Exploitation of Lichens as a Source of Novel Natural Products

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Abstract

Lichens can be described as an obligate mutualistic symbiosis, usually between an ascomycete fungus (the mycobiont) and either a green alga and/or cyanobacterium (the photobiont), in which a unique thallus structure is formed. Fungal species which follow this evolutionarily successful lifestyle are known to produce a diverse range of secondary metabolites, many of which are unique to lichens and are not found to be produced in other non-lichen fungal species. Lichen secondary metabolites, the majority of which are thought to be produced by the fungal partner, possess a variety of biological activities and, therefore, have the potential for biotechnological and industrial applications. However, the extremely slow growth rate associated with lichens hampers their biotechnological exploitation as it is neither feasible nor sustainable to produce lichen secondary metabolites on an industrial scale. Thus, alternative methods are required for the production of lichen secondary metabolites.

This study used two approaches in an attempt to overcome this problem. The first approach involved the use of UV mutagenesis to create mutants of various lichen mycobionts (*Xanthoria parietina*, *Lecanora chlarotera* and *Amandinea punctata*) possessing a faster growth rate. It was planned to analyse these mutants at the genomic level in order to obtain insights into the mutations supporting higher growth rates. However, although results obtained in this study showed that exposure of *X. parietina* ascospores to UV-C appeared to produce potentially faster-growing mutants with at least an incremental increase in growth rate, only pilot work was conducted in this study and further elucidation of the genetic basis of this increased growth rate is required.

The second approach used in this study to achieve the production and characterisation of lichen-derived secondary metabolites was that of the heterologous expression of lichen secondary metabolism genes in the non-lichen fungal expression platform strains *Aspergillus niger* ATNT16 and *Aspergillus oryzae* OP12. The heterologous expression of two types of lichen secondary metabolism genes, those encoding polyketide synthases and non-ribosomal peptide synthetase-like enzymes, was attempted. A number of polyketide products have been described

from lichens, the biosynthesis of some of which are restricted to lichen species. Conversely, no metabolites originating from NRPS-like enzymes have been described from lichens, but the enzymes are conserved.

The results obtained here demonstrate that heterologous expression of lichen secondary metabolism genes is an appropriate method for the production of certain lichen-derived secondary metabolites. When focussing on lichen-derived PKS genes, four genes from *Cladonia grayi*, two genes from *Evernia prunastri* and one gene from *Usnea longissima* were selected for expression studies. These studies resulted in the functional heterologous expression of two PKS genes from *C. grayi* (*Clagr3.6* and *Clagr3.21*), with the Clagr3.6 enzyme being identified as a 1,3,6,8-tetrahydroxynaphthalene synthase and the Clagr3.21 enzyme awaiting characterisation. However, it was observed that metabolite production was only successful for these lichen-derived genes at 23 °C, suggesting that these lichen secondary metabolism proteins are temperature-sensitive. Unfortunately, attempts to achieve the functional heterologous expression of the PKS genes from *E. prunastri* and *U. longissima* were unsuccessful.

Attempts to achieve the functional expression of NRPS-like enzymes focussed on two genes from *C. grayi* and three genes from *E. prunastri*. Similarly to the results obtained for PKS expression, the functional heterologous expression of the three NRPS-like enzymes from *E. prunastri* was not achieved. However, we were able to achieve the functional expression of both NRPS-like genes (*Clagr3.11* and *Clagr3.30*) selected from *C. grayi*. The Clagr3.30 enzyme was identified as a typical atromentin synthetase, but again was only active at a low temperature. Interestingly, the Clagr3.11 enzyme was found to produce three metabolites, which suggests that the enzyme possesses a flexible substrate specificity. The discovery of this enzyme gives the first example of an NRPS-like enzyme that is not only able to accept various substrates, but is also capable of producing metabolites from mixed substrates, including from non-natural substrates such as 4-chloro-phenylpyruvate; *in vitro* studies using the Clagr3.11 enzyme confirmed this. Further studies using Clagr3.11, in which the adenylation domain of this enzyme was fused with the thiolation and thioesterase domains of an *Aspergillus brasiliensis* atromentin synthetase, also resulted in successful metabolite production; this demonstrates that it is feasible to

use Clagr3.11 in domain fusion experiments. Furthermore, the Clagr3.11 enzyme differed from that of the Clagr3.30, Clagr3.6 and Clagr3.21 enzymes in the sense that metabolite production was achieved at both 23 °C and 28 °C, indicating a reduced temperature sensitivity of this enzyme. The discovery of the first NRPS-like enzyme with a flexible substrate specificity is particularly noteworthy, as it provides the opportunity to use this enzyme for the production of novel natural products not found in nature. This would perhaps allow the creation of natural products which possess enhanced or novel biological activities, which could therefore have pharmaceutical applications.

The successful heterologous production of these lichen-derived secondary metabolites at 23 °C, and the subsequent suggestion that lichen secondary metabolism proteins may be temperature-sensitive, implies that a relatively low growth temperature is of high importance for the successful heterologous production of lichen-derived metabolites. Further investigation of the heat sensitivity of lichens appeared to confirm this observation, with lichen primary metabolism proteins also appearing to show sensitivity to higher temperatures. This has important implications for future attempts to achieve metabolite production from lichen-derived genes in heterologous hosts, as the low temperatures favoured for production of lichen-derived proteins is not compatible with the growth of established expression platforms, such as *A. niger* and *A. oryzae*, currently used for heterologous protein production. Therefore, the development of a psychrotolerant expression platform may be of particular advantage for future studies involving the heterologous production of lichen-derived proteins.

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Table of Contents

Abbreviations

A	Adenylation (domain)
ACM	*Aspergillus* complete medium
ACP	Acyl carrier protein
ANOVA	Analysis of variance
AP	Alkaline phosphatase
AT	Acyltransferase (domain)
ATP	Adenosine triphosphate
AMM	*Aspergillus* minimal medium
BGC	Biosynthetic gene cluster
bZIP	Basic leucine zipper
C	Condensation (domain)
C_2	Carbon
$CaCl_2$	Calcium chloride
C-C	Carbon-carbon
CoA	Coenzyme A
Cys	Cysteine
DH	Dehydratase (domain)
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraaceticacid
ER	Endoplasmic reticulum
ER	Enoylreductase (domain)
FAS	Fatty acid synthase
gDNA	Genomic deoxyribonucleic acid
GOI	Gene of interest
h	Hours
HCl	Hydrochloric acid
His	Histidine
HPLC	High performance liquid chromatography

HR-PKS	Highly-reducing polyketide synthase
HSE	Heat shock element
HSP	Heat shock protein
HSR	Heat shock response
KCl	Potassium chloride
kDa	Kilodalton
KR	Ketoreductase (domain)
KS	Ketoacylsynthase (domain)
LB	Lysogeny broth medium
MAT	Malonyl-acetyl transferase (domain)
ME	Malt extract medium
MEYE	Malt extract yeast extract medium
$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
min	Minutes
m/z	Mass-to-charge ratio
N_2	Nitrogen
NaCl	Sodium chloride
NBT-BCIP	Nitro-blue tetrazolium chloride/5-bromo-4-chloro-3’-indolylphosphate
NR-PKS	Non-reducing polyketide synthase
NRPS	Non-ribosomal peptide synthetase
ORF	Open reading frame
PABA	Para-aminobenzoic acid
PBS	Phosphate-buffered saline
PCP	Peptidyl carrier protein
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIPES	Piperazine-N,N’-bis(2-ethanesulfonic acid)
PKS	Polyketide synthase
PP	Phosphopantetheine (domain)
PR-PKS	Partially-reducing polyketide synthase
PT	Product template (domain)

R	Reductase (domain)
ROS	Reactive oxygen species
rpm	Rotations per minute
s	Seconds
SAM	*S*-adenosyl-L-methionine
SEM	Standard error of the mean
SAT	Starter-unit acyltransferase
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SDW	Sterile distilled water
SM	Secondary metabolite
T	Thiolation (domain)
TAE	Tris-acetate-EDTA buffer
TBS	Tris-buffered saline
TBST	Tris-buffered saline-Tween 20
TE	Thioesterase (domain)
TE	Tris-EDTA buffer
Tris	Tris-hydroxymethyl-methylamine
tRNA	Transfer ribonucleic acid
UPR	Unfolded protein response
Uri	Uridine
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
YEPD	Yeast extract peptone dextrose medium
Zn	Zinc

Chapter 1: Introduction

1.1 Background – Ascomycete Fungi

Fungi are believed to have arisen between 0.5 and 1.5 billion years ago, and are described as heterotrophic organisms which are characterised by cell walls that contain chitin (Taylor and Berbee, 2006; Naranjo-Ortiz and Gabaldón, 2019a). The number of fungal species is believed to lie in the range of 2.2 to 3.8 million species, yet only 120,000 species of fungi have so far been named (Hawksworth and Lücking, 2017). The heterotrophic nature of fungi means they do not produce their own carbon and must, therefore, acquire the fixed carbon they require from other sources. In order to address this, various nutritional strategies have been adopted by different fungal species (Honegger, 2008a). These modes of nutrition include becoming a saprobe, entering into mutualistic symbioses or adopting a parasitic lifestyle (Honegger, 1993; Stajich *et al.*, 2009; Naranjo-Ortiz and Gabaldón, 2019b). The mode of nutrition for the common ancestor of Fungi is not known; however, parasitic species have been found in the earliest-diverging branch within the Fungi (James *et al.*, 2006). The amoeboid protist *Nuclearia* has been shown to be the sister group to Fungi, and is able to take up nutrients by ingestion. The evolution of filamentous growth in fungi, however, aids in attachment to substrates and uptake of nutrients by extracellular digestion and subsequent absorption (Medina *et al.*, 2003; James *et al.*, 2006; Steenkamp *et al.*, 2006). Some fungal taxonomic groups contain fungal species that have all adopted the same nutritional strategy, whereas other fungal taxa are composed of species which have adopted different modes of nutrition (Honegger, 1993). Lichenisation, that is the mutualistic symbiosis between a fungus and a green alga and/or a cyanobacterium, is an example of an ancient and successful nutritional strategy commonly adopted by fungi (Honegger, 1993; Honegger, 2008a; Honegger *et al.*, 2013).

The fungal kingdom is currently considered to be made up of nine phyla, of which the Ascomycota is the largest group, accounting for approximately two-thirds of all fungal species described (Lutzoni *et al.*, 2004; Schoch *et al.*, 2009; Stajich *et al.*, 2009; Naranjo-Ortiz and Gabaldón, 2019a). Fungi belonging to this phylum are

characterised by mating-induced, short-lived dikaryon hyphae (hyphae containing two nuclei) which, through karyogamy and meiosis, lead to the formation of sac-like structures known as asci; these asci contain sexual spores (James *et al.*, 2006; Stajich *et al.*, 2009; Naranjo-Ortiz and Gabaldón, 2019a). The Ascomycota is divided into three subphyla, known as the Pezizomycotina, Saccharomycotina and Taphrinomycotina (Stajich *et al.*, 2009; Spatafora *et al.*, 2017). The Pezizomycotina is the largest subphylum, and has an increased number of enzymes involved in secondary metabolism in comparison to other fungal species (James *et al.*, 2006; Arvas *et al.*, 2007; Wisecaver *et al.*, 2014). The vast majority of lichenised fungal species are found within the Pezizomycotina, and around 40% of the group is made up of lichens (Naranjo-Ortiz and Gabaldón, 2019a). Six of the thirteen classes that make up the Pezizomycotina can be found to contain lichenised fungi, with some of these classes (Arthoniomycetes, Coniocybomycetes, Lichinomycetes and Lecanoromycetes) only containing lichenised fungal species (Grube and Wedin, 2016; Spatafora *et al.*, 2017; Adl *et al.*, 2019; Naranjo-Ortiz and Gabaldón, 2019a).

As this thesis will focus on the production of lichen-derived secondary metabolites by either (i) increasing lichen growth rate or (ii) heterologous production in fungal expression platforms, the subsequent sections will go into further detail about the growth of lichens and fungal secondary metabolism.

1.2 Background – Lichens

1.2.1 The Lichen Symbiosis

Lichens are usually described as an obligate symbiosis in which there are two partners, the mycobiont (usually an ascomycete fungus) and the photobiont (a green alga and/or a cyanobacterium), which together form a unique structure known as the lichen thallus (Nash III, 2008a; Aschenbrenner *et al.*, 2016). An interaction between a fungus and an alga or cyanobacterium is only referred to as a lichen when the symbiosis is extracellular and the fungus is either the dominant partner or, as a minimum, an equal partner in the relationship. In addition to this, the mycobiont is the exhabitant in the majority of taxonomic groups. Of the lichen-forming fungi, approximately 85% form a symbiosis with a green alga (with the most common lichen photobiont being green algae of the genus *Trebouxia*), around 10% form a symbiosis

with a cyanobacterium and approximately 4% form a symbiotic relationship with both (Honegger, 1991b). Lichenisation is thought of as an ancient nutritional strategy for obtaining fixed carbon, with the thallus structure formed by the two partners of the symbiosis being traced back as early as the Devonian period, approximately 400 million years ago (Ahmadjian, 1993; Honegger, 2009; Honegger *et al.*, 2013).

The symbiotic nature of lichens was discovered by Schwendener in 1869, although this view was not widely accepted at the time, a discovery which took place some ten years before the defining of the term 'symbiosis' by de Bary as the co-existence of different organisms (de Bary, 1879). The degree to which the lichen symbiosis is obligate varies for the different symbiotic partners (Nash III, 2008a). The majority of lichen-forming fungal species are ecologically obligate, but physiologically facultative, biotrophs. This means that, although they can be cultured in the aposymbiotic state, the symbiotic phenotype is found almost exclusively in nature; the exception to this is germ tubes searching for a compatible photobiont. It is thought that this is due to the slow-growing free-living form of the fungal partner being unable to compete in nature. This is in contrast to photobionts, in which some genera are commonly found in both the free-living and symbiotic states, whereas others are rarely found in the free-living state and are thought to be poor competitors when not in the symbiotic state (Crittenden *et al.*, 1995; Honegger, 2001; Nash III, 2008a; Honegger, 2008a; Honegger, 2009).

The lichen thallus is a stable symbiotic system in which the fungal partner is described as heterotrophic and the photosynthetic partner is described as autotrophic (Nash III, 2008a). In the lichen symbiosis, the exhabitant mycobiont provides shelter for the inhabitant unicellular photobiont, whilst the photobiont fixes carbon via photosynthesis to provide an energy source for the fungal partner. The cells that make up the photobiont layer within the lichen thallus are positioned in a way that allows optimal levels of photosynthesis to occur (Ahmadjian, 1993; Aschenbrenner *et al.*, 2016). In addition to the provision of fixed carbon, cyanobacterial photobionts also carry out nitrogen fixation of atmospheric nitrogen, with the majority of the fixed nitrogen being utilised by the fungal partner (Millbank and Kershaw, 1973; Ahmadjian, 1993; Nash III, 2008b). The thallus is thought to provide protection for the photobiont, with the cortex of the thallus protecting against high levels of light, as well as from

drought and temperature extremes (Ahmadjian, 1993; Nash III, 2008a; Aschenbrenner *et al.*, 2016). Furthermore, the fungus is believed to provide water and minerals to the photobiont (Honegger, 1991b).

The classical view of the lichen symbiosis is that it is a mutualistic relationship in which both partners benefit from the symbiosis. This view has arisen as a result of the widespread distribution of lichens and the fact that photobiont cells in the thallus have a healthy appearance (Ahmadjian, 1993). However, this view has been challenged by those who instead deem the nature of the lichen symbiosis to be that of controlled parasitism, due to the fact that the fungal partner appears to receive most of the benefits, as well as the photosynthetic partner seeming to grow more slowly when in the lichenised state compared to when free-living (Ahmadjian, 1993; Nash III, 2008a).

However, the generally-accepted view that a lichen is a symbiosis between two partners has been contradicted by microbiome research which has found that lichens have bacterial associations that are specific to the host (Grube *et al.*, 2009; Aschenbrenner *et al.*, 2016). The biofilm-like colonisation of stress-tolerant lichen-associated bacterial communities on the surface of lichens, thought to be mainly controlled by the mycobiont, has been shown to have a high level of structure. These host-associated bacteria have been found to be resistant to abiotic stress, particularly osmotic stress and oxidative stress, through stress protection and metabolism changes. The discovery of these bacterial microbiomes implies that lichens should instead be described as multi-species symbioses, or complex microbial ecosystems, rather than dual symbioses (Grube *et al.*, 2009; Aschenbrenner *et al.*, 2016; Grube *et al.*, 2015; Cernava *et al.*, 2019).

Evidence from multi-omics approaches have provided evidence for the hypothesis that these host-associated bacterial communities functionally contribute to the lichen meta-organism, with all partners of the lichen symbiosis having specific functions within the lichen community (Grube *et al.*, 2015; Cernava *et al.*, 2017; Eymann *et al.*, 2017). The role of the lichen microbiome is thought to contribute to the survival and stability of the overall symbiosis, with lichen-associated bacterial communities being found to have roles in the supply of nutrients, stress resistance (both biotic and abiotic), support for photosynthesis and growth of the mycobiont and

photobiont, metabolite detoxification, and degradation of older thallus parts. These various identified functions suggest that lichen-associated bacteria interact with both the mycobiont and photobiont (Grube *et al.*, 2015; Cernava *et al.*, 2017). Further to this, it is thought that, in return for contributing to the survival of the lichen thallus and the symbiome as a whole, the cell wall components of the lichen-forming fungus and extracellular polysaccharides provide a source of nutrients for the lichen-associated microbiome (Grube *et al.*, 2009; Grube *et al.*, 2015). However, the lichen-associated microbiome has not been found to have a role in nitrogen fixation which is, instead, carried out by the cyanobiont (Eymann *et al.*, 2017).

The complexity of the lichen symbiosis is further increased due to interactions with lichenicolous fungi which occur on or in lichens (Nash III, 2008a). There are approximately 2300 species of lichenicolous fungi described, and are found within both the Ascomycota (96% of taxa) and Basidiomycota (4% of taxa) (Diederich *et al.*, 2018). Lichenicolous fungi, which are different from the dominant fungal partner, form an obligate association with their lichen host. The specificity of lichenicolous fungi to their host is thought to be high, with 95% of lichenicolous fungi appearing to associate with a single lichen genus (Lawrey and Diederich, 2003; Muggia and Grube, 2018). The relationship can either be saprophytic (the lichenicolous fungus colonises a dead lichen thallus) or parasitic (the living lichen host provides fixed carbon to the lichenicolous fungus) in nature (Lawrey and Diederich, 2003). Further to this, the nature of the parasitic relationships can vary, with the occurrence of commensalistic relationships that do not cause damage to the lichen host as well as more aggressive relationships which result in damage (i.e. lesions and discolourations) to the lichen thallus. The majority of lichenicolous species are weak parasites of either the mycobiont, the photobiont or, in some cases, both. In addition to these lichen parasites, there are lichenicolous fungi derived from lichens, which are searching for a photobiont and will eventually become independent from the lichen host. There are also examples of lichenicolous lichens which maintain a separate photobiont whilst colonising a lichen host; these types of lichenicolous lichens are not defined as parasites because they employ the same nutritional strategy as other lichens, obtaining nutrients from its photobiont rather than the lichen host it associates with (Lawrey and Diederich, 2003; Diederich *et al.*, 2018).

In further support for the concept of lichens as metacommunities, a study by Spribille *et al.* (2016) found that, in many common lichens across a range of taxonomic groups, the symbiosis also comprises a specific basidiomycete yeast in addition to the core relationship between the ascomycete fungus and its photosynthetic partner, providing further support for the concept of lichens as metacommunities. The basidiomycete yeast was identified as belonging to the class *Cystobasidiomycetes* and was found to be located in the cortex of the lichen thallus (Figure 1.1). The location of these yeast cells could perhaps suggest that more than one fungus is involved in the construction of the cortex, thus challenging the accepted notion that the lichen thallus is formed solely by the dominant fungal partner. In addition, the cortex is involved in thallus structure and transport as well as being responsible for the production of some secondary metabolites, and the location of the yeast cells within the cortex suggests that these basidiomycete yeasts could have a regulatory role in the lichen symbiosis. Furthermore, the presence of a basidiomycete yeast within the lichen thallus is thought to be correlated with variations in the lichen phenotype (Spribille *et al.*, 2016). However, further research also found the presence of *Cystobasidiomycete* yeasts in species of lichens that either possessed a partial cortex or did not possess a cortex at all, meaning that it is likely that these yeasts either exist as a superficial biofilm or inhabit the thallus without associating with the cortex (Černajová and Škaloud, 2019). Furthermore, the hypothesis postulated by Spribille *et al.* (2016) that basidiomycete yeasts are present within a diverse range of lichen taxa has been challenged in a study conducted by Lendemer *et al.* (2019), in which they failed to detect the presence of basidiomycete yeast in 97.3% of sampled lichen species. This, therefore, casts doubt upon the hypothesis that basidiomycete yeasts are an obligate component of the lichen metacommunity (Oberwinkler, 2017; Lendemer *et al.*, 2019). In addition to this, recent research has observed a much lower mycobiont specificity of cystobasidiomycete yeasts compared to the photobiont, therefore suggesting that the cystobasidiomycete yeasts are not as closely associated with the symbiosis (Mark *et al.*, 2020).

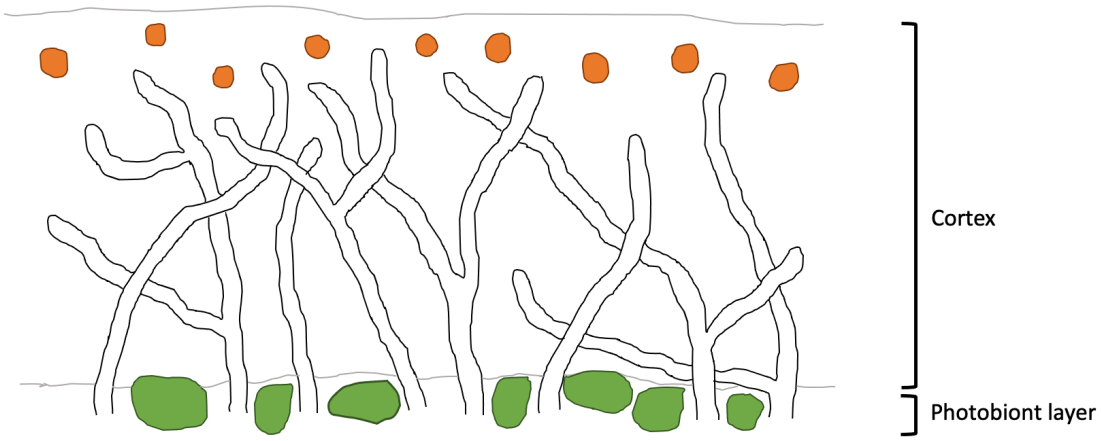


Figure 1.1. Location of basidiomycete yeast within lichen thallus. The yeast cells, shown in orange, are found interspersed with the hyphae of the ascomycete mycobiont in the cortex of the lichen thallus, surrounded by an extracellular polysaccharide matrix. Photobiont cells are shown in green. Diagram not to scale. Adapted from Jenkins and Richards (2019), and Spribille *et al.* (2020).

1.2.2 Lichen Systematics

Lichen-forming fungi, of which there are over 19,000 species, make up nearly half of known ascomycetes and approximately a fifth of all fungal species. Although the majority of fungal species that follow this evolutionarily successful lifestyle are ascomycetes, with 99% of lichen-forming fungi belonging to the subphylum Pezizomycotina, approximately 1% of lichen-forming fungal species are basidiomycetes (Stocker-Wörgötter, 2008; Honegger *et al.*, 2013; Lücking *et al.*, 2016). Historically, lichens were treated as a systematic unit and, as a result, were organised into their own group known as the 'Lichenes', a level equal to that of the ascomycetes and basidiomycetes (Tehler and Wedin, 2008). This now-obsolete classification was based upon the presence of the thallus that is produced as a result of the symbiosis, with lichens being further divided based upon the morphology of fruiting bodies (Honegger, 2008a; Tehler and Wedin, 2008; Lücking *et al.*, 2016). This view of lichens as a systematic unit predominated for the majority of the 20th Century. However, the classification of lichens as a separate group was greatly debated, specifically whether the lichens should be classified separately into their own group or instead classified in the fungal system. Over the course of the 20th Century, lichen-forming fungal species were gradually integrated into the fungal system. The classification of lichen species was increasingly based upon various morphological characteristics, namely the development of ascomata and the structure of the ascus. In addition to this, the

taxonomic classification of lichens was strongly influenced by the chemical characteristics of lichen species, through the use of secondary metabolites as chemical markers (Tehler and Wedin, 2008; Stocker-Wörgötter, 2008; Lücking *et al.*, 2016).

Lichens are regarded as being polyphyletic, that is lichenisation and delichenisation events have occurred independently multiple times in the phylogenetic tree (Gargas *et al.*, 1995; Lutzoni *et al.*, 2001). This observation is supported by the fact that various taxonomically-unrelated groups of fungi contain both lichenised and non-lichenised fungal species and, therefore, means that lichens cannot be regarded as a monophyletic group (Honegger, 1993; Nash III, 2008a; Tehler and Wedin, 2008). Although lichens are composed of at least two partners, the species name of a lichen is the same as that of the mycobiont. This species name does not refer to the photobiont, which has its own species name (Honegger, 2001).

1.2.3 Habitats

The success of the lichen symbiosis has meant that lichens are able to grow in most types of habitat, including terrestrial as well as freshwater and marine ecosystems (Nash III, 2008a; Pankratov *et al.*, 2017). Approximately 10% of terrestrial habitats are dominated by lichens, with these ecosystems (such as arctic, antarctic, desert and high alpine ecosystems) being those where vascular plants are at their physiological limits (Honegger, 2009). It is their adaptations to extreme environmental conditions that have enabled lichens to colonise almost all terrestrial regions, from tropical to polar regions. Further to this, lichens follow a poikilohydric lifestyle, meaning that their water status changes with that of the surrounding environment. However, lichens are able to obtain water from other sources, including fog and dew; under low-temperature and high-humidity conditions, lichens are also able to obtain moisture from non-saturated air (Nash III, 2008a; Stocker-Wörgötter, 2008; Aschenbrenner *et al.*, 2016). Furthermore, lichens have the ability to colonise almost any substrate type, including rocks, plants and bare soils in addition to man-made materials (Crittenden and Porter, 1991; Nash III, 2008a; Aschenbrenner *et al.*, 2016). However, even though lichens possess a diverse range of ecological adaptations, most species of lichen are known to be highly sensitive to any changes that may occur in their surrounding environment or micro-habitat. This adaptation to

specific ecological conditions means that lichens are rarely found to grow in non-native habitats (Boustie and Grube, 2005; Stocker-Wörgötter, 2008).

The success of the lichen symbiosis has resulted in both the mycobiont and the photobiont being found in ecosystems which they would otherwise rarely inhabit, or not be able to inhabit at all. The benefits of lichenisation to the photobiont include being able to occur in dry habitats when part of a lichen. This contrasts with the aposymbiotic forms of green algae and cyanobacteria, which are commonly found in aquatic or moist terrestrial habitats. In addition, the lichen symbiosis allows the photobiont to inhabit sites where there are high levels of light, as the fungal partner is able to reduce the intensity of light that the photobiont is exposed to (Nash III, 2008a).

1.2.4 Environmental Role of Lichens

Although lichens are found in the majority of terrestrial habitats, there is variation in their contribution to an ecosystem in terms of biomass (Kershaw, 1985; Nash III, 2008a; Seaward, 2008). The slow growth rate of lichens means that their contribution is quite small in the majority of ecosystems (Nash III, 2008a). However, lichen species possessing a faster growth rate have the ability to rapidly increase their biomass and can, therefore, significantly contribute to an ecosystem in terms of biomass. This enables lichens to be a major contributor in the formation and functioning of ecosystems, supporting a diverse range of organisms through the creation of food webs and by adding to energy flow and the cycling of minerals within the ecosystem (Nash III, 2008c; Seaward, 2008; Pankratov *et al.*, 2017).

The major long-term role of lichens is considered to be that of biological weathering agents, being involved both physically and chemically in the formation of soil (Syers and Iskandar, 1973; Seaward, 2008). Further to this, the majority of lichen species are able to obtain nutrients from the air, in addition to those lichen species with a cyanobacterial photobiont carrying out the fixation of atmospheric nitrogen. Nutrients that have been accumulated by lichens are then able to reach other members that make up the ecosystem through the processes of leaching and decomposition, as well as through consumption by animals (Nash III, 2008b; Nash III, 2008c; Asplund and Wardle, 2017). Lichens are also thought to provide habitats for a

range of invertebrate animals which may also utilise lichens as a source of food (Seaward, 2008; Asplund and Wardle, 2017).

It has also been suggested that the structural and functional differences that occur between lichens have a strong effect on how lichens interact with other members of the ecosystem, and thus impacts upon the role of lichens in the environment as community and ecosystem drivers. These functional differences include growth form, symbiont associations, water retention capacity, specific thallus mass, nutrient concentrations, secondary compounds and colour (Asplund and Wardle, 2017).

1.3 Development and Growth of Lichens

Lichens have the ability to grow in habitats in which sources of nutrients are poor and drought frequently occurs. These stress-tolerant symbioses are well known to be slow-growing and long-lived; these lichen characteristics are enabled through the balance of respiration and photosynthesis. It is thought that the fast growth of lichen-forming fungi would not provide a selective advantage for lichens when growing in their natural habitats, as this would result in the photobiont being outgrown by the mycobiont (Crittenden and Porter, 1991; Ahmadjian, 1993; Grube and Wedin, 2016). Observations of the various structures of lichens suggest that their growth patterns, in which the growth of the mycobiont and the photobiont is thought to occur in a coordinated manner, are controlled by complex regulatory mechanisms (Honegger, 1993). Furthermore, a recent genome analysis by Armaleo *et al.* (2019) of the lichen mycobiont *Cladonia grayi* and its algal photobiont *Asterochloris glomerata* reached the conclusion that lichen mycobionts are committed to the symbiosis. This means that the fungal partner has to adapt its growth rate to the carbon source provided by its photosynthetic partner.

1.3.1 Lichen Morphology

The unique vegetative structure that makes up the lichen, known as the thallus, is a complex structure which is not found in other fungal species that have not adopted the lichenised lifestyle (Aschenbrenner *et al.*, 2016; Grube and Hawksworth, 2007). Although it is believed that the fungal partner is the main determinant with

regards to the morphology of the thallus, the development of the lichen thallus only occurs once the symbiosis has been established (Jahns, 1988; Büdel and Scheidegger, 2008). Regardless of the morphology of a lichen, the thallus is required to allow optimal gain of carbon dioxide in order for net levels of photosynthesis carried out by the photobiont to be positive and, therefore, for growth of the lichen to occur. This means that levels of light reaching the photobiont, as well as diffusion of carbon dioxide to the photobiont, should be sufficient to allow photosynthesis to occur. In addition, the morphology of the lichen thallus is adapted to ensure that appropriate levels of water uptake or loss occur within a given habitat, again ensuring that sufficient levels of carbon dioxide reach the photobiont regardless of the hydration conditions (Ahmadjian, 1993; Büdel and Scheidegger, 2008).

Although the classification of lichen species is no longer based on the growth form of the lichen thallus, lichens can still be divided into groups relating to the growth form that they adopt (Jahns, 1973). The three main morphological growth types used to describe lichens are the crustose (form a flattened crust), foliose (leaf-like) and fruticose (shrub-like) growth forms (Büdel and Scheidegger, 2008). The structures of foliose and fruticose thalli, which are generally regarded as equal in their level of development, are more complex than the structure of crustose thalli. For this reason, crustose thalli can be described as primitive (Jahns, 1973). Crustose lichens form a strong attachment to the substrate via their lower surface, resulting in the thallus and substrate being almost inseparable from each other. The complexity of crustose thalli varies: some lichens lack an organised thallus, whereas the majority of crustose lichens form a stratified thallus. The thalli of foliose lichens, which only partially attach to the substrate, consist of flattened, leaf-like lobes and have distinct upper and lower surfaces. Foliose lichens vary in terms of thallus size and diversity. Fruticose lichen thalli are made up of strap-shaped or hair-like lobes which can either be flat or cylindrical; their thalli are either arranged radially or dorsiventrally (Jahns, 1973; Büdel and Scheidegger, 2008; Armstrong and Bradwell, 2011). There are also other groups which can be used to describe lichens, such as gelatinous lichens, however these additional groups can be included within the three main morphological groups previously mentioned (Büdel and Scheidegger, 2008).

In terms of their structure, the thalli of lichens are described as either homoiomerous or heteromerous (Jahns, 1973; Büdel and Scheidegger, 2008). Homoiomerous, non-stratified thalli have an even distribution of the fungal and photosynthetic partners (Figure 1.2A); the mycobiont is thought to grow over and encompass the photobiont cells found in the free-living state. Lichen species possessing this morphologically simple thallus structure are known as microlichens, and include thin crustose lichens as well as gelatinous crustose and gelatinous foliose lichen species (Honegger, 1991a; Honegger, 1993; Büdel and Scheidegger, 2008). The majority of lichens (including many species of crustose lichens), however, develop a heteromerous, internally-stratified thallus; lichen species adopting this thallus structure are usually termed macrolichens (Honegger, 1993; Büdel and Scheidegger, 2008). The thallus of a macrolichen, which rises above the substratum, is more evolved in terms of its morphology and is distinct from the morphology of lichen-forming fungi in the aposymbiotic state (Honegger, 1993; Ahmadjian, 1993). Heteromerous thalli can be divided into four main layers: the upper cortex, photobiont layer, medulla and lower cortex, as shown in Figure 1.2B (Büdel and Scheidegger, 2008).

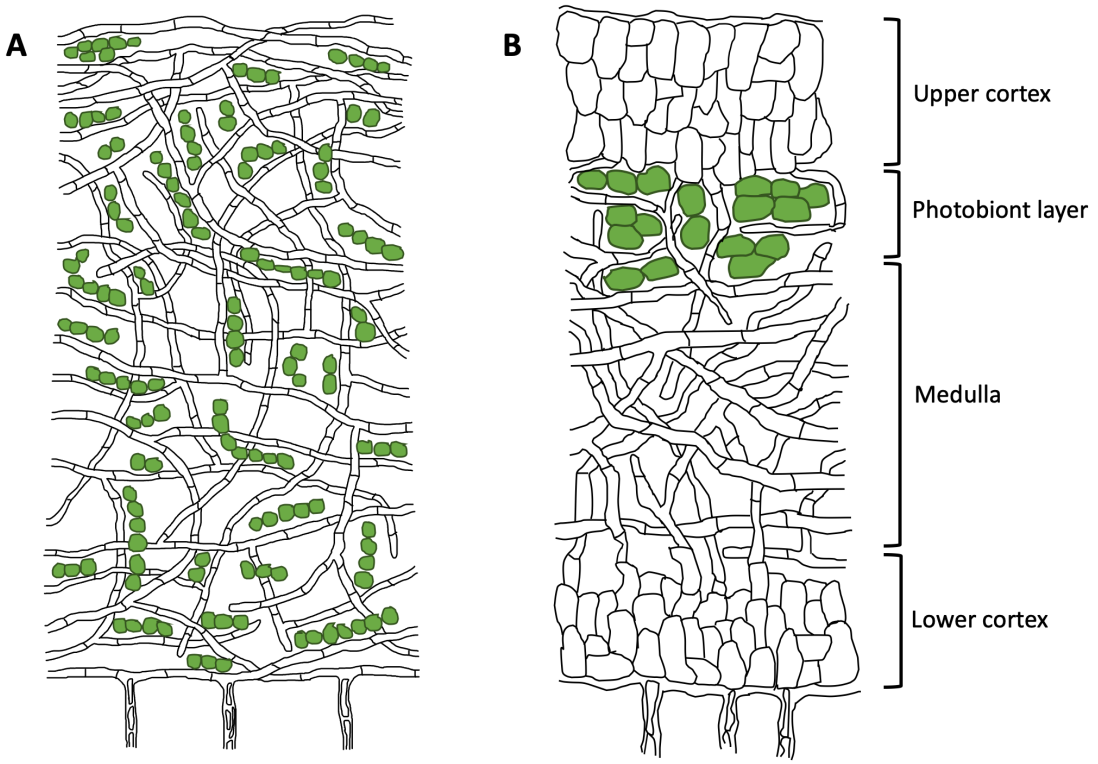


Figure 1.2. Structure of lichen thalli. Diagrams are not to scale. **(A)** Homoiomerous thallus. The cross-section shows the structure of the thallus from a *Collema* species in which the fungal hyphae are loosely interwoven and the photobiont cells are distributed evenly throughout the thallus. Adapted from Schneider (1897). **(B)** Heteromerous thallus. The cross-section shown demonstrates the typical structure of a foliose lichen thallus, with the photobiont cells shown in green. The upper cortex, medulla and lower cortex are made up of fungal hyphae; these hyphae are either loosely interwoven or are so densely interwoven that individual hyphae cannot be distinguished. Adapted from Smith *et al.* (2009), and Moore *et al.* (2011).

The majority of the thallus biomass is made up of the fungal partner and contains at least two types of fungal hyphae: loosely interwoven aerial hyphae and densely interwoven conglutinate hyphae (Crittenden and Porter, 1991; Honegger, 1993). The upper cortex and lower cortex are both composed of conglutinate fungal hyphae but differ in terms of their pigmentation, with the lower cortex usually being strongly pigmented (Büdel and Scheidegger, 2008). The upper and lower cortices are thought to possess a range of functions, including providing mechanical stability to the thallus, protecting the photobiont from high levels of light, and providing defence against herbivores. The cortical layers of the lichen thallus are also involved in the uptake of water; it is believed that the anatomical and morphological features, as well as the chemical composition, of the cortex influence

the rate of water uptake (Larson, 1984; Reutimann and Scheidegger, 1987; Kappen, 1988; Honegger, 1991b; Grube and Wedin, 2016). The medulla is made up of a layer of aerial hyphae, with the photobiont layer being found in the upper part of the medulla underneath the upper cortex (Jahns, 1973; Honegger, 1991b). There are high levels of airspace between the hyphae, meaning that the medullary layer constitutes the major component of the internal volume of the thallus (Büdel and Scheidegger, 2008). Secondary metabolites are often found to be deposited as crystals in the medullary layers of thalli; their presence results in the hyphal cells being hydrophobic, thus ensuring that the airspace between the hyphae remains during wet conditions and that gas exchange can occur within the thallus (Jahns, 1973; Honegger, 1986a; Honegger, 1991b; Rikkinen, 2002). In addition to these layers, lichen thalli also have attachment organs which generally develop from the lower cortex but can sometimes be found to develop from either the upper cortex or thallus margin. These attachment structures, which function to anchor the thallus to the substrate, include rhizines (hair-like structures composed of conglutinate hyphae) as well as basal hyphae and rhizoidal hyphae which penetrate to varying degrees into the substrate (Jahns, 1973; Jahns, 1988; Büdel and Scheidegger, 2008).

In cephalodiate lichen species, in which the lichen-forming fungus associates with a green alga and a cyanobacterium simultaneously, the secondary cyanobacterial photobiont is usually found in additional specialised structures known as cephalodia (Figure 1.3), although in some lichen species the cyanobacterial photobiont instead forms a second layer underneath the primary algal photobiont. The morphology of cephalodia varies depending on the lichen species, and can be found both internally in the medulla and externally on the upper or lower surfaces of the thallus. However, it is generally completely different to the morphology of the thallus formed with the primary algal photobiont (Jahns, 1973; Büdel and Scheidegger, 2008; Grube and Wedin, 2016).

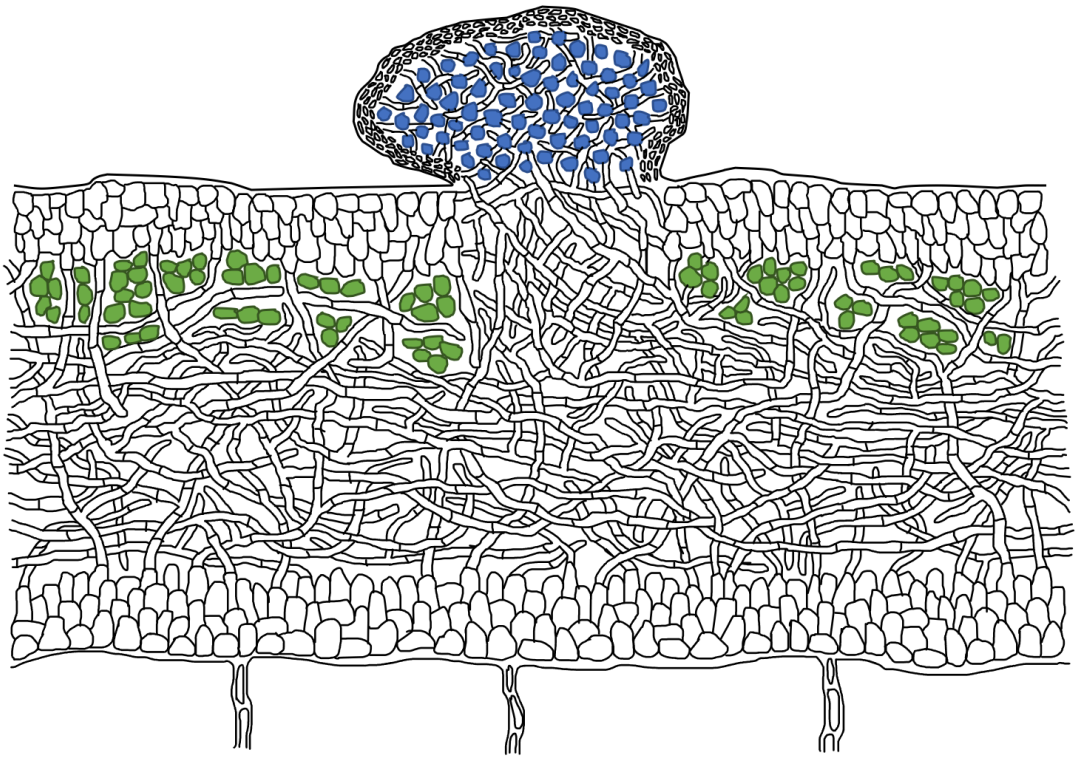


Figure 1.3. Structure of a cephalodiate thallus. Diagram not to scale. The cross-section shows the typical structure of a lichen thallus with a cephalodium located on the upper cortex. The cephalodium contains the cyanobacterial secondary photobiont and differs in structure to the main thallus. Adapted from Rikkinen (2002).

1.3.2 Morphogenesis

1.3.2.1 Photobiont Recognition and Compatibility

Due to the fact that most lichenised fungal species reproduce sexually, the lichen symbiosis has to be re-established after each reproductive cycle (Honegger, 2008b). The majority of lichen-forming fungal species display a moderate specificity in terms of the photobiont that they associate with (they accept several closely-related species as their photobiont), however many mycobionts have high levels of selectivity (Honegger, 1993). In this context, specificity describes the phylogenetic range of compatible partners, whereas selectivity refers to the availability of these compatible partners in natural habitats and the preferential, but not exclusive, association with a particular compatible partner (Ahmadjian, 1993; Yahr *et al.*, 2006; Honegger, 2008b).

It is thought that the variation in photobiont specificity that is present in species of lichen-forming fungi is associated with the morphology of the lichen thallus; lichen species that form more morphologically-advanced, internally-stratified thalli

(that is, foliose and fruticose thalli) appear to display higher levels of specificity than crustose lichen species that form less morphologically-advanced, non-stratified thalli (Honegger, 2008b). Furthermore, these varying levels of specificity correlate with the geographic range of a lichen species, with the same lichen species located in different habitats observed to associate with related photobiont species that are adapted to the particular climatic conditions of the habitat (Blaha *et al.*, 2006; Fernández-Mendoza *et al.*, 2011). This enables lichen species to expand their geographical range, and supports the hypothesis, described by Rodriguez *et al.* (2008), that the habitat conditions determine the preference of the lichen-forming fungal species for a particular photobiont (Yahr *et al.*, 2006; Fernández-Mendoza *et al.*, 2011, Grube and Wedin, 2016).

1.3.2.2 Relichenisation

The relichenisation process is thought to involve multiple developmental stages, which lead from recognition and initial contact of the mycobiont and photobiont cells to the development of a stratified thallus (Armaleo, 1991; Honegger, 2008b). However, the symbiotic phenotype characteristic of each lichen-forming fungal species only occurs when the mycobiont associates with a compatible photosynthetic partner (Honegger, 1993; Honegger, 2008b). The initial unspecific contact of mycobiont hyphae with either compatible or non-compatible photobiont cells leads to the formation of a non-stratified pre-thallus which will only develop into a fully stratified thallus with species-specific characteristics, when an association with a compatible photobiont has been formed (Honegger, 1993; Trembley *et al.*, 2002; Joneson and Lutzoni, 2009). As a result of the low probability of the mycobiont quickly coming into contact with a compatible photobiont, it is thought that the ability to form a pre-thallus with a non-compatible photobiont is a strategy adopted by lichen-forming fungal species to allow survival of the mycobiont until it is able to associate with a compatible photosynthetic partner (Honegger, 1993; Trembley *et al.*, 2002; Insarova and Blagoveshchenskaya, 2016).

In the early stages of entry into the lichen symbiosis, evidence has been found of changes in gene expression in each of the symbiotic partners. Gene expression is influenced both initially when the mycobiont and photobiont cells are physically

separate but in close proximity to each other, as well as after the mycobiont and photobiont cells come into contact (Trembley *et al.*, 2002; Joneson *et al.*, 2011). The exact stimuli and signal compounds required for the mycobiont to recognise the photobiont are not currently known, although it is believed that the mycobiont and photobiont both produce compounds which are involved in recognition prior to the contact stage of lichenisation (Honegger, 1993; Trembley *et al.*, 2002; Honegger, 2008b; Insarova and Blagoveshchenskaya, 2016). Regardless of the exact mechanisms by which recognition occurs, the mechanisms are initiated in the pre-contact stage of lichenisation (Meeßen and Ott, 2013). Recent work has found that during the second stage of lichenisation, in which the symbiotic partners come into contact with each other, lectins are involved in the recognition of a compatible partner. These glycoproteins cause the agglutination of cells from different origins. Recognition involves the production of lectins by the mycobiont which possess an arginase activity; one lectin is localised on the surface of the mycobiont hyphae (known as algal binding protein, ABP), and the other lectin is secreted. These fungal lectins bind to a polygalactosylated urease located on the cell wall of the photobiont. It is believed that the secreted lectins are involved in attracting the photobiont cells to the fungal mycelium, whereas ABP provides a link between the mycobiont hyphae and photobiont cells. Thus, the mycobiont is able to recognise a compatible partner by binding to a specific receptor on the photobiont cell wall. In addition to its role in the recognition of compatible partners, the photobiont also synthesises the lectin ligand as a defence mechanism in order to protect against enzymatic attack by the fungus (Vivas *et al.*, 2010; Singh and Walia, 2014; Insarova and Blagoveshchenskaya, 2016). Once this protection has been achieved, additional mechanisms of cell communication are required to produce the fully-differentiated thallus (Rikkinen, 2002; Vivas *et al.*, 2010).

Studies investigating the resynthesis of lichenisation have found that the mycobiont and photobiont cells are initially bound to each other via a non-specific mucilage that is secreted by peripheral fungal cells of the pre-thallus (Honegger, 1993; Trembley *et al.*, 2002; Honegger, 2008b). This is followed by the growth of mycobiont hyphae towards the photobiont cells and, subsequently, the mycobiont comes into contact with the photobiont. Once this contact has formed, there is an increased

incidence of hyphal branching and fungal appressoria are formed on the surface of the photobiont cells (Trembley *et al.*, 2002; Joneson and Lutzoni, 2009). This increased hyphal branching leads to the envelopment of the photobiont cells, and is followed by the incorporation and aggregation of the fungal and algal cells into an undifferentiated mass before the eventual development of a stratified thallus (Armaleo, 1991; Honegger, 1993; Joneson and Lutzoni, 2009). The later stages of lichenisation have not been described at the molecular level as a result of difficulties associated with achieving growth of the later developmental stages in the lab (Honegger, 2008b; Joneson *et al.*, 2011).

Studies of gene regulation during the early stages of relichenisation have shown that although the induction and repression of genes in both symbiotic partners is required, only a small proportion of genes are induced. However, these induced genes may still have an important role in the early stages of relichenisation. The majority of genes showing changes in expression during the early stages of relichenisation appear to be repressed, thus suggesting that down-regulation of genes is important in the lichenisation process (Trembley *et al.*, 2002; Honegger, 2008b; Joneson *et al.*, 2011). It has been proposed that the repression of genes occurs through DNA methylation, with studies observing that methylation of fungal DNA is correlated with being in the lichenised state (Armaleo and Miao, 1999). This method of gene repression to regulate the lichen symbiosis could perhaps provide an explanation as to how the partners of the symbiosis remain capable of growing in the aposymbiotic state (Trembley *et al.*, 2002).

1.3.2.3 Mycobiont-Photobiont Interface

The interface between the mycobiont and the photobiont is dependent upon the taxonomy of the partners that make up the symbiosis (Honegger, 2008b). The mycobiont-photobiont interface in the majority of lichen species involves haustoria, specialised hyphal branches formed by the mycobiont, which cover the surface of the photobiont cells (Ahmadjian, 1993; Rikkinen, 2002). This sealing of photobiont cells by hydrophobic fungal hyphae forms an apoplastic continuum between the symbiotic partners which allows the passive movement of water and dissolved nutrients between the mycobiont and photobiont. The water-repellent nature of the thallus

interior, thought to be conferred by hydrophobins located within the hyphal cell wall, is crucial to the functioning of the symbiosis as it enables the maintenance of continued gas exchange for the photobiont (Honegger, 1991b; Honegger, 1997; Dyer, 2002). The functions of haustoria also include the movement of carbohydrates from the photobiont to the mycobiont, as well as assisting in the positioning of photobiont cells within the lichen thallus (Honegger, 1997; Rikkinen, 2002).

