algal genomes (described in Chapter

2 and 3, respectively). Finally, expressions of each genes in the annotated genomes were compared between the symbiotic state and the non-symbiotic state. Genes up-regulated in the symbiotic state were considered to be involved in symbiotic interactions between the mycobiont and the photobiont (Chapter 4). Lichen symbiosis is often considered as mutualistic for the mycobiont's gain of photosynthetic products from the photobiont, and the photobiont's gain of protection from environmental stress. The results of the doctoral work indicate that the mycobiont benefits from photosynthetic products of the alga in the resynthesized thalli. Meanwhile, transfer of phosphorus and carbon dioxide from the mycobiont to the photobiont was suggested for the first time in terms of gene expression. Consequently, it appears that bidirectional transfer of nutrients is the basis of lichen symbiosis.

At the same time, these results raised new questions to be solved in future studies. The *U. hakonensis*-*Trebouxia* sp. system represents the symbiotic interaction of the mycobiont and the photobiont in resynthesized thalli of the lichen, and may not fully exhibit their interactions in nature. More than half of the genes that showed up-regulated expression in the symbiotic state had no similar sequences in the public database, and indicated as symbiosis related genes specific to the lichen. Including those, consistency in gene expression between resynthesized thalli and thalli in nature should be examined. Moreover, the association of *Sphingomonas* sp. with *Trebouxia* sp., revealed in this doctoral work, reminded us that lichen-associated bacteria should not be overlooked. They may play supportive roles to function the symbiosis when lichens are in their natural habitat. Therefore, investigation into the transcriptome of thalli in nature will elucidate the true nature of the symbiosis in *U. hakonensis*. Expansively, application of the methodology presented in this thesis to other lichen systems would reveal polyphyletic evolution of lichen symbiosis.

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