In lichen species containing an algal photobiont, there is a correlation between the morphology of the thallus and the type of haustoria formed by the mycobiont (Honegger, 1986b). Generally, mycobionts that form more morphologically-simple crustose thalli produce finger-like intracellular haustoria which pierce the cell wall of the alga and are in contact with the plasma membrane of the algal cell. This differs to mycobionts producing morphologically more complex stratified thalli, which instead produce one of three types of intraparietal haustoria that invade the cellulosic cell wall of the photobiont, but do not actually penetrate the cell wall. Lichens possessing a stratified crustose thallus produce haustoria formed from a tightly-adhering fungal hypha within the algal cell wall. The other two types of intraparietal haustoria are either found as an intracellular protrusion in which the fungal hypha is enveloped in the algal cell wall or, in the case of the most evolved haustorial type, as a stalked structure (Honegger, 1984; Honegger, 1986b; Honegger, 1991a). In addition to these two types of interaction between the mycobiont and photobiont, an intermediate interaction can also occur in which the mycobiont produces short, globose protrusions that are enveloped by the cell wall of the alga (Honegger, 2008b). In lichen species which associate with a cyanobacterial photobiont, a range of different interactions can occur at the mycobiont-photobiont interface (Rikkinen, 2002). These interactions include the positioning of cell walls next to each other or, as is the case for the majority of cyanobacterial lichen species producing stratified thalli, the formation of intragelatinous protrusions. Although some cyanolichen species are found to produce intracellular haustoria, the cell walls of the mycobiont and photobiont in cyanolichens do not usually form direct interactions, unlike in chlorolichens. Instead, the fungal hyphae penetrate the gelatinous sheath of the photobiont (Büdel and Rhiel, 1987; Honegger, 1997; Rikkinen, 2002).

1.3.2.4 Photosymbiodemes

Although the lichen thallus is predominantly made up of fungal mass and the growth form of the thallus is mainly determined by the fungal partner, it appears that the photobiont has an influence on the expression of the symbiotic phenotype, with the same mycobiont producing thalli with different morphologies depending on whether its photosynthetic partner is a green alga or a cyanobacterium. This ability of mycobionts to produce different morphotypes depending on its photobiont association, therefore, indicates that the symbiotic phenotype of the lichen-forming fungus is both caused and determined by the photobiont (Armaleo and Clerc, 1991; Crittenden and Porter, 1991; Honegger, 1993; Nash III 2008a; Büdel and Scheidegger, 2008; Honegger, 2008b).

Differing morphotypes are observed in cephalodiate lichenised fungal species that associate with algal and cyanobacterial photobionts simultaneously (tripartite lichens) as well as in lichen-forming fungal species that form consecutive symbiotic associations with green algae and cyanobacteria (termed photosymbiodemes) (Rikkinen, 2002; Honegger, 2008b). In the case of tripartite cephalodiate lichens, the simple morphology of the cephalodia, in which the secondary cyanobacterial photobiont is located, is notably different from the rest of the vegetative thallus which contains the primary green algal photobiont (Honegger, 1993). The morphology of the morphotype pairs formed by the mycobiont in photosymbiodemes, for example in *Peltigera* species, can either be similar (isomorphic) or dissimilar (heteromorphic), and are generally described in the literature as different species and sometimes even different genera (Brodo and Richardson, 1978; Honegger, 1993; Honegger, 2008b). The different morphotypes formed can be found as individual thalli (termed chlorosymbiodemes or cyanosymbiodemes, depending on the photobiont) or as intermixed thalli in which the two growth forms are joined together; these composite thalli are known as lichen chimerae (Armaleo and Clerc, 1991; Rikkinen, 2015).

1.3.3 Growth

1.3.3.1 Growth Patterns

The growth of the morphologically simple crustose lichens resembles the growth of moulds, involving the mycobiont growing over and enveloping photobiont cells either on or within the substrate where compatible photobiont cells are found (Honegger, 2008a; Honegger, 2008b). This differs considerably to the growth observed in the morphologically more-advanced foliose and fruticose lichens, in which the growth of the mycobiont and photobiont occur in a coordinated manner (Honegger, 1993; Honegger, 2008b). In this coordinated growth, the thallus can be divided into three zones, consisting of a pseudomeristematic marginal rim, a subapical elongation zone and a fully-differentiated thalline area (Honegger, 2008b). The majority of new cells are produced at the thallus margin, and the pseudomeristematic zone is typically characterised by high levels of cell division and small cell sizes of both the mycobiont and the photobiont (Hill, 1985; Hill, 1989; Hill, 1992; Honegger, 1993). Studies observing thallus growth after fragmentation have suggested that the fixed carbon required for growth to occur is not acquired from the centre of the thallus, but is instead produced in the thallus margins (Armstrong, 1974; Armstrong, 1979) The cells of the symbiotic partners grow to their full size in the elongation zone, with these fully-differentiated cells characterised by a low turnover rate (Honegger, 1993). In addition, a large number of oversized photobiont cells are found in the fully-differentiated, adult areas of the thallus (Hill, 1985; Hill, 1989). Growth of the lichen thallus mainly occurs at the margins and elongation zones but is not solely restricted to these areas, with the older regions of the thallus retaining their ability for expansion (Honegger, 2008b).

Cell division in photobiont cells is thought to be controlled by the mycobiont, with the cell cycle of the photosynthetic partner permitted in growing thalline areas and arrested in areas of the thallus where growth is not occurring. The regulatory mechanisms responsible for the coordinated growth observed in foliose and fruticose lichens are not well-studied, however various methods have been proposed which enable mycobiont control of the photobiont cell population. These include insufficient levels of fixed carbon (after loss to the mycobiont) and nutrients for cell division of the photobiont to occur, as well as the presence of inhibitory compounds produced by the

fungal partner (such as usnic acid and atranorin) preventing division of the photobiont cells (Hill, 1992; Ahmadjian, 1993; Honegger, 1993; Honegger, 2008b; Bačkor *et al.*, 2010; Lokajová *et al.*, 2014).

When the mycobiont of a lichen species is grown axenically, it does not produce the stratified thallus that is characteristic of lichens, but instead grows as a mass of undifferentiated hyphae (Crittenden and Porter, 1991). The stroma formed by the aposymbiotic fungus can sometimes display some resemblance to the characteristic structure of a lichen thallus, in which the fungal hyphae form a thallus-like structure consisting of conglutinate cells in the centre and aerial hyphae at the periphery. However, the symbiotic phenotype is much more complex and more highly differentiated (Jahns, 1973; Ahmadjian, 1993; Honegger, 2008a). This, as discussed previously, suggests that the presence of the photobiont causes the expression of the symbiotic phenotype in the mycobiont early in the process of lichenisation, with changes in gene expression occurring in both the fungal and photosynthetic partners. This results in a morphogenetic cascade that leads to the formation of the characteristic lichen thallus (Trembley *et al*., 2002; Joneson *et al.*, 2011).

1.3.3.2 Growth Requirements

The growth of a lichen is dependent upon the acquisition of carbon (the primary limiting factor associated with the growth capacity of a lichen) as well as other nutrients such as nitrogen and phosphorous, which are involved in the production of new nucleic acids, proteins and membranes (Palmqvist *et al.*, 2008; Nash III, 2008b). The micronutrients and macronutrients required by the lichen for primary metabolism are either positively-charged cations or negatively-charged anions. However, the other nutrients required by lichens, in addition to carbon and nitrogen, for growth are not well understood. This is due to the fact that whole lichens cannot currently be easily grown in culture. It has not been established whether the elements required for normal growth by most higher plants are also needed for growth in lichens (Nieboer *et al.*, 1978; Crittenden and Porter, 1991; Nash III, 2008c).

Carbon production in lichens occurs as a result of the photobiont carrying out photoassimilation during photosynthesis, whereas the acquisition of other minerals occurs across the entire lichen thallus through wet or dry deposition (Palmqvist *et al.*,

2008; Nash III, 2008c). Therefore, with the fungal partner being quantitatively predominant in the lichen thallus, it is thought that the mycobiont is responsible for the accumulation of the majority of the minerals required. However, there is an exception to this in terms of lichen species that contain a cyanobacterial photobiont, in which the cyanobacteria provide fixed nitrogen for the lichen (Millbank and Kershaw, 1969; Ahmadjian, 1993; Palmqvist *et al.*, 2008; Nash III, 2008b).

The fixed carbon that is required by the mycobiont to increase its biomass is synthesised by the photobiont through the process of photosynthesis, in which carbon dioxide is reduced to form carbohydrates. This, therefore, means that the growth capacity of a lichen is limited by the rate of photosynthesis carried out by the photobiont (Richardson *et al.*, 1968; Palmqvist *et al.*, 2008). The type of carbohydrate that is produced by the photosynthetic partner is dependent upon the type of photobiont that is present in the symbiosis; cyanobacteria produce glucose, whereas algal partners produce sugar alcohols known as polyols (either sorbitol, ribitol or erythritol) (Drew and Smith, 1966; Richardson and Smith, 1968; Richardson *et al.*, 1968; Galun, 1988).

The majority of these carbohydrates are then translocated from the photosynthetic partner to the fungal partner (Smith, 1978; Ahmadjian, 1993). Lichens do not possess specific cells or tissues which are involved in the translocation of water, metabolites and nutrients between the symbionts (Palmqvist, 2000). This, therefore, means that translocation between the partners differs depending on the lichen species, and is also affected by the chemical composition of the cell walls of the symbionts as well as by the nature in which they are integrated (Richardson *et al.*, 1968; Honegger, 1991b). Further to this, it has been suggested that the efficiency of carbohydrate translocation is affected by the hydration level of the thallus, with species-dependent variation in how the translocation efficiency is altered (Palmqvist *et al.*, 2008). The exact mechanisms triggering the translocation of the carbohydrates are unknown (Palmqvist, 2000). In addition, a specific glucose or polyol transporter has, as yet, not been identified in lichens, although Yoshino *et al.* (2019) have recently reported the conservation of polyol transporters in the Ascomycota and the expansion of polyol transporter genes during Lecanoromycete evolution (Honegger, 1991b; Palmqvist *et al.*, 2008; Yoshino *et al.*, 2019). Furthermore, transcriptome studies are

now providing insights into the nature of carbon transfer in lichens through their identification of transporters which are potentially significant in the translocation of carbon between the symbiotic partners (Wang *et al.*, 2014c). Once the carbohydrates have been taken up by the mycobiont, they are irreversibly converted into mannitol, making them unavailable for use by the photobiont, and are subsequently used by the mycobiont for growth (Richardson and Smith, 1966; Richardson and Smith, 1968; Galun, 1988; Lines *et al.*, 1989).

Nitrogen is crucial for the growth and survival of lichens, however its availability is usually a limiting factor (Sundberg *et al.*, 2001; Palmqvist and Dahlman, 2006; Nash III, 2008b). This is due to the fact that atmospheric nitrogen (N_2), the form in which the majority of nitrogen is found, cannot be readily processed by lichens. Instead, lichens utilise inorganic forms of nitrogen such as nitrate and ammonia. Therefore, the growth and survival of lichens containing a green algal photobiont is dependent on the availability of these inorganic nitrogen sources (Dahlman *et al.*, 2004; Nash III, 2008b). However, lichen species containing a cyanobacterial photobiont have the ability to directly use atmospheric nitrogen, as the cyanobacteria are able to fix atmospheric N_2, so are thought to be less dependent on these inorganic sources of nitrogen (Ahmadjian, 1993; Dahlman *et al.*, 2004; Nash III, 2008b; Rikkinen, 2015). This nitrogen-fixing activity is carried out within specialised cells, called heterocysts, that are formed by the photobiont; the fixed nitrogen is subsequently secreted by the photobiont to the mycobiont (Hitch and Millbank, 1975; Rikkinen, 2002; Nash III, 2008b). In tripartite lichens, almost all of the fixed nitrogen produced by the cyanobacterial photobiont is transferred to the mycobiont, with minimal amounts being transferred to the green algal photobiont (Millbank and Kershaw, 1969; Kershaw and Millbank, 1970; Rai and Bergman, 2002). The fixed nitrogen produced by the cyanobacterial photobiont is transferred to the mycobiont in the form of ammonia; this occurs through repression of glutamine synthetase activity within the heterocysts (Rai, 1990; Rikkinen, 2015). However, the mechanism by which ammonia is released from the cyanobiont and taken up by the mycobiont is not understood (Rai and Bergman, 2002). The transfer of ammonia between the symbionts, or the uptake of inorganic ammonia or nitrate (and subsequent conversion of nitrate to ammonium ions), is followed by assimilation of the ammonia by the

mycobiont to form glutamate. The glutamate is then converted to alanine before being transferred to the rest of the thallus (Ahmadjian, 1993; Rai and Bergman, 2002; Nash III, 2008b).

The main source of important macronutrients required for lichen growth is thought to be from atmospheric sources of inorganic salts (Crittenden and Porter, 1991). This dependence upon nutrients from atmospheric sources has occurred due to the fact that lichens do not have roots, so cannot absorb nutrients that are found in soil (Nieboer *et al.*, 1978; Nash III, 2008c). The atmospheric deposition of nutrients to lichens includes both wet deposition from precipitation (rainfall and snowfall) and occult precipitation (dew, mist and fog), and dry deposition (sedimentation, impaction and uptake of gases) (Knops *et al.*, 1991). As a result of the fact that levels of nutrients from atmospheric sources are relatively low compared to levels of nutrients found in soil, lichens have mechanisms which allow the concentration of nutrients (Nash III, 2008c). The most important mechanisms by which lichens accumulate minerals are considered to be the retention of ions at exchange sites located within cell walls (external to the cytoplasm of the mycobiont or photobiont) and mineral particulate trapping within intercellular spaces (Nieboer *et al.*, 1978; Knops *et al.*, 1991; Nash III 2008c). It has been observed that the growth form of the lichen influences the concentrations of nutrients found within the thallus (St. Clair *et al.*, 2002). However, although lichens derive the majority of their nutrients from atmospheric sources, nutrients derived from substrates, particularly cations, are also a potential source of nutrients. This is due to the fact many lichens grow on soil or rocks and their close contact with the substrate enables the direct diffusion of nutrients and, therefore, means that lichens could accumulate soluble nutrients from lithic sources (Nieboer *et al.*, 1978; Crittenden and Porter, 1991; Nash III, 2008c).

1.3.4 Reproduction

Similarly to the majority of fungal species, most lichen-forming fungi are able to reproduce both sexually and asexually through the abundant production of reproductive propagules by the individual symbiotic partners. These fruiting bodies contain either asexual conidia (found in conidiomata) or sexual ascospores (found in ascomata) which are derived solely from fungal origins. It is the fungal partner that

undergoes sexual reproduction fully and asexual reproduction to a certain extent. This contrasts with the photobiont, which possesses a reduced mode of reproduction when found in lichens; this suppression appears to be a result of the fungal partner controlling the physiology of the photobiont. When in the lichenised state, green algal photobionts do not produce sexual structures and reproduce clonally (Seymour *et al.*, 2005b; Büdel and Scheidegger, 2008; Grube and Wedin, 2016). The main challenge associated with lichenisation is the requirement that the symbiotic relationship must be re-established after each reproductive cycle; this re-establishment involves the fungal spores coming into contact with the correct photobiont (Honegger, 1993; Büdel and Scheidegger, 2008).

In addition to sexual and asexual reproduction, lichen-forming fungal species also have the ability to disperse vegetative symbiotic propagules, such as isidia and soredia, containing both the fungal and photosynthetic partners. This allows a new thallus to be formed without the need for relichenisation and, therefore, avoids the associated problems (Honegger, 1993; Seymour *et al.*, 2005b; Büdel and Scheidegger, 2008). However, of the various reproductive propagules formed, ascospores are most commonly produced by lichens. In fact, it appears that ascospores are the only method of dispersal in many lichen species (Murtagh *et al.*, 2000).

Sexual reproduction is thought to only occur in mature lichen thalli in which the stratified thallus structure is completely differentiated. Furthermore, reproductive structures are not produced when the fungal partner is grown in axenic culture (Honegger, 1993). Sexual reproduction in lichen-forming fungi occurs though one of two breeding systems: homothallism (self-fertilisation) or heterothallism (outcrossing with a compatible partner) (Murtagh *et al.*, 2000; Dyer, 2008). Although both breeding systems have been reported in various species of lichen-forming fungi, it is not known why different breeding systems are utilised (Murtagh *et al.*, 2000; Honegger *et al.*, 2004; Seymour *et al.*, 2005b; Wang *et al.*, 2014c). It has been suggested by Murtagh *et al.* (2000) that homothallism may have occurred in some species to provide a selective advantage in an environment where the opportunity to outcross does not frequently occur, and also to promote the establishment of a lichen population derived from a pioneer spore. They also hypothesise that genetic stability provides an advantage by enabling successful genotypes to persist in the extreme

habitats to which they are adapted. However, a subsequent study by Pizarro *et al.* (2019), in which widespread heterothallism was found in the Lecanoromycetes, suggests that this is the lichen ancestral state. They argue that although heterothallism is a high-risk strategy for lichens, it results in higher genetic diversity and a faster rate of adaptive evolution.

1.3.5 Isolation and Culturing

Difficulties in the isolation and culturing of lichens are well-known, with studies attempting the artificial synthesis of lichens frequently failing or achieving limited levels of success (Ahmadjian *et al.*, 1980; Crittenden and Porter, 1991; Ahmadjian, 1993; del Carmen Molina and Crespo, 2000; Joneson *et al.*, 2011). A low frequency of spore germination in the laboratory is a characteristic of many species of lichen-forming fungi (Crittenden *et al.*, 1995). In a study carried out by Crittenden *et al.* (1995), in which they attempted the isolation of more than 1000 lichen species, the failure in isolating lichen mycobionts from ascospores was attributed to either one of four reasons: germination of the ascospores did not occur, the ascospores were not discharged, ascospore germination occurred but growth was not sustained, or the discharged ascospores showed high levels of contamination. They suggested that a lack of ascospore viability, which would result in the ascospore failing to germinate, could be dependent on the availability of water and the physiological state of the lichen at the time of collection. In addition, it is thought that the presence or absence of dense tissues in fruiting bodies may affect isolation success, with the presence of dense tissues preventing the discharge of ascospores, or the lack of dense tissues in fruit bodies resulting in ascospores with an increased susceptibility to desiccation stress and, therefore, a lack of viability.

In addition to a lack of ascospore germination, the difficulties associated with the culturing of lichens could also be attributed to culture conditions (including nutrient availability and moisture conditions) and the length of the incubation period (the long period of time needed increases the risk of contamination). When compared with non-lichenised fungal species, lichen-forming fungi have more complex nutritional needs and a much slower growth rate (Armaleo, 1991; Jahns, 1993; Ahmadjian, 1993; del Carmen Molina and Crespo, 2000). Moreover, the discovery of

the lichen symbiosis as a holobiome consisting of multiple partners, rather than just the dominant fungal partner and photosynthetic partner, could provide an explanation as to why attempts to resynthesise lichens in laboratory conditions from axenic cultures of the mycobiont and photobiont have had limited success. As suggested by Hodkinson and Lutzoni (2009) and Spribille *et al.* (2016), these additional partners in the symbiosis may play a role in the formation of the lichen thallus and would, therefore, explain why axenic cultures of algae and fungi appear to show symbiotic interactions but the synthesis of a fully-differentiated lichen thallus is not regularly achieved in culture (Stocker-Wörgötter, 2001).

1.4 Secondary Metabolism

Fungi are well-known to produce a diverse range of secondary metabolites (SMs), with filamentous ascomycete fungi containing a high complexity and richness of SM pathways. The majority of fungal species that produce these SMs belong to the class Pezizomycotina (Jain and Keller, 2013; Bills and Gloer, 2016; Keller, 2019). However, the distribution of individual SMs are thought to be either species-specific or restricted to a small number of species (Keller *et al.*, 2005; Schimek, 2011). It is believed that the ecological success of filamentous fungi can be attributed to their expression of SMs, which enable the colonisation and persistence of a fungal species in its ecological niche (Fox and Howlett, 2008; Bills and Gloer, 2016).

SMs, also known as natural products, are compounds of a low molecular mass which, although not essential for the growth and survival of an organism, provide a selective advantage that enables the filamentous fungus to adapt to changes and stress factors in its environment (Schimek, 2011; Brakhage, 2013). These specialised metabolites, which possess a diverse array of biological activities, are known to function as pigments, toxins and antibiotics (Vining, 1990; Bills and Gloer, 2016). The production of SMs is thought to increase the survival of an organism by acting as inhibitors of competitors, mediating communication within and between species, and contributing to the virulence of pathogenic fungi (Keller and Hohn, 1997; Yim *et al.*, 2007; Fox and Howlett, 2008; Shwab and Keller, 2008; Scharf *et al.*, 2014; Macheleidt *et al.*, 2016). In addition, this range of biological activities displayed by SMs has

resulted in their use by humans as pharmaceuticals (Shwab and Keller, 2008; Brakhage, 2013; Keller, 2015).

1.4.1 Regulation of Fungal Secondary Metabolite Biosynthesis Gene Clusters

Unlike genes coding for primary metabolites, SM biosynthesis genes are frequently found to be clustered within the genomes of fungi in a manner reminiscent of bacterial operons (Keller and Hohn, 1997; Keller, 2015). These biosynthetic gene clusters (BGCs) are composed of contiguous genes located next to each other in the genome, with each gene involved in the production of a specific final metabolite (Schimek, 2011; Keller, 2019). The majority of genes found within a BGC are involved in the biosynthesis of the metabolite, with each BGC containing a minimum of a gene encoding a synthase and/or synthetase, which forms the backbone of the metabolite, as well as genes coding for enzymes involved in tailoring reactions that further modify the metabolite backbone (Shwab and Keller, 2008; Nützmann *et al.*, 2018; Keller, 2019). However, BGCs are usually also found to contain a gene coding for a cluster-specific transcription factor that is required for the expression of the other genes within the cluster. In addition, BGCs can also contain genes involved in self-protection against a toxic SM produced by a BGC (Keller *et al.*, 2005; Hoffmeister and Keller, 2007; Shwab and Keller, 2008; Keller, 2015; Keller, 2019).

The characteristic clustering of SM biosynthesis genes is thought to be important for the regulation of the genes within the cluster, by enabling their co-regulation, as well as providing a selective advantage for the biosynthesis gene cluster itself (Walton, 2000; Yu and Keller, 2005; Shwab and Keller, 2008). The co-regulation of genes within a BGC results in all the genes in that cluster being expressed or repressed at the same time and independently of other genes outside the cluster (Shwab and Keller, 2008). The regulation of SM biosynthesis gene clusters in fungi is complex and is thought to occur at multiple levels, with both regulation of specific gene clusters as well as a more global regulation of secondary metabolism (Brakhage, 2013; Macheleidt *et al.*, 2016). The tight transcriptional regulation of secondary metabolism within fungi ensures that SMs are only produced by the fungus when they are needed (Collemare and Seidl, 2019).

1.4.1.1 Local Regulation

The local regulation of secondary metabolism in fungi involves pathway-specific transcription factors, which are typically embedded within the cluster that they regulate (Brakhage, 2013; Keller, 2019). The most common type of cluster-specific transcription factors in fungi are Zn_2Cys_6 zinc cluster proteins; these transcription factors are sequence-specific, DNA-binding proteins which have only been identified in fungi (Shwab and Keller, 2008; Yin and Keller, 2011; Schimek, 2011; Macheleidt *et al.*, 2016; Shelest, 2017). Fungi are also found to have additional types of cluster-specific transcription factors: Cys_2His_2 zinc finger proteins are frequently found in fungi and, less frequently, basic leucine zipper (bZIP) and winged helix proteins. However, unlike Zn_2Cys_6 proteins, these other transcription factors are not fungal-specific and can be found in all eukaryotic organisms (Shwab and Keller, 2008; Yin and Keller, 2011; Macheleidt *et al.*, 2016). Cluster-specific transcription factors are typically thought to be positive regulators of gene clusters (Keller, 2019). It is thought that the expression of pathway-specific transcription factors is activated by signals and signal transduction cascades, however in most cases further investigation is needed to identify which signals and signal transduction cascades are involved (Brakhage, 2013).

1.4.1.2 Global Regulation

In addition to cluster-specific regulators, fungi also possess global transcription factors, also known as broad domain transcription factors; these are required for the regulation of clusters that do not possess specific transcription factors (Brakhage, 2013; Macheleidt *et al.*, 2016). Global transcription factors, which are also able to control the expression of gene clusters containing their own specific transcription factor through induction or repression of individual promoters within the cluster, provide a higher level of regulation which allows the control of a secondary metabolism BGC to be incorporated into wider regulatory networks (Hoffmeister and Keller, 2007; Brakhage, 2013). Broad domain transcription factors are expressed in response to environmental signals (such as temperature, light, pH, and carbon and nitrogen sources) and, therefore, enable the fungus to only produce SMs when they would provide an advantage (Shwab and Keller, 2008; Yin and Keller, 2011; Macheleidt

et al., 2016). Global transcription factors can either be positive or negative regulators of several SM gene clusters located throughout the genome, as well as regulators of other genes not related to secondary metabolism (Hoffmeister and Keller, 2007; Macheleidt *et al.*, 2016; Keller, 2019). The signals produced by the fungus in response to the environment usually involve Cys_2His_2 zinc finger proteins (Dowzer and Kelly, 1991; Tilburn *et al.*, 1995; Shwab and Keller, 2008; Yin and Keller, 2011).

In addition to transcriptional regulation of gene clusters, secondary metabolism in fungi is frequently also under the control of a global master regulator known as LaeA (Bok and Keller, 2004). LaeA, which is conserved across a number of filamentous fungi, is a nuclear protein that forms part of the velvet complex (a heterotrimeric complex consisting of VelB, VeA and LaeA proteins), which is known to coordinate sexual development and SM production within fungi in response to light (Bayram *et al.*, 2008; Bayram and Braus, 2012). LaeA regulation is specific to secondary metabolism and has not been observed to control the expression of genes located next to a cluster in the genome (Bok *et al.*, 2006a; Bok *et al.*, 2006b). Studies observing the effect of *laeA* disruption in various *Aspergillus* species found a loss of accumulation of produced SMs and that LaeA regulation occurs at the transcriptional level (Bok and Keller, 2004; Bok *et al.*, 2005; Bok *et al.*, 2006b; Perrin *et al.*, 2007; Kale *et al.*, 2008). It is believed that LaeA is a methyltransferase involved in the epigenetic regulation of secondary metabolism via chromatin remodelling. It has also been suggested that the mechanism occurs through control of the accessibility of binding factors to chromatin regions of the SM gene clusters by the formation of heterochromatin (Keller *et al.*, 2005; Bok *et al.*, 2006a; Bayram *et al.*, 2008).

Epigenetic regulation has been found to be important in the control of fungal secondary metabolism (Brakhage, 2013). It is thought that the arrangement of SM genes in the genome into clusters is important for the chromatin-mediated regulation of SM genes, as it enables the transcriptional control of SM genes via chromatin modification in a coordinated manner (Reyes-Dominguez *et al.*, 2010; Gacek and Strauss, 2012). Chromatin modifications, particularly post-translational modifications to histone tails, enable the activation of specific SM genes at particular locations in the genome (Strauss and Reyes-Dominguez, 2011; Gacek and Strauss, 2012; Brakhage, 2013). The two main enzymes carrying out post-transcriptional regulations involved

in secondary metabolism regulation are histone methyltransferases and histone acetyltransferases, and it has been suggested that the post-translational modifications carried out by these enzymes may either influence local chromatin structure or act as signals or binding sites for downstream transcriptional activators (Pfannenstiel and Keller, 2019).

1.4.2 Lichen Secondary Metabolites

Lichen SMs are found as extracellular compounds, many of which are deposited as crystals on the surfaces of hyphae and can be extracted using organic solvents (Stocker-Wörgötter, 2008). Most lichen secondary compounds are located in the medulla of the thallus, with additional SMs located in the thallus cortex. The restriction of certain compounds to either medullary or cortical regions is correlated with the functions of the metabolites and, therefore, means that the distribution of SMs within the lichen thallus is not even; it has also been found that the patterns in which secondary compounds are distributed within the thallus are specific to each taxon (Rundel, 1978; Nybakken and Gauslaa, 2007; Elix and Stocker-Wörgötter, 2008; Molnár and Farkas, 2010). Lichens produce a vast array of SMs, the majority of which have a fungal origin. They are thought to contribute significantly to the dry weight of a lichen thallus (in some cases making up as much as 10% of the dry weight) and, therefore, their production places high energy demands upon the lichen (Stocker-Wörgötter, 2008; Calcott *et al.*, 2018). The carbon that is needed to synthesise these compounds is, as mentioned previously, produced by the photosynthetic partner (Crittenden and Porter, 1991; Stocker-Wörgötter, 2008).

Many of the secondary products identified are unique to lichens, with many studies observing that the mycobiont within a lichen produces a different repertoire of SMs compared to when grown in axenic cultures or in co-cultures with the photobiont (Leuckert *et al.*, 1990; Yamamoto *et al.*, 1993; Brunauer *et al.*, 2006; Brunauer *et al.*, 2007; Fazio *et al.*, 2009; Elshobary *et al.*, 2016; Shanmugam *et al.*, 2016). It is thought that this may be a result of the interactions between the mycobiont and photobiont within the symbiosis having an influence on the SMs that the lichen produces (Calcott *et al.*, 2018). Studies of mycobionts growing in culture have also shown that the SMs produced by the mycobiont are affected by the carbon

source, with both carbohydrate type and concentration having an effect on expression profiles (Solhaug and Gauslaa, 2004; Brunauer *et al.*, 2006; Brunauer *et al.*, 2007; Fazio *et al.*, 2012; Elshobary *et al.*, 2016). However, in addition to these influences, the production of SMs in lichens has also been found to be affected by environmental factors, such as water availability and UV radiation, as well as by season and location (BeGora and Fahselt, 2001; Solhaug, *et al.*, 2003; Solhaug and Gauslaa, 2004; Bjerke *et al.*, 2004; Bjerke *et al.*, 2005a; Bjerke *et al.*, 2005b; McEvoy *et al.*, 2006; Nguyen *et al.*, 2013; Gauslaa *et al.*, 2013). Furthermore, since the discovery of lichens as holobiomes, it has been suggested that the presence or absence of additional bacteria and fungi within the holobiome may have an important influence on the production of SMs in lichens (Calcott *et al.*, 2018).

Most lichen SMs are produced via the acetyl-polymalonyl biosynthetic pathway, however some SMs are produced via alternative pathways, such as the shikimic acid or mevalonic acid biosynthetic pathways (Culberson, 1969; Elix and Stocker-Wörgötter, 2008; Stocker-Wörgötter, 2008). The products of these biosynthetic pathways are summarised in Figure 1.4. The majority of lichen SMs derived from the acetyl-polymalonyl pathway are aromatic phenolic compounds. These include depsides, depsidones, depsones, usnic acids and dibenzofurans, the most abundant of which are the depsides and depsidones and all of which are unique to lichens (Stocker-Wörgötter, 2008; Elix and Stocker-Wörgötter, 2008; Calcott *et al.*, 2018). Lichens can also produce polyaromatic polyketide compounds from a single polyketide chain, such as xanthones, anthraquinones, naphthaquinones and chromones (Elix and Stocker-Wörgötter, 2008). However, these groups of compounds are not as diverse as the phenolic polyketide compounds and these products (or analogous products) are often found to be produced in other free-living fungi and some higher plants (Calcott *et al.*, 2018; Stocker-Wörgötter, 2008; Nguyen *et al.*, 2013). In addition, although the genomes of lichen-forming fungal species have been found to contain SM gene clusters encoding non-ribosomal peptide synthetases, the products of these gene clusters are not thought to dominate in lichens due to the lower number of NRPS clusters compared to that of PKS clusters (Calchera *et al.*, 2019).

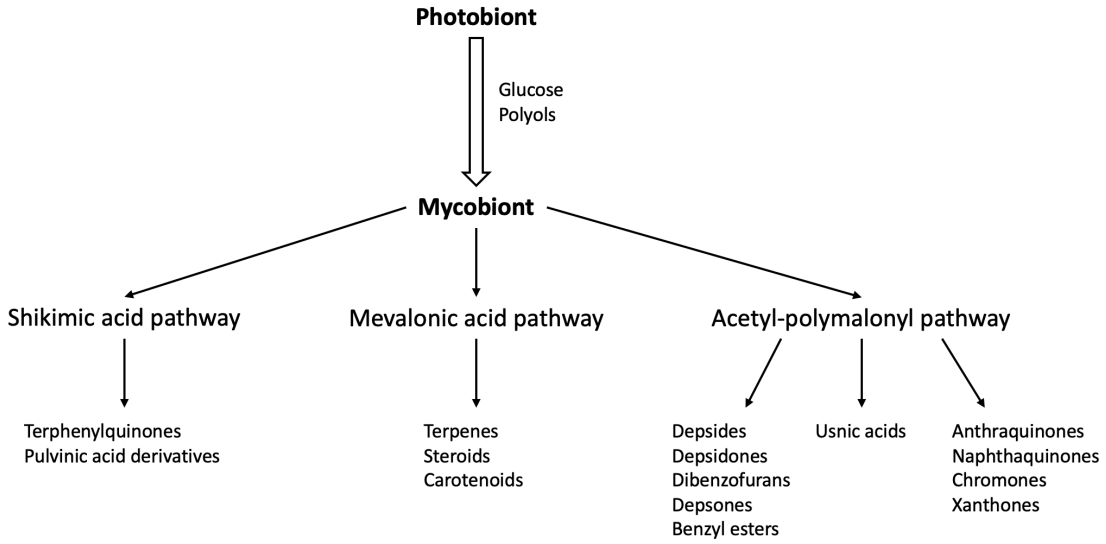


Figure 1.4. Biosynthesis of secondary metabolites in lichens. The carbon required to produce lichen SMs is transferred from the photobiont to the mycobiont and is then used in one of three biosynthetic pathways to produce lichen SMs. The majority of lichen SMs are polyketides synthesised via the acetyl-polymalonyl pathway. Adapted from Crittenden and Porter (1991) and Elix and Stocker-Wörgötter (2008).

1.4.3 Role of Secondary Metabolites in Lichens

It is thought that the production of SMs in lichens is influenced by the variable, and often extreme, environmental conditions in which the lichens are found, and that the biological functions of the SMs produced are the response of the lichen to changing ecological conditions (Stocker-Wörgötter, 2008). There is evidence to suggest that the SMs possess ecological and biological roles which are important in allowing the lichen to adapt to both abiotic and biotic factors (Lawrey, 1986; Rundel, 1978; Molnár and Farkas, 2010; Calcott *et al.*, 2018). Thus, the SMs produced by lichens provide a selective advantage for these long-lived and slow-growing symbioses, and may lead to increases in survival, productivity and reproductive success of the lichen (Rundel, 1978; Crittenden and Porter, 1991).

In addition to providing lichens with the ability to respond to abiotic and biotic factors, it has been suggested that fungal-derived SMs present in the lichen are used to control cell division in the photobiont (Calcott *et al.*, 2018). Several studies have found that both individual SMs and lichen extracts containing mixtures cause decreased growth in green algal photobionts (Bačkor *et al.*, 1998; Bud'ová *et al.*, 2006; Bačkor *et al.*, 2010; Lokajová *et al.*, 2014). This gives the mycobiont the ability to control the growth of the photobiont and, therefore, enables the maintenance of the

balance between the mycobiont and photobiont within the thallus (Lokajová *et al.*, 2014).

Pigmented cortical secondary compounds are involved in photoprotection within lichens by filtering the amount of UV that reaches the lichen and, therefore, protecting the lichen from high levels of UV radiation (Rundel, 1978; Rikkinen, 1995; Stocker-Wörgötter, 2008; Nguyen *et al.*, 2013). These UV-screening compounds, which form crystals on the surface of hyphae located in the upper cortex or the medulla of the thallus, include phenolic compounds such as depsides, depsidones and usnic acid, as well as anthraquinones, xanthones and shikimate-derived metabolites (Stocker-Wörgötter, 2008; Boustie *et al.*, 2011; Nguyen *et al.*, 2013). The lichen compounds reduce the amount of UV radiation reaching both the mycobiont and the photobiont through the absorption of both UV-A and UV-B radiation (Millot *et al.*, 2012; Nguyen *et al.*, 2013). This means that UV-induced biological damage is less likely to occur within the mycobiont hyphae or photobiont cells and the inhibition of photosynthesis is prevented (Rikkinen, 1995; Calcott *et al.*, 2018). In addition to their role in reducing UV levels, the pigmented compounds also have a general light-filtering effect, which provides more favourable conditions for photobionts that exhibit better growth at lower light intensities (Rundel, 1978).

In addition to metabolites that protect against UV radiation, lichens also produce compounds which are thought to convey oxidative stress tolerance to the lichen (Rundel, 1978; Lawrey, 1986; Molnár and Farkas, 2010). Various studies have found phenolic polyketides to display a strong antioxidant activity (Hidalgo *et al.*, 1994; Marante *et al.*, 2003; Odabasoglu *et al.*, 2004; Karakus *et al.*, 2009; Luo *et al.*, 2010). It has also been observed that the antioxidant activity of these compounds is more effective and they accumulate to higher amounts in lichen species which are subject to higher levels of oxidative stress (Luo *et al.*, 2009; Luo *et al.*, 2010). The antioxidant activity of these compounds has been shown to be related to their phenolic constituents, with the phenolic groups found in these SMs enabling the scavenging of free radicals (reactive oxygen and nitrogen species which can cause damage to cells). This, therefore, prevents the accumulation of free radical-induced cell damage within the lichen (Gulluce *et al.*, 2006; Molnár and Farkas, 2010). Further to this, certain lichen secondary compounds are thought to play a role in metal

homeostasis and pollution tolerance within lichens (Hauck and Huneck, 2007; Hauck, 2008; Hauck *et al.*, 2009). The metabolites are sensitive to the accumulation of heavy metals and are believed to control metal homeostasis by causing increases in the uptake of particular metal cations whilst reducing the adsorption levels of others (Białońska and Dayan, 2005; Molnár and Farkas, 2010). This enables lichens to increase their tolerance to heavy metals present in the environment, as well as preventing nutrient deficiencies of certain micronutrients for lichens growing in nutrient-poor environments (Hauck *et al.*, 2009; Molnár and Farkas, 2010).

Lichen secondary compounds are believed to play a role in protecting the lichen thallus against various other organisms (Rikkinen, 1995). The biologically active metabolites usually involved are mainly colourless or weakly-coloured polyketides (Stocker-Wörgötter, 2008). There is evidence to support the involvement of lichen SMs in defence against pathogens and parasitic fungi, as well as against herbivorous insects, molluscs and mammals (Rundel, 1978; Lawrey, 1986; Rikkinen, 1995; Halama and Van Haluwin, 2004; McEvoy *et al.*, 2007; Paudel *et al.*, 2008; Ranković *et al.*, 2008; Molnár and Farkas, 2010; Gauslaa *et al.*, 2013). Further to this, lichens can produce secondary compounds which have an allelopathic role within the lichen; these SMs inhibit the germination of seeds from vascular plants and can also prevent the germination of spores from mosses and other lichens (Rundel, 1978; Lawrey, 1986; Marante *et al.*, 2003; Romagni *et al.*, 2004; Calcott *et al.*, 2018). The ability of many lichen-derived secondary compounds to prevent the growth of a range of organisms, including plants, fungi, bacteria, protozoa and viruses suggests that they may be important in the protection of the long-lived and slow-growing lichen thallus against microbial decay in nature (Rundel, 1978; Rikkinen, 1995).

The genomes of lichens have been found to be rich in novel genes associated with polyketide biosynthesis, which therefore suggests that lichens have the potential to be sources of as-yet-undiscovered SMs and new polyketide products (Crittenden and Porter, 1991; Miao *et al.*, 2001; Müller, 2001; Boustie and Grube, 2005). Furthermore, lichen secondary compounds are recognised to possess multiple biological activities, with various screenings of lichen metabolites identifying cytotoxic, antitumour and antiviral activities, as well as compounds having antibacterial, antifungal, anti-inflammatory, antiproliferative, antipyretic and

analgesic effects (Müller, 2001; Stocker-Wörgötter, 2008; Molnár and Farkas, 2010). The diverse range of biological activities displayed by lichen SMs suggests that these compounds could potentially be used as novel pharmaceuticals. However, only a small number of lichen metabolites have been screened to determine their biological activities and therapeutic potential, and they have not been exploited in the pharmaceutical industry (Müller, 2001; Boustie and Grube, 2005; Molnár and Farkas, 2010).

1.4.4 Methods to Discover Products of Secondary Metabolite Biosynthesis Gene Clusters

Genomic studies have revealed the enormous biosynthetic potential of fungi, with the genomes of fungi found to contain high numbers of SM gene clusters (Brakhage and Schroeckh, 2011; Sanchez *et al.*, 2012). The realisation that the number of SM biosynthesis gene clusters vastly outweighs the number of known SMs for a given fungal species indicates that the biosynthetic potential of fungi remains under-explored (Van Lanen and Shen, 2006; Brakhage, 2013). However, there are challenges associated with the identification of SMs from fungi due to the fact that the cultivation of some fungi under laboratory conditions is difficult. In addition, many SM gene clusters have been found to be silent under standard laboratory conditions due to specific environmental triggers not being present under laboratory conditions, and these can be difficult to reproduce (Hertweck, 2009; Brakhage and Schroeckh, 2011; Lim *et al.*, 2012). Various strategies have been used to induce the expression of silent BGCs to identify potentially novel natural products that could have pharmaceutical applications (Lim *et al.*, 2012; Brakhage, 2013; Macheleidt *et al.*, 2016).

Approaches focussing on the culture conditions of a fungus have had success in activating the expression of some BGCs. These methods include simulation of the signals found in natural environments that lead to BGC expression, in addition to co-culturing the fungus with other microbes that cause the induction of BGC expression (Schroeckh *et al.*, 2009; Marmann *et al.*, 2014; Bertrand *et al.*, 2014; Netzker *et al.*, 2015). A simpler approach to induce the expression of SM biosynthesis gene clusters involves growing the fungus on different growth media (Frisvad, 2012). However,

although these strategies are successful in some instances, they do not trigger the expression of all SM gene clusters in a fungus (Pfannenstiel and Keller, 2019).

Alternative strategies have used genetic approaches to induce the expression of SM gene clusters (Rutledge and Challis, 2015). These can include the overexpression of pathway-specific transcription factors (which has the advantage of inducing the expression of all genes in the cluster at the same time) or swapping the endogenous promotor of an SM gene with a strong inducible promoter within the homologous host, of which both strategies have been successful in leading to the identification of previously undescribed SMs (Maiya *et al.*, 2006; Bergmann *et al.*, 2010; Brakhage, 2013). Additional genetic approaches involve global regulators of secondary metabolism, for example the overexpression or deletion of LaeA (Bok and Keller, 2004; Brakhage, 2013).

Further strategies to induce the expression of SM gene clusters focus on the epigenetic regulation of secondary metabolism. These methods involve the chemical or genetic manipulation of chromatin regulators in order to prevent chromatin modifications which lead to the silencing of secondary metabolism BGCs (Shwab *et al.*, 2007; Williams *et al.*, 2008; Henrikson *et al.*, 2009; Cichewicz, 2010; Pfannenstiel and Keller, 2019). An advantage to manipulating chromatin regulators is that induction of SM gene clusters can occur across the whole genome (Cichewicz, 2010; Rutledge and Challis, 2015).

In addition to the pleiotropic and pathway-specific strategies described above that take place in the homologous host, the identification of SMs from gene clusters can be achieved using heterologous expression (Itoh *et al.*, 2010; Zhang *et al.*, 2011; Rutledge and Challis, 2015). This involves the expression of an entire BGC in a heterologous host with low background metabolite levels and, therefore, carries the advantage of simplifying the identification of the metabolite produced by the BGC. Furthermore, heterologous expression provides the opportunity to identify SMs from fungal species which are not easy to cultivate and manipulate (Van Lanen and Shen, 2006; Rutledge and Challis, 2015).

1.5 Aims and Objectives of Thesis

The ultimate aim of this study is to achieve the production and characterisation of lichen-derived SMs. Although lichens produce a diverse range of SMs that possess a variety of biological activities, it is currently not feasible or sustainable to produce lichen-derived SMs on an industrial scale. It is hypothesised that using multiple approaches, including both increasing the growth rate of lichens and heterologously expressing lichen SM genes, improves their production for industrial exploitation. As lichens are an important source of potentially novel compounds, they could aid in meeting the increasing need for new compounds with pharmaceutical applications, especially antimicrobials. The investigation of the potential to produce lichen-derived SMs will be achieved through the following areas:

(1) Generation of faster-growing mutants of lichen mycobionts. The potential of using UV mutagenesis to create mutants of lichen mycobionts displaying a faster growth rate will be investigated. It is hypothesised that mutations can occur in genes associated with lichen growth rate and that, if successful, any mutants produced will possess a growth rate 2-3 times faster than that of mycobionts with a normal growth rate. The identification of mycobionts with an increased growth rate will enable subsequent genetic analysis of these mutants and the identification of the genes involved in the growth of lichen mycobionts. In addition, the creation of faster-growing lichen mutants would provide ideal strains that could be used in biotechnology to produce SMs on a larger scale.

(2) Production of lichen-derived SMs using fungal heterologous expression platforms. The suitability of fungal heterologous expression platforms to produce lichen-derived SMs will be investigated. A combination of bioinformatics and experimental work will be used to identify lichen SM biosynthetic gene clusters which will then be expressed in *Aspergillus* expression platforms. Any metabolites produced in these expression platforms will subsequently be characterised. It is anticipated that the heterologous expression of lichen secondary metabolism genes will enable the production of lichen SMs to be achieved in high quantities and will, therefore, provide an opportunity for lichen SMs to be produced sustainably on an industrial scale.

Chapter 2: Materials and Methods

General materials and methods are described in the following chapter. Materials and methods specific to individual chapters will be described in the relevant chapter.

2.1 Materials

2.1.1 Growth Media

Unless specified, all growth media were autoclaved at 117 °C for 30 min.

Aspergillus Complete Medium (ACM)

ACM was prepared according to Paoletti *et al.* (2005). Per litre: 10 g D-glucose (Sigma-Aldrich, UK), 1 g yeast extract (Oxoid, UK), 2 g peptone (Oxoid, UK), 1 g casamino acids (Sigma-Aldrich, UK), 0.075 g adenine (Sigma-Aldrich, UK), 10 ml *Aspergillus* vitamin solution, 20 ml *Aspergillus* salt solution were made up to the appropriate volume with distilled water and adjusted to pH 6.5. For solid media, 20 g agar/l (Sigma-Aldrich, UK) were added prior to autoclaving of media.

Aspergillus Minimal Medium (AMM(-N))

For **AMM(-N)G50Gln10 (GG10)**, per litre: 0.52 g potassium chloride (VWR International, UK), 0.52 g magnesium sulfate heptahydrate (Thermo Fisher Scientific, UK), 1.52 g potassium dihydrogen phosphate (Sigma-Aldrich, UK), 1 ml Hutner's trace elements (Hutner *et al.*, 1950), 9.91 g glucose monohydrate (Sigma-Aldrich, UK), and 1.46 g glutamine (Acros Organics, UK) were made up to the appropriate volume with distilled water and adjusted to pH 6.5. For solid media, 20 g bacteriological agar/l (Sigma-Aldrich, UK) were added prior to autoclaving of media. For **AMM(-N)G100Gln20 (GG20)** the same basic recipe was used as for GG10 medium, but per litre 19.82 g glucose monohydrate (Sigma-Aldrich, UK) and 2.92 g glutamine (Acros Organics, UK) were added. For **AMM(-N) starch 2%, 6 mM glucose (SG20)**, the main proportion of glucose from GG20 medium was replaced by 2% Difco soluble starch

(Thermo Fisher Scientific, UK) and, after autoclaving, 560 µl sterile 20% glucose solution was added.

Lysogeny Broth Medium (LB)

Per litre: 25 g LB broth powder (Melford, UK), 15 g agar (for solid media; Sigma-Aldrich, UK) was made up to the appropriate volume with distilled water, adjusted to pH 7.0 and autoclaved at 121 °C for 15 min.

Malt Extract Medium (ME)

Per litre: 17 g malt extract (Sigma-Aldrich, UK) was made up to the appropriate volume with distilled water, adjusted to pH 5.9 and autoclaved at 121 °C.

Malt Extract Yeast Extract Medium (MEYE)

Per litre: 20 g malt extract (Sigma-Aldrich, UK), 2 g yeast extract (Oxoid, UK), 20 g agar (Sigma-Aldrich, UK) was made up to the appropriate volume with distilled water and autoclaved at 121 °C for 15 min.

Yeast Extract Peptone Dextrose Medium (YEPD)

Per litre: 20 g peptone (Oxoid, UK), 10 g yeast extract (Oxoid, UK) were made up to 900 ml with distilled water and autoclaved at 121 °C for 15 min. After autoclaving, 100 ml sterile 20% sterile glucose solution was added.

2.1.2 Chemical Solutions and Buffers

All chemical solutions were autoclaved at 121 °C for 15 min, unless specified.

***Aspergillus* Salt Solution**

Per litre: 26 g potassium chloride (VWR International, UK), 26 g magnesium sulfate heptahydrate (Thermo Fisher Scientific, UK), 76 g potassium dihydrogen phosphate (Thermo Fisher Scientific, UK), 10 ml *Aspergillus* trace elements solution was made up to the appropriate volume with distilled water.

***Aspergillus* Trace Elements Solution**

Per litre: 40 mg sodium tetraborate decahydrate (VWR International, UK), 800 mg copper sulphate pentahydrate (Thermo Fisher Scientific, UK), 800 mg ferric orthophosphate monohydrate (Sigma-Aldrich, UK), 800 mg manganese sulphate tetrahydrate (Thermo Fisher Scientific, UK), 800 mg sodium molybdate dihydrate (Thermo Fisher Scientific, UK), 8 g zinc sulphate (Thermo Fisher Scientific, UK) was made up to the appropriate volume with distilled water.

***Aspergillus* Vitamin Solution**

Per litre: 400 mg para-aminobenzoic acid (Sigma-Aldrich, UK), 50 mg thiamine hydrochloride (Sigma-Aldrich, UK), 2 mg D-biotin (Sigma-Aldrich, UK), 100 mg nicotinic acid (Sigma-Aldrich, UK), 250 mg pyridoxine hydrochloride (Sigma-Aldrich, UK), 1.4 g choline chloride (Sigma-Aldrich, UK), 100 mg riboflavin (Sigma-Aldrich, UK) was made up to the appropriate volume with distilled water.

DNA Extraction Buffer

Per litre: 12.11 g Tris (adjusted to pH 8.0; Thermo Fisher Scientific, UK), 100 ml 0.5 M EDTA pH 8.0, 29.22 g sodium chloride was made up to the appropriate volume with distilled water and autoclaved. After autoclaving, 700 μl β-mercaptoethanol was added.

EDTA (Ethylenediaminetetraaceticacid)

0.5 M stock solution, per litre: 181.1 g EDTA was added to 600 ml distilled water and adjusted to pH 8.0 before being made up to the appropriate volume with distilled water.

TAE Buffer

50 X stock solution, per litre: 242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0 was made up to the appropriate volume with distilled water.

TE Buffer

Per litre: 10 ml 1 M Tris pH 7.5, 2 ml 0.5 M EDTA pH 8.0 was made up to the appropriate volume with distilled water.

Tween 80 Solution (0.1% v/v)

Per litre: 1 ml Tween 80 (Sigma-Aldrich, UK) was made up to the appropriate volume with distilled water.

2.2 Methods

2.2.1 Strains and Plasmids

All strains used in this study are shown in Table 2.1.

Strain	Genotype	Reference
A. niger A1144		FGSC, Kansas City, USA
A. niger A1144 Δ*pyrG*	*A. niger* A1144 Δ*pyrG*	Cruz-Leite *et al.* (2022), in press
A. niger ATNT16	TetOn:*terR_ble*	Geib and Brock, 2017
A. niger ATNT16 Δ*pyrG*x24	TetOn:*terR_ble*; Δ*pyrG*::*ptrA*	Geib and Brock, 2017
A. niger ATNT16 Δ*pyrG*x24 *orsA*:TE_L_*luciferase*_SM-X_URA	P*terA*:*orsA*:TE_L_*luciferase* _URA	This study
A. niger ATNT16 Δ*pyrG*x24 *orsA_luciferase*_SM-X_URA	P*terA*:*orsA_luciferase* _URA	This study
A. niger ATNT16 Δ*pyrG*x24 *orsA*:TE_L_*Me473_luciferase*_SM-X_URA	P*terA*:*orsA*:TE_L_*Me473_luciferase* _URA	This study
A. niger ATNT16 Δ*pyrG*x24 *orsA_Me473_luciferase*_SM-X_URA	P*terA*:*orsA*:TE_L_*Me473_luciferase* _URA	This study

A. niger ATNT16 Δ*pyrG*x24 *Me473*_SM_S- tag_X_URA	P*terA*:*Me473*_URA	This study
A. niger ATNT16 Δ*pyrG*x24 *O-MeT-c40*_SM_S- tag_X_URA	P*terA*:*O-MeT-c40*_URA	This study
A. niger ATNT16 Δ*pyrG*x24 *O-MeT-c41*_SM_S- tag_X_URA	P*terA*:*O-MeT-c41*_URA	This study
A. niger ATNT16 Δ*pyrG*x24 *PKS-c40*_SM-X_URA	P*terA*:*PKS-c40*_URA	This study
A. niger ATNT16 Δ*pyrG*x24 *PKS-c41*_SM-X_URA	P*terA*:*PKS-c41*_URA	This study
A. niger ATNT16 Δ*pyrG*x24 *UlPKS6*_SM-X_URA	P*terA*:*UlPKS6*_URA	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA*	TetOn:*terR_ble*;Δ*pyrG*::*ptrA;* Δ*pabaA*	Peres da Silva and Brock, unpublished
A. niger ATNT16 Δ*pyrG* Δ*pabaA Clagr3.6*_SM-X_URA	P*terA*:*Clagr3.6*_URA	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA Clagr3.6*_SM-X_URA *An-hacA*_SM-X_PABA	P*terA*:*Clagr3.6*_URA;*An-hacA*_ PABA	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA Clagr3.21*_SM-X_URA	P*terA*:*Clagr3.21*_URA	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA Clagr3.26*_SM-X_URA	P*terA*:*Clagr3.26*_URA	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA Clagr3.26*_SM-X_URA *An-hacA*_SM-X_PABA	P*terA*:*Clagr3.26*_URA;*An-hacA*_PABA	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA Clagr3.31*_SM-X_URA	P*terA*:*Clagr3.31*_URA	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA EpNRPSL1*_SM_S-tag_X_URA	P*terA*:*EpNRPSL1*_URA	This study

A. niger ATNT16 Δ*pyrG* Δ*pabaA* *EpNRPSL2*_SM_S-tag_X_URA	P*terA*:*EpNRPSL2*_URA	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA* *EpNRPSL3*_SM_S-tag_X_URA	P*terA*:*EpNRPSL3*_URA	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.11*_SM_S-tag_X_URA	P*terA*:*Clagr3.11*_URA	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.30*_SM_S-tag_X_URA	P*terA*:*Clagr3.30*_URA	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.30*_SM_S-tag_X_URA *An-hacA*_SM-X_PABA	P*terA*:*Clagr3.30*_URA;*An-hacA*_PABA	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.11*:T-TE$_{abrA}$_SM_S-tag_X_URA	P*terA*:*Clagr3.11*:T-TE$_{abrA}$_URA	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.11*:TE$_{abrA}$_SM_S-tag_X_URA	P*terA*:*Clagr3.11*:TE$_{abrA}$_URA	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1*	TetOn:*terR_ble*;Δ*pyrG*::*ptrA;* Δ*pabaA*;Δ*his1*	Peres da Silva and Brock, unpublished
A. niger ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* *Ep-pyrG_his1*_pJET1.2	*Ep-pyrG_his1*_pJET1.2	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* *Ep-his1*_URA_pJET1.2	*Ep-his1*_URA_pJET1.2	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* *Xp-pyrG_his1*_pJET1.2	*Xp-pyrG_his1*_pJET1.2	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* *Xp-pabaA_his1*_pJET1.2	*Xp-pabaA_his1*_pJET1.2	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* *Xp- his1*_URA_pJET1.2	*Xp- his1*_URA_pJET1.2	This study

A. niger ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1 Cg- pyrG_his1*_pJET1.2	*Cg- pyrG_his1*_pJET1.2	This study
A. niger ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1 Cg-pabaA_his1*_pJET1.2	*Cg-pabaA_his1*_pJET1.2	This study
A. oryzae OP12	P*amyB*:*terR_ptrA*	Geib *et al.* (2019)
A. oryzae OP12 *pyrG*$^-$	P*amyB*:*terR_ptrA*;*pyrG*$^-$	Geib *et al.* (2019)
A. oryzae OP12 *pyrG*$^-$ *orsA*:*TE*$_L$*_luciferase*_SM-X_URA	P*terA*:*orsA*:*TE*$_L$*_luciferase* _URA	This study
A. oryzae OP12 *pyrG*$^-$ *orsA_luciferase*_SM-X_URA	P*terA*:*orsA_luciferase* _URA	This study
A. oryzae OP12 *pyrG*$^-$ *orsA*:*TE*$_L$*_Me473_luciferase*_SM-X_URA	P*terA*:*orsA*:*TE*$_L$*_Me473_ luciferase* _URA	This study
A. oryzae OP12 *pyrG*$^-$ *orsA_Me473_luciferase*_SM-X_URA	P*terA*:*orsA_Me473_luciferase* _URA	This study
A. oryzae OP12 *pyrG*$^-$ *O-MeT-c40*_SM_S- tag_X_URA	P*terA*:*O-MeT-c40*_URA	This study
A. oryzae OP12 *pyrG*$^-$ *O-MeT-c41*_SM_S- tag_X_URA	P*terA*:*O-MeT-c41*_URA	This study
A. oryzae OP12 *pyrG*$^-$ *PKS-c40*_SM-X_URA	P*terA*:*PKS-c40*_URA	This study
A. oryzae OP12 *pyrG*$^-$ *PKS-c41*_SM-X_URA	P*terA*:*PKS-c41*_URA	This study
A. oryzae OP12 *pyrG*$^-$ *UlPKS6*_SM-X_URA	P*terA*:*UlPKS6*_URA	This study
A. oryzae OP12 pyrG$^-$ Δ*pabaA*	P*amyB*:*terR_ptrA*;*pyrG*$^-$; Δ*pabaA*	Wieder *et al.* (2022), in press

A. oryzae OP12 *pyrG*$^{-}$ Δ*pabaA* *Clagr3.6*_SM-X_URA	P*terA*:*Clagr3.6*_URA	This study
A. oryzae OP12 *pyrG*$^{-}$ Δ*pabaA* *Clagr3.21*_SM-X_URA	P*terA*:*Clagr3.21*_URA	This study
A. oryzae OP12 *pyrG*$^{-}$ Δ*pabaA* *Clagr3.26*_SM-X_URA	P*terA*:*Clagr3.26*_URA	This study
A. oryzae OP12 *pyrG*$^{-}$ Δ*pabaA* *Clagr3.31*_SM-X_URA	P*terA*:*Clagr3.31*_URA	This study
A. oryzae OP12 *pyrG*$^{-}$ Δ*pabaA* *EpNRPSL1*_SM_S-tag_X_URA	P*terA*:*EpNRPSL1*_URA	This study
A. oryzae OP12 *pyrG*$^{-}$ Δ*pabaA* *EpNRPSL2*_SM_S-tag_X_URA	P*terA*:*EpNRPSL2*_URA	This study
A. oryzae OP12 *pyrG*$^{-}$ Δ*pabaA* *EpNRPSL3*_SM_S-tag_X_URA	P*terA*:*EpNRPSL3*_URA	This study
A. oryzae OP12 *pyrG*$^{-}$ Δ*pabaA* *Clagr3.11*_SM_S-tag_X_URA	P*terA*:*Clagr3.11*_URA	This study
A. oryzae OP12 *pyrG*$^{-}$ Δ*pabaA* *Clagr3.30*_SM_S-tag_X_URA	P*terA*:*Clagr3.30*_URA	This study
A. oryzae OP12 *pyrG*$^{-}$ Δ*pabaA* *Clagr3.11*:T-TE$_{abrA}$_SM_S-tag_X_URA	P*terA*:*Clagr3.11*:T-TE$_{abrA}$_URA	This study
A. oryzae OP12 *pyrG*$^{-}$ Δ*pabaA* *Clagr3.11*:TE$_{abrA}$_SM_S-tag_X_URA	P*terA*:*Clagr3.11*:TE$_{abrA}$_URA	This study
A. oryzae OP12 *pyrG*$^{-}$ Δ*pabaA* *acyN*_SM_S-tag_X_URA	P*terA*:*acyN*_URA	Wieder *et al.* (2022), in press
A. oryzae OP12 pyrG^{-} Δ*pabaA* Δ*his1*	P*amyB*:*terR_ptrA*;*pyrG*$^{-}$;Δ*pabaA;*Δ*his1*	Peres da Silva and Brock, unpublished

*A. oryzae OP12 pyrG⁻ ΔpabaA Δhis1 Ep-pyrG_his1*_pJET1.2	*Ep-pyrG_his1*_pJET1.2	This study
*A. oryzae OP12 pyrG⁻ ΔpabaA Δhis1 Ep-his1*_URA_pJET1.2	*Ep-his1*_URA_pJET1.2	This study
*A. oryzae OP12 pyrG⁻ ΔpabaA Δhis1 Xp-pyrG_his1*_pJET1.2	*Xp-pyrG_his1*_pJET1.2	This study
*A. oryzae OP12 pyrG⁻ ΔpabaA Δhis1 Xp-pabaA_his1*_pJET1.2	*Xp-pabaA_his1*_pJET1.2	This study
*A. oryzae OP12 pyrG⁻ ΔpabaA Δhis1 Xp- his1*_URA_pJET1.2	*Xp- his1*_URA_pJET1.2	This study
*A. oryzae OP12 pyrG⁻ ΔpabaA Δhis1 Cg- pyrG_his1*_pJET1.2	*Cg- pyrG_his1*_pJET1.2	This study
*A. oryzae OP12 pyrG⁻ ΔpabaA Δhis1 Cg-pabaA_his1*_pJET1.2	*Cg-pabaA_his1*_pJET1.2	This study
A. brasiliensis CBS101740		CBS, Westerdijk, Netherlands

Table 2.1. Strains used in this study, along with corresponding genotype and reference.

All plasmids used in this study are shown in Table 2.2.

Plasmid	**Features**	**Reference**
SM-X_URA	SM-Xpress_URA Blaster	Geib *et al.* (2019)
*PKS-c40*_SM-X_URA	P*terA*:*PKS-c40*_SM-Xpress_ URA Blaster	This study
*PKS-c41*_SM-X_URA	P*terA*:*PKS-c41*_SM-Xpress_ URA Blaster	This study
*UlPKS6*_SM-X_URA	P*terA*:*UlPKS6*_SM-Xpress_ URA Blaster	This study
*Clagr3.6*_SM-X_URA	P*terA*:*Clagr3.6*_SM-Xpress_ URA Blaster	This study

*Clagr3.21*_SM-X_URA	P*terA*:*Clagr3.21*_SM-Xpress_ URA Blaster	This study
*Clagr3.26*_SM-X_URA	P*terA*:*Clagr3.26*_SM-Xpress_ URA Blaster	This study
*Clagr3.31*_SM-X_URA	P*terA*:*Clagr3.31*_SM-Xpress_ URA Blaster	This study
P2A_*luciferase*_SM-X_URA	P2A_*luciferase*_SM-Xpress_ URA Blaster	Geib and Brock, unpublished
*orsA:TE$_L$_luciferase*_SM-X_URA	P*terA*:*orsA:TE$_L$*_P2A_*luciferase*_ SM-Xpress_URA Blaster	This study
*orsA_luciferase*_SM-X_URA	P*terA*:*orsA*_P2A_*luciferase*_SM-Xpress_URA Blaster	This study
orsA:*TE$_L$_Me473_luciferase*_SM-X_URA	P*terA*:*orsA:TE$_L$*_P2A_*Me473*_ P2A_*luciferase*_SM_Xpress_URA Blaster	This study
*orsA_Me473_luciferase*_SM-X_URA	P*terA*:*orsA*_P2A_*Me473*_P2A_ *luciferase*_SM-Xpress_URA Blaster	This study
SM_S-tag_X_URA	Strep-tag_SM-Xpress_URA Blaster	Wieder *et al.* (2022), in press
*Me473*_SM_S- tag_X_URA	P*terA*:*Me473*_SM-Xpress_URA Blaster	This study
*O-MeT-c40*_SM_S- tag_X_URA	P*terA*:*O-MeT-c40*_SM-Xpress_URA Blaster	This study
*O-MeT-c41*_SM_S- tag_X_URA	P*terA*:*O-MeT-c41*_SM-Xpress_URA Blaster	This study
*EpNRPSL1*_SM_S-tag_X_URA	P*terA*:*EpNRPSL1*_SM-Xpress_ URA Blaster	This study
*EpNRPSL2*_SM_S-tag_X_URA	P*terA*:*EpNRPSL2*_SM-Xpress_	This study

	URA Blaster	
*EpNRPSL3*_SM_S-tag_X_URA	P*terA*:*EpNRPSL3*_SM-Xpress_ URA Blaster	This study
*Clagr3.11*_SM_S-tag_X_URA	P*terA*:*Clagr3.11*_SM-Xpress_ URA Blaster	This study
*Clagr3.30*_SM_S-tag_X_URA	P*terA*:*Clagr3.30*_SM-Xpress_ URA Blaster	This study
Clagr3.11:T-TE$_{abrA}$_SM_S-tag_X_URA	P*terA*:*Clagr3.11*:T-TE$_{abrA}$_SM-Xpress_URA Blaster	This study
Clagr3.11:TE$_{abrA}$_SM_S-tag_X_URA	P*terA*:*Clagr3.11*:TE$_{abrA}$_SM-Xpress_URA Blaster	This study
SM-X_PABA	SM-Xpress_*pabaA*	Peres da Silva and Brock, unpublished
*An-hacA*_SM-X_PABA	P*terA*:*An-hacA*_SM-Xpress_*pabaA*	This study
pJET1.2	pJET1.2/blunt	Thermo Fisher Scientific, UK
*Ep-pyrG_his1*_pJET1.2	*Ep-pyrG_his1*_pJET1.2	This study
*Ep-his1*_URA_pJET1.2	*Ep-his1*_URA Blaster_pJET1.2	This study
*Xp-pyrG_his1*_pJET1.2	*Xp-pyrG_his1*_pJET1.2	This study
*Xp-pabaA_his1*_pJET1.2	*Xp-pabaA_his1*_pJET1.2	This study
*Xp- his1*_URA_pJET1.2	*Xp-his1*_URA Blaster_pJET1.2	This study
*Cg- pyrG_his1*_pJET1.2	*Cg-pyrG_his1*_pJET1.2	This study
*Cg-pabaA_his1*_pJET1.2	*Cg-pabaA_his1*_pJET1.2	This study

Table 2.2. Plasmids used in this study, along with corresponding features and reference.

2.2.2 Culture Maintenance

Agar slopes were used for storage of *A. niger* ATNT16 and *A. oryzae* OP12 strains. Slopes were prepared by pouring 10 ml of molten GG10 agar (and any required supplements) into a 30 ml Universal tube and were left to set at an angle before being inoculated with conidia of the appropriate strain using a sterile 10 μl loop or, alternatively, a cotton swab. Slopes were incubated at 28 °C.

For long-term storage, conidial suspensions of *A. niger* ATNT16 and *A. oryzae* OP12 strains were filtered using a 40 μm cell strainer (Greiner Bio-One GmbH, Germany) to remove any debris. For each strain, 800 μl of spore suspension was added to a Microbank cryovial (Pro-Lab Diagnostics, UK) which contained cryobeads in a cryopreservative solution. The vials were agitated to ensure the beads were sufficiently covered with spores and the excess liquid was then removed from the vial. The resulting cryogenic stocks were stored at -80 °C.

2.2.3 DNA Extraction

DNA was extracted from *A. niger* and *A. oryzae* strains after a modified protocol from Dellaporta *et al.* (1983). In brief, strains were grown in liquid YEPD cultures (in addition to any required supplements) in a 25 ml volume at 28 °C, 150 rpm for 24 h. Mycelia were separated from culture filtrates by filtration over Miracloth (Merck, Darmstadt, Germany), washed in SDW, pressed dry with tissue and frozen in liquid nitrogen. Mycelia were stored at -80 °C until further use.

The mycelia were ground to a fine powder under liquid nitrogen and were transferred to a 2 ml reaction tube. 500 μl DNA extraction buffer, 60 μl 10% SDS and 2 μl RNase A (10 mg/ml, Sigma Aldrich) were added to the reaction tube before being vortexed and incubated at 65 °C for 15 min. After incubation, the reaction tube was placed on ice, 160 μl 5 M potassium acetate was added, and the reaction tube was incubated on ice for 5 min before centrifugation for 10 min at 13,000 rpm and 4 °C. The supernatant was transferred to a fresh 2 ml reaction tube, 700 μl isopropanol was added, and the reaction tube was inverted before being incubated at -20 °C for 20 min and subsequently centrifuged for 10 min at 10,000 rpm and 4 °C. The supernatant was removed, and the DNA was resuspended in 400 μl TE buffer; the reaction tube was

incubated at 60 °C until the DNA had dissolved and then 55 µl 3 M sodium acetate was added. The reaction tube was centrifuged for 5 min at 13,000 rpm and 4 °C, and the supernatant was subsequently transferred to a fresh 2 ml reaction tube. 500 µl isopropanol was added, and the reaction tube was inverted before being incubated at -20 °C for 10 min. The reaction tube was then centrifuged for 10 min at 10,000 rpm and 4 °C, and the supernatant was removed. The DNA pellet was washed with 400 µl 70% ethanol and was centrifuged again for 10 min at 10,000 rpm and 4 °C. The supernatant was removed, and the DNA pellet was dried for 5-10 min and then resuspended in 60 µl elution buffer. Genomic DNA was stored at 5 °C.

2.2.4 Species Identification

Specimens of *Xanthoria parietina*, *Lecanora chlarotera* and *Amandinea punctata* collected from various locations in the UK were identified by morphological characteristics, as described in Smith *et al.* (2009).

2.2.5 Kits and Enzymes for Molecular Biology

All oligonucleotides used in this study were synthesised by Eurogentec (Kaneka Eurogentec S.A., Seraing, Belgium) and are listed in Appendix 1. For the amplification of DNA, all PCR reactions were performed in a SpeedCycler^2 (Analytik Jena, Germany) in 10 µl volumes using either Phusion polymerase (cloning PCR; Thermo Fisher Scientific, UK) or Phire Hot Start II polymerase (colony PCR; Thermo Fisher Scientific, UK) with dNTPs from the Deoxynucleotide (dNTP) Solution Mix (New England Biolabs Ltd, Hitchin, UK). Restriction digests were carried out using FastDigest restriction enzymes (Thermo Fisher Scientific, UK), and FastAP (Thermo Fisher Scientific, UK) was used when dephosphorylation of the plasmid was required. For plasmid assembly, *in vitro* recombination was performed using the InFusion HD cloning kit (Takara Bio Europe SAS, Saint-Germain-en-Laye, France). *E. coli* DH5α cells, which were used to amplify plasmids, were made chemically competent using the Mix & Go *E. coli* Transformation Kit & Buffer Set (Zymo Research, Cambridge Bioscience, Cambridge, UK). DNA fragments and linearised plasmids used to construct the expression plasmids

were gel purified using the Monarch Gel Extraction kit (New England Biolabs Ltd, Hitchin, UK).

2.2.6 Agarose Gel Electrophoresis and Analysis

Agarose gels were made by dissolving agarose (Lonza, Rockland, Maine, USA) in 1× TAE buffer to a final concentration of 1% (w/v). In order to visualise DNA, ethidium bromide was added to a final concentration of 35 ng/μl. Gel images were taken using a BioRad Chemidoc XRS+ gel imaging system with the Quantity One 4.6.6 program (BioRad, Hemel Hempstead, Hertfordshire, UK).

2.2.7 Nucleic Acid Concentration Quantification

DNA concentrations were quantified using a Nanodrop ND-1000 spectrophotometer (Labtech, Ringmer, Sussex, UK). The quality and concentration of fungal genomic DNA was estimated from 0.8% agarose gels using Hyperladder 1 kb (Bioline, UK) as standard.

2.2.8 Plasmid Preparation

Liquid LB cultures were inoculated with *E. coli* DH5α colonies identified as containing the correct DNA insert for amplification and extraction of plasmid DNA. Cultures were inoculated by using a sterile toothpick to transfer the bacterial colony into 4.5 ml LB supplemented with ampicillin (final concentration 100 μg/ml) in a 30 ml Universal tube. Cultures were subsequently incubated at 37 °C, 150 rpm overnight. Plasmid DNA was then extracted using the Nucleospin Plasmid Miniprep kit (Macherey-Nagel, Düren, Germany).

2.2.9 Fungal Transformation

Liquid YEPD cultures in a 25 ml volume, supplemented with uridine (final concentration 10 mM) and para-aminobenzoic acid (final concentration 7.29 μM) where required, were inoculated with 400 μl of conidia suspension for each parental strain and incubated at 28 °C on a rotary shaker at 150 rpm to generate mycelia for protoplast transformation. After incubation for 22 h, mycelia were washed and

incubated for 1 h without agitation in citrate-phosphate buffer (pH 7.3) supplemented with dithiothreitol (final concentration 10 mM). Protoplasts were produced in 20 ml osmotic solution (0.6 M KCl, 10 mM phosphate buffer pH 5.8) containing 0.1 g lysing enzyme (Sigma-Aldrich) and 1.3 g VinoTaste Pro (Novozymes). The protoplasts were filtered, washed (wash solution: 0.6 M KCl, 0.1 M Tris/HCl pH 7.0) and resuspended in solution A (0.6 M KCl, 50 mM $CaCl_2$, 10 mM Tris/HCl pH 7.5). The protoplasts were then mixed with the respective plasmids and PEG solution (25% PEG 8000, 50 mM $CaCl_2$, 10 mM Tris/HCl pH 7.5), and subsequently incubated on ice for 30 min. Transformants were regenerated on plates containing GG10 agar with 1.2 M sorbitol as osmotic stabiliser and any additional supplementations as stated.

Chapter 3: UV Mutagenesis for Generation of Faster-Growing Lichen Mycobionts

3.1 Introduction

3.1.1 Lichen Growth Rates

Lichens are known to have slow growth rates, with many species of lichens exhibiting radial growth of only a few millimetres each year in the symbiotic state (Crittenden and Porter, 1991; Ahmadjian, 1993). There are various factors which affect the growth rate of lichens in nature, meaning that there are large amounts of variation present in lichen growth rates. These factors include the age and position of the thallus, environmental conditions, season and latitude. In addition, the amount of carbohydrates available from the photobiont also has an effect on the growth of the lichen (Benedict, 1990; Ahmadjian, 1993; Palmqvist *et al.*, 2008; Armstrong and Bradwell, 2010; Armstrong and Bradwell, 2011).

Variation in growth rate can also occur in a single lichen between the different lobes that make up the thallus, and it has been suggested that this variation may be a consequence of the differing genotypes of the lobes (Armstrong, 1984). Furthermore, although neighbouring lobes are influenced by proximity, carbohydrate exchange is unlikely to occur between lobes. This heterogeneous composition of the lichen thallus, in addition to the fact that the thallus is made up of two symbiotic partners and is therefore not physiologically integrated, means that it is not a coordinated structure. Thus, lichen growth is not uniform across the whole thallus (Armstrong, 1984; Ahmadjian, 1993). However, even though the lobes have different growth rates, the symmetry of the thallus is normally maintained due to fluctuations in growth of the lobes having an equalising effect (Honegger, 1993).

One key factor that can contribute to the observed low growth rate of lichens in nature is the fact that lichens can be physiologically inactive for long periods of time due to unsuitable environmental conditions. Thus, the lichen will only grow during short time periods when the surrounding environmental conditions are more suitable, and there is water available, for the lichen to be metabolically active. Thus, these long time periods when the lichen is metabolically inactive will be included when

measuring the average growth rate of the lichen over a certain period of time, and could result in an underestimation of the lichen growth rate (Crittenden and Porter, 1991). This might be particularly accentuated for lichens growing in extreme environments with limited annual periods of growth, for example in the Antarctic (Seymour *et al*., 2005a).

Further to the slow growth of lichens in nature, the cultured mycobionts of most lichen species have also been found to have slow growth rates. When grown on agar, cultured mycobionts tend to produce spherical colonies with a diameter of only 10-15 mm after one year. It is thought that this characteristic slow growth rate is an evolutionary adaptation resulting from the lichen symbiosis and is genetically controlled (Crittenden and Porter, 1991; Ahmadjian, 1993). For example, mycobionts of *Xanthoria elegans* from different worldwide sites exhibited different growth rates when grown under the same temperature conditions (Murtagh *et al.*, 2002). Crittenden and Porter (1991) found that the growth of cultured mycobionts on agar was slower than that of the source lichen-forming fungi found in nature; however, they also found that growing mycobionts in liquid culture resulted in higher growth rates than on agar media. A relationship between the growth rate of cultured mycobionts and the morphology of the corresponding lichen thallus has been suggested by Ahmadjian (1993), who found that mycobionts isolated from crustose or squamulose lichens had a faster growth rate than that of cultured mycobionts isolated from foliose lichens.

3.1.2 Physiological Factors Affecting Lichen Growth

There are a range of physiological factors that can have an effect on the growth of a lichen and lead to variations in growth rate. These variations include those related to the species and age of a lichen (Benedict, 1990). Some of the factors thought to influence lichen growth include competition with other organisms occupying the same substrate, the ratio of algal to fungal mass found within the lichen, as well as carbohydrate supply and diffusion (Pentecost, 1980; Armstrong, 1985; Benedict, 1990; Palmqvist, 2000; Armstrong and Bradwell, 2010).

Various indirect lichenometric studies of crustose lichens have found that smaller, younger thalli possess a faster growth rate than that of larger, and therefore

older, thalli; this indicates that lichen growth rates do not follow a linear relationship (Birkeland, 1981; Bull and Brandon, 1998; O'Neal and Schoenenberger, 2003; Armstrong and Bradwell, 2010). An important factor that may be implicated in the faster growth rate of younger thalli is that of the ratio of photobiont to mycobiont mass, with the ratio being higher in very young thalli (Clayden *et al.*, 2004).

In addition to the age of a lichen, it has been suggested that the movement of carbon (which is needed for growth) within the lichen thallus also has an effect on lichen growth rates (Aplin and Hill, 1979; Childress and Keller, 1980). It is thought that carbon translocation can be attributed to the differences in growth rate found between crustose and foliose lichens, with faster-growing foliose species having more efficient carbon translocation between the lobes of an integrated thallus compared to the translocation that occurs between the areolae and hypothallus of crustose species (Armstrong and Smith, 1987; Armstrong and Bradwell, 2010). Further to this, Benedict (2008) has suggested that the growth rate of crustose lichens decreases as the age of the thallus increases, as a result of carbon being used for the production of reproductive structures, such as apothecia, which therefore leads to a reduction in the translocation of carbon to the hypothallus to be used for radial growth.

3.1.3 Environmental Factors Affecting Lichen Growth

It has been suggested that one of the most important environmental factors limiting the growth rate of a lichen is that of thallus moisture content (Muir *et al.*, 1997; Hyvärinen and Crittenden, 1998), with Paterson *et al.* (1983) describing moisture availability as the most important environmental factor. This is due to the fact that, being poikilohydric organisms, lichens lack control of their water uptake and loss, and the thallus moisture content is therefore dependent upon the amount of water available in the environment (Palmqvist, 2000; Nash III, 2008a). The lichen thallus needs to have a certain minimal water content in order to be metabolically active and to allow photosynthesis to occur, with the levels of photosynthesis increasing linearly with thallus water content until an optimum thallus water content is reached. After this, increasing moisture content in the thallus has a detrimental effect on uptake of carbon dioxide and consequently limits the rate of photosynthesis taking place within the lichen (Green *et al.*, 2011). This means that growth of the lichen

thallus will only take place when the moisture level of the thallus is appropriate for photosynthesis to occur (Palmqvist, 2000). As such, the growth rate of a lichen is strongly correlated with the water availability in a particular environment (Benedict, 1990; Muir *et al.*, 1997).

Temperature and light availability are also thought to be important environmental factors that affect the growth rate of lichens (Armstrong, 2006; Armstrong and Bradwell, 2011). Similarly to water availability, temperature and light availability also have an effect on the rates of photosynthesis that take place within the lichen and, in turn, can limit the amount of carbon available to the lichen for growth (Benedict, 1990; Palmqvist *et al.*, 2008). When thallus moisture content is sufficient, light tends to be the primary limiting factor of lichen growth, with higher levels of light correlating to an increased growth rate (Muir *et al.*, 1997; Renhorn *et al.*, 1997; Palmqvist, 2000). Temperature is less important when considering the effect on lichen growth, particularly when considering lichen species found in temperate habitats (Palmqvist, 2000). However, it has been found that the higher temperatures associated with lichens found in tropical climates can increase respiration levels during dark periods which, in turn, leads to a reduction in net carbon (Zotz *et al.*, 1998; Palmqvist *et al.*, 2008).

Although water availability, temperature and light intensity as individual factors each have an effect on lichen growth rates, it is thought that the interaction of the three microclimatic factors also exhibits a considerable effect on the growth of lichens (Armstrong and Bradwell, 2011). This is due to the fact that thallus wetting results in carbon loss, through the loss of carbon dioxide as well as through rates of respiration increasing after the thallus has been resaturated (Smith and Molesworth, 1973). Therefore, in order for rates of photosynthesis to increase to a sufficient level that allows net carbon gain to be achieved and consequently enables the lichen to grow, the interactions between temperature, thallus moisture content and light availability are crucial to ensure that the lichen thallus maintains an appropriate moisture content in the light for a sufficient period of time (Armstrong, 1976; Armstrong and Bradwell, 2011).

3.1.4 Lichen Growth Rate and Their Exploitation

As discussed in the general introduction (Section 1.4), lichens produce a diverse range of SMs, some of which have potential commercial uses in the pharmaceutical and dye industries. However, the slow growth rates of lichens in nature has prevented their mass harvest due to sustainability issues, and the slow growth rate of mycobionts in axenic culture has proved a challenge for the economic production of metabolites in the biotechnology sector (Crittenden and Porter, 1991).

3.1.5 Chapter Objectives

The overall aim of this chapter was to investigate the growth rate of lichens by seeing if lichen sexual propagules could be exploited in an attempt to create mutants with faster growth rates. Such faster-growing mutants could then potentially provide insights into the genetic basis of slow growth rates in lichen-forming fungi as well as be used for the production of SMs on an industrial scale. Work was planned to involve:

(1) Creation of faster-growing strains. UV mutagenesis of various lichen mycobiont species will be used to create faster-growing strains. Mutants with a faster growth rate will be selected for using image analysis software.

(2) Genomic analysis of lichen growth rates. Mutants identified as possessing a relatively faster growth rate will be analysed at the genomic level in an attempt to provide insights into the genetic basis of growth rates in different lichens.

3.2 Materials and Methods

3.2.1 Species Origin and Storage

Thalli of *Xanthoria parietina*, *Lecanora chlarotera* and *Amandinea punctata* were collected from various locations in the UK, with collection details given in Appendix 2. All lichen samples were transported back to the laboratory in polythene bags in their naturally hydrated state. The lichen samples were trimmed so that only areas of thallus with apothecia remained and any other debris were removed. The samples were then air-dried at room temperature for 24 h before being sealed in fresh polythene bags and stored at -20 °C.

3.2.2 Isolation of Lichen Ascospores

Thalli of lichen species were taken from storage at -20 °C and were then washed in 100% ethanol for ~10 s to reduce surface contamination before being placed under a constant flow of water for 1 h, in order to rehydrate the thalli. The thalli were then rinsed in sterile distilled water (SDW) before being blotted dry with tissue. The rehydrated lichen thallus was subsequently attached to the lid of an empty sterile Petri dish with petroleum jelly via the underside of the thallus. Plates were incubated at 18 °C in the dark for 24 h with the thallus facing downwards to allow ascospores to discharge into the empty Petri dish. Ascospores were collected from the Petri dish using 1 ml of 0.1% Tween 80 (in SDW) and the resulting suspension was sonicated for 1 min without heating in an ultrasonic bath (USR 30 H; 160 W, 35 kHz) (Merck, Darmstadt, Germany) in order to break up any clumps of ascospores. The concentration of the spore suspension was then determined using an Improved Neubauer counting chamber.

3.2.3 UV Mutagenesis of Lichen Ascospores

For the UV mutagenesis of *X. parietina*, *L. chlarotera* and *A. punctata* ascospores, 50 μl of a suspension containing a known concentration of ascospores (see below for individual experiments) was spread onto a 9 cm Petri dish containing solid malt extract yeast extract (MEYE) medium supplemented with ampicillin and streptomycin (final concentration 100 μg/ml for each). Plates were then placed 45 cm under a Sylvania G15T8 germicidal UV-C lamp, the lid of the Petri dish was removed and the ascospores were exposed to UV for the appropriate amount of time before the lid was placed back onto the Petri dish. Plates were sealed with Parafilm and incubated at 18 °C in the dark. Plates were screened weekly for contamination and, where possible, contaminants were cut out of the agar under sterile conditions.

3.2.4 Determination of UV Exposure Time

The experiment was carried out in biological triplicate for each species, and agar plates were prepared in technical triplicate for each UV exposure time being tested. An ascospore suspension containing 5000 ascospores/ml was used when spreading the ascospores onto plates, and ascospores were exposed to UV-C for 0 s,

30 s, 60 s, 120 s, 300 s or 600 s. Plates were then incubated for 1-2 months (depending on the species) until colonies were visible and could be counted. Once the colonies had been counted on each plate, the average number of colonies was calculated for each exposure time and then converted to percentage survival (with the number of surviving colonies at 0 s of UV-C exposure being used as the 100% value control). A survival curve was then plotted on a graph for each species using GraphPad Prism version 9.2.0 (GraphPad Software, San Diego, California, USA), and the exposure time corresponding to a 5-10% survival rate was selected as the exposure time to use for subsequent UV mutagenesis experiments.

3.2.5 Generation and Identification of Faster-Growing Mutants

Once the UV exposure time had been determined for each lichen species, lichen ascospores were isolated, as described in Section 3.2.2, and exposed to UV-C for the appropriate length of time, as described in Section 3.2.3. For each UV mutagenesis experiment, ascospores from the same spore suspension were plated onto 50 plates which were to be exposed to UV, and three control plates which were not exposed to UV. The concentrations of the ascospore suspensions used to spread onto the control plates and treatment plates were calculated according to the survival rate of spores with or without UV treatment, to ensure that 20 surviving colonies would grow on each plate.

Plates were incubated for 15 weeks until colonies were readily visible but still maintained a mostly circular shape. Photographs of the plates were then taken using a Nikon D3200 camera fitted with an AF-S DX NIKKOR 35 mm 1:1.8G lens, and with the camera positioned 36 cm above the plate. Images were then analysed using a macro (provided by Franziska Wohlgemuth) run in Fiji (Schindelin *et al.*, 2012) in order to determine the area of each colony, as shown in Figure 3.1. Any colonies missed by the macro were manually included in the analysis, and any colonies where the edge of the colony could not be accurately determined (for example, if the colony was growing at the edge of the plate or if two colonies were growing directly next to each other) were discounted from the analysis.

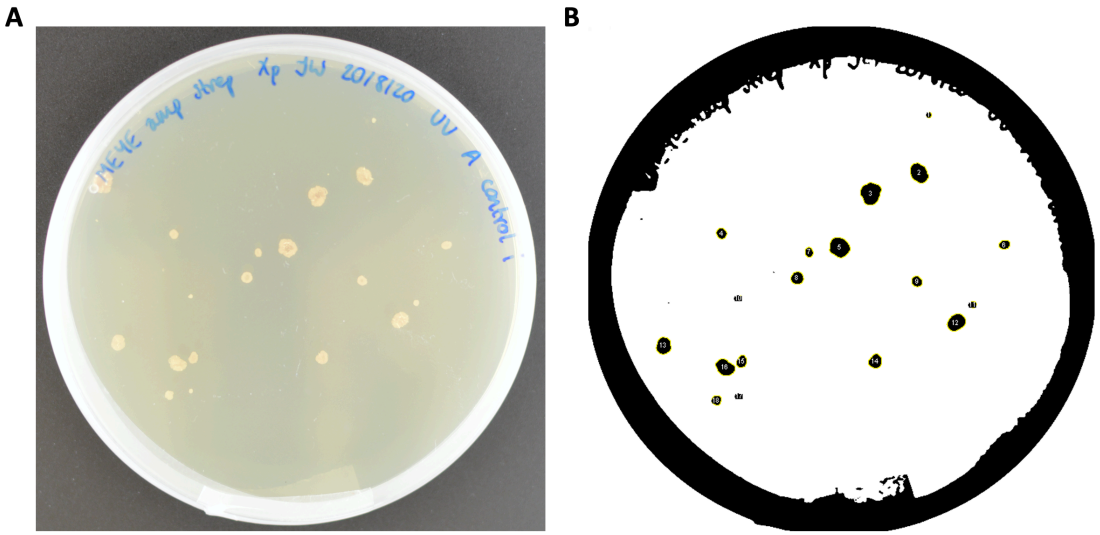


Figure 3.1. Image analysis of lichen mycobiont colonies. The macro was run on each raw image **(A)**, and a corresponding output image **(B)** was created in which colonies included in the analysis were outlined in yellow and assigned a number for identification purposes.

The average area of the colonies growing on the control plates was calculated, and this value was used as the average colony size of a lichen mycobiont with a normal growth rate. The areas of the colonies which underwent UV mutagenesis were then compared to the average value of the control colonies, as well as to the calculated standard deviation for the area of the colonies on the control plates, and colonies with an area more than three times greater than that of the average control colony were identified as potentially having a faster growth rate. Colonies identified as having a faster growth rate were subsequently cut out of the plate and transferred to another plate containing solid MEYE medium supplemented with ampicillin and streptomycin (final concentration 100 μg/ml for each) under sterile conditions, to ensure that these colonies did not end up growing into another colony on the original plate. These transfer plates were incubated at 18 °C in the dark.

3.3 Results

3.3.1 UV Exposure Time of Lichen Ascospores

In order to determine the appropriate UV exposure time of *X. parietina*, *L. chlarotera* and *A. punctata* ascospores for subsequent UV mutagenesis experiments, a known number of ascospores plated on solid MEYE supplemented with ampicillin and streptomycin were exposed to UV-C for various time periods ranging between 0

s and 600 s. Ascospore germination was used to indicate ascospore survival. A UV exposure time producing an ascospore survival rate of approximately 5-10% was determined as the exposure time that would be selected for subsequent UV mutagenesis experiments.

For *X. parietina* ascospores, a UV exposure time of 300 s was selected as the exposure time that would be used in subsequent *X. parietina* UV mutagenesis experiments (Figure 3.2A). For *L. chlarotera* ascospores, a UV exposure time of 216 s was selected as the exposure time to be used in subsequent *L. chlarotera* UV mutagenesis experiments (Figure 3.2B). The UV exposure time for *A. punctata* ascospores was identified as 280 s (Figure 3.2C); again, this was selected as the exposure time to be used in subsequent UV mutagenesis experiments.

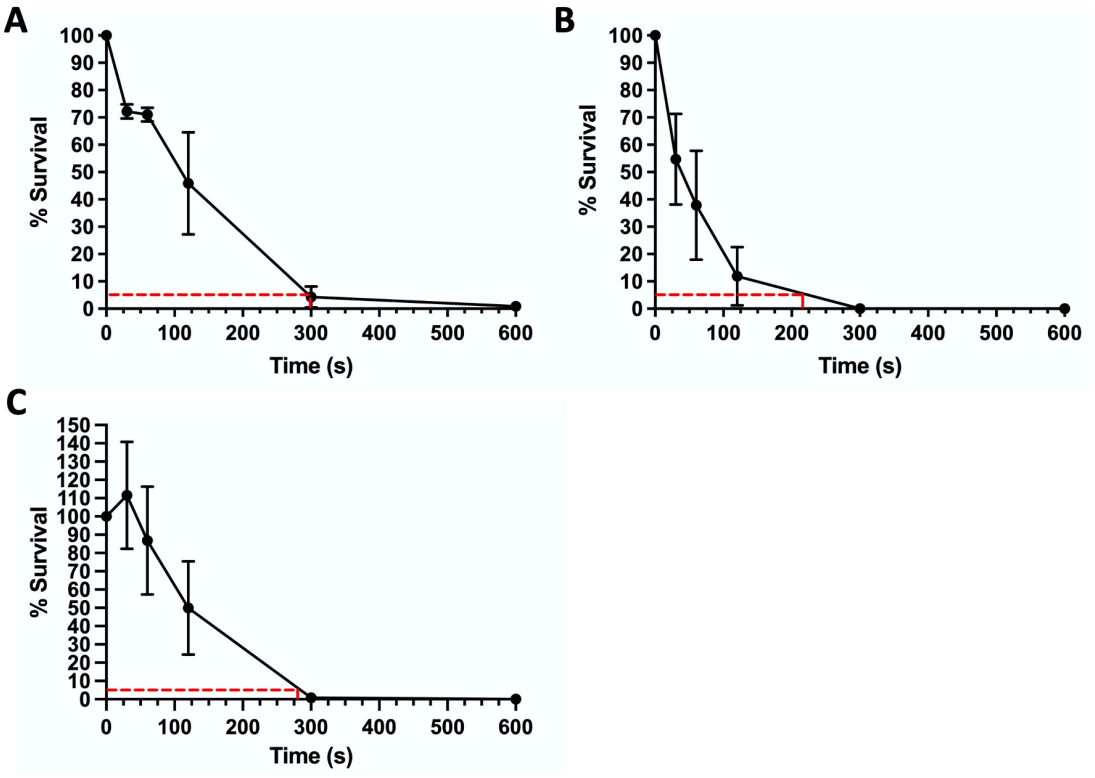


Figure 3.2. Percentage survival of *X. parietina* **(A)**, *L. chlarotera* **(B)** and *A. punctata* **(C)** ascospores after exposure to UV for 0-600 s. The red dashed line indicates the UV exposure time corresponding to a 5% survival rate for the ascospores. Error bars indicate SD.

3.3.2 Generation and Identification of Faster-Growing Mutants

3.3.2.1 *Xanthoria parietina*

In order to generate mutants of *X. parietina* possessing a relatively faster growth rate, a known number of ascospores were plated onto solid MEYE supplemented with ampicillin and streptomycin and exposed to UV-C for 300 s. The area of surviving colonies was calculated using image analysis software and those colonies having an area more than three times greater than that of the average area of colonies on control plates were identified as having a faster growth rate. These colonies identified as possessing a faster growth rate were subsequently isolated and transferred to fresh plates.

After incubation at 18 °C in the dark for 15 weeks, there were a total of 400 surviving *X. parietina* colonies on the treatment plates (full data shown in Appendix 3). Image analysis of colonies growing on the control plates showed that the average area of the colonies growing was 5996.86 pixels (Appendix 3). The standard deviation of the data obtained for the control plates was calculated as 4785.40 pixels. Of the surviving colonies growing on the treatment plates, image analysis identified six colonies as having an area that was more than three times greater than the average area of a control colony, as seen in Table 3.1 and Figure 3.3. The areas of these identified colonies were also greater than the average control colony area plus the calculated standard deviation (10,782.26 pixels). These colonies were then transferred to individual plates for subsequent analysis.

Plate Number	Colony Number	Area of Colony (pixels)
A-10	3	25,697
A-12	1	20,510
A-22	1	23,733
A-37	9	19,573
A-42	1	19,767
A-43	11	22,842

Table 3.1. Colonies of UV-exposed *X. parietina* identified as having an area more than three times greater than that of the average control non UV-exposed colony.

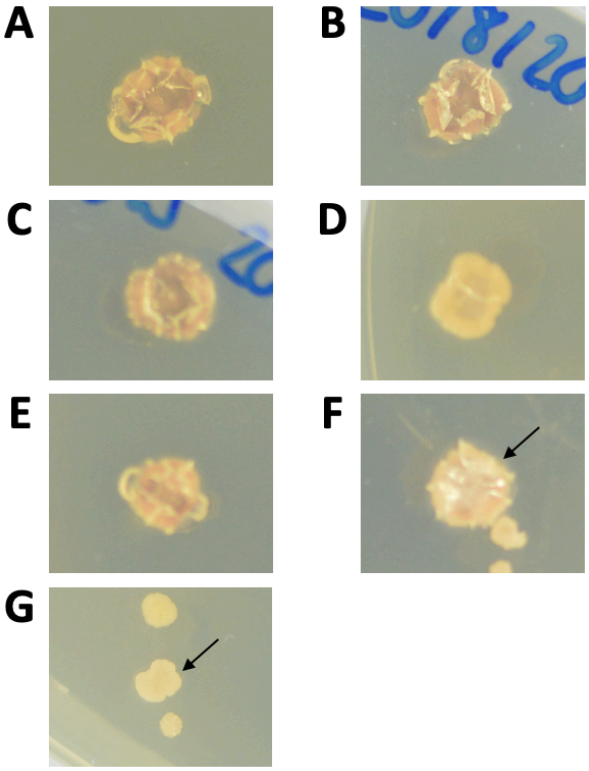


Figure 3.3. *X. parietina* mutants A-10.3 **(A)**, A-12.1 **(B)**, A-22.1 **(C)**, A-37.9 **(D)**, A-42.1 **(E)** and A-43.11 **(F)** identified as having an area more than three times greater than that of the average control colony, in comparison to a non-UV treated control colony **(G)** with a similar area (5770 pixels) to that of the average for the control colonies. For images with other colonies in close proximity, arrows indicate the identified colony.

3.3.2.2 *Lecanora chlarotera*

Repeated attempts were made to obtain sufficient ascospores of *L. chlarotera* for UV mutagenesis from the available material. However, great difficulties were encountered. As a result, and due to time constraints with other project work, the generation of faster-growing mutants was not carried out as part of this study.

3.3.2.3 *Amandinea punctata*

Repeated attempts were made to obtain sufficient ascospores of *A. punctata* for UV mutagenesis from the available material. However, great difficulties were encountered. As a result, and due to time constraints with other project work, the generation of faster-growing mutants was not carried out as part of this study.

3.4 Discussion

Lichens are well-known to possess extremely slow growth rates; this phenomenon has been observed both in nature and when grown in the laboratory under controlled conditions (Crittenden and Porter, 1991; Ahmadjian, 1993). Moreover, this slow growth rate has prevented the use of lichens for the production of SMs, and has consequently contributed to a lack of research into the many different SMs produced by various lichen species and their potential applications (Crittenden and Porter, 1991; Miao *et al.*, 2001).

The generation of faster-growing mutants of the mycobiont of various lichen species provides an opportunity to gain further insight into how the growth rates of lichens are regulated, as well as to potentially use these faster-growing strains in the production of lichen SMs in biotechnology. It was decided that this study would focus on the lichen species *X. parietina*, *L. chlarotera* and *A. punctata*, as the habitats of these lichens are found locally and, therefore, meant that lichen material should be readily available.

3.4.1 UV Exposure Time of Lichen Ascospores

The differing exposure times required to produce a 5% ascospore survival rate for each of the species indicates that the sensitivity of lichen ascospores to UV radiation is species-specific. The observed variability in UV tolerance between the ascospores of *X. parietina*, *L. chlarotera* and *A. punctata* could be a consequence of the fact that they are found in different natural environments, as each will be adapted to differing levels of UV radiation. This has been shown for the conidia (asexual spores) of various non-lichen fungal species (Braga *et al.*, 2001; Bidochka *et al.*, 2001; Singaravelan *et al.*, 2008; Luque *et al.*, 2012; Braga *et al.*, 2015). *X. parietina* is found to grow on a wide range of substrata, and has the ability to grow in both shaded habitats as well as in habitats in which the thallus is exposed to high levels of sunlight (Smith *et al.*, 2009); this suggests that *X parietina* ascospores will possess a reduced sensitivity to UV radiation in order to provide a selective advantage when growing in areas with high amounts of sunlight. Similarly, *A. punctata* is also found to grow on a wide range of substrata, including bark, wood and rocks, and is therefore also likely to be exposed to high levels of sunlight in these habitats (Smith *et al.*, 2009). *L.*

chlarotera, however, is usually found to grow on the bark of deciduous trees, a habitat which is more likely to be shaded and therefore receive lower levels of UV irradiation via sunlight (Smith *et al.*, 2009).

It can be assumed that those lichen species adapted to higher levels of UV irradiation will most likely have ascospores possessing adaptations which increase tolerance to UV radiation. Various mechanisms are employed by fungal species to prevent or reduce damage occurring, as well as to repair any damage that is caused (Braga *et al.*, 2015; Cortesão *et al.*, 2020). Studies focussing on the UV tolerance of conidia in non-lichenised fungal species have found that, in general, those species producing smaller and hyaline conidia display an increased sensitivity to UV radiation compared to those species producing larger and pigmented conidia (Al-Rubeai and El-Hassi, 1986; Braga *et al.*, 2001; Nascimento *et al.*, 2010). This principle could be applied to the ascospores of the lichen species used in this study to explain the variation in UV tolerance; *X. parietina* produces hyaline ascospores which are usually 12-16 x 7-9 µm and *A. punctata* produces brown ascospores that are 11.5-16 x 6-8 µm in size, whereas the ascospores of *L. chlarotera* are hyaline with a size generally in the range of 11-13 x 6.5-7.5 µm (Smith *et al.*, 2009). The brown pigment seen in *A. punctata* ascospores has not been identified. The conidia of most fungal species contain DHN-melanin which is responsible for pigmentation; however, ascospores may contain alternative pigments, such as asperthecin in *A. nidulans*, which are responsible for the pigmentation of fungal ascospores (Brown and Salvo, 1994; Braga *et al.*, 2015; Geib *et al.*, 2016; Palmer *et al.*, 2021). The larger size of *X. parietina* and *A. punctata* ascospores, as well as the brown pigmentation found in *A. punctata* ascospores, could in part explain why these two species have a similar UV exposure time to produce a 5% ascospore survival rate, and also why they appear to be more tolerant of UV than *L. chlarotera* ascospores.

3.4.2 Generation and Identification of Faster-Growing Mutants

The difficulties in obtaining ascospores from *L. chlarotera* and *A. punctata* material could perhaps be a consequence of when the later samples for UV mutagenesis work were collected, as lichen material from different sources (Appendix 2) were used for the initial exposure time work and the mutagenesis work. Lichens are

known to discharge their ascospores after wetting of the thallus has occurred, with a close correlation between rainfall and ascospore discharge being observed (Ostrofsky and Denison, 1980). Therefore, if a period of rainfall had occurred in the days preceding the point at which samples were collected, the majority of ascospores would have already been discharged prior to the lichen samples being collected. This would then mean that subsequent attempts to isolate a sufficient number of ascospores for experimental treatment would be unsuccessful.

When carrying out image analysis of the *X. parietina* treatment plates, it became apparent that there was variation present in the number of colonies growing on each plate despite best efforts for each plate to contain the same number of colonies. This observed variation in colony number could be due to the inherent variability present in the germination rates of ascospores from different thalli, or different apothecia, of the same species (Kofler, 1970). The variable colony numbers seen on the treatment plates could then have had an impact on the observed growth rates of colonies, as those colonies growing on plates with a higher colony number would have been subject to increased competition for nutrients, as well as having less space to grow. This could, therefore, mean that colonies with a potentially faster growth rate would not have been able to grow to their full potential. Consequently, the smaller area of these colonies would have obscured their faster growth rate, and these colonies would not have been identified in the analysis as possessing a faster growth rate.

The above caution aside, it was nevertheless of great interest to identify six UV mutants of *X. parietina* with greater than 3-fold elevated growth rates compared to the controls which had not undergone any UV exposure. This follows on from previous work with the lichen-forming fungus *Cladonia grayi*, for which a faster growing strain (which had arisen spontaneously during laboratory culture) had been selected for use in further experimental work (Daniele Armaleo, Duke University, USA). UV mutagenesis is a favourable method for exploring the genetic basis of traits whose underlying genetic basis is unknown, as mutagenesis can cause multiple mutations throughout the genome, which might either impact on a trait of interest due to a point mutation or synergy of multiple mutations (Jung and Till, 2021). In classical genetic

work, UV mutants have been used to reveal the genetic basis of traits such as pigment production (Caten, 1979).

It is acknowledged that there appears to be large amounts of variation in the areas of colonies growing on control plates, with one control colony in fact having an area more than three times greater than that of the average control colony area, and an additional five control colonies having an area greater than the average control colony plus the standard deviation value. Furthermore, the data obtained for the treatment plates also show a wide range of values for the areas of the colonies. This perhaps suggests that the six *X. parietina* mutants identified as having a faster growth rate do not in fact possess mutations as a result of UV exposure which are leading to an increased growth rate, and instead are a result of the large variation in colony size seen for colonies growing on both the control plates and treatment plates. Nevertheless, the areas of the six *X. parietina* mutants identified, when compared to the area of the average control colony, still suggest that these colonies appear to have an elevated growth rate, and it is worth continuing to study these identified colonies in order to determine the genetic basis of their apparent faster growth rate.

Considering filamentous ascomycetes in general, growth rate is a complex process, and is likely to be under multifactorial control. Various transcriptional studies have found that the growth and morphology of filamentous fungal species are controlled by diverse cell signalling networks (Meyer *et al.*, 2009; Harris *et al.*, 2009; Yin *et al.*, 2017). The major signalling cascade pathways in filamentous fungi include PKA/cAMP signalling, MAPK cascades and calcium ion responses, and these signalling cascades go on to activate transcription factors involved in the regulation of filamentous growth. Examples of such transcription factors include BrlA, StuA, FlbA and CrzA (Cairns *et al.*, 2019).

Given this background, various hypotheses and speculations can be made about the causal reasons for the slow growth rate of lichen-forming fungi and the possible reasons for the elevated growth rate in the UV-induced mutants of *X. parietina.* There are various processes taking place within the organism that will have an effect on the growth of a lichen. For example, production of SMs requires carbon, which will therefore mean that less carbon is available for lichen growth and, therefore, provides one contributory factor for the relatively low growth rates of

lichens (Honegger, 1993; Stocker-Wörgötter, 2008). This means that a faster growth rate might be achieved in the mycobiont, via any one of multiple mutations in genes needed for secondary metabolite production. As such, single point mutations in secondary metabolite pathways genes introduced into the mycobiont genome as a result of UV mutagenesis may have contributed to produce faster-growing mutants. However, given that it was hoped to produce faster growing strains for secondary metabolite production, such mutations would not be desirable.

Furthermore, UV mutagenesis is a random process and might have induced mutations in regulatory genes for growth such as those described above for *Aspergillus* species. Thus, the increased growth rate might have been due to altered transcriptional control by a mutated version of a transcription factor. By contrast, it is noted that there was no guarantee that the mutations introduced into the mycobiont genome would have the desired effect on the growth rate of the mycobiont and could in fact result in mutants with a slower growth rate. Such mutations could provide insights into genes whose overexpression might be used to increase lichen growth rate. However, such slow growing mutants were not targeted in the present study.

Further to this, the growth of each biont is thought to be regulated by its partner within the lichen symbiosis, as it is not thought to be advantageous for the mycobiont to outgrow its photosynthetic partner. This interaction of the lichen symbionts adds even more complexity in terms of the growth of lichens and suggests that lichens have evolved a slow growth rate because it provides a selective advantage (Ahmadjian, 1993; Honegger, 1993). In addition, mycobionts have been argued to be committed to the lichen symbiosis due to significant changes in their genome relative to non-lichenised fungi, and have adapted their growth rate accordingly (Armaleo *et al.*, 2019). Therefore, it is possible that the elevated growth rate seen in the UV-induced mutants of *X. parietina* might be due to mutation of one or more such genes involved with the lichen symbiosis (e.g. Joneson *et al*., 2011), with the mutation allowing more relaxed growth.

3.5 Conclusions

Due to time constraints and difficulties obtaining experimental material it was only possible to conduct pilot work in the present study. Nevertheless, the limited results obtained in this chapter suggest that the UV mutagenesis of lichen ascospores provides a suitable method to generate mutants of lichen mycobionts possessing at least an incremental increase in growth rate. UV mutagenesis was particularly applicable in the present studies because so little is known about genetic factors responsible for the slow growth rate of lichen-forming fungi. However, it is acknowledged that UV mutagenesis is a non-targeted method of introducing mutations into the mycobiont genome and, as a consequence, there is no way of specifically targeting those genes that might be involved in the regulation of lichen growth. Such directed work would require classic GM or CRISPR approaches not yet available for lichens (Miao *et al.*, 2001). Furthermore, the non-specific nature of UV-induced mutations also means that there is no way of preventing mutations being introduced in other genes unrelated to lichen growth which could have a detrimental effect on other essential processes within the mycobiont.

Future work following on from the work undertaken in this chapter could involve conducting genomic analysis on the six *X. parietina* colonies perceived as being faster-growing. This could allow insights to be obtained into the genes that are perhaps implicated in the regulation of growth in lichens. However, it is cautioned that due to the multifactorial nature of growth, it is unlikely that identifying a single point mutation(s) affecting the mycobiont growth rate will be easy or straightforward. In addition to this, future work could also include carrying out further UV mutagenesis of the *X. parietina* colonies identified as potentially possessing a faster growth rate, in order to see if the growth rate of these mutants can be increased further.

Chapter 4: Heterologous Expression of Lichen Polyketide Synthases

4.1 Introduction

4.1.1 Biosynthesis of Polyketides

The majority of polyketides produced by lichens are aromatic compounds made up of monoaromatic subunits, which are synthesised by a polyketide synthase (PKS). Two, or sometimes more, of these monoaromatic subunits are joined together to form the core structure of the polyketide product (Elix and Stocker-Wörgötter, 2008; Calcott *et al.*, 2018). The core structures of polyketides can then be further modified via a set of common reactions that occur after biosynthesis of the core structure has taken place (Stocker-Wörgötter, 2008). These tailoring reactions include oxidation, *O*-methylation, chlorination, halogenation, esterification and decarboxylation (Mosbach, 1969; Elix and Stocker-Wörgötter, 2008; Stocker-Wörgötter *et al.*, 2013; Millot *et al.*, 2016). The modification of the core structures results in the production of a diverse range of secondary compounds, examples of which are shown in Figure 4.1. Lichens can also produce additional aromatic compounds via the acetyl-polymalonyl biosynthetic pathway, known as anthraquinones, naphthaquinones, chromones and xanthones. However, these polycyclic SMs are produced via the internal cyclisation of a longer folded single polyketide chain, rather than deriving from two or more polyketide chains (Culberson and Elix, 1989; Stocker-Wörgötter, 2008; Elix and Stocker-Wörgötter, 2008; Calcott *et al.*, 2018).

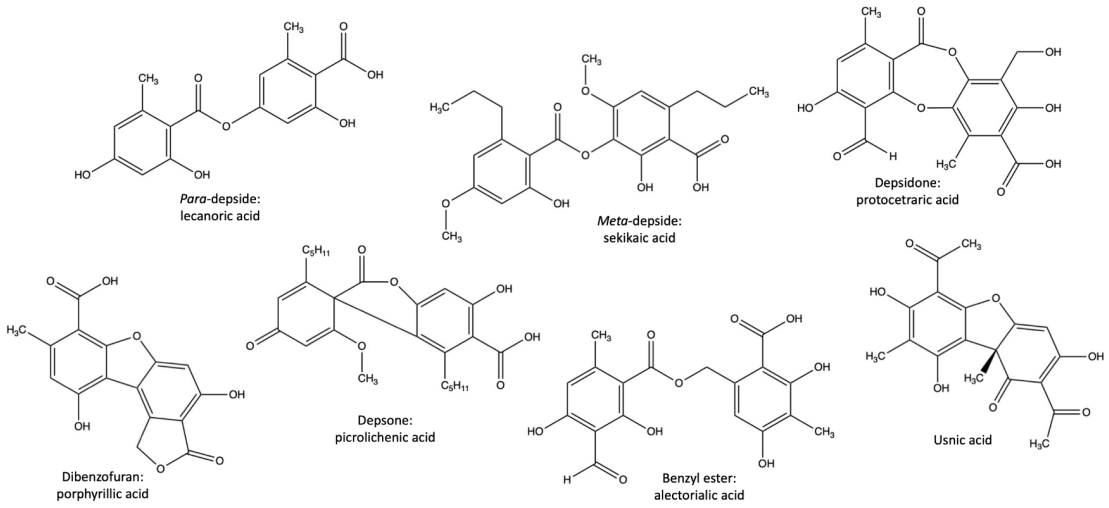


Figure 4.1. Examples of lichen phenolic polyketides. The core structures of lichen phenolic polyketide products are composed of either orcinol, β-orcinol or methylphloroacetophenone monoaromatic subunits joined together by ester, ether or carbon-carbon bonds. An example of each of the major groups of lichen phenolic polyketides is shown. Adapted from Elix and Stocker-Wörgötter (2008) and Calcott *et al.* (2018).

The majority of lichen aromatic polyketides are thought to be made up of two or three orcinol or β-orcinol monomers (Elix and Stocker-Wörgötter, 2008). However, the dibenzofuran-like usnic acid and its derivatives differ in the sense that they are composed of two methylphloroacetophenone monomers (Millot *et al.*, 2016). Even though these groups of secondary compounds differ in their basic building blocks, they are all thought to be produced in a similar way, via a non-reducing polyketide synthase (NR-PKS) which uses its active site in an iterative manner (Stocker-Wörgötter, 2008; Elix and Stocker-Wörgötter, 2008). Both the orsellinate-type and methylphloroacetophenone monoaromatic subunits are tetraketides composed of one acetyl-CoA and three malonyl-CoA extender units. The tetraketide is cyclised via a condensation reaction in order to produce either an orcinol, β-orcinol or methylphloroacetophenone precursor (Figure 4.2). These precursors are then used to form the core structures of lichen secondary compounds (Mosbach, 1969; Stocker-Wörgötter, 2008; Millot *et al.*, 2016). It is thought that the β-methyl groups found in β-orcinol and methylphloroacetophenone are introduced by a *C*-methyltransferase domain integrated into the PKS before the tetraketide precursor is cyclised (Elix and Stocker-Wörgötter, 2008; Nguyen *et al.*, 2013; Calcott *et al.*, 2018).

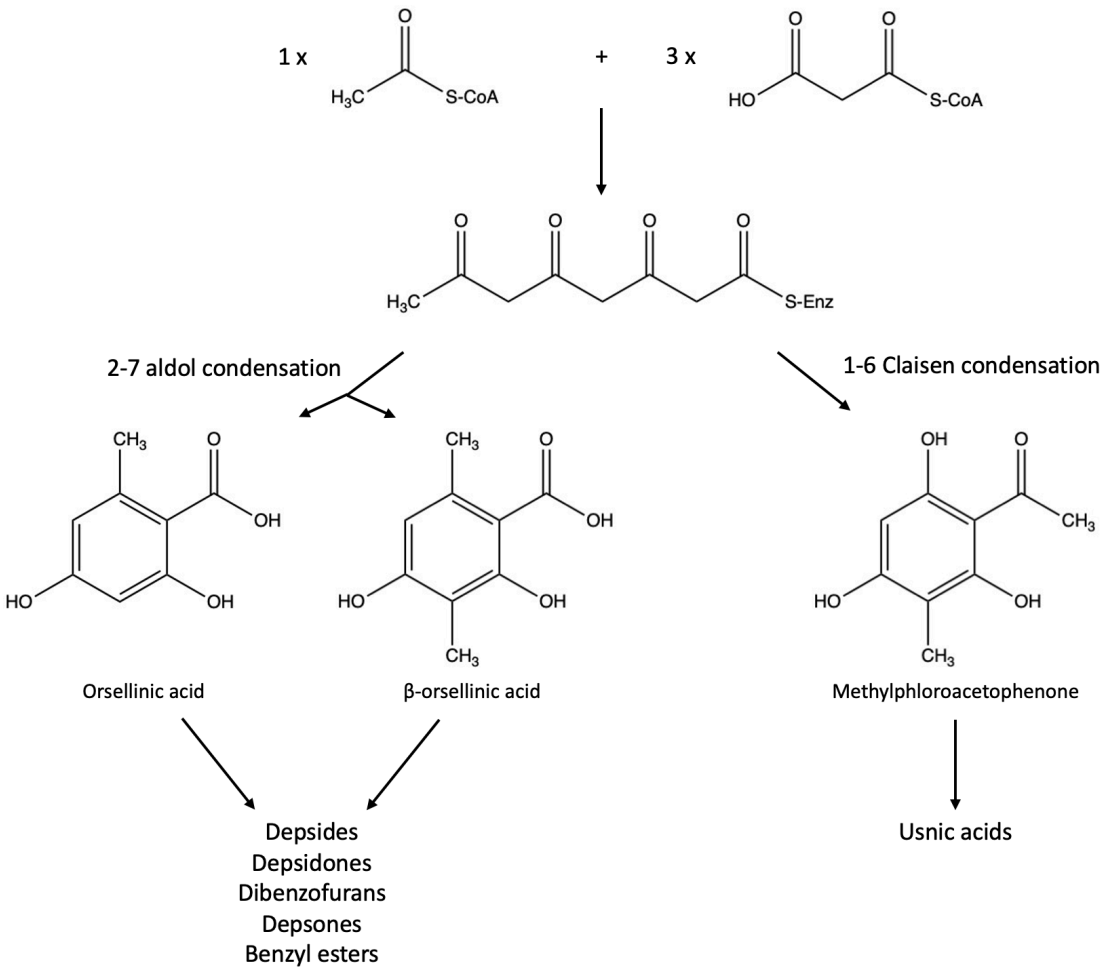


Figure 4.2. Cyclisation of tetraketides. The tetraketide precursor can either be cyclised via an enzyme-catalysed Claisen-type condensation reaction or a non-enzymatic aldol condensation reaction. A Claisen-type condensation will result in the production of a methylphloroacetophenone precursor, whereas an aldol condensation will lead to the production of an orsellinate-type precursor. These monoaromatic subunits form the core structure of phenolic polyketide SMs. Adapted from Stocker-Wörgötter (2008), Millot *et al.* (2016) and Calcott *et al.* (2018).

After cyclisation has taken place, the core structures of the aromatic polyketides are produced by the joining of the monoaromatic subunits with either ester, ether or carbon-carbon bonds; the type of bond present differs depending on the type of polyketide product (Mosbach, 1969; Elix and Stocker-Wörgötter, 2008). In the case of depsides, the core structure is formed when the carboxyl group of one subunit forms an ester bond with the hydroxyl group of a second subunit; the carboxyl group can be joined to either a *para* or *meta* hydroxyl group, thus forming either a *para*-depside or *meta*-depside, respectively (Mosbach, 1969; Stocker-Wörgötter, 2008; Calcott *et al.*, 2018). It is believed that *meta*-depsides are formed from *para*-depsides as a result of *C*-hydroxylation of the *para*-depside prior to a rapid

intramolecular rearrangement to form the *meta*-depside (Elix and Gaul, 1986). Variation in the structures of depsides can be found in the length and level of oxidation of their side chains in addition to the level of methylation of the hydroxyl- and carboxyl-groups (Stocker-Wörgötter, 2008).

Depsidones are formed when two orcinol or β-orcinol moieties are joined together with ester and ether bonds (Mosbach, 1969; Nguyen *et al.*, 2013). Due to the presence of a depside and its corresponding depsidone in the same lichen, it has been suggested that *para*-depsides are the precursors of depsidones (Culberson, 1964; Stocker-Wörgötter, 2008). The first hypothesis for the formation of depsidones proposed that they result from the intramolecular oxidative coupling of *para*-depsides via phenoxy radicals; however, it was found that the synthesis of depsidones is more complex (Seshadri, 1944; Stocker-Wörgötter, 2008). Studies involving the biomimetic synthesis of depsidones found that intramolecular rearrangements are required to form a depsidone; this resulted in a second hypothesis which suggested that depsidones are formed from a *meta*-depside intermediate that undergoes a Smiles rearrangement (Elix *et al.*, 1987). However, the most recent hypothesis regarding depsidone formation does not incorporate the ideas proposed in the first two hypotheses and instead proposes that depsidones are formed from the oxidation, by dioxygenase, of their corresponding *para*-depsides and subsequent cyclisation of the intermediate (Stocker-Wörgötter, 2008).

In addition to being the precursors of *meta*-depsides and depsidones, *para*-depsides are also believed to be biosynthetic intermediates of dibenzofurans, depsones and diphenyl ethers (Culberson and Elix, 1989; Elix and Stocker-Wörgötter, 2008). These compounds contain different bonds joining the two monoaromatic subunits (Elix and Stocker-Wörgötter, 2008). The core structure of dibenzofurans is formed by the oxidative coupling of the two orsellinic acid moieties, either through the joining of the carboxyl group of one subunit to the hydroxyl group of the other subunit or through the formation of a carbon-carbon bond between the two subunits (Huneck and Yoshimura, 1996; Millot *et al.*, 2016). Depsones contain a carbon-carbon bond in addition to an ester bond that joins the monoaromatic subunits (Huneck and Yoshimura, 1996; Elix and Stocker-Wörgötter, 2008). Diphenyl ethers are formed through the joining of the two subunits via an ether bond (Huneck and Yoshimura,

1996; Nguyen *et al.*, 2013). Importantly, while the production of these SMs appears common to various lichen species, the biosynthetic pathways are only deduced by the structures of the metabolites. The biochemical characterisation of PKSs and tailoring enzymes has not yet been successful, which leaves room for further speculations on the biosynthesis of these compounds (Bertrand *et al.*, 2018).

4.1.2 Polyketide Synthases

PKSs are large multifunctional protein complexes which usually contain a core of coordinated active sites. They are responsible for the production of the polyketide backbone and are encoded by a single PKS gene (Bingle *et al.*, 1999; Stocker-Wörgötter, 2008). In fungi, PKS genes are frequently found in metabolite biosynthesis gene clusters within the genome. The genes that make up a biosynthesis gene cluster are sequentially arranged, that is they are located adjacent to each other on a chromosome (Keller and Hohn, 1997; Elix and Stocker-Wörgötter, 2008). As well as the gene coding for the PKS, the clusters contain additional genes that are also involved in biosynthesis of the SM. These additional genes may include cytochrome P-450 monooxygenases, methyltransferases, oxidases, reductases, fatty acid synthetases and dehydrogenases (Keller and Hohn, 1997; Miao *et al.*, 2001; Brakhage, 2013).

There are three main types of PKS which are known to differ in their structures; they can be found in both prokaryotic and eukaryotic organisms (Hopwood and Sherman, 1990; Stocker-Wörgötter, 2008). Type I and type II PKSs are mainly found in bacteria and fungi, whereas type III PKSs dominate in higher plants; however, type III PKSs have been identified in some fungal species. Both type I and II PKSs use substrates which are joined to an acyl carrier protein (ACP) by a thioester bond; this is carried out via multifunctional enzymes (all the active sites are present in one protein) in type I PKSs or groups of monofunctional enzymes (each protein subunit has one active site) in type II PKSs. Type III PKSs, however, do not have an ACP moiety so instead use substrates which are coenzyme A esters rather than ACP-linked (Hopwood, 1997; Miao *et al.*, 2001; Stocker-Wörgötter, 2008; Hashimoto *et al.*, 2014).

It is believed that the synthesis of most polyketides in fungi involves iterative type I PKSs, which are analogous to fatty acid synthases (FASs) found in vertebrates and possess a similar structure to that of FASs (Hopwood, 1997; Bingle *et al.*, 1999; Elix and Stocker-Wörgötter, 2008). Iterative PKSs consist of single modules (i.e. they are single-protein complexes) that have all the domains needed and repeatedly use their active sites to synthesise the polyketide product (Miao *et al.*, 2001; Schmitt *et al.*, 2005; Stocker-Wörgötter, 2008). Iterative type I PKSs contain a single copy of a highly conserved ancestral ketoacyl synthase (KS) domain which is used repeatedly to sequentially condense C_2 molecules to form the growing polyketide chain. This decarboxylative condensation is the main step in forming the carbon backbone of the polyketide (Miao *et al.*, 2001; Boustie and Grube, 2005; Elix and Stocker-Wörgötter, 2008).

In addition to a KS domain, fungal PKSs can also contain an acyltransferase (AT) domain, dehydratase (DH) domain, ketoreductase (KR) domain, enoylreductase (ER) domain, an ACP (also known as a phosphopantetheine attachment site or PP domain) domain and a thioesterase (TE) domain, as shown in Figure 4.3. The domain architecture of a PKS depends upon whether it is a highly reducing PKS (HR-PKS), partially reducing PKS (PR-PKS) or non-reducing PKS (NR-PKS) (Hopwood and Sherman, 1990; Bingle *et al.*, 1999; Stocker-Wörgötter, 2008; Herbst *et al.*, 2018). All of these domains are found in FASs, whereas only the KS, AT and ACP domains are essential in PKSs (Elix and Stocker-Wörgötter, 2008; Brakhage, 2013). The KR, DH and ER domains have activities associated with a cycle of reactions resulting in the removal of a keto group. This involves the reduction of the keto-group to a hydroxyl-group, followed by the dehydration of the hydroxyl-group to an enoyl-group, and then reduction of the enoyl-group to produce an alkanoyl-group (Hopwood and Sherman, 1990; Elix and Stocker-Wörgötter, 2008). The three domains responsible for this cycle of reactions are non-essential in PKSs, meaning that they are only present in HR-PKSs or PR-PKSs where the polyketide product requires these domains for keto-group reductions (Stocker-Wörgötter, 2008; Herbst *et al.*, 2018).

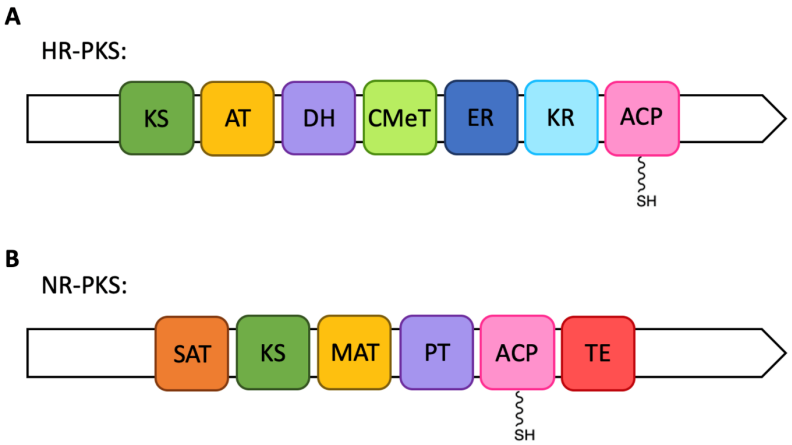


Figure 4.3. Domain organisation of a HR-PKS (A) and NR-PKS (B). In HR-PKSs, the AT domain is responsible for the selection of both acetyl-starter and malonyl-extender units. In NR-PKSs, the SAT (starter-unit acyltransferase) domain, which is unique to NR-PKSs, is responsible for loading a starter unit for polyketide extension and the MAT (malonyl-acetyl transferase) domain is responsible for the selection of malonyl-extender units. Transfer of the starter unit to the KS domain initiates extension of the polyketide backbone. The KS domain, which determines the length of the polyketide chain, carries out decarboxylative condensations of extender units which have been selected by the AT domain and subsequently loaded onto the ACP domain. In the case of HR-PKSs, the polyketide intermediate can then undergo various modifications, such as reduction, dehydration or methylation. NR-PKSs, however, lack these modifying domains and instead carry out aromatic cyclisation reactions of the polyketide intermediate using the PT (product template) domain, which is structurally related to the DH domain. Following cyclisation, the polyketide product is transferred to the TE domain which mediates product release. HR-PKSs commonly lack a *C*-terminal releasing domain, and instead need an additional enzyme to mediate release of the polyketide product (Chooi and Tang, 2012; Brakhage, 2013; Herbst *et al.*, 2018). Adapted from Stocker-Wörgötter (2008), Brakhage (2013) and Herbst *et al.* (2018).

The variations in domain composition of PKSs results in a diverse range of polyketides, each possessing a different structure and complexity (Elix and Stocker-Wörgötter, 2008). As a result of either all or some of the three non-essential reducing domains in a PKS, the polyketide product will either be fully oxidised, partially reduced or fully reduced (Hopwood, 1997; Schmitt *et al.*, 2005; Elix and Stocker-Wörgötter, 2008). Lichens are known to have both reducing and non-reducing types of PKS, as they are able to produce both types of polyketides. Reduced anthraquinones, known as anthrones, are examples of reduced polyketides produced in lichens, and depsides, depsidones and dibenzofurans are examples of fully oxidised polyketides that are found in lichens (Schmitt *et al.*, 2005; Stocker-Wörgötter, 2008).

4.1.3 Heterologous Expression Systems

Although lichens produce many unique secondary compounds possessing a diverse range of biological activities, the potential use of these metabolites as novel pharmaceuticals has not been exploited (Crittenden and Porter, 1991; Verma and Behera, 2015). There are a range of reasons which contribute to it being neither feasible nor sustainable to produce lichen SMs on an industrial scale. These include the slow growth rate of lichens in nature and in culture, difficulties with the resynthesis of lichens in culture and the fact that axenic cultures of mycobionts are found to produce different compounds to that of the mycobiont when part of the lichen symbiosis (Miao *et al.*, 2001; Boustie and Grube, 2005; Stocker-Wörgötter, 2015). In addition to the growth rate of lichens preventing their use in biotechnology, the slow growth of lichens, along with being unsuitable for molecular manipulation and under-represented in genome sequencing studies, has prevented the characterisation of the metabolites produced by lichen PKS genes (Miao *et al.*, 2001; Stocker-Wörgötter and Elix, 2002; Verma and Behera, 2015; Calcott *et al.*, 2018). The lack of an available transformation system for lichen-forming fungi means that alternative methods must be used to express lichen SM genes and characterise the metabolites that they produce. Heterologous expression is an example of an alternative method which provides the opportunity to study lichen SM genes, as well as depicting a potential method to produce lichen SMs in larger quantities (Sinnemann *et al.*, 2000; Miao *et al.*, 2001; Geib and Brock, 2017).

Heterologous expression can be used as a tool to further our understanding of the transcription of lichen PKS genes, as well as enabling the sustainable production of lichen secondary compounds in biotechnology through the expression of lichen PKSs in other fast-growing and genetically-tractable ascomycetous fungi. Furthermore, the use of heterologous expression to produce lichen SMs possessing various biological activities could result in the identification of lichen secondary compounds which can be used as novel pharmaceuticals. The use of heterologous expression systems to improve our understanding of PKS gene transcription in lichens also provides the opportunity to design new hybrid molecules which could have pharmaceutical applications (Miao *et al.*, 2001; Stocker-Wörgötter, 2008).

The heterologous host used to study lichen PKS genes should ideally have a low and well-characterised endogenous chemical profile (Miao *et al.*, 2001). In addition, for structure and biological activity determination of a metabolite, the heterologous expression should result in high amounts of the gene product (Geib and Brock, 2017). To produce high yields of the gene product, high-level expression of target genes is at least one prerequisite. This can be done by creating expression constructs which contain a strong host promoter or by generating multiple-copy integrations of the target gene, or a combination of the two strategies (Verdoes *et al.*, 1993; Miao *et al.*, 2001; Fleißner and Dersch, 2010; Gressler *et al.*, 2015; Geib and Brock, 2017).

The P*amyB*/TerR (P2) expression system in *Aspergillus oryzae* (Gressler *et al*., 2015; Geib *et al.*, 2019) and the *Aspergillus* Tet-on/TerR (ATNT) expression system in *Aspergillus niger* (Geib and Brock, 2017) are heterologous fungal expression platforms which can be used to express SM biosynthetic gene clusters from lichens. Both expression systems are based on the transcriptional regulator TerR from the *Aspergillus terreus* terrein biosynthetic pathway, and its target *terA* promoter (P*terA*) (Figure 4.4). In the P2 (*A. niger*) and OP12 (A*. oryzae*) system, the expression of *terR* is under the control of the amylase promoter (P*amyB*) from *A. oryzae* which is induced in the presence of starch and maltose and hexose sugars (Gressler *et al.*, 2015). The ATNT expression system, which was developed from the P2 system, replaced P*amyB* so that the transcription of *terR* is instead controlled by a tetracycline-controlled reverse transactivator (the Tet-on system). Tet-on is expressed from the constitutive *fraA* promoter, but only binds to the responsive element in the presence of doxycycline (Geib and Brock, 2017). However, in both systems the induction level of P*terA* is dependent upon the transcription level of *terR*. In addition, in both the P2 and ATNT expression systems, the coupled expression results in higher expression levels than observed from direct gene expression. This is a result of the amplification of the

activity of the promoter controlling *terR* at P*terA* (Gressler *et al.*, 2015; Geib and Brock, 2017).

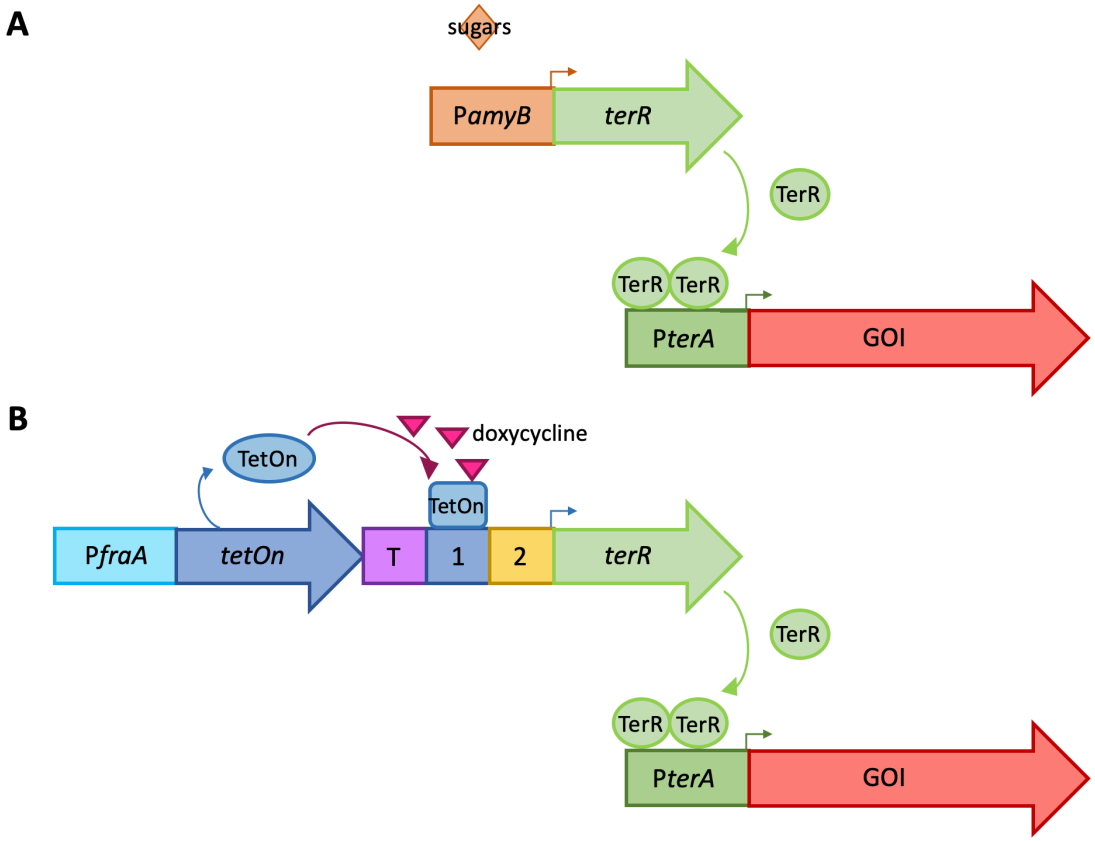


Figure 4.4. Schematic representations of the expression systems. (A) P2 expression system. P*amyB*, which is induced in the presence of sugars, controls the expression of *terR*. This leads to the production of TerR that binds to its target P*terA* and leads to transcription of the gene of interest (GOI). **(B)** ATNT expression system. Transcription of the reverse transactivator is controlled by the constitutive promoter *fraA* and is terminated by the *ergA* terminator (T). In the presence of doxycycline the reverse transactivator binds to the *tet-on* responsive element (1), leading to the transcription of *terR* from the minimal *gpdA* promoter (2). Transcription of the GOI is controlled in the same way as the P2 system, through the binding of TerR to P*terA*. Adapted from Gressler *et al.* (2015) and Geib and Brock (2017).

4.1.4 Previous Approaches of Heterologous Lichen Gene Expression

A study by Sinnemann *et al.* (2000) has provided evidence that heterologous expression can be used to study lichen genes. In this study, the *pyrG* gene from *Solorina crocea*, encoding an orotidine 5'-monophosphate decarboxylase involved in pyrimidine biosynthesis, was successfully cloned and expressed in the heterologous host *Aspergillus nidulans*, either under the control of its native promoter or under the control of the *A. nidulans trpC* promoter. A temperature effect on the strains containing the *S. crocea pyrG* gene was seen, in which growth of the transformants

was only observed at 24 °C and was slower than that of the controls; this was suggested to be an adaptation to lower temperatures. Although heterologous expression was successful when *S. crocea pyrG* was under the control of its native promoter, the higher transformation efficiency seen when the gene was under the control of the *A. nidulans trpC* promoter supports the idea that the use of expression constructs containing strong host promoters might aid the study of lichen genes (Sinnemann *et al.*, 2000; Miao *et al.*, 2001). However, this expression trial by Sinnemann *et al.* (2000) remained for some time as the only successful example of the heterologous expression of a lichen gene in an ascomycete host, with various attempts to heterologously express different types of PKS genes from multiple lichen species proving unsuccessful (Chooi *et al.*, 2008; Gagunashvili *et al.*, 2009; Armaleo *et al.*, 2011; Wang *et al.*, 2016; Bertrand and Sorensen, 2019a; Bertrand and Sorensen, 2019b). These expression trials achieved transcription (including intron splicing) of the PKS gene in the expression platform but no *de novo* biosynthesis of a polyketide product was observed. This, therefore, suggested that a systematic problem exists when trying to express lichen PKSs in *Ascomycota* hosts (Abdel-Hameed *et al.*, 2018).

The lack of success in achieving the functional heterologous expression of a lichen SM gene in an ascomycete host has also contributed to the fact that, at the onset of this study, no lichen biosynthetic gene cluster had been definitively linked to an SM with experimental proof, either through heterologous expression or gene knockout (Bertrand *et al.*, 2018; Bertrand and Sorensen, 2019a). However, several lichen SMs have been putatively assigned to a gene cluster. The first cluster was that of grayanic acid in *Cladonia grayi*, through a combination of phylogenetic analysis, PKS domain arrangement prediction and comparing mRNA levels with metabolites produced (Armaleo *et al.*, 2011). This was followed by the putative identification of two further SM gene clusters in *Cladonia uncialis* using prediction of the PKS domain arrangement and the functions of associated tailoring enzymes; these gene clusters encode usnic acid and 6-hydroxymellein (Abdel-Hameed *et al.*, 2016a; Abdel-Hameed *et al.*, 2016b). More recently, the functional heterologous expression of a lichen SM gene has been achieved, albeit not in an *Aspergillus* host. Kim *et al.* (2021) managed to achieve the heterologous expression of *atr1* from *Stereocaulon alpinum* in the plant-pathogenic fungus *Ascochyta rabiei*, a dothideomycete. They found that *atr1*

produces the depside 4-*O*-demethylbarbatic acid and is involved in the production of atranorin, a cortical substance commonly found in macrolichens.

4.1.5 Chapter Objectives

The aim of this chapter is to achieve the heterologous production of lichen polyketides in the expression platforms *A. niger* ATNT16 Δ*pyrG*x24 and *A. oryzae* OP12 *pyrG*$^{-}$. This will be attempted using two approaches, as described below.

(1) Heterologous production of lichen polyketides using a synthetic biology approach. The *A. niger* ATNT16 Δ*pyrG*x24 and *A. oryzae* OP12 *pyrG*$^{-}$ expression platforms will initially be used for the heterologous expression of genes from various fungal species for the production of lichen polyketides possessing a simple molecular structure. Once this is achieved, the same expression platforms can then be used for the heterologous production of more complex lichen polyketide products.

(2) Heterologous expression of PKS genes identified from lichen species. The *A. niger* ATNT16 Δ*pyrG*x24 and *A. oryzae* OP12 *pyrG*$^{-}$ expression platforms will be used for the heterologous expression of selected PKS genes identified from various lichen species. HPLC analysis will be carried out to determine if any metabolites are being produced, and subsequent analysis will be performed to characterise the metabolite being produced.

4.2 Materials and Methods

4.2.1 Species and Strains

The expression strains used in this study were either *A. niger* ATNT16 Δ*pyrG*x24 (Geib and Brock, 2017), *A. niger* ATNT16 Δ*pyrG* Δ*pabaA*, *A. oryzae* OP12 *pyrG*$^{-}$ (Geib *et al.*, 2019) or *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA*. A sample of the thallus of *E. prunastri* was obtained from Nottingham, UK and a sample of the thallus of *U. longissima* was obtained from Oregon, USA. Genomic DNA was extracted from the whole lichen thallus of each species as described in Section 2.2.4. Genomic DNA from

Cladonia grayi was provided by Daniele Armaleo (Duke University, North Carolina, USA).

4.2.2 Preparation of Conidial Suspensions

Conidial suspensions of *A. niger* ATNT16 Δ*pyrG*x24 and *A. oryzae* OP12 *pyrG*$^{-}$ were obtained by growing each strain on slopes in Universal tubes containing solid *Aspergillus* minimal medium with 50 mM glucose and 10 mM glutamine (AMM(-N)G50Gln10, denoted GG10) and any required supplementations. After incubation at 28 °C, conidial suspensions were made by scraping conidia into 6 ml phosphate-buffered saline (PBS) using a sterile cotton swab.

4.2.3 Generation of Strains for Production of Lichen Polyketides

Sequences of all oligonucleotides used in this study are listed in Appendix 1. Plasmid maps of the principle plasmids used in this study are shown in Appendix 4.

4.2.3.1 Production of Orsellinic Acid, Lecanoric Acid, Everninic Acid and Evernic Acid

4.2.3.1.1 Lichen Polyketide-Producing Strains

Four different polycistronic expression constructs were created for the production of lichen-derived SMs using the P2A_*luciferase*_SM-X_URA plasmid, which contains a luciferase reporter gene that results in luminescent colonies when the gene of interest is expressed. The construct for the production of orsellinic acid (*orsA*:*TE*$_L$_*luciferase*_SM-X_URA) contained the *orsA* gene from *A. nidulans* without its thioesterase domain fused to the thioesterase domain from *A. terreus terA* (Gressler *et al.*, 2015), and was separated from the luciferase gene by a P2A coding sequence. The *orsA* and *terA* thioesterase domain fusion was amplified from plasmid *orsA*:*TE(terA)*$_L$_SM-X_URA using oligonucleotide 1 that contained an overlap to the *NcoI*-restricted P2A_*luciferase*_SM-X_URA and oligonucleotide 2 that also contained an overhang to the *NcoI*-restricted P2A_*luciferase*_SM-X_URA plasmid. The construct for the production of lecanoric acid (*orsA*_*luciferase*_SM-X_URA) contained the *orsA* gene from *A. nidulans* with its own thioesterase domain separated from the luciferase gene by a P2A coding sequence. The *orsA* gene was amplified from plasmid

P*terA*:*orsA*:T*orsA*_pCRIV (Gressler *et al.*, 2015) using oligonucleotide 1 and oligonucleotide 3 containing an overlap to the *NcoI*-restricted P2A_*luciferase*_SM-X_URA plasmid. The construct for the production of everninic acid (*orsA*:*TE$_L$*_*Me473*_*luciferase*_SM-X_URA) contained the fusion of *orsA* without its thioesterase domain with the *A. terreus terA* thioesterase domain as well as the *Me473 O*-methyltransferase gene from *Armillaria mellea*. The genes were separated by P2A coding sequences. The *orsA* and *terA* thioesterase domain fusion was amplified from the same plasmid as for the orsellinic acid expression construct using oligonucleotide 1 and oligonucleotide 4 which contained an overhang to the P2A sequence. The *Me473* gene was amplified from plasmid pMD01 pET28 (kindly provided by Dirk Hoffmeister, Jena, Germany) with oligonucleotide 5 containing an overhang to the P2A sequence and oligonucleotide 6 which contained an overlap to the *NcoI*-restricted P2A_*luciferase*_SM-X_URA plasmid. The construct for the production of evernic acid (*orsA*_*Me473*_*luciferase*_SM-X_URA) contained the full *orsA* gene from *A. nidulans* as well as the *Me473* gene from *A. mellea*. The genes were separated by P2A coding sequences. The *orsA* gene was amplified from the same plasmid as that used for the lecanoric acid expression construct using oligonucleotide 1 and oligonucleotide 7 which contained an overlap to the P2A sequence towards *Me473*. The *Me473* gene was amplified from plasmid pMD01 pET28 with oligonucleotide 5 and oligonucleotide 6.

Each construct was assembled via *in vitro* recombination using the InFusion HD cloning kit (Takara/Clontech) by mixing the *NcoI*-restricted P2A_*luciferase*_SM-X_URA plasmid and gel-purified PCR fragments. The assembled plasmids were then amplified in *E. coli* DH5α cells. Colony PCR was used for the selection of positive clones using oligonucleotides 8 and 9 for *orsA*:*TE$_L$*_*luciferase*_SM-X_URA, oligonucleotides 10 and 9 for *orsA*_*luciferase*_SM-X_URA, oligonucleotides 8 and 11 for *orsA*:*TE$_L$*_*Me473*_*luciferase*_SM-X_URA, and oligonucleotides 10 and 11 for *orsA*_*Me473*_*luciferase*_SM-X_URA. The plasmids were purified and each plasmid was checked for correct assembly using restriction analyses. The isolated plasmids were then used for the transformation of *A. niger* ATNT16 Δ*pyrG*x24 and *A. oryzae* OP12 *pyrG*$^-$ (for transformation media, top agar was supplemented with luciferin (final

concentration 133 μg/ml) for both strains and bottom agar was supplemented with doxycycline (final concentration 10 μg/ml) for *A. niger* ATNT16 Δ*pyrG*x24).

Liquid cultures of *Aspergillus* minimal medium with 100 mM glucose and 20 mM glutamine (AMM(-N)G100Gln20, denoted GG20) in a 25 ml volume were supplemented with doxycycline (final concentration 10 μg/ml) for *A. niger* ATNT16 Δ*pyrG*x24 strains. Liquid malt extract medium (ME) cultures, also in a volume of 25 ml, were used for *A. oryzae* OP12 *pyrG*$^{-}$ strains. Respective media were inoculated with conidia suspensions of transformants for each expression construct. Cultures were incubated at 28 °C on a rotary shaker at 150 rpm. The mycelia were removed by filtration over Miracloth (Merck, Darmstadt, Germany) and the culture filtrates were then extracted with ethyl acetate, dried and solved in methanol and analysed by high performance liquid chromatography (HPLC) for SM production.

4.2.3.1.2 *O*-Methyltransferase-Expressing Strains

Expression constructs containing an *O*-methyltransferase gene were created for the generation of *O*-methyltransferase-expressing strains using the SM_S-tag_X_URA plasmid, which contains a Strep-tag sequence that can be used for the addition of the tag at either the *N*- or *C*-terminus of a protein.

An expression construct containing the *Me473* gene from *A. mellea* with a *C*-terminal Strep-tag was generated. The *Me473* gene was amplified from plasmid pMD01 pET28 using oligonucleotide 12 which contained an overhang to the *NsiI*-restricted SM_S-tag_X_URA plasmid and oligonucleotide 13 containing an overlap to the Strep-tag sequence. The *NsiI*-restricted SM_S-tag_X_URA plasmid and gel-purified PCR fragment were assembled via *in vitro* recombination, amplified in *E. coli* DH5α cells and purified. Positive clones were selected by colony PCR using oligonucleotides 14 and 15. The isolated plasmid was then checked for correct assembly via restriction analyses and subsequently used in the transformation of *A. niger* ATNT16 Δ*pyrG*x24 (for transformation media, top agar was supplemented with luciferin (final concentration 133 μg/ml) and bottom agar was supplemented with doxycycline (final concentration 10 μg/ml)).

The genome of the lichen *Evernia prunastri* was analysed using antiSMASH 4.0 (Blin *et al.*, 2017) for SM biosynthetic gene clusters which met the minimum requirements for that of the evernic acid biosynthetic gene cluster. Of the 98 SM biosynthetic gene clusters identified in the *E. prunastri* genome, two clusters appeared as the most promising candidates; these were clusters 40 and 41. Two expression constructs were created for the generation of strains expressing the *O*-methyltransferases from clusters 40 and 41. The first construct contained the *E. prunastri O*-methyltransferase gene from cluster 40 (*O-MeT-c40*) with an *N*-terminal Strep-tag. The *O-MeT-c40* gene was amplified from *E. prunastri* gDNA using oligonucleotide 16 containing an overlap to the Strep-tag sequence and oligonucleotide 17 which contained an overhang to the *NcoI*-restricted SM_S-tag_X_URA plasmid. The second construct contained the *E. prunastri O*-methyltransferase gene from cluster 41 (*O-MeT-c41*) with an *N*-terminal Strep-tag. The *O-MeT-c41* gene was amplified from *E. prunastri* gDNA using oligonucleotide 18 which contained an overhang to the Strep-tag sequence and oligonucleotide 19 containing an overlap to the *NcoI*-restricted SM_S-tag_X_URA plasmid. The *NcoI*-restricted linearised plasmid and gel-purified PCR fragments were assembled by *in vitro* recombination, amplified in *E. coli* DH5α cells and purified. Positive clones were then selected by colony PCR using oligonucleotides 20 and 15 for *O-MeT-c40*_SM_S-tag_X_URA and oligonucleotides 21 and 15 for *O-MeT-c41*_SM_S-tag_X_URA. Correct assembly of the isolated plasmids was checked using restriction analyses and the plasmids were then subsequently used in the transformation of *A. niger* ATNT16 Δ*pyrG*x24 and *A. oryzae* OP12 *pyrG*$^{-}$.

4.2.3.2 *Evernia prunastri PKS-c40* and *PKS-c41*

Two expression constructs were generated for the expression of the PKS genes from clusters 40 and 41 in the *E. prunastri* genome (identified in Section 4.2.3.1.2) using the SM-X_URA plasmid. The PKS from cluster 40 (*PKS-c40*) was amplified from *E. prunastri* gDNA by splitting the PKS into two fragments using oligonucleotide 22 which contained an overhang to the *NcoI*-restricted SM-X_URA plasmid, overlapping nucleotides 23 and 24, and oligonucleotide 25 which also contained an overlap to the *NcoI*-restricted SM-X_URA. The PKS from cluster 41 (*PKS-c41*) was also amplified from

E. prunastri gDNA, again by splitting the PKS into two fragments, using oligonucleotide 26 which contained an overhang to the *NcoI*-restricted plasmid, overlapping oligonucleotides 27 and 28, and oligonucleotide 29, again containing an overhang to the *NcoI*-restricted SM-X_URA plasmid.

Each construct was assembled using the *NcoI*-restricted SM-X_URA plasmid and the gel-purified PCR fragments via *in vitro* recombination, and was subsequently amplified and purified. Colony PCR was used for the selection of positive clones using oligonucleotides 30 and 15 for *PKS-c40*_SM-X_URA, and oligonucleotides 31 and 15 for *PKS-c41*_SM-X_URA. Each isolated plasmid was checked for correct assembly using restriction analyses. The isolated plasmids were then used for the transformation of *A. niger* ATNT16 Δ*pyrG*x24 and *A. oryzae* OP12 *pyrG*$^{-}$ (for transformation, bottom agar was supplemented with doxycycline (final concentration 10 μg/ml) for *A. niger* ATNT16 Δ*pyrG*x24).

Liquid GG20 cultures in a 25 ml volume supplemented with doxycycline (final concentration 10 μg/ml) for *A. niger* ATNT16 Δ*pyrG*x24 strains and liquid ME cultures, also in a volume of 25 ml, for *A. oryzae* OP12 *pyrG*$^{-}$ strains were inoculated with conidial suspensions of transformants for each expression construct; cultures were incubated at 28 °C on a rotary shaker at 150 rpm. The mycelia were removed by filtration over Miracloth (Merck, Darmstadt, Germany) and the culture filtrates were then analysed by HPLC for SM production. In addition, liquid GG20 cultures in a 25 ml volume supplemented with doxycycline (final concentration 10 μg/ml) for *A. niger* ATNT16 Δ*pyrG*x24 strains and liquid AMM (-N) starch 2%, 6 mM glucose cultures, also in a volume of 25 ml, for *A. oryzae* OP12 *pyrG*$^{-}$ strains were inoculated with pooled conidial suspensions of transformants for each expression construct, and the cultures were incubated at 18 °C on a rotary shaker at 150 rpm. The mycelia and culture filtrates were separated, and culture filtrates were extracted and analysed by HPLC for SM production.

4.2.3.3 *Usnea longissima UlPKS6*

An expression construct (*UlPKS6*_SM-X_URA) was created for the generation of strains expressing the *UlPKS6* gene identified in *Usnea longissima* by Wang *et al.* (2014b) using the SM-X_URA plasmid. The *UlPKS6* gene was amplified from *U. longissima* gDNA by splitting the PKS into two fragments using oligonucleotide 32 containing an overhang to the *NcoI*-restricted SM-X_URA plasmid, overlapping oligonucleotides 33 and 34 located in the middle of *UlPKS6* and oligonucleotide 35 which contained an overlap to the *NcoI*-restricted SM-X_URA plasmid.

The *NcoI*-restricted linearised plasmid and two gel-purified PCR fragments were mixed and the plasmid was assembled by *in vitro* recombination, amplified in *E. coli* DH5α cells and purified. Positive clones were then selected by colony PCR using oligonucleotides 36 and 15. The isolated plasmid was checked for correct assembly by restriction analyses and was subsequently used to transform *A. niger* ATNT16 Δ*pyrG*x24 and *A. oryzae* OP12 *pyrG*$^{-}$ (for transformation media, bottom agar was supplemented with doxycycline (final concentration 10 μg/ml) for *A. niger* ATNT16 Δ*pyrG*x24).

A liquid GG20 culture in a 25 ml volume supplemented with doxycycline (final concentration 10 μg/ml) was inoculated with a conidial suspension containing conidia from a pool of all *A. niger* ATNT16 Δ*pyrG*x24 *UlPKS6*_SM-X_URA transformants. A liquid ME culture in a 25 ml volume was inoculated with a conidial suspension containing conidia from a pool of all *A. oryzae* OP12 *pyrG*$^{-}$ *UlPKS6*_SM-X_URA transformants. Cultures were incubated at 28 °C on a rotary shaker at 150 rpm. The mycelia and culture filtrates were separated, and culture filtrates were then extracted and analysed for SM production by HPLC. Subsequently, liquid GG20 cultures in a 25 ml volume supplemented with doxycycline (final concentration 10 μg/ml) for *A. niger* ATNT16 Δ*pyrG*x24 strains and liquid AMM (-N) starch 2%, 6 mM glucose cultures, also in a volume of 25 ml, for *A. oryzae* OP12 *pyrG*$^{-}$ strains were inoculated with pooled conidial suspensions of all transformants, and the cultures were incubated at 18 °C on a rotary shaker at 150 rpm. The mycelia and culture filtrates were separated, and culture filtrates were extracted and analysed by HPLC for SM production.

4.2.3.4 *Cladonia grayi Clagr3.6*, *Clagr3.21*, *Clagr3.26* and *Clagr3.31*

Expression constructs were generated for the expression of four PKS genes, *Clagr3.6*, *Clagr3.21*, *Clagr3.26* and *Clagr3.31*. These were identified in the *Cladonia grayi* genome by selecting candidates with similarity to the THN synthase *PKS1* from *Colletotrichum lagenarium*. These genes were inserted into the SM-X_URA plasmid, and were amplified from *C. grayi* gDNA by splitting each PKS gene into two fragments. The *Clagr3.6* gene was amplified using oligonucleotide 37 containing an overlap to the *NcoI*-restricted SM-X_URA plasmid, overlapping oligonucleotides 38 and 39 located in the middle of the *Clagr3.6* sequence, and oligonucleotide 40 which contained an overhang to the *NcoI*-restricted SM-X_URA plasmid. In the same way, the *Clagr3.21* gene was amplified using oligonucleotide 41 with an *NcoI*-restricted SM-X_URA overhang, overlapping oligonucleotides 42 and 43, and oligonucleotide 44 with an overlap to the *NcoI*-restricted SM-X_URA plasmid. The *Clagr3.26* gene was amplified using oligonucleotide 45 containing an overhang to the *NcoI*-restricted SM-X_URA plasmid, overlapping oligonucleotides 46 and 47, and oligonucleotide 48 which also contained an overhang to the *NcoI*-restricted SM-X_URA plasmid. The *Clagr3.31* gene was amplified using oligonucleotide 49 with an overlap to the *NcoI*-restricted SM-X_URA plasmid, overlapping oligonucleotides 50 and 51 located in the middle of the *Clagr3.31* gene, and oligonucleotide 52 which also contained an overlap to the *NcoI*-restricted SM-X_URA plasmid.

For each construct, the *NcoI*-restricted linearised SM-X_URA plasmid and the two gel-purified fragments for each gene were mixed and assembled by *in vitro* recombination, amplified in *E. coli* DH5α cells and purified. Positive clones were subsequently selected by colony PCR using oligonucleotides 53 and 15 for *Clagr3.6*_SM-X_URA, oligonucleotides 54 and 15 for *Clagr3.21*_SM-X_URA, oligonucleotides 55 and 15 for *Clagr3.26*_SM-X_URA, and oligonucleotides 56 and 15 for *Clagr3.31*_SM-X_URA; each plasmid was checked for correct assembly by restriction analyses. The resulting *Clagr3.6*_SM-X_URA, *Clagr3.21*_SM_X_URA, *Clagr3.26*_SM-X_URA and *Clagr3.31*_SM-X_URA plasmids were then used to transform *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* (for transformation media, bottom agar was supplemented with para-aminobenzoic acid (final concentration 7.29 μM) and doxycycline (final concentration 10 μg/ml) for *A.*

niger ATNT16 Δ*pyrG* Δ*pabaA*, and para-aminobenzoic acid (final concentration 7.29 μM) for *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA*).

Liquid GG20 cultures in a 25 ml volume were supplemented with 7.29 μM para-aminobenzoic acid and doxycycline (final concentration 10 μg/ml) for *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* strains and liquid ME cultures, also in a 25 ml volume, were supplemented with 7.29 μM para-aminobenzoic acid for *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* strains. Media were inoculated with either individual or pooled transformant conidia suspensions for each expression construct. Cultures were incubated in parallel at both 23 °C and 28 °C on a rotary shaker at 150 rpm. The mycelia were removed from culture filtrates by filtration over Miracloth (Merck, Darmstadt, Germany), and the culture filtrates were then analysed by HPLC for SM production.

4.2.4 Transformant Analysis

Transformation plates were incubated at 28 °C, and transformants were streaked twice onto fresh GG10 agar plates for purification. Transformants containing the luciferase reporter were screened for bioluminescence and all transformants were checked by diagnostic PCR after extracting genomic DNA using oligonucleotides 57 and 15.

4.2.5 *O*-Methyltransferase *In Vitro* and *In Vivo* Assays

For *in vitro* analysis of *O*-methyltransferase activity of *A. niger* ATNT16 Δ*pyrG*x24 *O-MeT-c40*_SM_S-tag_X_URA and *A. niger* ATNT16 Δ*pyrG*x24 *O-MeT-c41*_SM_S-tag_X_URA transformants, liquid YEPD cultures in a volume of 30 ml supplemented with doxycycline (final concentration 10 μg/ml) were inoculated with conidia suspensions of transformants for the preparation of cell-free extracts. After incubation at 28 °C on a rotary shaker at 150 rpm, mycelia were harvested from the cultures, washed in SDW, pressed dry with tissue and frozen in liquid nitrogen. The mycelia were then ground to a fine powder under liquid nitrogen and resuspended in Tris buffer (150 mM NaCl, 50 mM Tris, 10 % glycerine, pH 7.5). Any debris were removed by centrifugation at 13,000 rpm for 10 min. Protein concentrations of the cell-free extracts were determined using the Bradford assay and the cell-free extracts

were subsequently used in an *in vitro* assay to determine *O*-methyltransferase activity. The *in vitro* assay was set up in a 3 ml volume with 1 mg protein, 1 mM $MgCl_2$, 500 μM *S*-adenosyl-L-methionine (SAM) and 20 μl lecanoric acid (about 5 mg/ml in methanol). The assays were incubated at 28 °C and then analysed by HPLC for SM production.

For *in vivo* analysis of *O*-methyltransferase activity, liquid GG20 cultures in a volume of 25 ml were supplemented with doxycycline (final concentration 10 μg/ml) and inoculated with conidial suspensions of *A. niger* ATNT16 Δ*pyrG*x24 transformants. For the *in vivo* analysis of Me473 activity, conidia from the orsellinic acid-producing strain *A. niger* ATNT16 Δ*pyrG*x24 *orsA*:*TE*$_L$_*luciferase*_SM-X_URA 3 were co-incubated with pools of *A. niger* ATNT16 Δ*pyrG*x24 *Me473*_S-tag_SM-X_URA transformant conidia. For the *in vivo* analysis of O-MeT-c40 and O-MeT-c41 activities, conidia from the lecanoric acid-producing strain *A. niger* ATNT16 Δ*pyrG*x24 *orsA_luciferase*_SM-X_URA 4-2.1 were co-incubated with pools of *A. niger* ATNT16 Δ*pyrG*x24 *O-MeT-c40*_SM_S-tag_X_URA transformant conidia or *A. niger* ATNT16 Δ*pyrG*x24 *O-MeT-c41*_SM_S-tag_X_URA transformant conidia. Cultures were incubated at 28 °C on a rotary shaker at 150 rpm. The mycelia and culture filtrates were separated by filtration over Miracloth (Merck, Darmstadt, Germany), and culture filtrates were then analysed for SM production by HPLC.

4.2.6 Metabolite Extraction and Analysis

Metabolites were extracted from culture filtrates by mixing with an equal volume of ethyl acetate and removing the overlaying solvent layer. The extracts were then filtered over anhydrous sodium sulfate and evaporated under reduced pressure. Metabolites were extracted from *in vitro* assays by mixing with an equal volume of ethyl acetate, collecting the overlaying organic layer and repeating the procedure once; the two fractions were combined and evaporated under reduced pressure. The resulting dried extracts were then dissolved in methanol and subjected to HPLC analysis using a Dionex UltiMate3000 (Thermo Fisher Scientific) and an Eclipse XDB-C18 column (4.6x150 mm, 5 μm; Agilent) kept at 40 °C. A binary solvent system of water containing 0.1% formic acid (solvent A) and 100% methanol (solvent B) was used

with a flow rate of 1 ml/min: 0.5 min 10% B, 15 min 90% B, 17 min 90% B, 17.5 min 100% B, 22 min 100% B, 23 min 10% B, 25 min 10% B.

4.3 Results

4.3.1 Production of Lichen Polyketides

4.3.1.1 Lichen Polyketide-Producing Strains

For the production of the SMs orsellinic acid and lecanoric acid that are frequently found in lichens, two constructs were generated. One construct contained the *orsA* gene from *A. nidulans* with its thioesterase domain replaced by the thioesterase domain from *A. terreus terA* for the production of orsellinic acid (Figure 4.5A). The second construct contained the entire *A. nidulans orsA* gene with its own thioesterase domain for the production of lecanoric acid (Figure 4.5B). The genes were fused to the P2A peptide sequence (Geib and Brock, 2017) that is followed by a firefly luciferase gene. This allows a pseudo-polycistronic gene expression of the PKS gene in combination with the luciferase gene and bioluminescence of transformants can be used for detection of transformants with full-length integration of the expression construct. These constructs were used to transform *A. niger* ATNT16 Δ*pyrG*x24 and *A. oryzae* OP12 *pyrG*$^{-}$ strains, and positive transformants were identified by luminescence screening. Positive transformants were cultivated under inducing conditions and extracts of culture filtrates were analysed using HPLC to detect the metabolites produced. As shown in Figure 4.5, the strains generated produced orsellinic acid (Figure 4.5I) and lecanoric acid (Figure 4.5J), which indicates that the luciferase reporter did not interfere with the activity of the PKSs, and that the luciferase expression system is compatible with PKS expression. The orsellinic acid and lecanoric acid metabolites produced by these strains were verified by comparison with the results of Gressler *et al.* (2015), in which strains expressing the same *orsA*:TE_L or *orsA* constructs also produced orsellinic acid or lecanoric acid, respectively.

For the production of everninic acid and evernic acid, two expression constructs were generated. The first construct contained the *A. mellea Me473 O*-methyltransferase gene in addition to the *A. nidulans orsA* gene with its thioesterase domain replaced by the *A. terreus terA* thioesterase domain and was used for the production of everninic acid (Figure 4.5C). The second construct contained the full-

length *A. nidulans orsA* gene as well as the *A. mellea Me473* gene with the aim to produce evernic acid (Figure 4.5D). These constructs were used in the transformation of *A. niger* ATNT16 Δ*pyrG*x24 and *A. oryzae* OP12 *pyrG*$^{-}$ strains, and positive transformants were identified by luminescence screening. The positive transformants were then cultivated in liquid media under inducing conditions and extracts of culture filtrates were analysed by HPLC. As shown in Figure 4.5, only orsellinic acid and lecanoric acid were produced and neither everninic acid (Figure 4.5K) nor evernic acid (Figure 4.5L) were detected even though strains expressing the full expression constructs were generated. This indicates that either (a), the methyltransferase is not functionally produced in the heterologous hosts or (b) that the methyltransferase cannot act on orsellinic or lecanoric acid as substrate, but requires the protoilludene moiety attached to orsellinic acid as the enzyme is part of the melleolide biosynthesis gene cluster from *A. mellea* (Engels *et al.*, 2021).

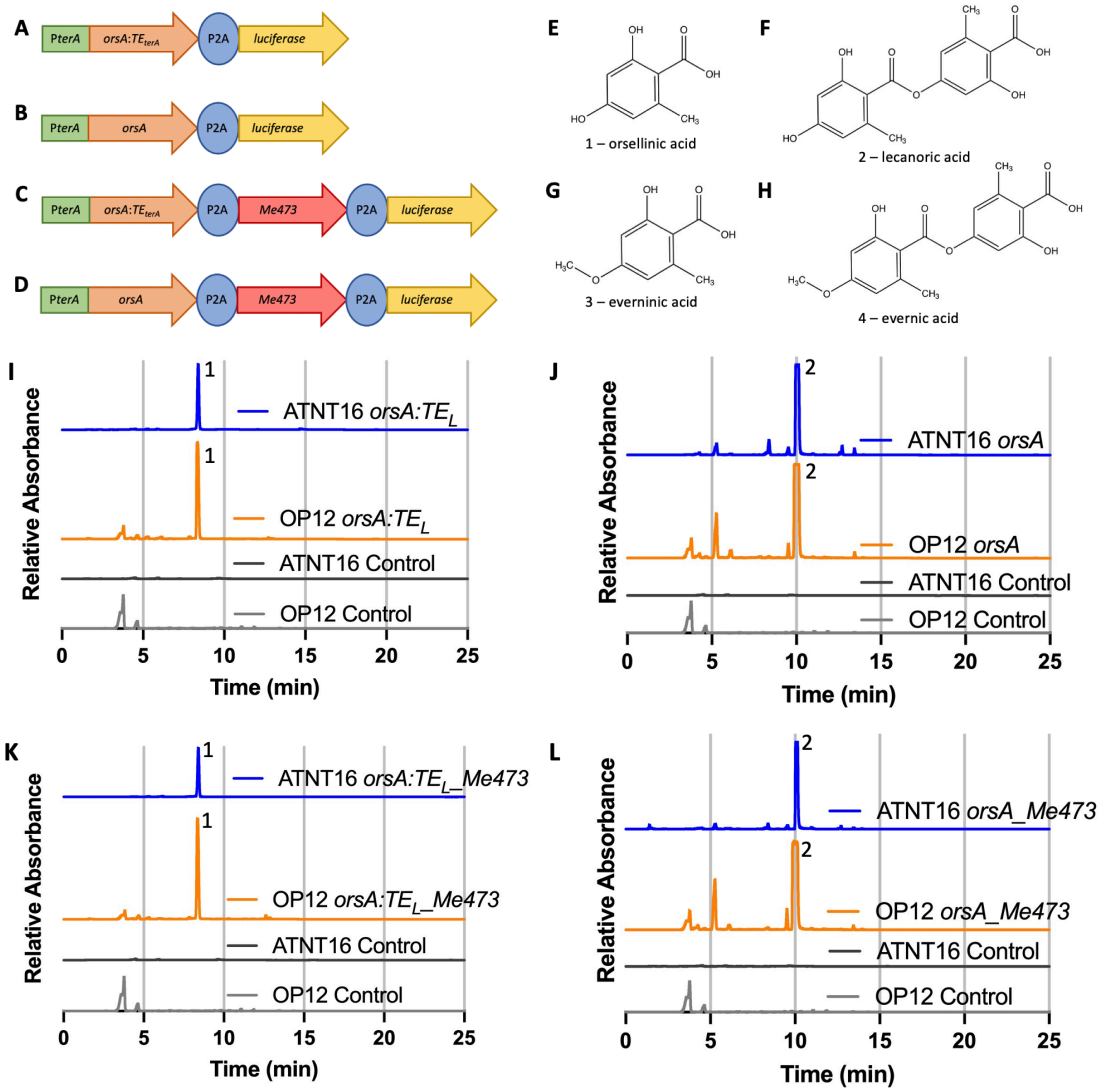


Figure 4.5. Production of lichen-derived SMs using polycistronic expression constructs. **(A-D)** Schematic representations of the polycistronic expression constructs for the production of orsellinic acid **(A)**, lecanoric acid **(B)**, everninic acid **(C)** and evernic acid **(D)**. **(E-H)** Structures of the SMs. **(I-L)** HPLC analysis of culture filtrates of transformants. **(I)** ATNT16 Δ*pyrG*x24 *orsA*:*TE*$_L$_*luciferase*_SM-X_URA 3 and OP12 *pyrG- orsA:TE*$_L$_*luciferase*_SM-X_URA 5, showing production of orsellinic acid (1), compared to ATNT16 and OP12 controls. **(J)** ATNT16 Δ*pyrG*x24 *orsA_luciferase*_SM-X_URA 4-2.1 and OP12 *pyrG- orsA_luciferase*_SM-X_URA 4-2.3, showing production of lecanoric acid (2), compared to ATNT16 and OP12 controls. **(K)** ATNT16 Δ*pyrG*x24 *orsA*:*TE*$_L$_*Me473_luciferase*_SM-X_URA 16 and OP12 *pyrG- orsA:TE*$_L$_*Me473_luciferase*_SM-X_URA 18, showing production of orsellinic acid (1), compared to ATNT16 and OP12 controls. **(L)** ATNT16 Δ*pyrG*x24 *orsA_Me473_luciferase*_SM-X_URA 4-2.1 and OP12 *pyrG- orsA_Me473_luciferase*_SM-X_URA 4-2.1, showing production of lecanoric acid (2), compared to ATNT16 and OP12 controls. The orsellinic acid and lecanoric acid metabolites were verified by comparison with the results of Gressler *et al.* (2015), in which orsellinic acid and lecanoric acid were characterised.

4.3.1.2 *O*-Methyltransferase-Expressing Strains

To exclude that the functional co-expression of the *A. mellea* methyltransferase was hindered by the pseudo-polycistronic gene expression, a second approach was used to produce everninic acid. This strategy involved the generation of an expression construct containing Me473 with a *C*-terminal Strep-tag (Figure 4.6A) which was then used in the transformation of the *A. niger* ATNT16 Δ*pyrG*x24 strain. Positive transformants were identified by PCR screening and then co-incubated with the orsellinic acid-producing strain *A. niger* ATNT16 Δ*pyrG*x24 *orsA*:TE_L_*luciferase*_SM-X_URA 3 under inducing conditions. HPLC analysis of extracts of the culture filtrates was then carried out to identify the metabolites produced. Figure 4.6D shows that no everninic acid was detected.

As a result of the lack of *O*-methyltransferase activity observed in Me473, alternative *O*-methyltransferases were subsequently tested to determine if they had *O*-methyltransferase activity. Due to the fact that we were trying to methylate lichen-derived metabolites, putative *O*-methyltransferases were identified from a lichen species. As one of the major SMs produced by *E. prunastri* is evernic acid, its genome was analysed using antiSMASH 4.0 (Blin *et al.*, 2017) in order to identify a putative evernic acid biosynthesis gene cluster using the minimum requirements of an NR-PKS and a SAM-dependent *O*-methyltransferase. Two clusters, 40 and 41, were identified as the most promising candidates for the evernic acid biosynthetic gene cluster. The *O*-methyltransferases from these gene clusters were used to generate expression constructs (Figure 4.6B and 4.6C) which were then used to transform *A. niger* ATNT16 Δ*pyrG*x24 and *A. oryzae* OP12 *pyrG*$^-$ strains. Positive transformants were then identified by PCR screening for full-length integration of the construct.

For the *in vivo* synthesis of evernic acid, conidia from an *A. niger* ATNT16 Δ*pyrG*x24 *orsA*_*luciferase*_SM-X_URA strain which produces lecanoric acid were co-incubated with conidia from *A. niger* ATNT16 Δ*pyrG*x24 *O-MeT-c40*_SM_S-tag_X_URA or *A. niger* ATNT16 Δ*pyrG*x24 *O-MeT-c41*_SM_S-tag_X_URA strains under inducing conditions. HPLC analysis of the *in vivo* assays for both *O*-methyltransferases showed that evernic acid was not produced in either assay (Figures 4.6E), again indicating that

either no functional enzymes had been produced or that the *O*-methyltransferase was unable to methylate lecanoric acid.

For the *in vitro* synthesis of evernic acid, cell-free extracts containing either O-MeT-c40 with an *N*-terminal Strep-tag or O-MeT-c41 with an *N*-terminal Strep-tag (from *A. niger* ATNT16 Δ*pyrG*x24 *O-MeT-c40*_SM_S-tag_X_URA or *A. niger* ATNT16 Δ*pyrG*x24 *O-MeT-c41*_SM_S-tag_X_URA strains generated) were used in *in vitro* assays with lecanoric acid as the substrate and SAM as the methyl group donor. HPLC analysis of the *in vitro* assays for both *O*-methyltransferases showed that evernic acid was not produced in either assay (Figures 4.6F), indicating that neither *O*-methyltransferase was able to methylate lecanoric acid. However, we did not attempt to purify either the O-MeT-c40 or O-MeT-c41 enzymes from the respective cell-free extracts, meaning it is therefore possible that the amount of active methyltransferase in the extract was too low for any enzyme activity to be observed.

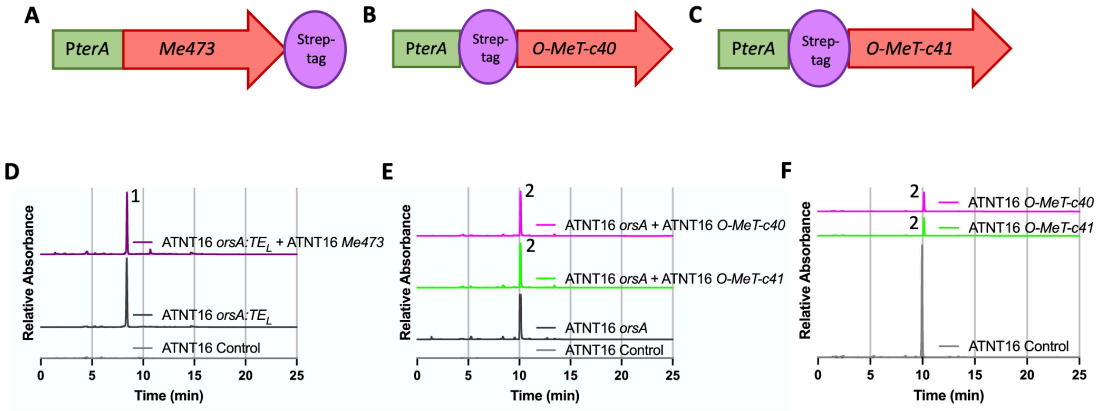


Figure 4.6. Analysis of *O*-methyltransferase activity. **(A-C)** Schematic representations of the expression constructs for the production of Me473 with *C*-terminal Strep-tag **(A)**, O-MeT-c40 with *N*-terminal Strep-tag **(B)**, and O-MeT-c41 with *N*-terminal Strep-tag **(C)**. **(D)** HPLC analysis of culture filtrate of ATNT16 Δ*pyrG*x24 *orsA*:*TE*$_L$_*luciferase*_SM-X_URA 3 and ATNT16 Δ*pyrG*x24 *Me473*_S-tag_SM-X_URA 1,3 co-incubation, showing production of orsellinic acid (1), compared to ATNT16 Δ*pyrG*x24 *orsA*:*TE*$_L$_*luciferase*_SM-X_URA 3 and ATNT16 control. **(E)** HPLC analysis of culture filtrates of ATNT16 Δ*pyrG*x24 *orsA_luciferase*_SM-X_URA 4-2.1 co-incubation with ATNT16 Δ*pyrG*x24 *O-MeT-c40*_SM_S-tag_X_URA 2-1.5,6,7 or ATNT16 Δ*pyrG*x24 *O-MeT-c41*_SM_S-tag_X_URA 1-1.2,3,5, with both showing production of lecanoric acid (2), compared to ATNT16 Δ*pyrG*x24 *orsA_luciferase*_SM-X_URA 4-2.1 and ATNT16 control. **(F)** HPLC analysis of O-MeT-c40 and O-MeT-c41 *in vitro* assays using ATNT16 Δ*pyrG*x24 *O-MeT-c40*_SM_S-tag_X_URA 2-1.5,6,7 or ATNT16 Δ*pyrG*x24 *O-MeT-c41*_SM_S-tag_X_URA 1-1.2,3,5 transformants, with both showing lecanoric acid (2), compared to ATNT16 control. Despite the use of multiple *O*-methyltransferases, methylation of orsellinic acid or lecanoric acid was not observed.

4.3.2 Strains Expressing Lichen Polyketide Synthases

4.3.2.1 *Evernia prunastri PKS-c40* and *PKS-c41*

Although the expression of the two methyltransferases from *E. prunastri* did not result in the production of evernic acid, we aimed for the characterisation of the accompanied PKS genes. For this purpose, expression constructs were generated (Figure 4.7A and 4.7B) which contained the PKS gene from each of the clusters named *PKS-c40* and *PKS-c41*. The resulting expression constructs were used in the transformation of *A. niger* ATNT16 Δ*pyrG*x24 and *A. oryzae* OP12 *pyrG*$^{-}$ strains. Positive transformants were then identified by PCR screening for full-length integration of the construct and transformants were cultivated under inducing conditions at 28 °C. Culture filtrates were analysed by HPLC. Figure 4.7C and 4.7D show that, except for some background metabolites from the expression platform strains, no metabolites were produced by strains expressing either *PKS-c40* or *PKS-c41*.

Transformants were then cultivated under inducing conditions but grown at a lower temperature (18 °C) in order to see if this had any effect on the production of metabolites. Culture filtrates were analysed by HPLC in the same way as previously to identify any new metabolites. Similar to the incubation at 28 °C, Figures 4.7E and 4.7F show that no metabolites of significant intensity were produced by any of the transformants at the lower temperature. This implies that no functional PKSs were produced in the heterologous hosts.

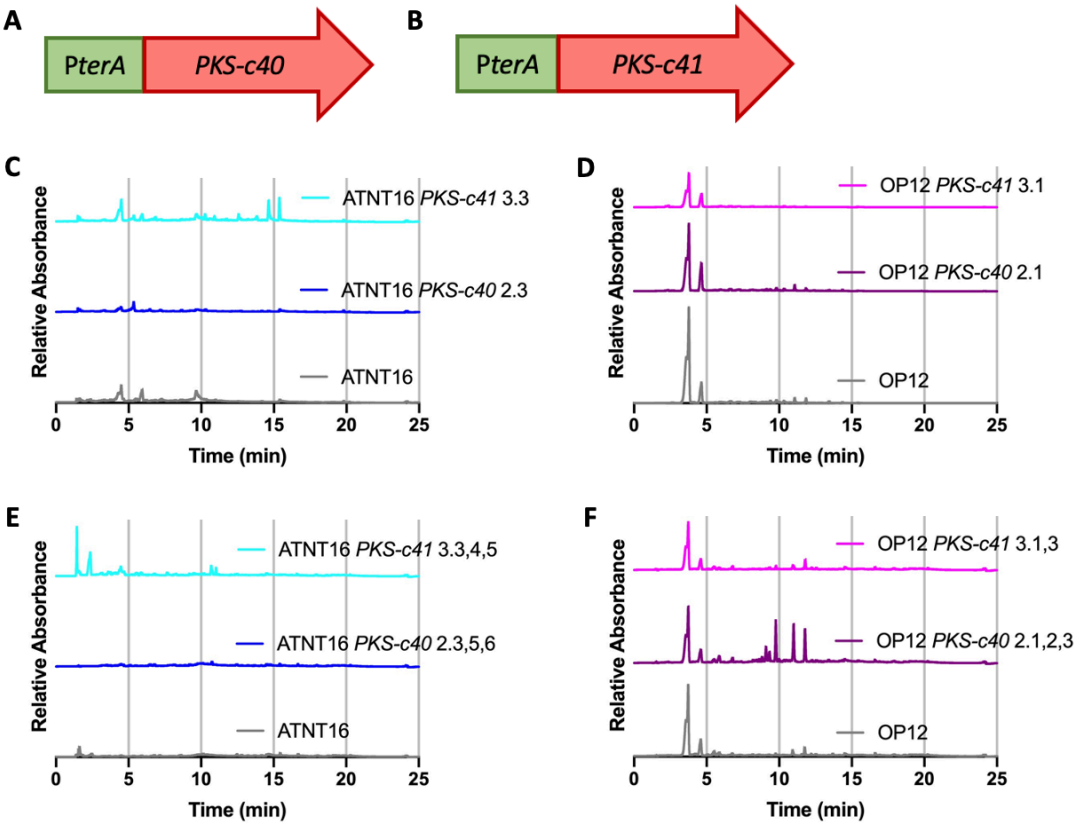


Figure 4.7. Metabolite production from *PKS-c40* and *PKS-c41*. **(A-B)** Schematic representations of the expression constructs containing the PKS gene from cluster 40 **(A)** and cluster 41 **(B)**. **(C)** HPLC analysis of culture filtrates of ATNT16 Δ*pyrG*x24 *PKS-c40*_SM-X_URA 2.3 and ATNT16 Δ*pyrG*x24 *PKS-c41*_SM-X_URA 3.3 grown at 28 °C compared to ATNT16 control. **(D)** HPLC analysis of culture filtrates of OP12 *pyrG*$^{-}$ *PKS-c40*_SM-X_URA 2.1 and OP12 *pyrG*$^{-}$ *PKS-c41*_SM-X_URA 3.1 grown at 28 °C compared to OP12 control. **(E)** HPLC analysis of culture filtrates of ATNT16 Δ*pyrG*x24 *PKS-c40*_SM-X_URA 2.3,5,6 and ATNT16 Δ*pyrG*x24 *PKS-c41*_SM-X_URA 3.3,4,5 grown at 18 °C compared to ATNT16 control. **(F)** HPLC analysis of culture filtrates of OP12 *pyrG*$^{-}$ *PKS-c40*_SM-X_URA 2.1,2,3 and OP12 *pyrG*$^{-}$ *PKS-c41*_SM-X_URA 3.1,3 grown at 18 °C compared to OP12 control. Despite a varying intensity of background metabolites, the reduction of the growth temperature does not result in a new dominating metabolite in any of the samples.

4.3.2.2 *Usnea longissima UlPKS6*

Wang *et al.* (2014b) identified three NR-PKSs from *U. longissima* (*UlPKS2*, *UlPKS4* and *UlPKS6*) and assigned putative metabolites to these PKSs. In order to functionally characterise the product of *UlPKS6*, an expression construct was generated containing the *UlPKS6* gene (Figure 4.8A) and subsequently used in the transformation of *A. niger* ATNT16 Δ*pyrG*x24 and *A. oryzae* OP12 *pyrG*$^{-}$ strains. Positive transformants were identified by screening for full-length integration of the construct and conidia from a pool of all *A. niger* ATNT16 Δ*pyrG*x24 *UlPKS6*_SM-X_URA

transformants or all *A. oryzae* OP12 *pyrG*⁻ *UlPKS6*_SM-X_URA transformants were cultivated under inducing conditions. Extracts of the culture filtrates were analysed by HPLC in order to determine if any metabolites were produced. As shown in Figure 4.8B, no metabolites appear to be produced by the *A. niger* ATNT16 Δ*pyrG*x24 *UlPKS6*_SM-X_URA or *A. oryzae* OP12 *pyrG*⁻ *UlPKS6*_SM-X_URA transformants.

The pools of transformants were then cultivated under inducing conditions at 18 °C in order to see if this resulted in the production of a metabolite. Culture filtrates were analysed by HPLC to identify if any metabolites produced. Figure 4.8C shows that no reproducible peaks for novel metabolites were detected in any of the transformants when grown at a lower temperature.

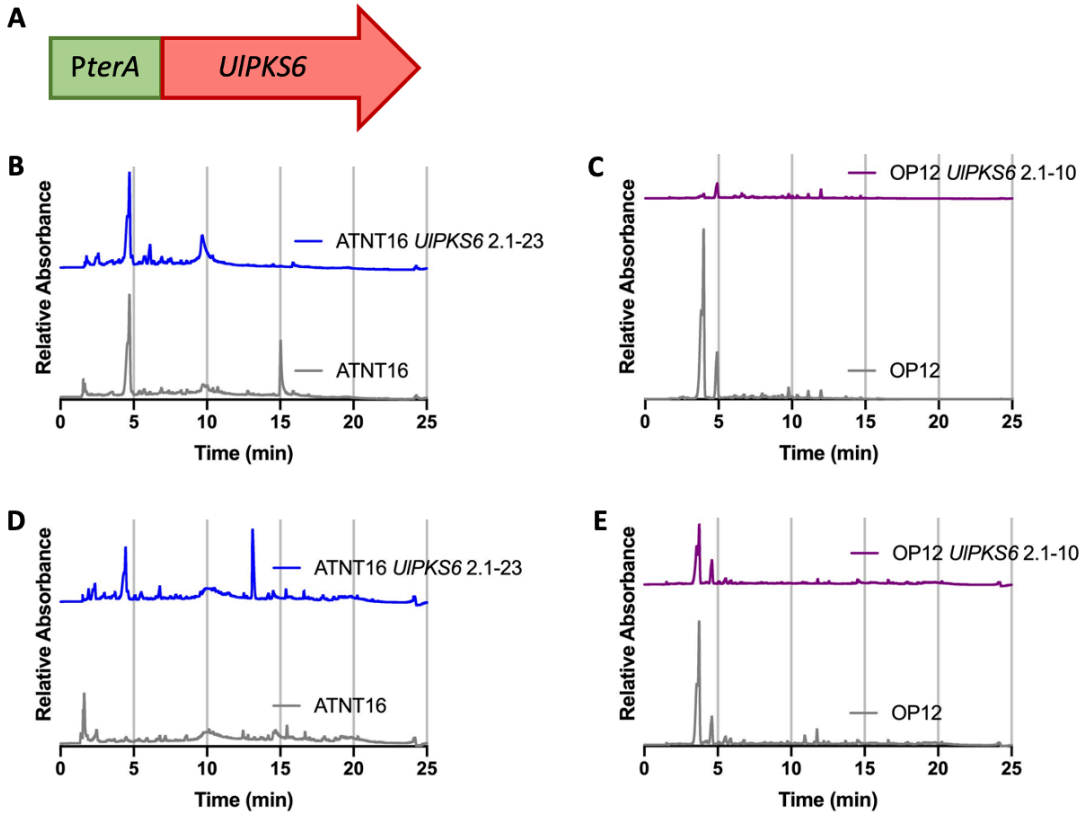


Figure 4.8. (A) Schematic representation of the construct for the expression of *UlPKS6*. **(B)** HPLC analysis of culture filtrate of ATNT16 Δ*pyrG*x24 *UlPKS6*_SM-X_URA 2.1-23 transformants grown at 28 °C compared to ATNT16 control. **(C)** HPLC analysis of culture filtrate of OP12 *pyrG*⁻ *UlPKS6*_SM-X_URA 2.1-10 transformants grown at 28 °C compared to OP12 control. **(D)** HPLC analysis of culture filtrate of ATNT16 Δ*pyrG*x24 *UlPKS6*_SM-X_URA 2.1-23 transformants grown at 18 °C compared to ATNT16 control. The peak produced by the ATNT16 transformants was not reproducible. **(E)** HPLC analysis of culture filtrate of OP12 *pyrG*⁻ *UlPKS6*_SM-X_URA 2.1-10 transformants grown at 18 °C compared to OP12 control.

4.3.2.3 *Cladonia grayi Clagr3.6*, *Clagr3.21*, *Clagr3.26* and *Clagr3.31*

A set of four putative non-reducing PKS genes, *Clagr3.6*, *Clagr3.21*, *Clagr3.26* and *Clagr3.31*, were selected from the *C. grayi* genome. In order to identify the metabolites produced by these SM genes, four expression constructs containing either one of the PKS encoding genes were generated (Figure 4.9A, 4.9B, 4.9C and 4.9D). These expression constructs were subsequently used in the transformation of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* strains. Resulting positive transformants were identified by PCR screening for full-length integration of the construct and cultivated under inducing conditions at 28 °C. Culture filtrates were analysed by HPLC to determine if any metabolites were produced. Figure 4.9E and 4.9F show that no metabolites were detected by strains expressing any of the four PKS genes at 28 °C.

To test whether some temperature dependency on metabolite production can be observed, transformants were grown under inducing conditions at 23 °C. Culture filtrates were analysed by HPLC, and Figure 4.9G and 4.9H show that for strains expressing *Clagr3.26* or *Clagr3.31* there was still no metabolite production at 23 °C. However, metabolite production was observed at 23 °C for *A. niger* and *A. oryzae* strains expressing *Clagr3.6*, with a peak occurring at 7.75 min. This peak has a shoulder, indicating that two metabolites are present. When compared to the metabolite profile of a heterologously expressed THN synthase (PKS1) from *Sporothrix schenckii* (Peres da Silva *et al.*, unpublished results), the metabolites matched in retention time and UV/Vis profile to 1,3,6,8-tetrahydroxynaphthalene and 3,4-dihydro-3,6,8-trihydroxy-3-methylisocoumarin. This indicates that *Clagr3.6* encodes a functional THN synthase, but only when expressed at low temperature. In addition, a yet uncharacterised metabolite was also observed at 23 °C for *A. oryzae* strains expressing *Clagr3.21*, with a peak occurring at 11.6 min. However, in the case of *Clagr3.21,* no metabolite production was observed for *A. niger* strains at 23 °C. While the expression of PKS genes in heterologous expression platforms may require further optimisation, it shows that at least some lichen-derived PKSs can be functionally produced in aspergilli, although a low incubation temperature appears to be a prerequisite and is studied in subsequent chapters.

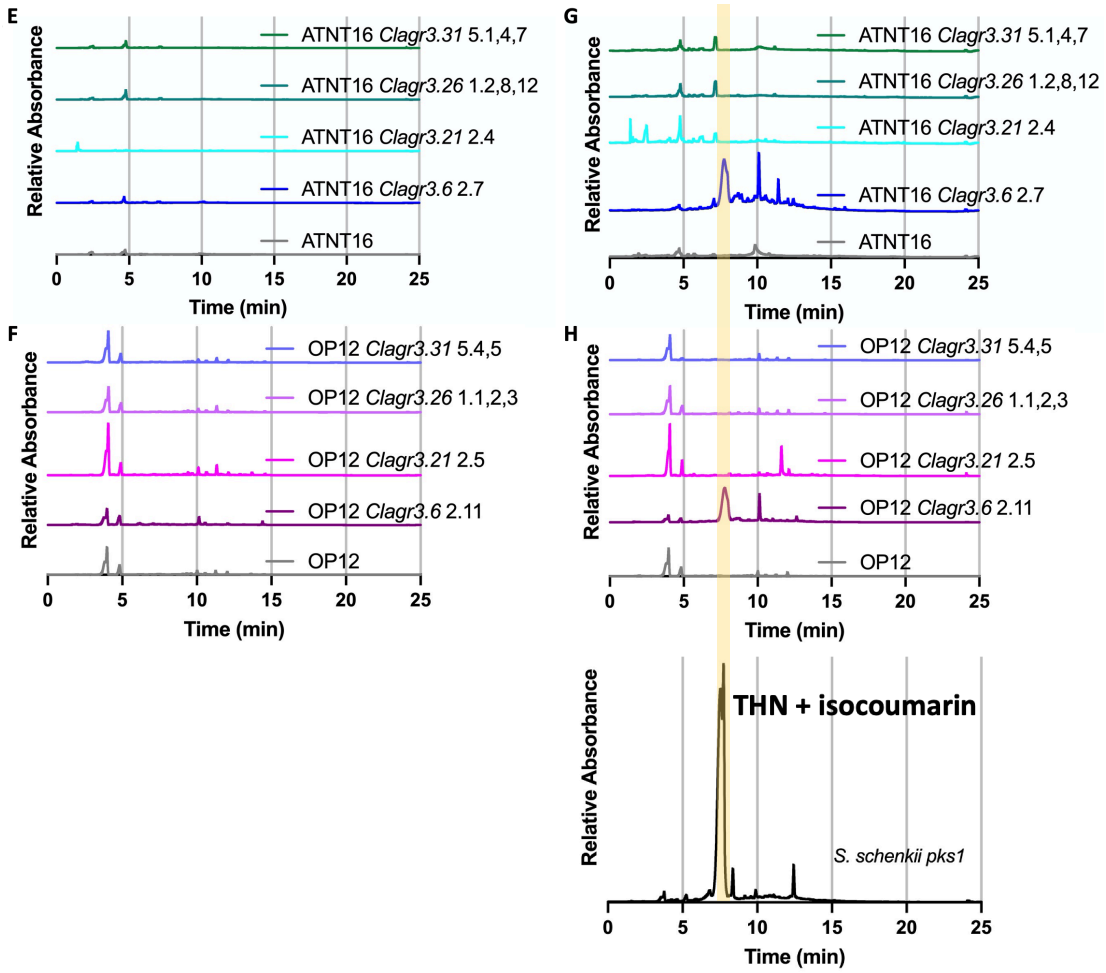


Figure 4.9. Metabolite production from *Clagr3.6*, *Clagr3.21*, *Clagr3.26* and *Clagr3.31*. (A-D) Schematic representations of the expression constructs containing the PKS genes *Clagr3.6* **(A)**, *Clagr3.21* **(B)**, *Clagr3.26* **(C)** and *Clagr3.31* **(D)**. **(E)** HPLC analysis of culture filtrates of ATNT16 Δ*pyrG* Δ*pabaA Clagr3.6*_SM-X_URA 2.7, ATNT16 Δ*pyrG* Δ*pabaA Clagr3.21*_SM-X_URA 2.4, ATNT16 Δ*pyrG* Δ*pabaA Clagr3.26*_SM-X_URA 1.2,8,12 and ATNT16 Δ*pyrG* Δ*pabaA Clagr3.31*_SM-X_URA 5.1,4,7 grown at 28 °C compared to ATNT16 control. **(F)** HPLC analysis of culture filtrates of OP12 *pyrG*$^-$ Δ*pabaA Clagr3.6*_SM-X_URA 2.11, OP12 *pyrG*$^-$ Δ*pabaA Clagr3.21*_SM-X_URA 2.5, OP12 *pyrG*$^-$ Δ*pabaA Clagr3.26*_SM-X_URA 1.1,2,3 and OP12 *pyrG*$^-$ Δ*pabaA Clagr3.31*_SM-X_URA 5.4,5 grown at 28 °C compared to OP12 control. **(G)** Same as **(E)**, but *A. niger* strains were grown at 23 °C. **(H)** Same as **(F)**, but *A. oryzae* strains were grown at 23 °C. The retention times of the peaks obtained in **(G)** and **(H)**, as highlighted by the yellow bar, correspond to the retention times of 1,3,6,8-tetrahydroxynaphthalene and 3,4-dihydro-3,6,8-trihydroxy-3-methylisocoumarin characterised by Peres da Silva *et al.* (unpublished results).

4.4 Discussion

At the beginning of this work, no successful example of a *de novo* biosynthesis of a lichen SM via the heterologous expression of a lichen SM gene had been described (Chooi *et al.*, 2008; Gagunashvili *et al.*, 2009; Armaleo *et al.*, 2011; Wang *et al.*, 2016; Bertrand and Sorensen, 2019a; Bertrand and Sorensen, 2019b). However, the

successes of Sinnemann *et al.* (2000) and Kim *et al.* (2021) proved that lichen genes were suitable to be expressed in non-lichen fungal expression platforms. Due to the fact that lichens possess a strongly enriched number of PKS gene clusters in their genome, it was decided initially to focus on the heterologous production of lichen polyketides (Stocker-Wörgötter, 2008).

4.4.1 Production of Lichen Polyketides

The attempts to produce orsellinic acid and lecanoric acid using genes from the non-lichenised fungal species *A. nidulans* and *A. terreus* confirmed that the *A. niger* ATNT16 Δ*pyrG*x24 and *A. oryzae* OP12 *pyrG*$^{-}$ expression platforms appear as generally suitable to produce metabolites found in lichen species. Furthermore, the use of the luciferase screening system confirmed that the reporter gene can be used in combination with PKS genes for pseudo-polycistronic gene expression, and eases the screening for transformants with full-length integration of the PKS gene.

However, the lack of success in achieving the methylation of these metabolites using *A. niger* ATNT16 Δ*pyrG*x24 or *A. oryzae* OP12 *pyrG*$^{-}$ transformant strains containing the *A. mellea Me473 O*-methyltransferase gene indicates that Me473 is not able to methylate either orsellinic acid or lecanoric acid. This could be due to the fact that the Me473 *O*-methyltransferase is normally involved in the production of melleolides in the basidiomycete *A. mellea*, the unique structures of which are composed of an orsellinic acid moiety joined to a tricyclic sesquiterpene (protoilludane) alcohol via an ester bond (Arnone *et al.*, 1986; Wick *et al.*, 2016; Engels *et al.*, 2021). The structures of orsellinic acid and lecanoric acid do not possess the protoilludane moiety found in melleolides and are, therefore, less bulky in their structures. This difference in the structures of melleolides compared to orsellinic acid and lecanoric acid could have an effect on the positioning of the substrate when interacting with the Me473 protein. Therefore, if either orsellinic acid or lecanoric acid are not correctly positioned, methylation of the substrate will not occur.

In addition, methylation of lecanoric acid was also not observed for either O-MeT-c40 or O-MeT-c41, suggesting that both *O*-methyltransferases were either inactive or use a different substrate. However, the apparent lack of activity could have also occurred as a result of a missing direct interaction between the *O*-

methyltransferase and the TE domain of *A. nidulans orsA*. This, therefore, suggests that the use of a PKS and an *O*-methyltransferase from the same gene cluster might be required for a direct interaction between the enzymes. Alternatively, the use of cell-free extracts rather than purified enzyme could have meant that the amount of active enzyme was too low for *O*-methyltransferase activity to be observed. Purification of O-MeT-c40 or O-MeT-c41 would result in higher quantities of potentially active enzyme, and could therefore result in observable methyltransferase activity. Assays using purified O-MeT-c40 or O-MeT-c41 should be carried out in future in order to determine if this is the case.

4.4.2 Expression of Lichen Polyketide Synthases

The lack of success in achieving metabolite production from *A. niger* ATNT16 and *A. oryzae* OP12 transformant strains containing *PKS-c40* or *PKS-c41* from *E. prunastri*, *UlPKS6* from *U. longissima*, or *Clagr3.26* or *Clagr3.31* from *C. grayi* is in agreement with the attempts of other research groups to achieve the functional heterologous expression of lichen PKS genes in alternative fungal expression platforms. However, the successful heterologous expression of two *C. grayi* PKS genes, *Clagr3.6* and *Clagr3.21*, albeit with an apparent strict temperature dependency, in two *Aspergillus* strains achieved in this study demonstrates that the expression of lichen PKS genes is, in principle, possible in *Aspergillus* expression platform strains.

The successful heterologous expression of only two PKS genes from the four *C. grayi* PKS genes selected in this study implies that the choice of expression platform is critical for functional expression of a lichen SM gene to occur, as one expression platform does not seem to be appropriate for all SM genes from the same lichen species. Further to this, it appears that the successful heterologous expression of a PKS gene in one expression platform does not automatically guarantee that heterologous expression will be achieved in a different expression platform. This is evidenced by our attempts to heterologously express the *Clagr3.21* gene, which was successful in *A. oryzae* OP12 but not in *A. niger* ATNT16. Therefore, the functional expression of lichen SM genes appears to be dependent upon the combination of lichen SM gene and *Aspergillus* expression platform chosen.

Furthermore, the observed temperature dependency of the *Clagr3.6* and *Clagr3.21* PKS genes suggests that optimisation of the culture conditions is required, and that an alternative cold-adapted or slow-growing fungal expression platform may perhaps be more suitable for the heterologous expression of lichen genes. If the proteins produced by a lichen are adapted to lower temperatures, the use of a fungal expression platform strain which optimally grows at 28 °C would likely lead to the incorrect folding of any lichen proteins produced and their subsequent degradation, regardless of the 28 °C incubation temperature being suitable for the expression platform. Thus, it is important to consider both the natural habitat of the lichen species under investigation, as well as the cultivation temperature of the chosen expression platform. The use of a cold-adapted fungal expression platform would be more suitable for the heterologous expression of lichen genes from species which are adapted to lower temperatures.

The inability to achieve the functional expression of some lichen SM genes could also be linked to the nutritional strategies of lichens, which differs from that of non-lichenised fungal species. The carbon source required by the mycobiont is produced by the photobiont and is provided in the form of polyols by algal photobionts (Richardson and Smith, 1968; Richardson *et al.*, 1968; Honegger, 2008a). This may result in the product of a lichen PKS not being formed in a non-lichen expression platform if the polyketide product requires substrates not found in non-lichenised fungal species, as the non-lichen expression platform will not have access to the polyol building blocks available to the lichen mycobiont in nature. Previous studies have found that both the type of carbon source and the amount of carbon supplied had an effect on the SM profile of various mycobionts (Solhaug and Gauslaa, 2004; Brunauer *et al.*, 2006; Brunauer *et al.*, 2007; Elshobary *et al.*, 2016). Therefore, future studies could investigate carbon supplementation when attempting the heterologous expression of lichen PKSs, in order to determine if the supplementation of the growth medium with polyols results in the production of lichen polyketide metabolites.

4.5 Conclusions

The results obtained in this chapter demonstrate that the *A. niger* ATNT16 and *A. oryzae* OP12 strains used in these studies are suitable for the functional heterologous expression of certain SM genes identified from lichen PKS gene clusters. Specifically, the *A. niger* ATNT16 expression platform was found to be suitable for the functional expression of the *Clagr3.*6 gene from *C. grayi*, and the *A. oryzae* OP12 strain was found to be a suitable expression platform for the expression of the *Clagr3.6* and *Clagr3.21* PKS genes from *C. grayi*. These results are significant in the sense that they contradict the hypotheses put forward by other research groups that there is a systematic problem with the heterologous expression of lichen SM genes in *Ascomycota* hosts. However, the results obtained in this study do suggest that the combination of expression platform and lichen SM gene is critical for successful heterologous expression of the gene of interest. Furthermore, optimisation of the cultivation conditions needs to be considered.

Future work associated with the studies carried out in this chapter should focus on the optimisation of cultivation conditions for the heterologous expression of lichen genes in aspergilli, as well as the development of expression platforms using cold-adapted or slow-growing fungal species. Future work specifically concerning the *C. grayi Clagr3.21* gene should also focus on the characterisation of the metabolite produced by this gene. In addition, the carbon supplementation of the transformant strains should be investigated to determine if the form in which carbon is supplied to the transformant strains has an effect on the production of lichen polyketides as observed in axenic mycobiont cultures.

Chapter 5: Heterologous Expression of Lichen Non-Ribosomal Peptide Synthetase-Like Enzymes

5.1 Introduction

5.1.1 Non-Ribosomal Peptide Synthetase-Like Enzymes

Non-ribosomal peptide synthetase (NRPS)-like enzymes can be found in fungal genomes either by themselves or located within a biosynthesis gene cluster (Geib *et al.*, 2019). They are involved in the production of a large number of metabolites which can have a range of biological activities, many of which are implicated in fungal development and environmental competition, as well as in nutrition and virulence (Marahiel *et al.*, 1997; Geib *et al.*, 2019). In addition, a number of metabolites produced by NRPS-like enzymes have also been found to have pharmaceutical applications; for example, prenylated butyrolactones have anti-inflammatory, antiparasitic and antitumour activities, and asterriquinones display antitumour and insulin-mimicking activities (Kim *et al.*, 2007; Li *et al.*, 2012; Dewi *et al.*, 2015; Guo *et al.*, 2016; da Silva *et al.*, 2017; Hühner *et al.*, 2018; Geib *et al.*, 2019). Biochemical information on the importance and catalytic properties of NRPS-like enzymes was scarce due to a lack of research being carried out on this class of enzymes (Schneider *et al.*, 2007). However, more recently the characterisation of various NRPS-like enzymes in different fungal species has shed light on the importance of the metabolites produced by these enzymes (Geib *et al.*, 2019).

True NRPS enzymes contain at least one module which consists of an adenylation (A) domain which is responsible for the recognition and activation of amino acids via adenylation, a thiolation (T) domain (also known as a peptidyl carrier protein, or PCP) which is involved in the formation of a 4'-phosphopantetheinyl linker in order to attach the aminoacyl moiety to the enzyme, and a condensation (C) domain which is responsible for the formation of a peptide bond between two amino acids, as shown in Figure 5.1A (Stachelhaus *et al.*, 1996; Conti *et al.*, 1997; Stachelhaus *et al.*, 1998; Weber *et al.*, 2000; Geib *et al.*, 2019). NRPS-like enzymes are similar to NRPSs in the sense that they possess similar catalytic A and T domains as those found in NRPSs, but they are monomodular enzymes which lack the C domain found in true

NRPS genes (van Dijk *et al.*, 2016). Instead, NRPS-like enzymes contain an A domain, a T domain, and either a thioesterase (TE) domain for non-reducing enzymes (Figure 5.1B) or a reductase (R) domain for reducing enzymes located at the *C*-terminus (Yeh *et al.*, 2012; Sun *et al.*, 2016; Hühner *et al.*, 2019). Although they utilise different mechanisms, the R domain and the TE domain are responsible for the formation and release of a product (Wang *et al.*, 2014a; van Dijk *et al.*, 2016; Geib *et al.*, 2019).

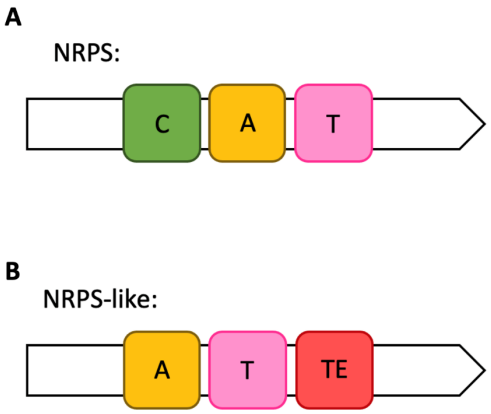


Figure 5.1. Domain organisation of an NRPS enzyme (A) and a non-reducing NRPS-like enzyme (B). NRPSs and NRPS-like enzymes differ in their domain structure, with both enzymes containing an A and a T domain but NRPS-like enzymes lacking the C domain that is found in true NRPSs. Adapted from Sun *et al.* (2016) and Hühner *et al.* (2019).

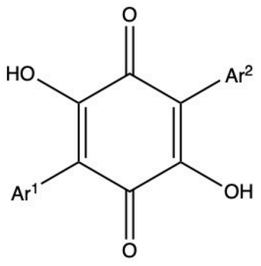
There are a number of examples of NRPS-like enzymes which have been identified in various fungal species, therefore supporting the idea that NRPS-like enzymes are found throughout the fungal kingdom. The first fungal NRPS-like enzyme with a characterised corresponding product was TdiA from *A. nidulans*, which is ultimately responsible for the production of terrequinone A (Bok *et al.*, 2006b; Balibar *et al.*, 2007; Schneider *et al.*, 2007). Subsequently, studies have been conducted for other fungal species; for example, *A. terreus* possesses various NRPS-like enzymes which direct the biosynthesis of γ-butyrolactones, aspulvinone E, phenguignardic acid and atromentin (Guo *et al.*, 2013; Geib *et al.*, 2016; Sun *et al.*, 2016; Hühner *et al.*, 2018). In addition, a number of other NRPS-like enzymes have been characterised in fungi, such as MicA in *A. nidulans* which is a microperfuranone synthetase, as well as AbrA in *A. brasiliensis*, AtrA in *Tapinella panuoides*, GreA in *Suillus grevillei*, and various InvA NRPS-like enzymes in *Paxillus involutus*, all of which are atromentin synthetases

(Schneider *et al.*, 2008; Wackler *et al.*, 2012; Yeh *et al.*, 2012; Braesel *et al.*, 2015; Geib *et al.*, 2019).

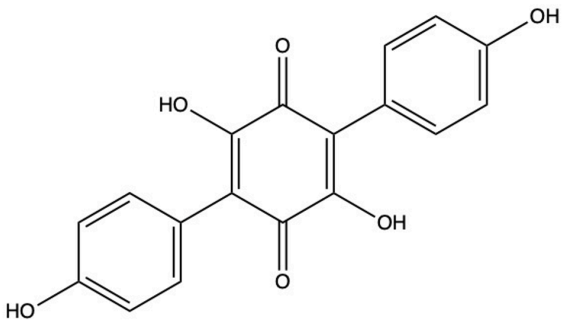
5.1.2 Biosynthesis of Metabolites from Non-Ribosomal Peptide Synthetase-Like Enzymes

Due to the fact that NRPS-like enzymes lack a C domain, they do not form peptide bonds between amino acids (Yeh *et al.*, 2012; Geib *et al.*, 2019). Instead, NRPS-like enzymes that contain a TE domain are thought to carry out the condensation of two identical aromatic α-keto acids. The activated α-keto acids are attached to the T domain via its 4'-phosphopantetheine arm, whereby the first α-keto acid is activated by the A domain and then transferred to the TE domain; this α-keto acid is then bound to the TE domain as an oxoester. After this, a second α-keto acid is activated by the A domain and tethered to the T domain as a thioester. The condensation of the two bound α-keto acids is believed to be carried out by the TE domain and results in the production of various interconnecting core ring structures; these are known as terphenylquinones and furanones (Figure 5.2) (Balibar *et al.*, 2007; Pauly *et al.*, 2014; Geib *et al.*, 2016; Geib *et al.*, 2019; Hühner *et al.*, 2019). In contrast, those NRPS-like enzymes which possess an R domain at their *C*-terminus carry out the reduction of substrates without condensation to produce aryl-aldehydes (Wang *et al.*, 2014a). In order for NRPS-like enzymes to become catalytically active, it is necessary that the inactive *apo* protein undergoes a post-translational modification to be converted into its active *holo* form. This involves the transfer of the 4'-phosphopantetheinyl moiety of coenzyme A to a conserved serine residue found in the T domain (Marahiel *et al.*, 1997; Balibar *et al.*, 2007).

Terphenylquinones:

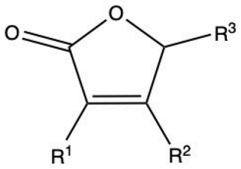


Core structure

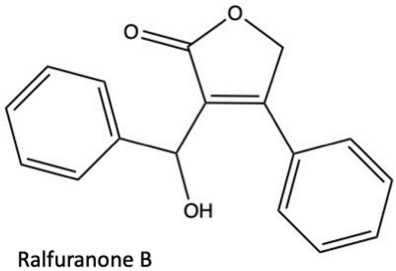


Atromentin

Furanones:



Core structure



Ralfuranone B

Figure 5.2. Core structures of metabolites produced by NRPS-like enzymes. The core structures of terphenylquinones and furanones are shown, along with an example of a metabolite containing each of the core structures. Adapted from Geib *et al.* (2016).

It is thought that the major substrates of non-reducing NRPS-like enzymes found in fungi are the α-keto acids of the aromatic amino acids tryptophan, tyrosine and phenylalanine (Figure 5.3) (Hühner *et al.*, 2018). Each NRPS-like enzyme appears to be specific for a single aromatic α-keto acid that it can accept as substrate, meaning that only a single product can be formed by the NRPS-like enzyme. It has been established that within the primary sequence of the A domain, there are ten key amino acid residues, known as the non-ribosomal code, which line the active site and are thought to be implicated in substrate recognition, as well as to contribute to substrate specificity (Stachelhaus *et al.*, 1999; Wackler *et al.*, 2012). However, even though non-reducing NRPS-like enzymes have a strong preference for accepting only

a single α-keto acid as substrate, the core structures that they produce result in the formation of a diverse range of metabolites (Geib *et al.*, 2019).

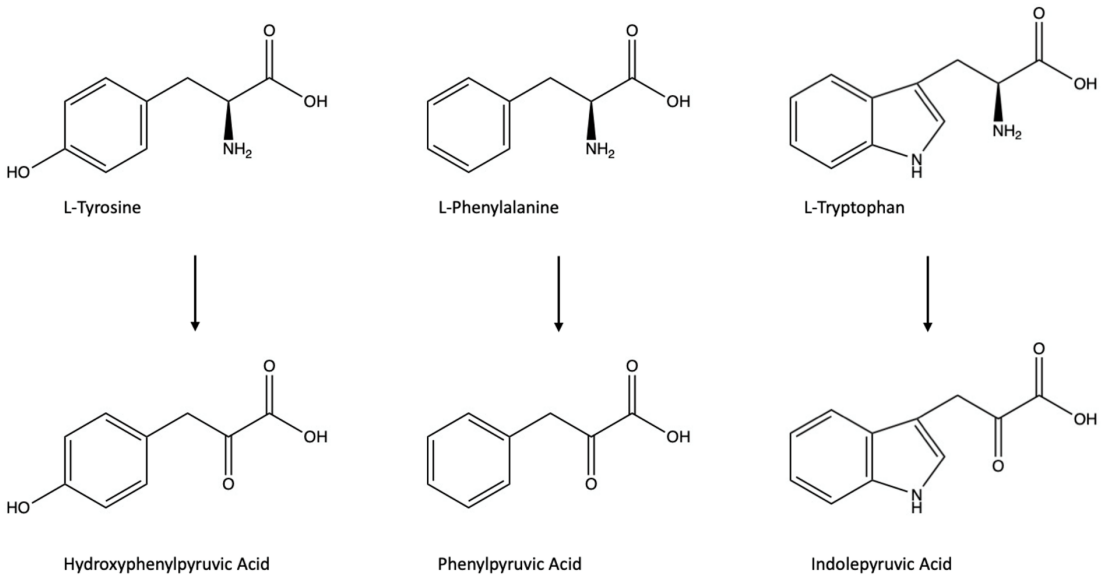


Figure 5.3. The structures of the three aromatic amino acids are shown, along with their corresponding α-keto acid. The amino acid undergoes deamination in order to produce the α-keto acid; this process is carried out by a transaminase enzyme in which the amine group is exchanged for a keto group. The α-keto acids can then be used as substrates by NRPS-like enzymes. Adapted from Bok *et al.* (2006b) and Hühner *et al.* (2018).

The core structure of terphenylquinones is produced as a result of two symmetrical nucleophilic attacks: one occurs at the C3 of one α-keto acid and at the C1 of the other α-keto acid, and vice versa. This results in the formation of two new C-C bonds (Geib *et al.*, 2016). The formation of the core structure of furanones differs from that of terphenylquinones, whereby the butenolide core structure is formed by one of two pathways. The first pathway involves the formation of a terphenylquinone intermediate which is subsequently oxidatively cleaved and lactonized, as well as possibly decarboxylated. The second pathway involves the formation of a single C-C bond as a result of a direct aldol condensation of two building blocks; an intramolecular lactonization then takes place, followed by hydrolysis and then decarboxylation (Schüffler *et al.*, 2011; Pauly *et al.*, 2014; Geib *et al.*, 2016).

The core structure formed by a non-reducing NRPS-like enzyme is determined by the action of the TE domain (Balibar *et al.*, 2007; Geib *et al.*, 2019; Hühner *et al.*, 2019). However, the exact mechanism used by the TE domain is currently not well

understood, due to the fact that only a small number of non-reducing NRPS-like enzymes have been studied and characterised so far and no crystal structures have been elucidated. It has been suggested that the formation of a core ring structure is determined by a specific sequence motif, in which a triad consisting of serine, asparagine and histidine followed by a branched-chain aliphatic amino acid (leucine or isoleucine) and two proline residues leads to the formation of a quinone ring structure. Furanone core ring structures are instead thought to be formed by the presence of a triad made up of serine, aspartate and histidine. However, predictions of thioesterase chemistry based upon sequence pattern analysis remain difficult to determine (Braesel *et al.*, 2015; Geib *et al.*, 2016; Geib *et al.*, 2019). Once the core structure has been formed by the NRPS-like enzyme, the peptide backbone can then undergo further modification (Fischbach and Walsh, 2006). Modifications of the peptide backbone can include prenylation, hydroxylation and cyclisation. Thus, NRPS-like enzymes produce a range of scaffolds for the production of a myriad of natural products (Winkelblech *et al.*, 2015; Payne *et al.*, 2017; Rudolf *et al.*, 2017; Tang *et al.*, 2017; Hühner *et al.*, 2018).

5.1.3 Previous Heterologous Expression of Lichen Non-Ribosomal Peptide Synthetase-Like Enzymes

Although various fungal species have been found to contain a number of NRPS-like enzyme encoding genes in their genomes, research into the characterisation of these genes and the metabolites that they produce have, until recently, been limited (Schneider *et al.*, 2007). As mentioned in previous sections, studies carried out in recent times have now seen the characterisation of various NRPS-like enzymes in a range of fungal species; however, these studies have focussed on non-lichen fungal species (Bok *et al.*, 2006b; Balibar *et al.*, 2007; Schneider *et al.*, 2007; Schneider *et al.*, 2008; Wackler *et al.*, 2012; Yeh *et al.*, 2012; Guo *et al.*, 2013; Braesel *et al.*, 2015; Geib *et al.*, 2016; Sun *et al.*, 2016; Hühner *et al.*, 2018; Geib *et al.*, 2019). To date, there have not been any attempts described that study the NRPS-like enzymes found in lichenised fungal species using heterologous expression of these SM genes in non-lichen fungal expression platforms. Instead, studies by other research groups have focussed on the heterologous expression of lichen PKSs (Chooi *et al.*, 2008;

Gagunashvili *et al.*, 2009; Armaleo *et al.*, 2011; Wang *et al.*, 2016; Bertrand and Sorensen, 2019a; Bertrand and Sorensen, 2019b; Kim *et al.*, 2021).

Even though there have not yet been any heterologous expression studies involving lichen NRPS-like enzymes, there has been an increasing interest in the sequencing of genomes of lichen species (Park *et al.*, 2013a; Park *et al.*, 2013b; Park *et al.*, 2014a; Park *et al.*, 2014b; Park *et al.*, 2014c; Wang *et al.*, 2014c; Meiser *et al.*, 2017; Bertrand *et al.*, 2018; Dal Grande *et al.*, 2018; Wang *et al.*, 2018; Armstrong *et al.*, 2018; Armaleo *et al.*, 2019; McKenzie *et al.*, 2020). These genome sequences have revealed that, similar to non-lichen fungal species, lichenised fungal species contain high numbers of SM genes in their genomes, including genes coding for NRPS-like enzymes in their genomes. For example, 15 NRPS-like genes (including reducing and non-reducing enzymes) have been identified in the *C. grayi* genome, and the genome of *Cladonia uncialis* has been found to contain eight NRPS-like genes (Bertrand *et al.*, 2018; Armaleo *et al.*, 2019). The NRPS-like genes identified in the genomes of these lichen species could therefore be the focus of future studies, both to determine if the heterologous expression of lichen NRPS-like genes is feasible and, subsequently, to characterise the metabolites produced by these genes.

5.1.4 Chapter Objectives

Due to the low success rate achieved in the previous chapter in terms of functionally expressing lichen PKS genes in a heterologous host, we decided to attempt the heterologous expression of lichen-derived SMs encoded by a different class of SM-producing enzymes, in this case non-reducing NRPS-like enzymes. This class of enzymes was chosen due to the fact that the molecular mass of these enzymes is comparably much smaller than that of PKSs (about 110 kDa compared to >200 kDa), as well as the fact that the biosynthesis of products encoded by non-reducing NRPS-like enzymes does not require accessory enzymes (unlike for some PKSs).

Therefore, the aim of this chapter is to achieve the heterologous expression of lichen NRPS-like enzymes in *Aspergillus* heterologous hosts. The *A. niger* ATNT16 and *A. oryzae* OP12 expression platforms will be used for the heterologous expression of a range of genes encoding for NRPS-like enzymes identified from various lichen

species. HPLC analysis will subsequently be undertaken to determine if any metabolites are being produced.

5.2 Materials and Methods

5.2.1 Species and Strains

The expression strains used in this study were either *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* or *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA*. As stated in Section 4.2.1, a sample of the thallus of *E. prunastri* was collected from Nottingham, UK; genomic DNA was subsequently extracted from the whole lichen thallus as described in Section 2.2.4. Genomic DNA obtained from *C. grayi* was provided by Daniele Armaleo (Duke University, North Carolina, USA). Genomic DNA from *Aspergillus brasiliensis* was obtained from the wild-type strain CBS101740.

5.2.2 Preparation of Conidial Suspensions

Conidial suspensions of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* were obtained by growing each strain on slopes in Universal tubes containing solid GG10 medium and any necessary supplementations. After incubation at 28 °C, conidial suspensions of the strains were generated by scraping conidia into 6 ml PBS using a sterile cotton swab.

5.2.3 Generation of Strains Expressing Lichen Non-Ribosomal Peptide Synthetase-Like Enzymes

Sequences of all oligonucleotides used in this study are listed in Appendix 1. Plasmid maps of the principle plasmids used in this study are shown in Appendix 4.

5.2.3.1 *Evernia prunastri EpNRPSL1*, *EpNRPSL2* and *EpNRPSL3*

Three different expression plasmids were generated for the heterologous expression of three NRPS-like enzymes identified in the *E. prunastri* genome using the SM_S-tag_X_URA plasmid, in which each NRPS-like enzyme received an *N*-terminal Strep-tag. The *EpNRPSL1* gene was amplified from *E. prunastri* gDNA using oligonucleotide 58 which contained an overhang to the Strep-tag sequence, and

oligonucleotide 59 which contained an overhang to the *NcoI*-restricted SM_S-tag_X_URA plasmid. In the same way, the *EpNRPSL2* gene was amplified from *E. prunastri* gDNA using oligonucleotide 60 which overlapped with the Strep-tag sequence, and oligonucleotide 61 which overlapped with the *NcoI*-restricted SM_S-tag_X_URA plasmid. Finally, the *EpNRPSL3* gene was amplified from *E. prunastri* gDNA using oligonucleotides 62 and 63, which contained overhangs to the Strep-tag sequence and the *NcoI*-restricted SM_S-tag_X_URA plasmid, respectively.

Each of the constructs were generated by combining the *NcoI*-restricted SM_S-tag_X_URA plasmid and the gel-purified PCR fragment for each gene, and assembling by *in vitro* recombination. The resulting constructs were then amplified in *E. coli* DH5α cells and purified. Positive clones for each construct were selected using colony PCR with oligonucleotides 64 and 15 for *EpNRPSL1*_SM_S-tag_X_URA, oligonucleotides 65 and 15 for *EpNRPSL2*_SM_S-tag_X_URA, and oligonucleotides 66 and 15 for *EpNRPSL3*_SM_S-tag_X_URA. The isolated plasmids were then checked for correct assembly using restriction analyses. The resulting plasmids were subsequently used for the transformation of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA*. For transformation, media was supplemented with doxycycline (final concentration 10 μg/ml) for *A. niger* ATNT16 Δ*pyrG* Δ*pabaA*, and para-aminobenzoic acid (final concentration 7.29 μM) for both strains.

Liquid GG20 cultures in a 25 ml volume supplemented with 7.29 μM para-aminobenzoic acid and 10 μg/ml doxycycline for *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* strains and 25 ml liquid malt extract (ME) cultures supplemented with 7.29 μM para-aminobenzoic acid for *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* strains were inoculated with conidia suspensions of transformants from each expression construct. Cultures were incubated at 23 °C and 28 °C in parallel on a rotary shaker at 150 rpm. Mycelia were subsequently removed by filtration over Miracloth (Merck, Darmstadt, Germany), and the culture filtrates were analysed by HPLC for SM production.

5.2.3.2 *Cladonia grayi Clagr3.11* and *Clagr3.30*

Expression constructs were generated for the heterologous expression of two genes encoding putative non-reducing NRPS-like enzymes, *Clagr3.11* and *Clagr3.30*,

that were identified in the *C. grayi* genome. These expression constructs were generated using the SM_S-tag_X_URA plasmid, in which each NRPS-like enzyme received an *N*-terminal Strep-tag. The *Clagr3.11* gene was amplified from *C. grayi* gDNA using oligonucleotides 67 and 68, which overlapped with the Strep-tag sequence and the *NcoI*-restricted SM_S-tag_X_URA plasmid, respectively. The *Clagr3.30* gene was also amplified from *C. grayi* gDNA using oligonucleotide 69 which contained an overlap with the Strep-tag sequence, and oligonucleotide 70 which contained an overhang to the *NcoI*-restricted SM_S-tag_X_URA plasmid.

Each construct was generated using the *NcoI*-restricted SM_S-tag_X_URA plasmid and the gel-purified PCR fragment. The plasmid and PCR fragment for each gene were mixed, assembled by *in vitro* recombination, amplified in *E. coli* DH5α cells and isolated. Resulting positive clones were selected by colony PCR using oligonucleotides 71 and 15 for *Clagr3.11*_SM_S-tag_X_URA, and oligonucleotides 72 and 15 for *Clagr3.30*_SM_S-tag_X_URA. Each plasmid was then checked for correct assembly by restriction analyses. The *Clagr3.11*_SM_S-tag_X_URA and *Clagr3.30*_SM_S-tag_X_URA plasmids were subsequently used for the transformation of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA*. For transformation, media was supplemented with 10 μg/ml doxycycline and 7.29 μM para-aminobenzoic acid for *A. niger* ATNT16 Δ*pyrG* Δ*pabaA*, and only 7.29 μM para-aminobenzoic acid for *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA*.

In order to induce metabolite production, 25 ml liquid GG20 cultures supplemented with 7.29 μM para-aminobenzoic acid and 10 μg/ml doxycycline for *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* strains, and 25 ml liquid ME cultures supplemented with 7.29 μM para-aminobenzoic acid for *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* strains were inoculated with conidia suspensions of transformants for each expression construct. The liquid cultures were incubated at 23 °C and 28 °C in parallel at 150 rpm on a rotary shaker. The mycelia were separated from culture filtrates by filtration over Miracloth (Merck, Darmstadt, Germany), and the culture filtrates or mycelia were extracted with ethyl acetate and subsequently analysed by HPLC for metabolite production.

In an attempt to direct the production towards certain metabolites, 25 ml liquid ME cultures supplemented with 7.29 μM para-aminobenzoic acid and either 5

mM tyrosine or 5 mM phenylalanine were inoculated with *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.11*_SM_S-tag_X_URA 2.4 transformant conidia. The liquid cultures were incubated at 23 °C on a rotary shaker at 150 rpm. As before, mycelia were separated from culture filtrates by filtration over Miracloth (Merck, Darmstadt, Germany) and the mycelia were analysed by HPLC for metabolite production.

5.2.4 Generation of Strains Expressing Hybrid Non-Ribosomal Peptide Synthetase-Like Enzymes

Two expression constructs were created using the SM_S-tag_X_URA plasmid containing hybrid genes, in which various domains from *C. grayi Clagr3.11* were replaced by the respective domains of the *A. brasiliensis abrA* gene to assemble hybrid NRPS-like enzymes with an *N*-terminal Strep-tag. One expression construct, *Clagr3.11*:T-TE$_{abrA}$_SM_S-tag_X_URA, contained the A domain of *Clagr3.11* fused to the T and TE domains from *abrA*, whilst the other expression construct, *Clagr3.11*:TE$_{abrA}$_SM_S-tag_X_URA, contained the A and T domains from *Clagr3.11* fused to the TE domain from *abrA*. For the generation of the *Clagr3.11*:T-TE$_{abrA}$_SM_S-tag_X_URA expression plasmid, the *Clagr3.11* A domain was amplified from the *Clagr3.11*_SM_S-tag_X_URA plasmid using oligonucleotide 67, which overlapped with the Strep-tag sequence, and oligonucleotide 73 which contained an overlap to the *abrA* T domain sequence. The *abrA* T and TE domains were amplified from *A. brasiliensis* wild-type gDNA using oligonucleotide 74, which overlapped with the *Clagr3.11* A domain sequence, and oligonucleotide 75 which contained an overhang to the *NcoI*-restricted SM_S-tag_X_URA plasmid. For the *Clagr3.11*:TE$_{abrA}$_SM_S-tag_X_URA plasmid, the *Clagr3.11* A and T domains were amplified from the *Clagr3.11*_SM_S-tag_X_URA plasmid using oligonucleotide 67, which contained an overhang to the Strep-tag sequence, and oligonucleotide 76 which overlapped with the *abrA* TE domain sequence. The *abrA* TE domain was amplified from *A. brasiliensis* wild-type gDNA using oligonucleotide 77, which contained an overlap to the *Clagr3.11* T domain sequence, and oligonucleotide 75 which overlapped with the *NcoI*-restricted SM_S-tag_X_URA plasmid.

Each expression construct was assembled via *in vitro* recombination by mixing the *NcoI*-restricted SM_S-tag_X_URA plasmid and the gel-purified PCR fragments, and

was subsequently amplified in *E. coli* DH5α cells and isolated. Positive clones were selected by colony PCR using oligonucleotides 78 and 15 for *Clagr3.11*:T-TE$_{abrA}$_SM_S-tag_X_URA and oligonucleotides 78 and 15 for *Clagr3.11*:TE$_{abrA}$_SM_S-tag_X_URA. The isolated plasmids were checked for correct assembly using restriction analyses and then used in the transformation of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA*. For transformation media, agar was supplemented with 7.29 μM para-aminobenzoic acid for both strains, as well as 10 μg/ml doxycycline for *A. niger* ATNT16 Δ*pyrG* Δ*pabaA*.

In order to induce metabolite production, 25 ml liquid GG20 cultures supplemented with 7.29 μM para-aminobenzoic acid and 10 μg/ml doxycycline for *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* strains, and 25 ml liquid ME cultures supplemented with 7.29 μM para-aminobenzoic acid for *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* strains were inoculated with conidia suspensions of transformants from each expression construct. Liquid cultures were incubated at 23 °C and 28 °C in parallel on a rotary shaker at 150 rpm. Mycelia and culture filtrates were separated by filtration over Miracloth (Merck, Darmstadt, Germany) and the culture filtrates were analysed by HPLC for SM production.

In order to direct metabolite production towards polyporic acid, 25 ml liquid ME cultures supplemented with 7.29 μM para-aminobenzoic acid and 5 mM phenylalanine were inoculated with conidia suspensions of *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* transformants for each expression construct. Liquid cultures were again incubated at 23 °C and 28 °C in parallel at 150 rpm on a rotary shaker. Mycelia and culture filtrates were separated by filtration over Miracloth (Merck, Darmstadt, Germany) and the culture filtrates or mycelia were analysed by HPLC for SM production.

5.2.5 Transformant Analysis

Transformation plates were incubated at 28 °C, and transformants were streaked twice onto fresh GG10 agar plates for purification. All selected transformants were screened by diagnostic PCR after gDNA extraction using oligonucleotides 57 and 15 in order to confirm the full-length integration of the gene of interest.

5.2.6 Enzyme Purification

For purification of Clagr3.11, an *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* strain producing Clagr3.11 with an *N*-terminal Strep-tag was selected. The strain was grown for 46.5 h at room temperature in 1500 ml GG20 supplemented with 7.29 µM para-aminobenzoic acid, 15 µg/ml doxycycline, 1 mM tyrosine and 1 mM phenylalanine. Mycelium was harvested over Miracloth (Merck, Darmstadt, Germany) and pressed dry. Approximately 6 g mycelium was ground to a fine powder under liquid nitrogen and suspended in buffer W (100 mM Tris/HCl pH 8.0, 150 mM NaCl) supplemented with 1 mM EDTA. Cell debris were removed by 8 min centrifugation at 4 °C and 4000 rpm, followed by 2 min centrifugation at 4 °C and 13,000 rpm. The supernatant was filtered over a 0.45 µm filter (Sartorius, Göttingen, Germany) and was applied to a 1 ml Strep-Tactin Superflow gravity flow column (IBA Lifesciences GmbH, Göttingen, Germany) previously equilibrated with buffer W. After washing the column with five column volumes of buffer W containing 1 mM EDTA, Clagr3.11 was eluted using buffer W supplemented with 2.5 mM desthiobiotin. Eluates were concentrated using a centrifugal filter device with a 30 kDa cut-off. Enzyme purity was analysed by SDS-PAGE using a NuPAGE 4-12% Bis-Tris gel in a MOPS-buffered running system (Invitrogen, Thermo Fisher Scientific, UK) and stained with Coomassie PhastGel Blue R-350 (GE Healthcare) staining solution.

For purification of AcyN, an *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* strain producing AcyN with an *N*-terminal Strep-tag was selected. The strain was grown for 27 h at 28 °C in two cultures of 50 ml ME supplemented with 7.29 µM para-aminobenzoic acid. Mycelium was harvested over Miracloth (Merck, Darmstadt, Germany) and pressed dry. Approximately 4 g mycelium was ground to a fine powder under liquid nitrogen and suspended in buffer W supplemented with 1 mM EDTA. Enzyme purification was carried out as described above.

5.2.7 Clagr3.11 and AcyN *In Vitro* Assays

In a 5 ml reaction containing 100 mM PIPES pH 7.5, 6 mM ATP and 8 mM $MgCl_2$, 0.2 mg of purified Clagr3.11 or AcyN enzyme was incubated with either 2.5 mM *p*-hydroxyphenylpyruvate, 2.5 mM phenylpyruvate, or a combination of the two substrates. These combinations were 2.5 mM *p*-hydroxyphenylpyruvate and 2.5 mM

phenylpyruvate, 2.5 mM *p*-hydroxyphenylpyruvate and 1 mM phenylpyruvate, or 1 mM *p*-hydroxyphenylpyruvate and 2.5 mM phenylpyruvate. Reactions without the enzyme served as controls. After incubation for 24 h at room temperature for Clagr3.11 or 28 °C for AcyN, reactions were acidified to pH 3 with HCl resulting in protein denaturation and PIPES-buffer precipitation. The precipitate was separated from the supernatant by centrifugation, and both supernatant and pellet were extracted with ethyl acetate, dried and solved in methanol for HPLC analysis.

5.2.8 Clagr3.11 Feeding Experiments

To identify if the Clagr3.11 enzyme could be used to produce novel metabolites, an *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* strain containing the *Clagr3.11*_SM_S-tag_X_URA plasmid was grown in liquid cultures supplemented with 4-chloro-phenylalanine. For *in vivo* analysis of Clagr3.11 substrate specificity, liquid ME cultures in a 25 ml volume supplemented with 7.29 µM para-aminobenzoic acid, 2.5 mM 4-chloro-phenylalanine and either 1 mM tyrosine or 1 mM phenylalanine were inoculated with *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.11*_SM_S-tag_X_URA 2.4 transformant conidia. Liquid cultures were for 36.5 h incubated at 23 °C on a rotary shaker at 150 rpm. Mycelia and culture supernatants were separated by filtration over Miracloth (Merck, Darmstadt, Germany), extracted and were analysed by HPLC for SM production. Mass spectrometry was subsequently used (courtesy of Ben Pointer-Gleadhill, University of Nottingham, UK) to confirm the chemical structures of any metabolites produced.

5.2.9 Metabolite Extraction and Analysis

Metabolites were extracted from culture filtrates by mixing with an equal volume of ethyl acetate and removing the overlying solvent layer. Metabolites were extracted from mycelia via homogenisation in ethyl acetate by placing in an ultrasonic bath (USR 30 H; 160 W, 35 kHz) (Merck, Darmstadt, Germany) without heating for ~10 min. The extracts were filtered over anhydrous sodium sulfate and evaporated under reduced pressure. The resulting residues were then solved in methanol.

For *in vitro* assays, the supernatant and pellet were separated after acidification of the reaction. Metabolites were extracted from the supernatant by

mixing with an equal volume of ethyl acetate, collecting the overlaying organic layer and repeating the procedure once. The two fractions were combined and evaporated under reduced pressure. Metabolites were extracted from the pellet by adding ethyl acetate, mixing and collecting the organic layer. This was repeated once and, again, the two fractions were combined before evaporation under reduced pressure. The resulting residues from supernatant and pellet extractions were then solved in methanol.

The samples solved in methanol subsequently underwent HPLC analysis using a Dionex UltiMate3000 (Thermo Fisher Scientific, UK) and an Eclipse XDB-C18 column (4.6x150 mm, 5 μm; Agilent, California, USA) kept at 40 °C. A binary solvent system of water containing 0.1% formic acid (solvent A) and 100% methanol (solvent B) was used with a flow rate of 1 ml/min: 0.5 min 10% B, 15 min 90% B, 17 min 90% B, 17.5 min 100% B, 22 min 100% B, 23 min 10% B, 25 min 10% B.

5.3 Results

5.3.1 Strains Expressing Lichen Non-Ribosomal Peptide Synthetase-Like Enzymes

5.3.1.1 *Evernia prunastri EpNRPSL1*, *EpNRPSL2* and *EpNRPSL3*

Three putative non-reducing NRPS-like enzymes, *EpNRPSL1*, *EpNRPSL2* and *EpNRPSL3*, were identified in the genome of *E. prunastri*. In order to identify the metabolites produced by these SM genes, three expression constructs were created in which each of the NRPS-like genes contained an *N*-terminal Strep-tag (Figure 5.4A, 5.4B and 5.4C). These expression plasmids were then used for the transformation of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* strains. The resulting transformants were screened by PCR for full-length integration of the construct before cultivation under inducing conditions at 23 °C and 28 °C in parallel. Culture filtrates were subsequently analysed by HPLC in order to identify if any metabolites were produced. Figure 5.4D, 5.4E, 5.4F and 5.4G show that, except for background metabolites from the expression platform strains, no additional metabolites were produced by transformant strains expressing any of the three NRPS-like genes at either 23 °C or 28 °C. This indicates that these genes are either non-functional or not functionally produced in the heterologous expression system.

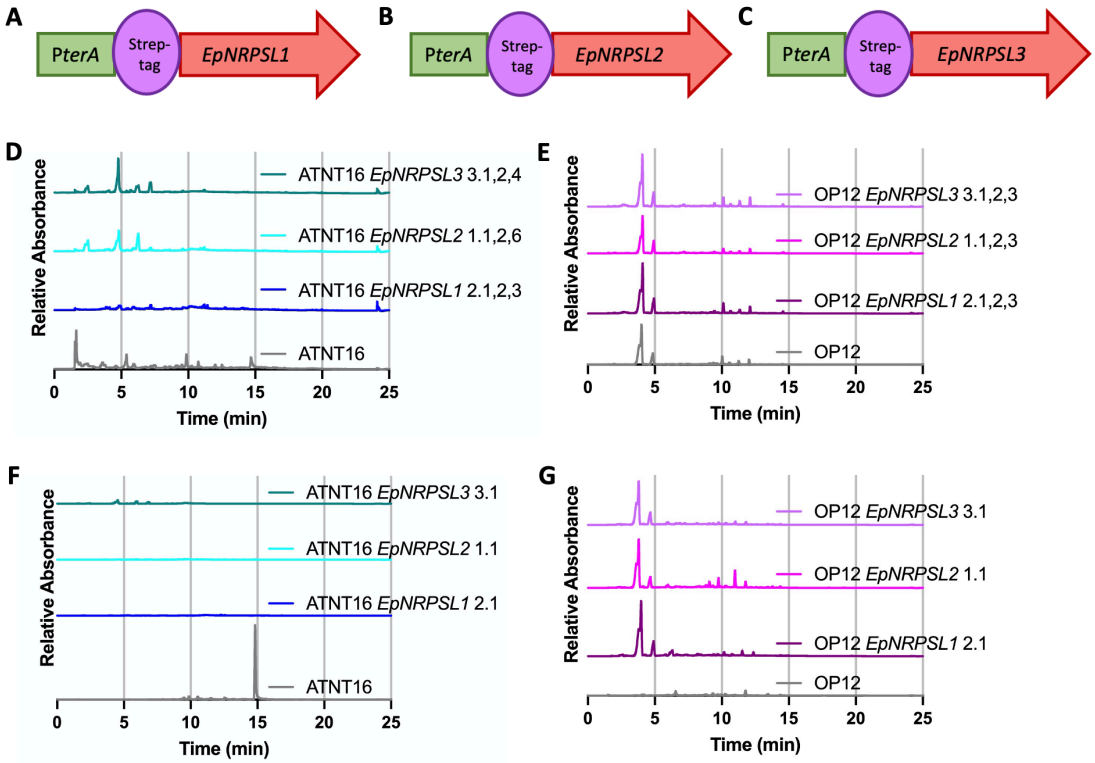


Figure 5.4. Metabolite production from *EpNRPSL1*, *EpNRPSL2* and *EpNRPSL3*. (A-C) Schematic representations of the expression constructs containing the NRPS-like genes *EpNRPSL1* **(A)**, *EpNRPSL2* **(B)** and *EpNRPSL3* **(C)**. **(D)** HPLC analysis of culture filtrates of ATNT16 Δ*pyrG* Δ*pabaA EpNRPSL1*_SM_S-tag_X_URA 2.1,2,3, ATNT16 Δ*pyrG* Δ*pabaA EpNRPSL2*_SM_S-tag_X_URA 1.1,2,6 and ATNT16 Δ*pyrG* Δ*pabaA EpNRPSL3*_SM_S-tag_X_URA 3.1,2,4 grown at 23 °C compared to ATNT16 control. **(E)** HPLC analysis of culture filtrates of OP12 *pyrG*$^-$ Δ*pabaA EpNRPSL1*_SM_S-tag_X_URA 2.1,2,3, OP12 *pyrG*$^-$ Δ*pabaA EpNRPSL2*_SM_S-tag_X_URA 1.1,2,3 and OP12 *pyrG*$^-$ Δ*pabaA EpNRPSL3*_SM_S-tag_X_URA 3.1,2,3 grown at 23 °C compared to OP12 control. **(F)** HPLC analysis of culture filtrates of ATNT16 Δ*pyrG* Δ*pabaA EpNRPSL1*_SM_S-tag_X_URA 2.1, ATNT16 Δ*pyrG* Δ*pabaA EpNRPSL2*_SM_S-tag_X_URA 1.1 and ATNT16 Δ*pyrG* Δ*pabaA EpNRPSL3*_SM_S-tag_X_URA 3.1 grown at 28 °C compared to ATNT16 control. **(G)** HPLC analysis of culture filtrates of OP12 *pyrG*$^-$ Δ*pabaA EpNRPSL1*_SM_S-tag_X_URA 2.1, OP12 *pyrG*$^-$ Δ*pabaA EpNRPSL2*_SM_S-tag_X_URA 1.1 and OP12 *pyrG*$^-$ Δ*pabaA EpNRPSL3*_SM_S-tag_X_URA 3.1 grown at 28 °C compared to OP12 control. No metabolites other than background metabolites were detected.

5.3.1.2 *Cladonia grayi Clagr3.11* and *Clagr3.30*

Two putative non-reducing NRPS-like genes, *Clagr3.11* and *Clagr3.30*, were identified in the genome of *C. grayi*. Expression constructs containing each of the NRPS-like genes with an *N*-terminal Strep-tag were created in order to determine the metabolites produced by each of the genes (Figure 5.5A and 5.5B). The two expression constructs were subsequently used in the transformation of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* and *A. oryzae* OP12 *pyrG*$^-$ Δ*pabaA* strains. Resulting positive transformants were identified by screening for full-length integration of the construct and were then

cultivated under inducing conditions; transformants were grown at 23 °C and 28 °C in parallel. Culture filtrates or mycelia were analysed by HPLC to determine if any metabolites were being produced.

Figure 5.5C and 5.5D show that both *A. niger* and *A. oryzae* strains expressing *Clagr3.11* or *Clagr3.30* produced metabolites at 23 °C, which is consistent with metabolite production from expression of the *Clagr3.6* and *Clagr3.21* PKS genes described in the previous chapter. *A. niger* strains expressing *Clagr3.30* revealed a metabolite peak at 12.1 min which corresponds to atrofuranic acid, and *A. oryzae* strains expressing *Clagr3.30* are producing a peak with a retention time of 9.6 min, which corresponds to atromentin. Therefore, Clagr3.30 is an atromentin synthetase. The results obtained from strains expressing *Clagr3.11* are particularly striking, however. This is due to the fact that *A. oryzae* transformants expressing *Clagr3.11* produced three peaks, indicating the production of three metabolites rather than one. Thereby, the peak at 9.6 min corresponds to atromentin, the peak at 11.6 min corresponds to ascocorynin, and the peak at 13.2 min corresponds to polyporic acid. This implies that the Clagr3.11 enzyme has a more flexible substrate specificity and seems to utilise different combinations of substrates in parallel. Similarly, extraction of the mycelium of *A. niger* transformants expressing *Clagr3.11* also revealed the production of three metabolites, with a peak at 12.1 min for atrofuranic acid, and peaks at 13.5 min and 14.0 min which correspond to the monohydroxylated and unhydroxylated derivatives, respectively. The retention times of the peaks obtained here for atromentin, ascocorynin and polyporic acid correspond with the retention times of peaks obtained for standards of atromentin, ascocorynin and polyporic acid, as shown in Figure 5.5. In addition, the retention times of the peaks obtained here for atrofuranic acid correspond with the retention time of atrofuranic acid obtained from an *A. niger* ATNT16 $melA_{(A)}$:$atrA_{At(T\text{-}TE)}$ strain as characterised in Geib *et al.* (2019) and shown in Figure 5.5. Furthermore, Figure 5.5E and 5.5F show that *A. niger* and *A. oryzae* strains expressing *Clagr3.11* are also able to produce metabolites when transformants were cultivated at 28 °C. However, no metabolite production is observed at 28 °C for *A. niger* or *A. oryzae* strains expressing *Clagr3.30*, which is again consistent with the thermosensitive metabolite production observed in the previous chapter for the PKSs *Clagr3.6* and *Clagr3.21*.

To further confirm the flexible substrate specificity of the Clagr3.11 enzyme, *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.11*_SM_S-tag_X_URA 2.4 was subsequently grown at 23 °C in liquid cultures supplemented with either 5 mM tyrosine or 5 mM phenylalanine. Mycelia extracts were analysed by HPLC to determine the metabolites produced. Figure 5.5G shows that all three metabolites are produced when fed with phenylalanine, whereby atromentin is less dominating. When supplemented with tyrosine, atromentin and ascocorynin are the major products. Therefore, it appears that supplementing the liquid culture with an aromatic amino acid changes the ratio of the metabolites produced, with tyrosine feeding pushing metabolite production towards the hydroxylated products, whereas phenylalanine feeding pushes metabolite production towards that of polyporic acid. This demonstrates that metabolite production can be directed towards a particular metabolite by increasing the pool of its associated α-keto acid.

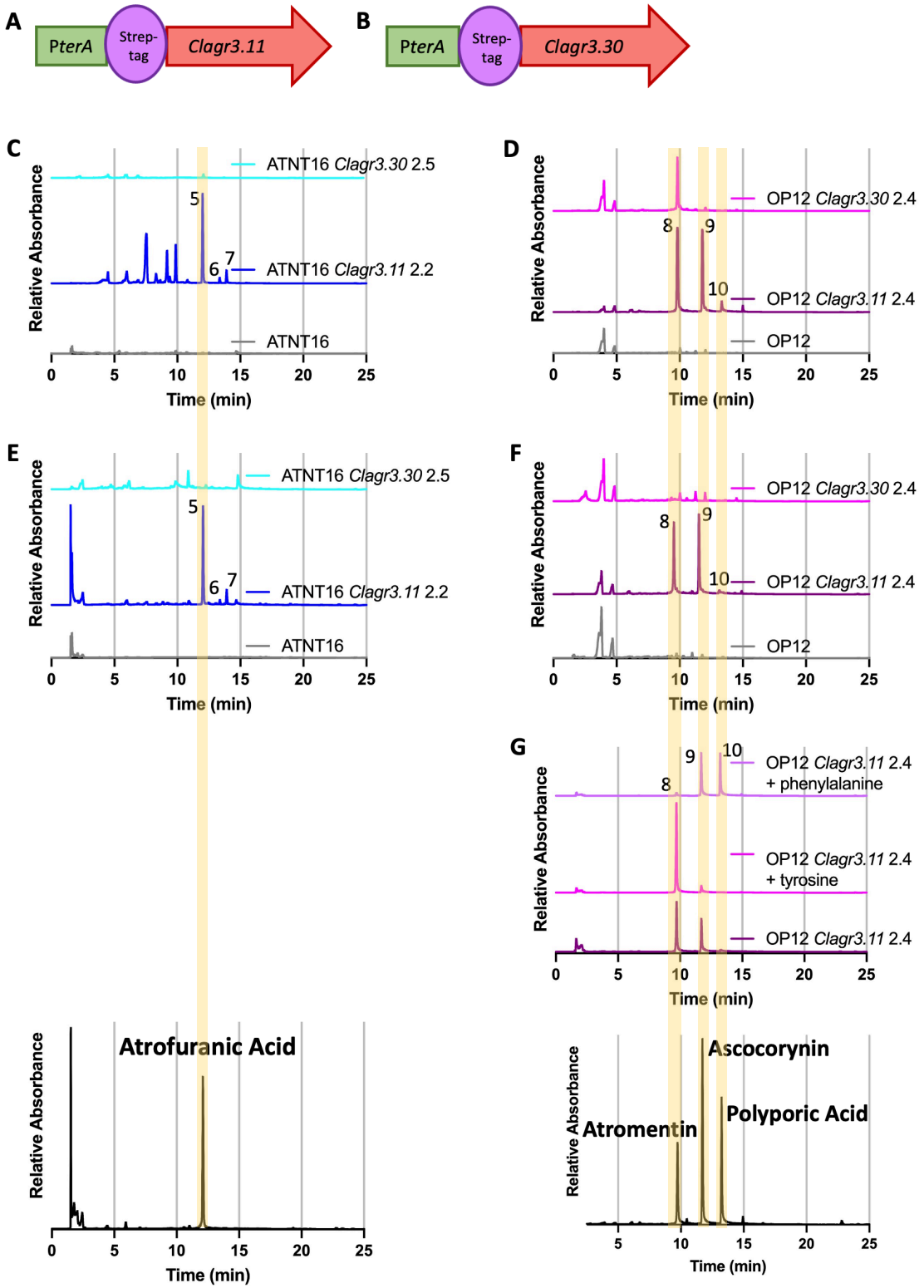


Figure 5.5. Metabolite production from *Clagr3.11* and *Clagr3.30*. 5 = atrofuranic acid, 6 = monohydroxylated compound, 7 = unhydroxylated compound, 8 = atromentin, 9 = ascocorynin, 10 = polyporic acid. **(A-B)** Schematic representations of the expression constructs containing the NRPS-like genes *Clagr3.11* **(A)** and *Clagr3.30* **(B)**. **(C)** HPLC analysis of culture filtrates of ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.11*_SM_S-tag_X_URA 2.2, showing production of atrofuranic acid and two additional metabolites, and ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.30*_SM_S-tag_X_URA 2.5,

showing production of atrofuranic acid, grown at 23 °C compared to ATNT16 control. **(D)** HPLC analysis of culture filtrates of OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.11*_SM_S-tag_X_URA 2.4, showing production of atromentin, ascocorynin and polyporic acid, and OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.30*_SM_S-tag_X_URA 2.4, showing production of atromentin, grown at 23 °C compared to OP12 control. **(E)** Same as **(C)**, but *A. niger* strains were grown at 28 °C. Production of atrofuranic acid and two additional metabolites is seen for the *Clagr3.11* strain, but no metabolite production is seen for the *Clagr3.30* strain. **(F)** Same as **(D)**, but *A. oryzae* strains were grown at 28 °C. Production of atromentin, ascocorynin and polyporic acid is seen for the *Clagr3.11* strain, but no metabolite production is seen for the *Clagr3.30* strain. **(G)** HPLC analysis of the mycelia of OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.11*_SM_S-tag_X_URA 2.4 supplemented with 5 mM tyrosine or 5 mM phenylalanine grown at 23 °C compared to OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.11*_SM_S-tag_X_URA 2.4 without supplementation. Production of atromentin, ascocorynin and polyporic acid is seen with phenylalanine supplementation, and production of atromentin and ascocorynin is seen with tyrosine supplementation. The retention time of the atrofuranic acid peaks obtained here were verified by comparison with the results of Geib *et al.* (2019), in which atrofuranic acid was characterised. The retention times of the peaks obtained here for atromentin, ascocorynin and polyporic acid correspond with the retention times of the respective standards.

5.3.2 Clagr3.11 *In Vitro* Assays

In order to confirm that the Clagr3.11 enzyme alone was responsible for the production of atromentin, ascocorynin and polyporic observed in the *in vivo* metabolite analysis after expression in *A. oryzae*, *in vitro* experiments were carried out in which the purified Clagr3.11 enzyme was mixed with various combinations of *p*-hydroxyphenylpyruvate and phenylpyruvate as substrate. HPLC analysis of the *in vitro* assays showed that atromentin is the exclusive product when 2.5 mM *p*-hydroxyphenylpyruvate was used as sole substrate (Figure 5.6A), and polyporic acid was the only product with 2.5 mM phenylpyruvate as sole substrate (Figure 5.6B). In the reaction containing an equal ratio of *p*-hydroxyphenylpyruvate and phenylpyruvate as substrate, atromentin and ascocorynin were produced (Figure 5.6C). This was also the case for the reaction containing a higher concentration of *p*-hydroxyphenylpyruvate compared to phenylpyruvate (Figure 5.6D). The reaction containing a higher concentration of phenylpyruvate compared to *p*-hydroxyphenylpyruvate as substrate resulted in the production of atromentin, ascocorynin and polyporic acid (Figure 5.6E). This, therefore, confirms that the Clagr3.11 enzyme has a relaxed substrate specificity, as it is able to accept both *p*-hydroxyphenylpyruvate and phenylpyruvate as substrate.

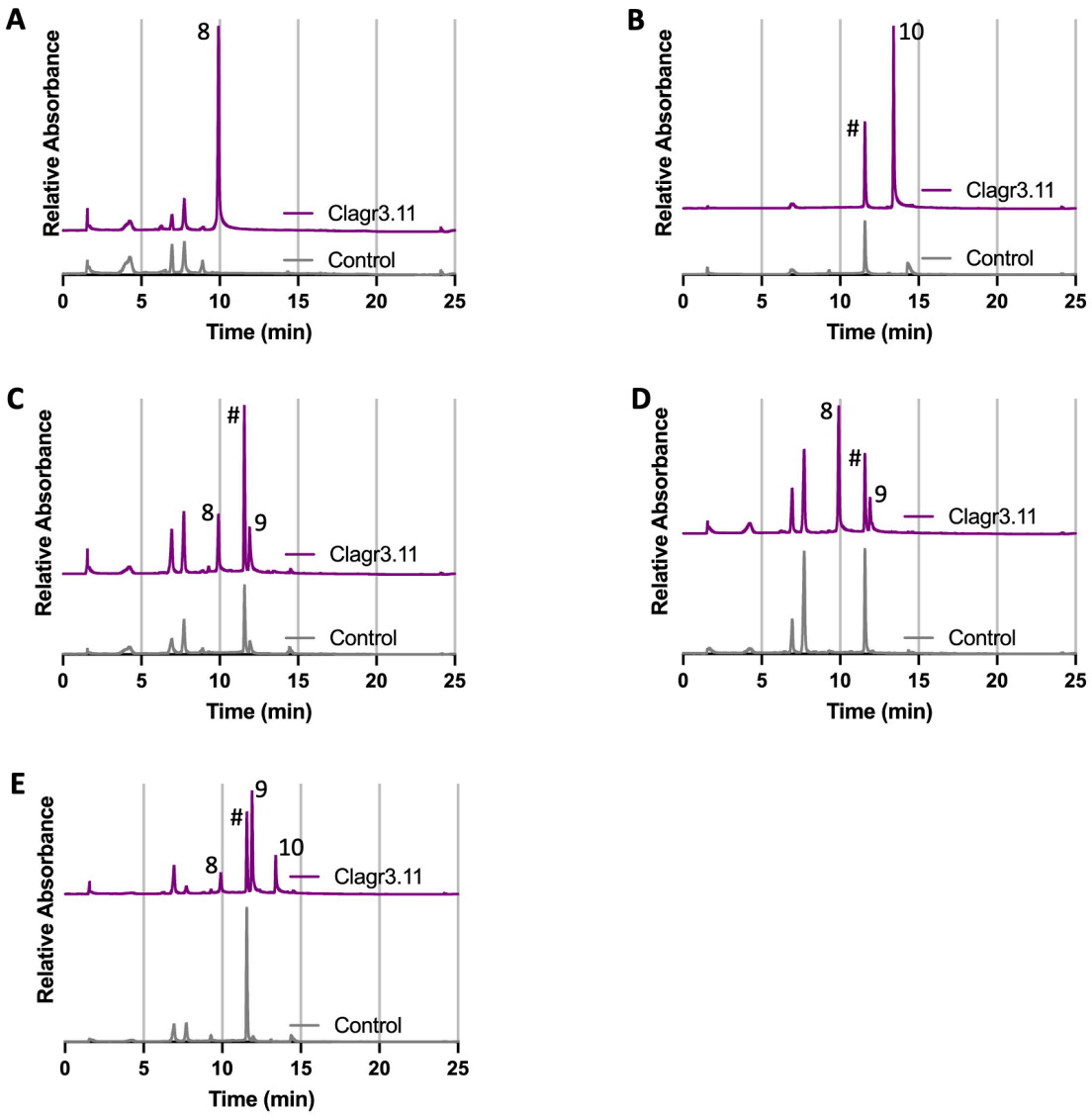


Figure 5.6. HPLC analysis of Clagr3.11 *in vitro* assays compared to control with either 2.5 mM *p*-hydroxyphenylpyruvate **(A)**, 2.5 mM phenylpyruvate **(B)**, 2.5 mM *p*-hydroxyphenylpyruvate and 2.5 mM phenylpyruvate **(C)** 2.5 mM *p*-hydroxyphenylpyruvate and 1 mM phenylpyruvate **(D)**, or 1 mM *p*-hydroxyphenylpyruvate and 2.5 mM phenylpyruvate **(E)** as substrate. 8 = atromentin, 9 = ascocorynin, 10 = polyporic acid, # = phenylpyruvate. Atromentin is present in the extract with *p*-hydroxyphenylpyruvate as the sole substrate, and polyporic acid is present in the extract with phenylpyruvate as the sole substrate. For the extracts with a combination of the two substrates, polyporic acid is only present in addition to atromentin and ascocorynin when the substrate ratio is skewed towards phenylpyruvate.

However, when focussing on the reactions in which a combination of *p*-hydroxyphenylpyruvate and phenylpyruvate were used as substrates, polyporic acid is only produced in the reaction in which the concentration of phenylpyruvate is dominating. This suggests that the Clagr3.11 enzyme favours *p*-

hydroxyphenylpyruvate, as polyporic acid is not produced when *p*-hydroxyphenylpyruvate and phenylpyruvate are in a 1:1 ratio.

5.3.3 AcyN *In Vitro* Assays

In order to compare the ascocorynin biosynthetic pathway of Clagr3.11 to that of another NRPS-like enzyme, *in vitro* assays using the AcyN enzyme from *Ascocoryne sarcoides* were carried out (Wieder *et al.*, 2022, in press). When the *acyN* gene is expressed in *A. oryzae*, the only product detected in HPLC analyses is polyporic acid. Therefore, it was of interest to investigate a potential production of ascocorynin and/or atromentin when purified AcyN is challenged with various mixtures of *p*-hydroxyphenylpyruvate and phenylpyruvate. Thus, purified AcyN enzyme was mixed with the same combinations of *p*-hydroxyphenylpyruvate and phenylpyruvate as those used in the Clagr3.11 *in vitro* assays. HPLC analysis of the *in vitro* assays showed that when *p*-hydroxyphenylpyruvate was used as the sole substrate, a trace amount of atromentin was detected in the supernatant extract (Figure 5.7A). However, polyporic acid was the sole product detected in the assays containing either phenylpyruvate alone or in combination with *p*-hydroxyphenylpyruvate (Figure 5.7B). No atromentin or ascocorynin was detected. The peaks for atromentin and polyporic acid correspond to the retention times for atromentin and polyporic acid standards, as shown in Figure 5.7. This confirms that AcyN is a polyporic acid synthetase, and that ascocorynin production in *A. sarcoides* requires an additional enzyme for monohydroxylation of polyporic acid. Thus, the biosynthetic pathway for ascocorynin in *A. sarcoides* differs to that of the one-enzyme biosynthetic pathway found in *C. grayi*. In addition, these results confirm the unprecedented substrate flexibility of the A domain of the Clagr3.11 enzyme.

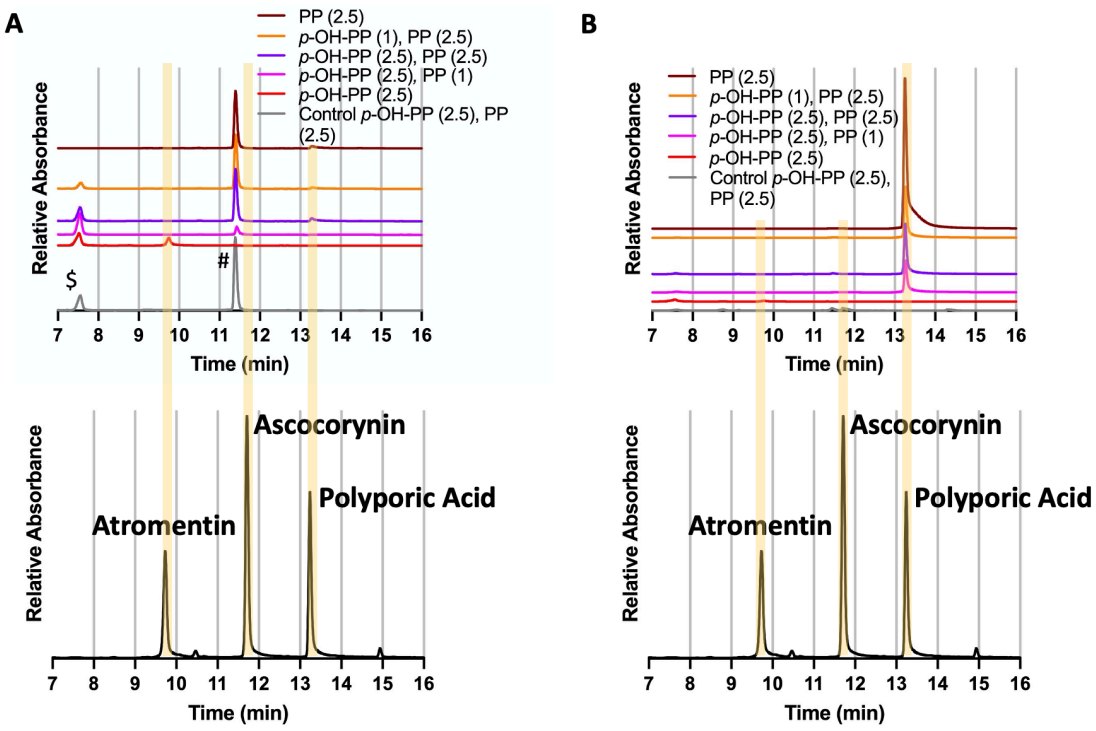


Figure 5.7. HPLC analysis of supernatant extracts **(A)** and pellet extracts **(B)** from AcyN *in vitro* assays. Numbers in brackets denote substrate concentration in [mM]. PP = phenylpyruvate, *p*-OH-PP = *p*-hydroxyphenylpyruvate, $ = *p*-hydroxyphenylpyruvate, # = phenylpyruvate. (**A**) In the supernatant extracts, trace amounts of atromentin are present when *p*-hydroxyphenylpyruvate is used as the sole substrate. In addition, small amounts of polyporic acid are present in the supernatant fraction when phenylpyruvate is used as the sole substrate or a combination of the two substrates is used. **(B)** In the pellet extracts, polyporic acid is present when phenylpyruvate is used as the sole substrate or combination with *p*-hydroxyphenylpyruvate. Ascocorynin is not detected in either of the samples. The retention times of the peaks obtained here for atromentin and polyporic acid correspond with the retention times of the respective standards.

5.3.4 Clagr3.11 Feeding Experiments

To determine if the Clagr3.11 enzyme can be used for the production of novel metabolites not found in nature, the *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.11*_SM_S-tag_X_URA 2.4 transformant strain was grown under inducing conditions at 23 °C in which liquid cultures were supplemented with 2.5 mM 4-chloro-phenylalanine in addition to either 1 mM tyrosine or 1 mM phenylalanine. Culture filtrates or mycelia were analysed by HPLC to determine if any chlorinated metabolites were produced. Figure 5.8A shows that when grown in a liquid culture supplemented with 4-chloro-phenylalanine and tyrosine, *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.11*_SM_S-tag_X_URA 2.4 is able to produce chlorinated ascocorynin (peak at 13.2 min) in addition to atromentin (peak at 9.6 min) and ascocorynin (peak at 11.6 min).

Furthermore, Figure 5.8B shows that *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.11*_SM_S-tag_X_URA 2.4 in a liquid culture supplemented with 4-chloro-phenylalanine and phenylalanine is able to produce chlorinated ascocorynin (peak at 13.2 min) and chlorinated polyporic acid (peak at 14.4 min), as well as atromentin (peak at 9.6 min), ascocorynin (peak at 11.6 min) and polyporic acid (peak at 13.2 min). Mass spectrometry confirmed that the metabolite detected at 13.2 min has an *m/z* of 341.0223 and the metabolite detected at 14.4 min has an *m/z* of 325.0273, thus corresponding with that of chlorinated ascocorynin and chlorinated polyporic acid, respectively. These results show that the Clagr3.11 enzyme is able to use 4-chloro-phenylpyruvate as a substrate in addition to *p*-hydroxyphenylpyruvate and phenylpyruvate. However, in these experiments no double-chlorinated metabolite was detected, which may be due to a lower affinity for 4-chloro-phenylpyruvate compared to the other substrates or an inability to form the quinone core when both side chains are chlorinated.

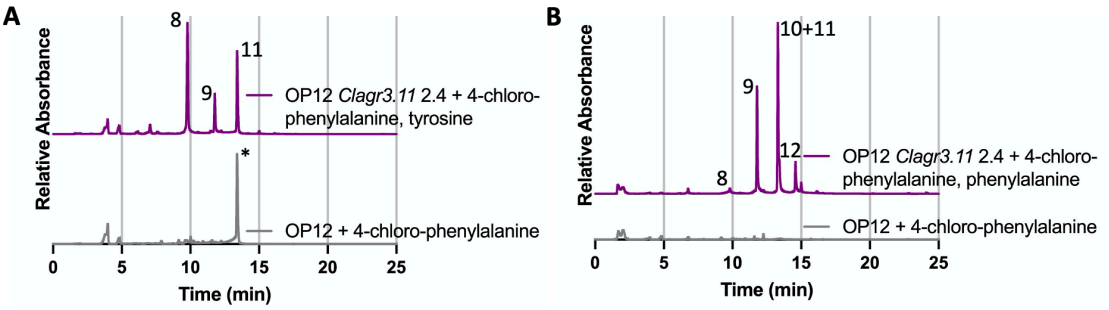


Figure 5.8. Metabolite production from *Clagr3.11* supplemented with 4-chloro-phenylalanine. 8 = atromentin, 9 = ascocorynin, 10 = polyporic acid, 11 = chlorinated ascocorynin, 12 = chlorinated polyporic acid, * = 4-chloro-phenylpyruvate. **(A)** HPLC analysis of culture filtrate of OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.11*_SM_S-tag_X_URA 2.4 supplemented with 2.5 mM 4-chloro-phenylalanine and 1 mM tyrosine grown at 23 °C compared to OP12 control, showing production of chlorinated ascocorynin. **(B)** HPLC analysis of the mycelium of OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.11*_SM_S-tag_X_URA 2.4 supplemented with 2.5 mM 4-chloro-phenylalanine and 1 mM phenylalanine grown at 23 °C compared to OP12 control, showing production of chlorinated ascocorynin and chlorinated polyporic acid.

5.3.5 Strains Expressing Hybrid Non-Ribosomal Peptide Synthetase-Like Enzymes

In order to determine which domain of Clagr3.11 was responsible for the relaxed substrate specificity of the enzyme, two expression constructs were created containing hybrid genes of *Clagr3.11* with *A. brasiliensis abrA* (Figure 5.9A and 5.9B).

One construct contained the A domain of *Clagr3.11* fused to the T and TE domains of *abrA* (Figure 5.9C), and the other construct contained the A and T domains of *Clagr3.11* with the TE domain of *abrA* (Figure 5.9D). For each fusion site, highly conserved residues were selected in the stretch between the A and T domains or the T and TE domains, respectively. Each of the hybrid NRPS-like genes possessed an *N*-terminal Strep-tag. These expression constructs were then used for the transformation of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* strains, and resulting positive transformants were identified by screening for full-length integration of the construct. Transformants were cultivated under inducing conditions at 23 °C and 28 °C in parallel, and culture filtrates were subsequently analysed by HPLC to determine the metabolites that were produced.

Figure 5.9E, 5.9F, 5.9G and 5.9H show that the metabolites produced by the original Clagr3.11 enzyme were also produced by the hybrid NRPS-like enzymes at both 23 °C and 28 °C. *A. oryzae* transformants expressing *Clagr3.11*:T-TE$_{abrA}$ or *Clagr3.11*:TE$_{abrA}$ produced a metabolite with a peak at 9.6 min for atromentin, a peak at 11.6 min for ascocorynin and a peak at 13.2 min for polyporic acid at 23 °C. At 28 °C, these strains were producing metabolites with peaks corresponding to atromentin and ascocorynin, but were not producing polyporic acid. *A. niger* strains expressing *Clagr3.11*:T-TE$_{abrA}$ or *Clagr3.11*:TE$_{abrA}$ were producing metabolites with a peak at 12.1 min for atrofuranic acid, and peaks at 13.5 min and 14.0 min for the monohydroxylated and unhydroxylated derivatives, respectively, at both 23 °C and 28 °C.

Due to the lack of polyporic acid production by *A. oryzae* strains at 28 °C, in addition to the very low amounts of polyporic acid produced at 23 °C, these strains were subsequently cultivated under inducing conditions in which liquid cultures were supplemented with 5 mM phenylalanine. Strains were grown at 23 °C and 28 °C in parallel, and the culture filtrates or mycelia were analysed by HPLC to determine the metabolites that were produced. This phenylalanine feeding was carried out to shift the substrate pool towards phenylpyruvate and direct metabolite production towards that of polyporic acid. Figure 5.9I and 5.9J confirm that the *A. oryzae* strain expressing *Clagr3.11*:T-TE$_{abrA}$ is able to produce polyporic acid in addition to atromentin and ascocorynin at both 23 °C and 28 °C. The *A. oryzae* strain expressing *Clagr3.11*:TE$_{abrA}$,

however, is able to produce polyporic acid at 23 °C but polyporic acid production is not observed at 28 °C.

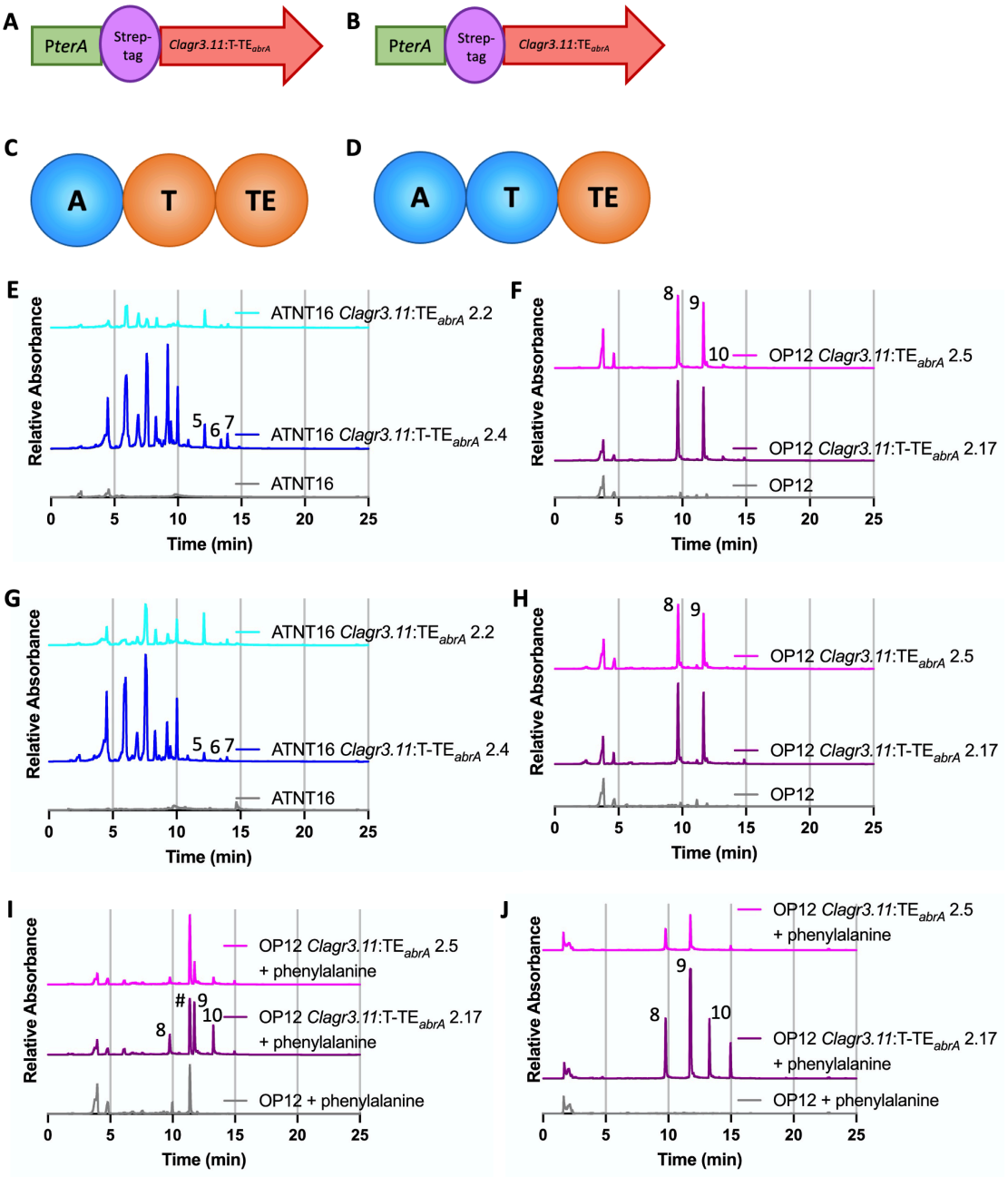


Figure 5.9. Metabolite production from *Clagr3.11*:T-TE$_{abrA}$ and *Clagr3.11*:TE$_{abrA}$. 5 = atrofuranic acid, 6 = monohydroxylated compound, 7 = unhydroxylated compound, 8 = atromentin, 9 = ascocorynin, 10 = polyporic acid, # = phenylpyruvate. **(A-D)** Schematic representations of the expression constructs containing the hybrid NRPS-like genes *Clagr3.11*:T-TE$_{abrA}$ **(A)**, which contains the *Clagr3.11* A domain fused to the *abrA* T and TE domains **(C)**, and *Clagr3.11*:TE$_{abrA}$ **(B)**, which contains the *Clagr3.11* A and T domains fused to the *abrA* TE domain **(D)**. **(E)** HPLC analysis of culture filtrates of ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.11*:T-TE$_{abrA}$_SM_S-tag_X_URA 2.4 and ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.11*:TE$_{abrA}$_SM_S-tag_X_URA 2.2 grown at 23 °C compared to ATNT16 control. Production of atrofuranic acid and two additional metabolites is observed. **(F)** HPLC analysis of culture filtrates of OP12 *pyrG*$^-$

Δ*pabaA Clagr3.11*:T-TE$_{abrA}$_SM_S-tag_X_URA 2.17 and OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.11*:TE$_{abrA}$_SM_S-tag_X_URA 2.5 grown at 23 °C compared to OP12 control. Production of atromentin, ascocorynin and polyporic acid is observed. **(G)** Same as **(E)**, but *A. niger* strains were grown at 28 °C. Production of atrofuranic acid and two additional metabolites is observed. **(H)** Same as **(F)**, but *A. oryzae* strains were grown at 28 °C. Production of atromentin and ascocorynin is observed. **(I)** HPLC analysis of culture filtrates of OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.11*:T-TE$_{abrA}$_SM_S-tag_X_URA 2.17 and OP12 *pyrG*$^{-}$ Δ*pabaA Clagr3.11*:TE$_{abrA}$_SM_S-tag_X_URA 2.5 supplemented with 5 mM phenylalanine grown at 23 °C compared to OP12 control. Production of atromentin, ascocorynin and polyporic acid is observed. **(J)** Same as **(I)**, but *A. oryzae* strains were grown at 28 °C. Production of atromentin, ascocorynin and polyporic acid is observed for the *Clagr3.11*:T-TE$_{abrA}$ strain, and production of atromentin and ascocorynin is observed for the *Clagr3.11*:TE$_{abrA}$ strain. Strains expressing the hybrid genes are able to produce the same metabolites as strains expressing *Clagr3.11*.

5.4 Discussion

Although there have been a number of attempts by other research groups to achieve the heterologous expression of lichen SM genes in non-lichen fungal hosts, these have all focussed on the expression of PKS genes and have largely been unsuccessful (Chooi *et al.*, 2008; Gagunashvili *et al.*, 2009; Armaleo *et al.*, 2011; Wang *et al.*, 2016; Bertrand and Sorensen, 2019a; Bertrand and Sorensen, 2019b; Kim *et al.*, 2021). The successful heterologous expression of the PKS genes *Clagr3.6* and *Clagr3.21* from *C. grayi* achieved in the previous chapter demonstrates that the *A. niger* ATNT16 and *A. oryzae* OP12 expression platforms can act as suitable heterologous hosts for the expression of lichen SM genes. However, due to the low success rate observed in the previous chapter in terms of achieving the heterologous expression of lichen PKS genes, the aim of this chapter was to attempt the heterologous expression of NRPS-like genes from various lichen species in the *A. niger* ATNT16 and *A. oryzae* OP12 expression platforms. To date, no successful attempts on the heterologous expression of lichen NRPS-like enzyme encoding genes in non-lichen fungal expression platforms have been described.

5.4.1 Expression of Lichen Non-Ribosomal Peptide Synthetase-Like Enzymes

The lack of success in achieving the functional expression of either *EpNRPSL1*, *EpNRPSL2* or *EpNRPSL3* from *E. prunastri* is reminiscent of the results obtained in the previous chapter for *E. prunastri* PKS genes. Thus, the results obtained here further support the suggestion made previously that the choice of expression platform is

crucial to achieve the functional heterologous expression of a lichen SM gene in a non-lichen fungal expression platform.

However, studies described in this chapter resulted in the successful heterologous expression of *Clagr3.11* and *Clagr3.30* in the *A. niger* ATNT16 and *A. oryzae* OP12 expression platforms. To our knowledge, these are the first examples of a successful heterologous expression of a lichen NRPS-like gene in an *Ascomycota* host. Results show that *A. oryzae* strains expressing *Clagr3.30* are able to produce the terphenylquinone atromentin, therefore meaning that Clagr3.30 is an atromentin synthetase. *A. niger* strains expressing *Clagr3.30* produced atrofuranic acid rather than atromentin, which is in agreement with a cross-chemistry in *A. niger* directing metabolite production towards atrofuranic acid rather than atromentin (Geib *et al.*, 2019). Similar to Clagr3.30, Clagr3.11 also appears to act as an atromentin synthetase, as *A. oryzae* strains expressing *Clagr3.11* were found to produce atromentin and *A. niger* strains expressing *Clagr3.11* were found to produce atrofuranic acid. However, the strains expressing *Clagr3.11* were found to produce three metabolites in total rather than just the one metabolite that was expected. *A. oryzae* strains produced ascocorynin and polyporic acid in addition to atromentin, and *A. niger* strains produced a monohydroxylated compound and an unhydroxylated compound (the equivalents of ascocorynin and polyporic acid) in addition to atrofuranic acid. Thus, the Clagr3.11 enzyme is the first NRPS-like enzyme to be described with a flexible substrate specificity. This result was unexpected, as all NRPS-like enzymes characterised so far only produce a single metabolite product (Schneider *et al.*, 2007; Yeh *et al.*, 2012; Geib *et al.*, 2016; Geib *et al.*, 2019; Wieder *et al.*, 2022, in press).

The flexible substrate specificity of Clagr3.11 enables the biosynthesis of its metabolites either through the condensation of two identical aromatic α-keto acids, or the condensation of two different α-keto acids. Thus, the enzyme is able to carry out the condensation of two *p*-hydroxyphenylpyruvate subunits to produce atromentin, two phenylpyruvate subunits to produce polyporic acid, or one *p*-hydroxyphenylpyruvate subunit and one phenylpyruvate subunit to produce ascocorynin. Therefore, the biosynthetic pathway for ascocorynin production in *C. grayi* differs to that in *A. sarcoides*. While the production of ascocorynin in *A. sarcoides* also suggested the presence of an NRPS-like enzyme that condenses two different α-

keto acids to produce ascocorynin (Quack *et al.*, 1982), our experiments confirmed that AcyN is highly specific in the use of a single type of substrate. AcyN is a true polyporic acid synthetase which is highly specific for phenylpyruvate as its substrate. A monooxygenase, MO6277, located outside of the ascocorynin biosynthesis gene cluster then carries out the mono-hydroxylation of polyporic acid to produce ascocorynin (Wieder *et al.*, 2022, in press). The studies on Clagr3.11 show that different pathways can result in ascocorynin production, but the benefits from and biological importance of terphenylquinone production in either *C. grayi* or *A. sarcoides* remains speculative. However, these metabolites may act as anti-feeding compounds as especially polyporic acid has been identified as a cause of mushroom poisoning from consumption of *Hapalopilus rutilans* by inhibition of the dihydroorotate dehydrogenase (Kraft *et al.*, 1998). In addition, it is unclear why *C. grayi* possesses two NRPS-like enzymes that are capable of producing atromentin: one highly specific atromentin synthetase and one enzyme with relaxed substrate specificity. It also raises questions on the evolution of these two enzymes in *C. grayi*.

The observed temperature dependency of the *C. grayi* NRPS-like enzymes is also consistent with the temperature sensitivity observed for *C. grayi* PKSs as described in the previous chapter. Again, this suggests that optimisation of cultivation conditions is required, and that a cold-adapted fungal expression platform may be more suitable for the heterologous expression of lichen-derived NRPS-like enzymes. The Clagr3.11 enzyme, however, differs in the sense that metabolite production was observed at 28 °C in addition to 23 °C, although it appeared that metabolite production was more pronounced at 23 °C compared to 28 °C. The ability of Clagr3.11 to achieve metabolite production at 28 °C, something not seen for Clagr3.30 or for the Clagr3.6 and Clagr3.21 PKSs studied in the previous chapter, suggests that this enzyme is unique. Not only does the enzyme display a relaxed substrate specificity, but it is also functional at a wider range of temperatures.

5.4.2 Substrate Specificity of Clagr3.11 Enzyme

The ability of Clagr3.11 to produce atromentin, ascocorynin and polyporic acid confirms that the enzyme possesses a flexible substrate specificity by which it accepts various substrates and produces metabolites from mixed substrates. Further to this,

we have also demonstrated that Clagr3.11 is capable of using non-natural substrates, such as 4-chloro-phenylpyruvate. This flexible substrate specificity is unprecedented, as all other NRPS-like enzymes studied so far are only able to accept one α-keto acid as substrate to form one product (Schneider *et al.*, 2007; Yeh *et al.*, 2012; Geib *et al.*, 2016; Geib *et al.*, 2019; Wieder *et al.*, 2022, in press). The full spectrum of non-natural substrates activated by the A domain of Clagr3.11 has not been studied yet, but it is likely that at least substrates with bromo- and fluoro-atoms in the *para* position of the phenyl-ring may also be used.

Due to the fact that the A domain is responsible for substrate selection in NRPS-like enzymes, we assumed that this domain would be responsible for the relaxed substrate specificity in Clagr3.11 (Hühner *et al.*, 2019). Hybrid genes containing domains from Clagr3.11 and AbrA produced atromentin, ascocorynin and polyporic acid. As AbrA as native enzyme solely produces atromentin when expressed in *A. oryzae* (Geib *et al*., 2019), this result confirms that the Clagr3.11 A domain is responsible for the flexible substrate specificity. While an exact quantification of metabolites is hampered by a different distribution in culture filtrates and mycelium, it appears that the fusion of Clagr3.11 with the more thermostable AbrA leads to increased metabolite production at 28 °C compared to that of Clagr3.11, with the *Clagr3.11*:T-TE$_{abrA}$ fusion appearing to be more thermostable than the *Clagr3.11*:TE$_{abrA}$ fusion. This could be due to the fact that the *Clagr3.11*:T-TE$_{abrA}$ hybrid NRPS-like enzyme contains two thermostable domains from AbrA compared to the *Clagr3.11*:TE$_{abrA}$ hybrid NRPS-like enzyme which only has one.

The flexible substrate specificity observed in Clagr3.11, in addition to confirmation that the enzyme is suitable to be used in domain swapping studies, provides the opportunity for this enzyme to be used in the production of novel metabolites not found in nature. This provides the scope for a wide range of natural products to be created using the A domain of the Clagr3.11 enzyme. These novel metabolites could have the potential to possess a diverse range of enhanced or novel biological activities, and could therefore have diverse pharmaceutical, and other, applications.

5.5 Conclusions

The results obtained in this chapter confirm that the *A. niger* ATNT16 and *A. oryzae* OP12 expression platforms are suitable for the functional expression of lichen NRPS-like genes, as demonstrated by the successful heterologous expression of both *Clagr3.11* and *Clagr3.30* from *C. grayi*. The functional expression of the *Clagr3.11* and *Clagr3.30* genes from *C. grayi* are the first examples of the successful heterologous expression of a lichen NRPS-like enzyme in an *Ascomycota* host. Furthermore, the results obtained for the *Clagr3.11* NRPS-like gene are particularly significant due to the fact the Clagr3.11 enzyme appears to have a relaxed substrate specificity. All NRPS-like enzymes that have been analysed so far are highly specific in terms of the substrate they accept, leading to the formation of only one product. However, the Clagr3.11 enzyme appears to be an exception to this rule, as it can accept more than one α-keto acid as substrate and can, therefore, produce more than one metabolite. Thus, the Clagr3.11 enzyme is the first example of an NRPS-like enzyme with a relaxed substrate specificity.

Future work following on from the results obtained in this study should focus on using the Clagr3.11 enzyme to create novel natural products not found in nature. This could be attempted either by using non-natural substrates or through domain swapping experiments with other NRPS-like enzymes. These novel metabolites can then be assessed for any biological activities and the potential applications of these. Future work should also focus on the identification of the two metabolites produced by *A. niger* Clagr3.11 strains in addition to atrofuranic acid. Preliminary analyses indicate that atrofuranic acid acts as an inhibitor of the *Mycobacterium tuberculosis* InhA protein that is a clinically validated target (Manjunatha *et al*., 2015). Thus, the opportunity to produce a range of atrofuranic acid derivatives by exploitation of the Clagr3.11 A domain may result in the production of novel drugs with increased anti-tuberculosis activity.

Chapter 6: Heat Sensitivity of Lichens

6.1 Introduction

6.1.1 Stress Response in Lichens

Lichens differ from many other eukaryotic organisms in their ability to tolerate extreme environmental conditions; this capability to survive stresses that would lead to the death of other less specialised organisms means that lichens can be described as extremophiles. Environmental stresses include extremes of temperature, high light intensities, low nutrient availability and desiccation. Other abiotic stresses experienced by lichens include air pollutants (such as sulfur dioxide), as well as water oversaturation or submergence. The distribution of lichens in an environment is dependent on the suitability of a given habitat for the lichen; the level of stress is a result of the interaction between substrate and environment (MacFarlane and Kershaw, 1980; Beckett *et al.*, 2008; Grube, 2010). In addition to abiotic stresses, lichens can also experience biotic stresses. These include pathogens, insect predation and other competing lichens or higher plants (Beckett *et al.*, 2008; Grube, 2010). Each of the different types of stresses experienced by lichens are believed to cause specific types of damage to the lichen. For example, desiccation can cause damage to the lichen cytoskeleton, as well as causing leaky membranes and changing protein structure. Temperature stress is thought to result in a different type of membrane damage when the lichen is exposed to colder temperatures, whereas exposure of the lichen to heat leads to the denaturation of proteins. However, even though different stresses experienced by the lichen results in various types of damage, most stresses are believed to result in ROS formation (Beckett *et al.*, 2008).

The particular environmental stress to which lichens are exposed will vary according to the specific species of lichen, with different species having contrasting ecologies and niches and therefore exposure to different stressors. Nevertheless, a few general observations can be made. The tolerance of lichens to biotic and abiotic stresses is dependent on adaptive mechanisms, although these are not yet fully understood (Beckett *et al.*, 2008; Grube 2010). These mechanisms are either processes which protect the lichen from stress or are repair processes which the lichen

uses in response to damage caused by stress. This protection and repair in lichens is linked with the ability of the lichen to offset the increased production of ROS, such as superoxide, hydroxyl radicals, hydrogen peroxide and singlet oxygen (Kranner *et al.*, 2008; Grube, 2010; Weissman *et al.*, 2005). Adaptations to stress can include the accumulation of pigments in the cell wall or non-chromogenic SMs located in the cytoplasm which act as UV screens, as well as the presence of metabolites which inactivate ROS (Avalos and Carmen Limón, 2015; Braga *et al.*, 2015; Cordero and Casadevall, 2017; Cortesão *et al.*, 2020). Further to this, in response to any DNA damage, various mechanisms including homologous recombination, non-homologous end-joining, nucleotide excision repair, mismatch repair and photoreactivation are utilised by fungi in order to repair any damage induced by UV radiation, and are most likely to be utilised by lichen mycobionts (Sinha and Häder, 2002; Goldman and Kafer, 2004).

The main mechanism to prevent ROS production is that of non-photochemical quenching, in which excess energy absorbed by photosystem II is dissipated as heat (Kranner *et al.*, 2008; Gasulla *et al.*, 2021). ROS formation can also be prevented in lichens through the inactivation of photosynthesis, with the decoupling of photosystems I and II occurring at the onset of desiccation and their recoupling once the lichen thallus becomes rehydrated (Bilger *et al.*, 1989). ROS scavenging in lichens can take place either through enzymatic or non-enzymatic antioxidants. Antioxidant enzymes include peroxidases, catalases and superoxide dismutase, as well as auxiliary enzymes such as mono- and dehydroascorbate reductases and glutathione reductase. Non-enzymatic antioxidants, which are of low-molecular weight, include ascorbate, glutathione and tocopherols (Kranner *et al.*, 2008; Beckett *et al.*, 2008; Gasulla *et al.*, 2021). It is generally accepted that ROS scavenging involves a combination of enzymatic and non-enzymatic antioxidants, as was suggested by Foyer and Halliwell (1976) for plants, although it is not currently known whether lichens do indeed produce ascorbate (Kranner *et al.*, 2005; Beckett *et al.*, 2008).

In addition to preventing ROS formation and ROS scavenging, lichens are also thought to produce sugars and dehydrins, late embryogenesis abundant-like proteins, in order to tolerate desiccation. Sugars such as sucrose and trehalose are believed to lead to the cytoplasm forming a ‘glass phase’ as a result of their ability to form

hydrogen bonds; this process is known as vitrification, and enables the cytoplasm to possess the properties of a liquid whilst having the viscosity of a solid. The glass phase prevents cells from collapsing as a result of desiccation by filling space, as well as causing potentially harmful chemical reactions to take place at a much slower pace due to the high viscosity severely reducing molecular mobility (Beckett *et al.*, 2008; Kranner *et al.*, 2008; Gasulla *et al.*, 2021). Further to this, the non-reducing sugars produced by lichens are also thought to stabilise proteins and membranes during desiccation by forming hydrogen bonds that substitute for water (Crowe *et al.*, 1984). Dehydrins are believed to provide stability to both macromolecular and cellular structures in the cytoplasm when desiccated by forming a molecular network that can prevent cytoplasmic proteins from denaturing or cellular membranes from fusing (Close, 1997; Beckett *et al.*, 2008). Furthermore, abiotic stresses have also been found to lead to the formation of osmolytes (Honegger *et al.*, 1993). These osmolytes, which consist of the polyols normally found in lichens, prevent the shrinkage of both fungal and algal protoplasts which would otherwise occur in the event of desiccation. Polyols can also play a role in the stabilisation of macromolecules during desiccation, as their hydroxyl groups can be used as substitutes for the hydrogen bonds found in water (Grube, 2010; Gasulla *et al.*, 2021).

6.1.2 Proteostasis

Protein homeostasis, also known as proteostasis, is a cell-autonomous process which ensures that the correct folding of proteins can be maintained when an organism experiences various stresses (Weindling and Bar-Nun, 2015). These stresses, which protein folding can be especially sensitive to, can either be endogenous or exogenous (Rutkowski and Kaufman, 2004). Protein folding can be facilitated through a range of mechanisms, including the prevention of protein synthesis, the induction of molecular chaperones in order to assist with correct protein folding, and the upregulation of the degradation of terminally misfolded proteins (Ellgaard *et al.*, 1999; Rutkowski and Kaufman, 2004; Weindling and Bar-Nun, 2015). Thus, these mechanisms work together to minimise the accumulation and aggregation of incorrectly-folded proteins (Bertolotti *et al.*, 2000). It is believed that the stress response pathways in fungi which activate proteostasis are coordinated in a network

and are compartment-specific, with the heat shock response (HSR) taking place in the nucleus and cytosol, and the unfolded protein response (UPR) taking place either in the endoplasmic reticulum (ER) or in mitochondria (Liu and Chang, 2008; Weindling and Bar-Nun, 2015).

The HSR, which primarily responds to stress conditions in the cytosol, can be activated by a variety of stresses in addition to heat stress, including oxidative stress, osmotic stress, ethanol exposure and glucose starvation. In *Saccharomyces cerevisiae*, HSR is thought to be regulated by a single transcription factor, heat shock factor 1 (Hsf1), which binds to the promoter region of its target genes as a homo-trimer; these promoter elements, known as heat shock elements (HSEs), are functionally conserved and are found in a wide range of target genes. HSEs are made up of a variable number of contiguous copies of the repeating sequence nGAAn arranged in alternating orientation (Sorger *et al.*, 1987; Sorger, 1991; Hahn *et al.*, 2004; Eastmond and Nelson, 2006; Liu and Chang, 2008; Weindling and Bar-Nun, 2015). Heat shock proteins (HSPs), which are induced by HSR, are a superfamily of conserved proteins which are implicated in maintaining the functionality and structure of cellular macromolecules. They are classified depending on their molecular weight and mode of action, being described as either large HSPs or small HSPs. Large HSPs, which are ATP-dependent, assist in the correct folding of nascent proteins and the refolding of denatured proteins, whereas ATP-independent small HSPs prevent the aggregation of misfolded proteins by binding with their hydrophobic surface (Zapun *et al.*, 1999; Hahn *et al.*, 2004; Eastmond and Nelson, 2006; Garrido *et al.*, 2012; Zuo *et al.*, 2016; Gasulla *et al.*, 2021). In addition to HSPs, Hsf1 is also believed to regulate a wide range of other genes, including those involved in protein degradation, carbohydrate metabolism, energy generation and cell wall integrity maintenance (Yamamoto *et al.*, 2005).

The function of the UPR is to check newly-synthesised proteins in the secretory pathway to ensure their correct glycosylation, folding and maturation; the use of chaperones and foldases to assist with protein folding, in addition to the use of quality control mechanisms, ensures that only correctly-folded proteins are released (Gething and Sambrook, 1992; Ellgaard *et al.*, 1999; Zapun *et al.*, 1999; Weindling and Bar-Nun, 2015). There are various stresses which can lead to the disruption of protein folding, and the UPR of the ER is triggered when the number of unfolded proteins found within

the ER increase to an extent that the folding capacity of the ER is exceeded, and there is an increased need for chaperones and foldases (Mulder *et al.*, 2004; Rutkowski and Kaufman, 2004; Davé *et al.*, 2006; Ron and Walter, 2007). In *S. cerevisiae*, the species in which the UPR was first studied, the ER membrane protein Ire1 has an endoribonucleolytic activity which causes the non-conventional splicing of *HAC1* mRNA within the cytoplasm. Hac1 is a basic leucine zipper (bZIP)-type transcription factor which is constitutively expressed but is unable to be translated as a result of a 252-nucleotide unconventional intron which forms a 3' hairpin; this hairpin is removed in a non-spliceosomal manner, in which Ire1 cleaves the intron borders of *HAC1* mRNA and the tRNA ligase Rlg1 rejoins the exons. The Ire1 protein is able to sense the accumulation of unfolded proteins through its interaction with the chaperone BiP (Sidrauski *et al.*, 1996; Kawahara *et al.*, 1997; Sidrauski and Walter, 1997; Gonzalez *et al.*, 1999; Bertolotti *et al.*, 2000; Rüegsegger *et al.*, 2001; Rutkowski and Kaufman, 2004; Mulder *et al.*, 2004; Davé *et al.*, 2006; Mulder and Nikolaev, 2009; Weindling and Bar-Nun, 2015). Upon translation of Hac1, the transcription factor moves to the nucleus and subsequently binds to UPR elements in their promoter region. UPR elements include ER chaperones, as well as parts of the ER-associated degradation machinery (Cox and Walter, 1996; Weindling and Bar-Nun, 2015). A similar pathway has also been found in filamentous fungi, whereby the HacA (or Hac1) transcription factor was identified in *A. niger*, *A. nidulans*, *Aspergillus fumigatus* and *Trichoderma reesei*; these *hacA/hac1* genes are the functional homologues of the *HAC1* gene in *S. cerevisiae*. These *hac* genes possess a 20-nucleotide unconventional intron (Figure 6.1) which is also spliced from the *hac* mRNA in order to induce UPR. Mulder *et al.* (2004) confirmed that the sequences at the intron borders that had been found to be conserved in yeast *HAC1* and mammalian *XBP1* are also conserved in *A. niger hacA*. This suggests that *A. niger hacA* is also spliced by an Ire1-like protein. However, the *hac* transcripts in these filamentous fungi are also found to be truncated (Figure 6.1), with the 5' end being shorter, which leads to increased transcript levels (Saloheimo *et al.*, 2003; Mulder *et al.*, 2004; Davé *et al.*, 2006).

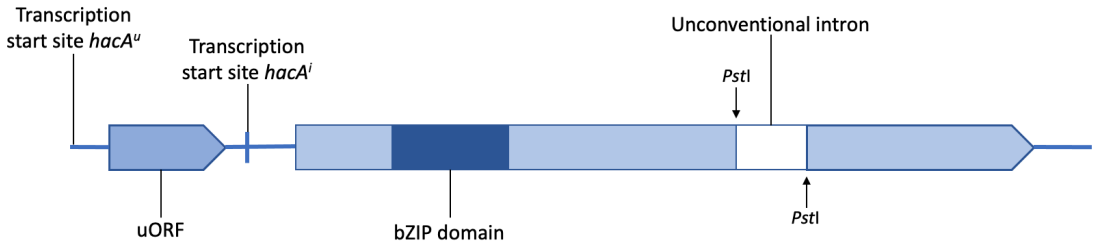


Figure 6.1. Main features of *A. niger hacA* mRNA. HacA is a bZIP-type transcription factor, meaning that it contains a leucine-zipper dimerisation motif downstream of the DNA-binding domain. The unspliced mRNA ($hacA^u$) encodes a protein of 436 amino acids, whereas the spliced mRNA ($hacA^i$) encodes a protein of 342 amino acids. This is due to the fact that splicing causes a 222-amino acid sequence in the *C*-terminal region to be replaced with a different 128-amino acid coding sequence. The region of *hacA* removed during truncation of the 5' end includes an open reading frame encoding a 44-amino acid peptide. Adapted from Mulder *et al.* (2004).

Although the UPR and HSR take place in different cellular compartments, they are in fact believed to be directly linked, with UPR being found to regulate HSR (Weindling and Bar-Nun, 2015). In addition to this, Weindling and Bar-Nun (2015) also found that intact UPR is required for HSR, and that this involves the sirtuin Sir2. This is due to the fact that UPR upregulates Sir2 and, not only is Sir2 required for the activation of Hsf1 by HSR, but is also needed for the activation of Hsf1 by UPR. Further to this, Sir2 is able to attenuate the UPR pathway, thus preventing any deleterious consequences of prolonged UPR by ensuring that UPR only occurs transiently.

6.1.3 Previous Approaches of Heterologous Expression of a Lichen *pyrG*

As discussed in Section 4.1.4, Sinnemann *et al.* (2000) achieved the functional expression of the *pyrG* gene from the lichen *S. crocea* in the heterologous host *A. nidulans*. The *pyrG* gene, encoding an orotidine 5'-monophosphate decarboxylase, is a gene involved in the primary metabolism of fungi as it is involved in pyrimidine biosynthesis; the enzyme is responsible for catalysing the decarboxylation of orotidine monophosphate to uridine monophosphate (Traut and Temple, 2000). Fungal strains lacking the gene encoding an orotidine 5'-monophosphate decarboxylase are auxotrophic for uridine or uracil (Weidner *et al.*, 1998). Uridine, or more specifically uridine monophosphate, is required for the biosynthesis of pyrimidine nucleotides. This means that the enzyme is essential and fungal strains lacking an orotidine 5'-monophosphate decarboxylase require uridine supplementation in order to be able

to survive. However, this auxotrophy can be complemented through the heterologous expression of a *pyrG* gene (Weidner *et al.*, 1998; Traut and Temple, 2000; Sinnemann *et al.*, 2000).

In the study conducted by Sinnemann *et al.* (2000), the *S. crocea pyrG* was found to complement the uridine auxotrophy of the *A. nidulans* heterologous host, both with the *pyrG* gene under the control of its native promoter or under the control of the *A. nidulans trpC* promoter. However, they observed a temperature effect on the strains expressing *S. crocea pyrG*, as growth of the transformants without uridine supplementation was only observed at 24 °C and not at higher assay temperatures. Further to this, the growth of the transformants was slower than that of the controls. In contrast, supplementation with uridine resulted in the strains expressing *S. crocea pyrG* growing equally as well as the host at either 24 °C, 33 °C or 37 °C. Sinnemann *et al.* (2000) went on to suggest that the observed temperature effect could be assigned to the PyrG protein rather than to the *A. nidulans* heterologous host, as *A. nidulans* is able to grow normally at all three incubation temperatures used in this study. Furthermore, the fact that the same growth behaviour was observed in strains containing the native *S. crocea pyrG* promoter and the *A. nidulans trpC* promoter implies that the temperature effect on growth was not due to the regulation of the gene. It was therefore suggested that the proposed temperature sensitivity of the PyrG enzyme was a result of an adaptation of *S. crocea* to the lower temperatures found in its natural environment. However, to date, this hypothesis has not been confirmed through studies of additional primary metabolism genes from *S. crocea* or other lichen species.

6.1.4 Chapter Objectives

Due to the apparent heat sensitivity of lichen proteins observed in previous chapters, the main aims of this chapter are to investigate the heat sensitivity of lichens and to achieve the co-expression of lichen secondary metabolism genes with a native transcription factor in the *A. niger* ATNT16 expression platform. These will be attempted as described below:

(1) Investigation of the heat sensitivity of lichen sexual propagules. Ascospores from various lichen species will be exposed to both wet and dry heat treatments at a range of temperatures in order to determine their heat sensitivity.

(2) Investigation of the heat sensitivity of lichen primary metabolism proteins. The *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1* expression platforms will be used for the heterologous expression of selected primary metabolism genes identified from various lichen species in order to complement one of the auxotrophies of the respective *A. niger* or *A. oryzae* strains. The heat sensitivity of these primary metabolism proteins will then be assessed.

(3) Co-expression of lichen secondary metabolism genes with native transcription factor. *A. niger* ATNT16 strains expressing selected lichen genes encoding PKSs or NRPS-like enzymes will be co-expressed with the *hacA* transcription factor identified from *A. niger*. HPLC analysis will be carried out to determine the effect of the overexpression of the native *hacA* on the heterologous production of lichen SMs.

6.2 Materials and Methods

6.2.1 Species and Strains

Samples of *Xanthoria parietina*, *Lecanora chlarotera* and *Amandinea punctata* thalli used in heat shock experiments were collected from various locations in the UK, with collection details given in Appendix 2. All lichen samples were transported back to the laboratory in polythene bags in their naturally hydrated state. The lichen samples were then trimmed so only areas of thallus with apothecia remained and any other debris were removed. The samples were subsequently air-dried at room temperature for 24 h before being sealed in fresh polythene bags and stored at -20 °C before further analysis. Ascospore suspensions of *A. nidulans* 2-3 and *A. nidulans* 2-137 were provided by Wenyue Du (University of Nottingham, UK).

The expression strains used in this study for the expression of lichen primary metabolism genes were either *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* or *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1*. As stated in Section 4.2.1, a sample of the thallus of *E. prunastri* was collected from Nottingham, UK; genomic DNA was subsequently

extracted from the whole lichen thallus as described in Section 2.2.4. Likewise, a sample of the thallus of *X. parietina* was collected from Nottingham, UK and genomic DNA was extracted from the whole lichen thallus as described in Section 2.2.4. Genomic DNA obtained from *C. grayi* was kindly provided by Daniele Armaleo (Duke University, North Carolina, USA).

For the co-expression of lichen SM genes with the native *A. niger hacA*, *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.6*_SM-X_URA, *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.26*_SM-X_URA and *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.30*_SM_S-tag_X_URA strains created in the previous chapters were used. Genomic DNA from *A. niger* was obtained from an A1144 Δ*pyrG* strain.

6.2.2 Preparation of Conidial Suspensions

Conidial suspensions of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1*, *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1*, *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.6*_SM-X_URA, *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.26*_SM-X_URA and *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.30*_SM_S-tag_X_URA were obtained by growing each strain on slopes in Universal tubes containing solid GG10 medium and any necessary supplementations. After incubation at 28 °C, conidial suspensions of the strains were generated by scraping conidia into 6 ml PBS using a sterile cotton swab.

6.2.3 Isolation of Lichen Ascospores

Thalli of lichen species stored at -20 °C were washed in 100% ethanol for ~10 s to reduce surface contamination before being placed under a constant flow of water for 1 h, in order to rehydrate the thallus. The thalli were then rinsed in SDW before being blotted dry with tissue. The rehydrated lichen thallus was subsequently attached to the lid of an empty sterile Petri dish with petroleum jelly via the underside of the thallus. Plates were incubated at 18 °C in the dark for 24 h with the thallus facing downwards to allow ascospores to discharge into the empty Petri dish. Ascospores were collected from the Petri dish using 1 ml SDW + 0.1% Tween 80 and the resulting suspension was sonicated without heating for 1 min in an ultrasonic bath (USR 30 H; 160 W, 35 kHz) (Merck, Darmstadt, Germany) in order to break up any clumps of

ascospores. The concentration of the spore suspension was then determined using an Improved Neubauer chamber.

6.2.4 Heat Treatment of Ascospores

For the wet heat shock of *X. parietina*, *L. chlarotera*, *A. punctata* and *A. nidulans* ascospores, 200 μl aliquots of a 5000 ascospores/ml suspension in 1.5 ml Eppendorf tubes were centrifuged at 11,000 rpm for 5 min to pellet the ascospores. The supernatant was removed, the pellet was resuspended in 200 μl SDW, and the 200 μl aliquot was transferred to a 0.2 ml PCR tube before undergoing a 30 min heat treatment using a thermal cycler. Subsequently, 50 μl aliquots were spread onto 9 cm Petri dishes containing solid MEYE for lichen ascospores or solid ACM for *A. nidulans* ascospores, supplemented with ampicillin and streptomycin (final concentration 100 μg/ml for each), in technical triplicate. Plates were sealed with Parafilm and incubated at 18 °C in the dark for approximately two months for lichen species, and at 28 °C for 72 h for *A. nidulans* strains, until colonies could be counted. Plates containing lichen species were screened weekly for contamination and contaminants were cut out of the agar where possible under sterile conditions. The experiment was carried out in biological triplicate for each species.

For the dry heat shock of *X. parietina*, *L. chlarotera*, *A. punctata* and *A. nidulans* ascospores, 200 μl aliquots of a 5000 ascospores/ml suspension in 1.5 ml Eppendorf tubes were centrifuged at 11,000 rpm for 5 min to pellet the ascospores. The supernatant was removed, and the pellet was dried for 10 min in a sterile laminar flow hood before undergoing a 30 min heat treatment in a heat block. The pellet was then resuspended in 200 μl SDW and 50 μl aliquots were subsequently spread onto 9 cm Petri dishes containing solid MEYE for lichen ascospores or solid ACM for *A. nidulans* ascospores, supplemented with ampicillin and streptomycin (final concentration 100 μg/ml for each), in technical triplicate. Plates were then sealed with Parafilm and incubated at 18 °C in the dark for approximately two months for lichen species, and at 28 °C for 72 h for *A. nidulans* strains, until colonies could be counted. Plates containing lichen species were screened weekly for contamination and contaminants

were cut out of the agar where possible under sterile conditions. The experiment was carried out in biological triplicate for each species.

Before being converted to percentages, the original data for the germination of *X. parietina*, *L. chlarotera*, *A. punctata* and *A. nidulans* ascospores for each type of heat treatment were analysed using GraphPad Prism version 9.2.0 (GraphPad Software, San Diego, California, USA) by carrying out a one-way ANOVA with $\alpha = 0.05$.

6.2.5 Generation of Strains Expressing Lichen Primary Metabolism Genes

Sequences of all oligonucleotides used in this study are listed in Appendix 1. Plasmid maps of the principle plasmids used in this study are shown in Appendix 4.

6.2.5.1 *Evernia prunastri pyrG* and *his1*

Expression constructs were created for the expression of two putative primary metabolism genes, *pyrG* and *his1*, from *E. prunastri*. These expression constructs were created using the pJET1.2 plasmid (Thermo Fisher Scientific, UK), with each plasmid also containing a selection marker. For the plasmid containing *Ep-pyrG* the selection marker was the *his1* gene, and for the plasmid containing *Ep-his1* the selection marker was *pyrG*. The *Ep-pyrG* gene (including promoter and terminator regions) was amplified from *E. prunastri* gDNA using oligonucleotides 79 and 80, and the *Ep-his1* gene was amplified from *E. prunastri* gDNA using oligonucleotides 81 and 82. For the selection markers, *his1* was excised from the SM-X_His plasmid by carrying out a restriction digest with *NotI*, and *pyrG* was excised from the SM-X_URA plasmid by carrying out a restriction digest with *NotI* which excised the *pyrG*-containing URA Blaster.

Each construct was created by mixing the linearised pJET1.2 plasmid with the gel-purified PCR fragment of the gene of interest using the CloneJET PCR Cloning kit (Thermo Fisher Scientific, UK). The assembled plasmids were then amplified in *E. coli* DH5α cells. Positive clones were selected by colony PCR, using oligonucleotides 83 and 84 for *Ep-pyrG*_pJET1.2 and oligonucleotides 85 and 84 for *Ep-his1*_pJET1.2. The plasmids were purified, and each plasmid was checked for correct assembly using restriction analyses. The resulting isolated plasmids were then linearised using the *NotI* restriction enzyme. The *NotI*-restricted plasmids were mixed with the gel-purified

PCR fragment of the appropriate selection marker and assembled by restriction ligation using the Rapid DNA Ligation kit (Roche, Sigma-Aldrich, UK). The assembled plasmids were amplified and purified as before, and resulting positive clones were selected by colony PCR using oligonucleotides 86 and 87 for *Ep-pyrG_his1*_pJET1.2 and oligonucleotides 88 and 89 for *Ep-his1*_URA_pJET1.2. Again, each plasmid was checked for correct assembly by restriction analyses. The *Ep-pyrG_his1*_pJET1.2 and *Ep-his1*_URA_pJET1.2 plasmids were subsequently used for the transformation of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1*. For transformation, media was supplemented with 10 mM uridine and 7.29 μM para-aminobenzoic acid for strains expressing *Ep-pyrG_his1*_pJET1.2, or 7.29 μM para-aminobenzoic acid and 2.5 mM histidine for strains expressing *Ep-his1*_URA_pJET1.2.

6.2.5.2 *Xanthoria parietina pyrG*, *pabaA* and *his1*

Three expression constructs were created for the expression of the putative primary metabolism genes *pyrG*, *pabaA* and *his1* from *X. parietina*. Again, these constructs were created using the pJET1.2 plasmid and contained a selection marker in addition to the gene of interest. The constructs containing *Xp-pyrG* or *Xp-pabaA* contained *his1* as the selection marker, and the construct containing *Xp-his1* contained *pyrG* as the selection marker. The *Xp-pyrG* gene was amplified from *X. parietina* gDNA using oligonucleotides 90 and 91, the *Xp-pabaA* gene was amplified from *X. parietina* gDNA using oligonucleotides 92 and 93, and the *Xp-his1* gene was amplified from *X. parietina* gDNA using oligonucleotides 94 and 95. As before for the selection markers *his1* was excised from the SM-X_His plasmid by carrying out a restriction digest with *NotI*, and *pyrG* was excised from the SM-X_URA plasmid by carrying out a restriction digest with *NotI* which excised the *pyrG*-containing URA Blaster.

Each construct was generated using the linearised pJET1.2 plasmid and the gel-purified PCR fragments of the gene of interest and selection marker. The plasmid and PCR fragments were mixed together and assembled using the CloneJET PCR Cloning kit (Thermo Fisher Scientific, UK), amplified in *E. coli* DH5α cells and purified. Positive clones were identified by colony PCR using oligonucleotides 96 and 84 for *Xp-pyrG*_pJET1.2, oligonucleotides 97 and 84 for *Xp-pabaA*_pJET1.2, oligonucleotides 98

and 84 for *Xp-his1*_pJET1.2, oligonucleotides 86 and 87 for *Xp-pyrG_his1*_pJET1.2, oligonucleotides 86 and 87 for *Xp-pabaA_his1*_pJET1.2, and oligonucleotides 88 and 89 for *Xp-his1*_URA_pJET1.2. Each plasmid was checked for correct assembly using restriction analyses. The *Xp-pyrG_his1*_pJET1.2, *Xp-pabaA_his1*_pJET1.2 and *Xp-his1*_URA_pJET1.2 plasmids were then used for the transformation of the *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1* strains. For transformation, media was supplemented with 10 mM uridine and 7.29 μM para-aminobenzoic acid for strains expressing *Xp-pyrG_his1*_pJET1.2 and *Xp-pabaA_his1*_pJET1.2, or 7.29 μM para-aminobenzoic acid and 2.5 mM histidine for strains expressing *Xp-his1*_URA_pJET1.2.

6.2.5.3 *Cladonia grayi pyrG* and *pabaA*

Two expression constructs were generated for the expression of the putative primary metabolism genes *pyrG* and *pabaA* from *C. grayi*. In the same way as before, the constructs were generated using the pJET1.2 plasmid and included a selection marker in addition to the gene of interest. The constructs containing *Cg-pyrG* or *Cg-pabaA* both contained *his1* as the selection marker. The *Cg-pyrG* gene was amplified from *C. grayi* gDNA using oligonucleotides 99 and 100, and the *Cg-pabaA* gene was amplified from *C. grayi* gDNA using oligonucleotides 101 and 102. As previously, the *his1* selection marker was excised from the SM-X_His plasmid by carrying out a restriction digest with *NotI*.

Each expression construct was created using the linearised pJET1.2 plasmid and the gel-purified PCR fragments of the gene of interest and selection marker. In the same way as before, the plasmid and PCR fragments were mixed together and assembled, amplified in *E. coli* DH5α cells and purified. Colony PCR was used to identify positive clones, using oligonucleotides 103 and 84 for *Cg-pyrG*_pJET1.2, oligonucleotides 104 and 84 for *Cg-pabaA*_pJET1.2, oligonucleotides 86 and 87 for *Cg-pyrG_his1*_pJET1.2, and oligonucleotides 86 and 87 for *Cg-pabaA_his1*_pJET1.2. Again, each plasmid was checked for correct assembly by restriction analyses. The *Cg-pyrG_his1*_pJET1.2 and *Cg-pabaA_his1*_pJET1.2 plasmids were subsequently used for the transformation of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* and *A. oryzae* OP12 *pyrG*$^{-}$

Δ*pabaA* Δ*his1*. For transformation, media was supplemented with 10 mM uridine and 7.29 μM para-aminobenzoic acid.

6.2.6 Assessing Temperature Dependency of Lichen Primary Metabolism Proteins

A. niger ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1* positive transformants confirmed to contain each of the primary metabolism gene expression constructs were grown on GG10 agar plates with and without supplementation in parallel at a range of temperatures (18 °C, 23 °C and 28 °C) in order to determine the heat sensitivity of the primary metabolism proteins. Conidial suspensions at a concentration of $1x10^5$ ascospores/ml were created for each transformant, and 3 μl of each suspension was spotted onto plates; conidial suspensions of *A. niger* ATNT16 wild-type and *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1*, or *A. oryzae* OP12 wild-type and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1* were also spotted on plates as controls. Suspensions of strains expressing *Ep-pyrG*, *Xp-pyrG* or *Cg-pyrG* were spotted onto plates with and without uridine supplementation in parallel, strains expressing *Xp-pabaA* or *Cg-pabaA* were spotted onto plates with and without para-aminobenzoic acid supplementation in parallel, and strains expressing *Ep-his1* or *Xp-his1* were spotted onto plates with and without histidine supplementation in parallel. Plates were incubated at their respective temperature, and pictures were taken of the plates after seven days using a Nikon D3200 camera fitted with an AF-S DX NIKKOR 35 mm 1:1.8G lens in order to record that complementation of the auxotrophic phenotype had occurred, as well as to determine the heat sensitivity of the lichen primary metabolism proteins.

6.2.7 Dot Blot

To determine the heat sensitivity of the Clagr3.11, Clagr3.30 and Clagr3.11:T-TE_{abrA} proteins, liquid ME cultures in a 25 ml volume supplemented with 7.29 μM para-aminobenzoic acid were inoculated with conidial suspensions of *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* *Clagr3.11*_SM_S-tag_X_URA 2.4, *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* *Clagr3.30*_SM_S-tag_X_URA 2.4 and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* *Clagr3.11*:T-TE_{abrA}_SM_S-tag_X_URA 2.17 transformants. Liquid cultures were incubated at 23 °C

and 28 °C in parallel on a rotary shaker at 150 rpm. Mycelia were harvested over Miracloth (Merck, Darmstadt, Germany) and pressed dry. The mycelia were then ground to a fine powder under liquid nitrogen and suspended in TBS buffer (40 mM Tris-HCl pH 7.5, 150 mM NaCl). Cell debris were removed by two rounds of 5 min centrifugation at 4 °C and 13,000 rpm. The resulting cell-free extracts were adjusted to 2 mg/ml, 1 mg/ml and 0.5 mg/ml of total protein, and 2.5 µl of each was spotted onto a dry nitrocellulose membrane. Purified Clagr3.11 and MelA (containing a His-tag) proteins at a concentration of 0.025 mg/ml were also spotted onto the membrane as controls.

The membrane was allowed to dry fully before being incubated in 20 ml 1x blocking solution (Roche, Sigma-Aldrich, UK) in TBS for 60 min. The membrane was then washed three times in TBST buffer (40 mM Tris-HCl pH7.5, 150 mM NaCl, 0.05% Tween20) for 5 min each, before being incubated in 10 ml TBST containing 20 µg/ml avidin for 15 min. The membrane was then incubated for 40 min with the Strep-Tactin AP conjugate (IBA Lifesciences GmbH, Göttingen, Germany) in a 1:4000 dilution before being washed twice with TBST and twice with TBS (1 min incubation for each). The membrane was subsequently equilibrated with detection buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$) before being stained with NBT-BCIP (45 µl nitro-blue tetrazolium chloride (75 mg/ml in 70% *N,N*-dimethylformamide) and 35 µl 5-bromo-4-chloro-3'-indolylphosphate (50 mg/ml in 100% *N,N*-dimethylformamide) in 10 ml detection buffer). The membrane was then washed with SDW.

6.2.8 Generation of Strains Expressing Native *hacA* and Lichen Secondary Metabolism Genes

An expression construct was created for the co-expression of the native *A. niger hacA* gene with either *Clagr3.6*, *Clagr3.26* or *Clagr3.30* from *C. grayi*. The expression construct containing *hacA* was created using the SM-X_PABA plasmid. The *hacA* gene was amplified from *A. niger* A1144 Δ*pyrG* gDNA in two fragments using oligonucleotide 105 which overlapped with the *NcoI*-restricted SM-X_PABA plasmid, overlapping oligonucleotides 106 and 107, and oligonucleotide 108 which also overlapped with the *NcoI*-restricted SM-X_PABA plasmid. The amplification of *hacA*

excluded a 20-bp intron, thus ensuring that the HacA transcription factor would be expressed in its active form.

The *NcoI*-restricted SM-X_PABA plasmid and the two gel-purified PCR fragments were mixed together, and the plasmid was assembled by *in vitro* recombination, amplified in *E. coli* DH5α cells and purified. Positive clones were subsequently selected by colony PCR using oligonucleotides 109 and 15. The isolated plasmid was checked for correct assembly by restriction analyses and was then used to transform either *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.6*_SM-X_URA 2.7, *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.26*_SM-X_URA 1.2 or *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* *Clagr3.30*_SM_S-tag_X_URA 2.5. For transformation media, bottom agar was supplemented with 10 μg/ml doxycycline.

Liquid GG20 cultures in a 25 ml volume supplemented with 10 μg/ml doxycycline was inoculated with individual transformant conidial suspensions for each strain. Cultures were incubated at 23 °C and 28 °C in parallel at 150 rpm on a rotary shaker. The mycelia and culture filtrates were separated by filtration over Miracloth (Merck, Darmstadt, Germany) and the culture filtrates were then analysed by HPLC for metabolite production.

6.2.9 Transformant Analysis

Transformation plates were incubated at 28 °C, and transformants were streaked twice onto fresh GG10 agar plates for purification. All selected transformants were checked by diagnostic PCR after gDNA extraction using oligonucleotides 79 and 84 for strains expressing *Ep-pyrG_his1*_pJET1.2, oligonucleotides 81 and 84 for strains expressing *Ep-his1*_URA_pJET1.2, oligonucleotides 90 and 84 for strains expressing *Xp-pyrG_his1*_pJET1.2, oligonucleotides 92 and 84 for strains expressing *Xp-pabaA_his1*_pJET1.2, oligonucleotides 94 and 84 for strains expressing *Xp-his1*_URA_pJET1.2, oligonucleotides 99 and 84 for strains expressing *Cg-pyrG_his1*_pJET1.2, oligonucleotides 104 and 84 for strains expressing *Cg-pabaA_his1*_pJET1.2, and oligonucleotides 57 and 15 for strains expressing *An-hacA*_SM-X_PABA in order to confirm the full-length integration of the gene of interest.

6.2.10 Metabolite Extraction and Analysis

Metabolites were extracted from culture filtrates by mixing with an equal volume of ethyl acetate and removing the overlying solvent layer. Metabolites were extracted from mycelia via homogenisation in ethyl acetate by placing in an ultrasonic bath (USR 30 H; 160 W, 35 kHz) (Merck, Darmstadt, Germany) without heating for ~10 min. The extracts were filtered over anhydrous sodium sulfate and evaporated under reduced pressure. The resulting residues were then solved in methanol.

The samples solved in methanol subsequently underwent HPLC analysis using a Dionex UltiMate3000 (Thermo Fisher Scientific, UK) and an Eclipse XDB-C18 column (4.6x150 mm, 5 μm; Agilent, California, USA) kept at 40 °C. A binary solvent system of water containing 0.1% formic acid (solvent A) and 100% methanol (solvent B) was used with a flow rate of 1 ml/min: 0.5 min 10% B, 15 min 90% B, 17 min 90% B, 17.5 min 100% B, 22 min 100% B, 23 min 10% B, 25 min 10% B.

6.3 Results

6.3.1 Heat Sensitivity of Lichen Ascospores

To investigate the resistance of *X. parietina*, *L. chlarotera* and *A. punctata* ascospores to heat stress, ascospores of each species were subjected to either a wet heat treatment or a dry heat treatment for 30 min at various temperatures ranging between 20 °C and 100 °C before being incubated on plates containing solid MEYE supplemented with ampicillin and streptomycin (with ascospores not exposed to any heat treatment used as a control). The percentage germination of ascospores (relative to numbers observed in the control) was used to determine the resistance of the ascospores to the heat treatment, with the presence of a colony on the plate indicating the germination of an ascospore and, therefore, resistance of the ascospore to the heat treatment. A one-way ANOVA was carried out on the original data for each lichen species, to analyse the effects of temperature for each type of heat treatment on the percentage germination of ascospores.

For all three species of lichen, ascospore germination only occurred up to 35 °C after a wet heat treatment, whereas after a dry heat treatment ascospore germination occurred up to 80 °C for *X. parietina* and *L. chlarotera*, and up to 70 °C for *A. punctata* (Figure 6.2). These results demonstrate that lichen ascospores are more

resistant to dry heat than wet heat, and that *X. parietina* and *L. chlarotera* ascospores appear to be more resistant to dry heat than *A. punctata* ascospores as germination continued to occur at the higher 80 °C temperature for the former species. When considering the results for *X. parietina* ascospores undergoing a dry heat treatment, indeed it appeared that the heat treatment actually promoted (activated) germination up to 40 °C, as the percentage germination of ascospores increased relative to the control; beyond 40 °C, though, the heat treatment then seemed to become detrimental to the ascospores, with the percentage germination of ascospores decreasing with each successive elevation in temperature. This activating effect of a dry heat treatment up to 40 °C was not apparent in the results for *L. chlarotera* or *A. punctata* ascospores.

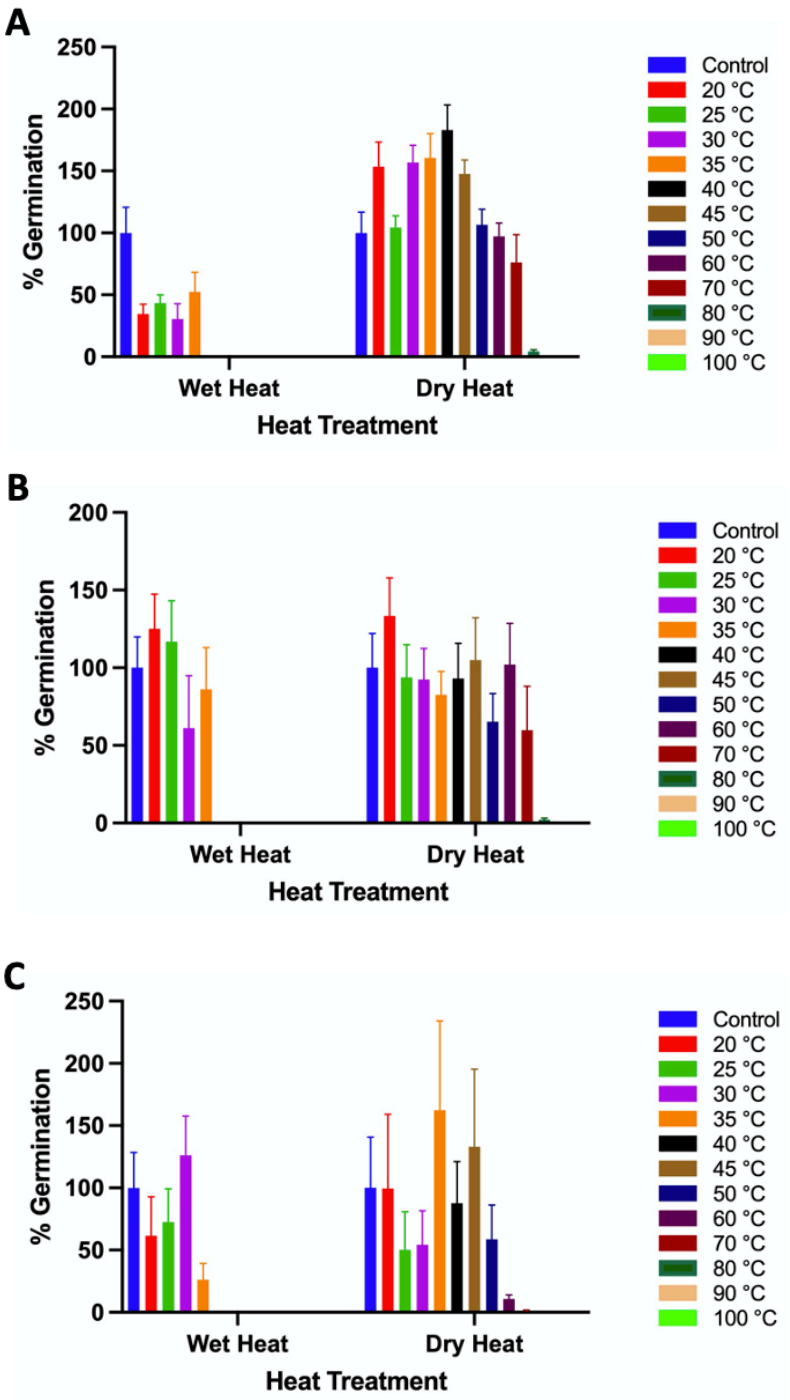


Figure 6.2. Percentage germination (relative to numbers observed in the control) of *X. parietina* **(A)**, *L. chlarotera* **(B)** and *A. punctata* **(C)** ascospores after wet heat or dry heat treatments. Ascospore germination is expressed relative to the control, in which ascospores did not undergo a heat treatment. Error bars indicate SEM.

As shown in Figure 6.2A for *X. parietina*, there was a significant effect of temperature on ascospore germination for both wet heat (one-way ANOVA: $F(12, 103) = 13.31$, $p < 0.0001$) and dry heat (one-way ANOVA: $F(12, 101) = 19.60$, $p <$

0.0001). Similar to *X. parietina*, there was a significant effect of temperature on the germination of *L. chlarotera* ascospores for both wet heat (one-way ANOVA: $F(12, 101) = 9.539$, $p < 0.0001$) and dry heat (one-way ANOVA: $F(12, 100) = 4.171$, $p < 0.0001$) (Figure 6.2B). Further to this, as shown in Figure 6.2C, temperature had a significant effect on the germination of *A. punctata* ascospores for wet heat (one-way ANOVA: $F(12, 102) = 6.928$, $p < 0.0001$) and dry heat (one-way ANOVA: $F(12, 98) = 2.278$, $p = 0.0134$). Therefore, the significant effect of temperature on ascospore germination in all three lichen species for both types of heat treatment indicate that the ascospores of these lichen species are sensitive to heat, with the ascospores being unable to withstand temperatures above 35 °C for wet heat and above 70 °C or 80 °C for dry heat.

When comparing the heat sensitivity of the ascospores of the three lichen species to that of two strains of *A. nidulans*, 2-3 and 2-137, it appeared that the *A. nidulans* ascospores are in fact more resistant to heat than the ascospores of *X. parietina*, *L. chlarotera* and *A. punctata*. This is due to the fact that ascospore germination occurs up to 50 °C after a wet heat treatment for both *A. nidulans* strains, and up to 90 °C after a dry heat treatment for both strains (Figure 6.3). However, these results are in agreement with those from the lichen species in the sense that the ascospores of both *A. nidulans* strains appear to be more resistant to dry heat than to wet heat, with the ascospores being able to germinate after exposure to extreme temperatures during dry heat treatments. As shown in Figure 6.3A, the effect of temperature on ascospore germination for *A. nidulans* 2-3 was significant for both wet heat (one-way ANOVA: $F(12, 104) = 7.613$, $p < 0.0001$) and dry heat (one-way ANOVA: $F(12, 104) = 7.026$, $p < 0.0001$). The significant effect of temperature on ascospore germination was also observed for *A. nidulans* 2-137, again for both wet heat (one-way ANOVA: $F(12, 104) = 6.856$, $p < 0.0001$) and dry heat (one-way ANOVA: $F(12, 104) = 10.85$, $p < 0.0001$) (Figure 6.3B).

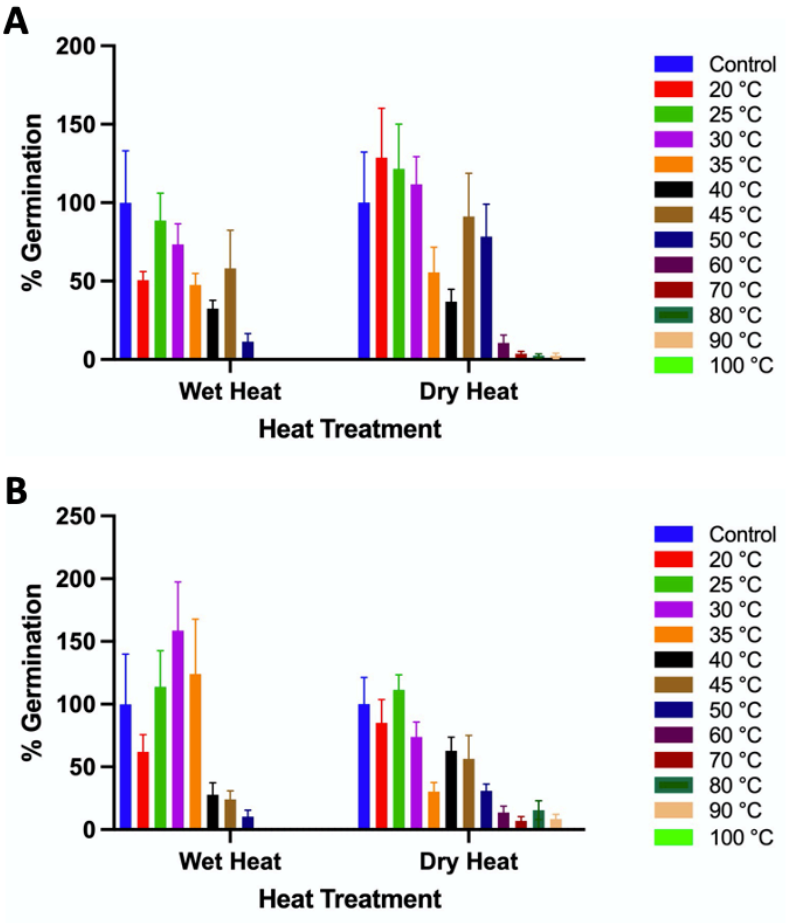


Figure 6.3. Percentage germination (relative to numbers observed in the control) of *A. nidulans* 2-3 **(A)** and *A. nidulans* 2-137 **(B)** ascospores after wet heat or dry heat treatments. Ascospore germination is expressed relative to the control, in which ascospores did not undergo a heat treatment. Error bars indicate SEM.

6.3.2 Heat Sensitivity of Lichen-Derived Proteins

6.3.2.1 Expression of Lichen Primary Metabolism Genes

Expression constructs were created containing either the *E. prunastri pyrG*, *E. prunastri his1*, *X. parietina pyrG*, *X. parietina pabaA*, *X. parietina his1*, *C. grayi pyrG* or *C. grayi pabaA* primary metabolism genes. These constructs were then used to transform *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* and *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1* strains. Subsequent transformants in which the full construct was integrated were then grown at assay temperatures of 18 °C, 23 °C and 28 °C on GG10 plates with and without supplementation in parallel in order to determine the heat sensitivity of the proteins produced by these primary metabolism genes.

6.3.2.1.1 Temperature Dependency of *Evernia prunastri pyrG* and *his1*

When considering the heterologous expression of *E. prunastri pyrG* in *A. niger* ATNT16, complementation of the uridine auxotrophy was successful in one out of four of the selected transformants, with this single transformant able to grow without uridine supplementation (Figure 6.4). This transformant was able to grow at all three assay temperatures, however the growth of the transformant appeared reduced compared to that of the wild-type, indicating that the *E. prunastri pyrG* was less efficient or active than the host *A. niger pyrG*. Of the four *A. oryzae* OP12 transformants expressing *E. prunastri pyrG*, successful complementation was achieved in three of the four transformants (Figure 6.5). Similar to the *A. niger* ATNT16 transformant, the *A. oryzae* OP12 strains were able to grow at all three temperatures, but again showed a reduced growth rate compared to the wild-type.

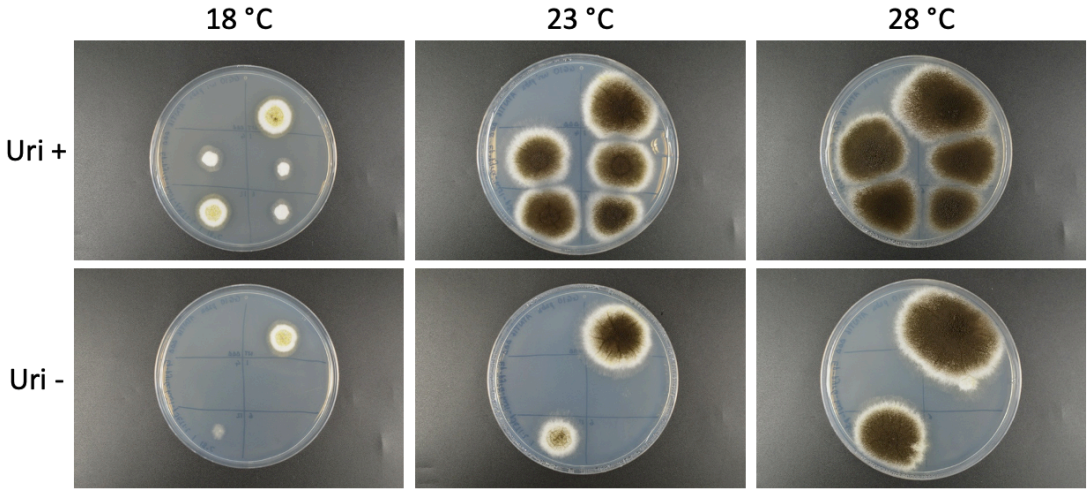


Figure 6.4. Growth of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1 Ep-pyrG_his1*_pJET1.2 transformants. The ATNT16 wild-type positive control is located on the right of the top row, and the ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* negative control is located on the left of the top row. The bottom two rows correspond to transformant strains.

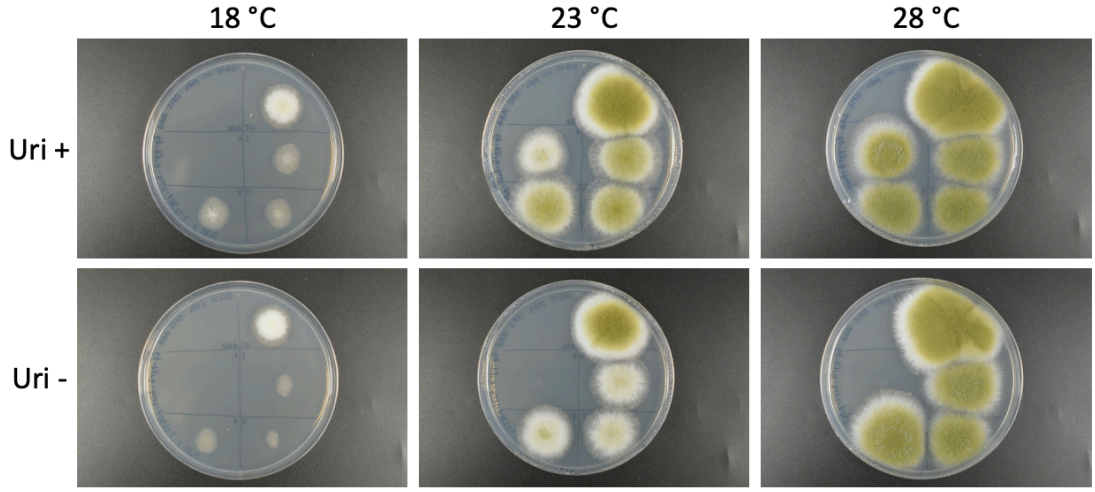


Figure 6.5. Growth of *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1 Ep-pyrG_his1*_pJET1.2 transformants. The OP12 wild-type positive control is located on the right of the top row, and the OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1* negative control is located on the left of the top row. The bottom two rows correspond to transformant strains.

The heterologous expression of *E. prunastri his1* in *A. niger* ATNT16 resulted in the successful complementation of the histidine auxotrophy in three of the four selected transformants (Figure 6.6). It was very noteworthy that the *E. prunastri* His1 protein displayed a pronounced sensitivity to heat, as all three transformants were able to grow at 18 °C without histidine supplementation, with two of the transformants showing similar growth rates to that of the wild-type. However, at 23 °C, only two of the transformants are able to grow, and the heat sensitivity of the protein was evident in the reduced growth rate of the transformants compared to the wild-type. This heat sensitivity was even more pronounced at 28 °C, with the only transformant that was able to grow showing a severely reduced growth rate. The heat sensitivity of the *E. prunastri* His1 protein was even more apparent when expressed in *A. oryzae* OP12 (Figure 6.7). At 18 °C, three transformants were able to grow but showed a reduced growth rate compared to that of the wild-type. At 23 °C, only one of these transformants was capable of growth, however the colony was barely established. When incubated at 28 °C, none of the transformants were able to grow,

thus showing that that *E. prunastri* His1 protein is sensitive to and inactive at higher temperatures.

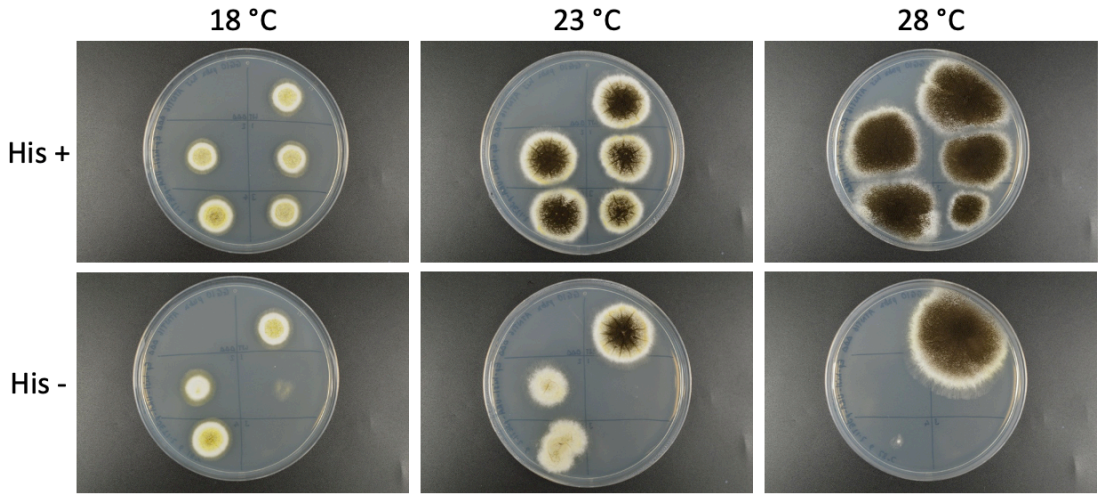


Figure 6.6. Growth of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1 Ep-his1*_URA_pJET1.2 transformants. The ATNT16 wild-type positive control is located on the right of the top row, and the ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* negative control is located on the left of the top row. The bottom two rows correspond to transformant strains.

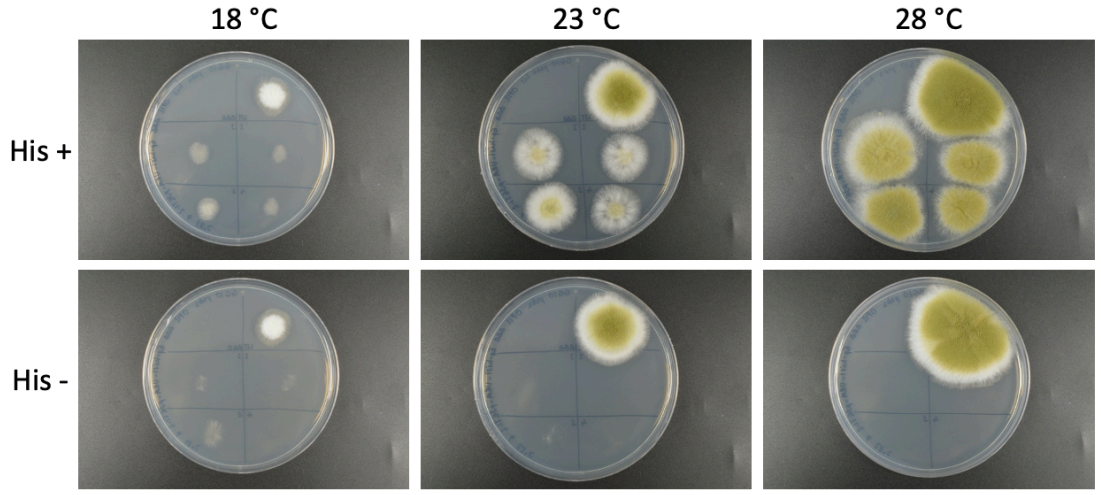


Figure 6.7. Growth of *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1 Ep-his1*_URA_pJET1.2 transformants. The OP12 wild-type positive control is located on the right of the top row, and the OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1* negative control is located on the left of the top row. The bottom two rows correspond to transformant strains.

6.3.2.1.2 Temperature Dependency of *Xanthoria parietina pyrG*, *pabaA* and *his1*

Of the four *A. niger* ATNT16 transformant strains expressing *X. parietina pyrG*, none of the transformants were able to grow at any of the three assay temperatures (Figure 6.8). This suggests that successful complementation of the uridine auxotrophy was not achieved, therefore meaning that the transformants were unable to grow in the absence of uridine, despite the presence of the *X. parietina pyrG*. This was also the case for the heterologous expression of *X. parietina pyrG* in *A. oryzae* OP12, as none of the selected transformants were able to grow without uridine supplementation at any of the three assay temperatures (Figure 6.9). This, again, suggests that complementation of the uridine auxotrophy was not achieved.

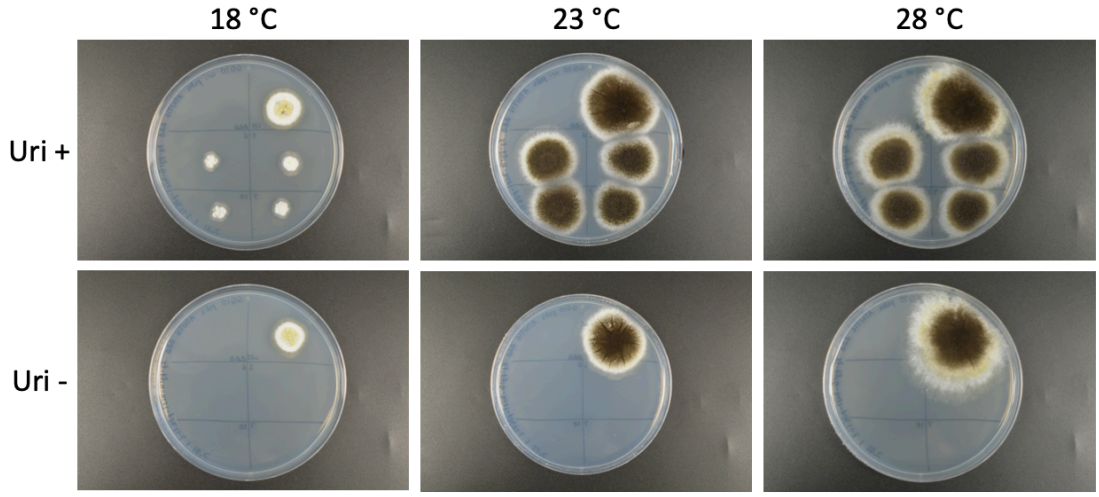


Figure 6.8. Growth of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* *Xp-pyrG_his1*_pJET1.2 transformants. The ATNT16 wild-type positive control is located on the right of the top row, and the ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* negative control is located on the left of the top row. The bottom two rows correspond to transformant strains.

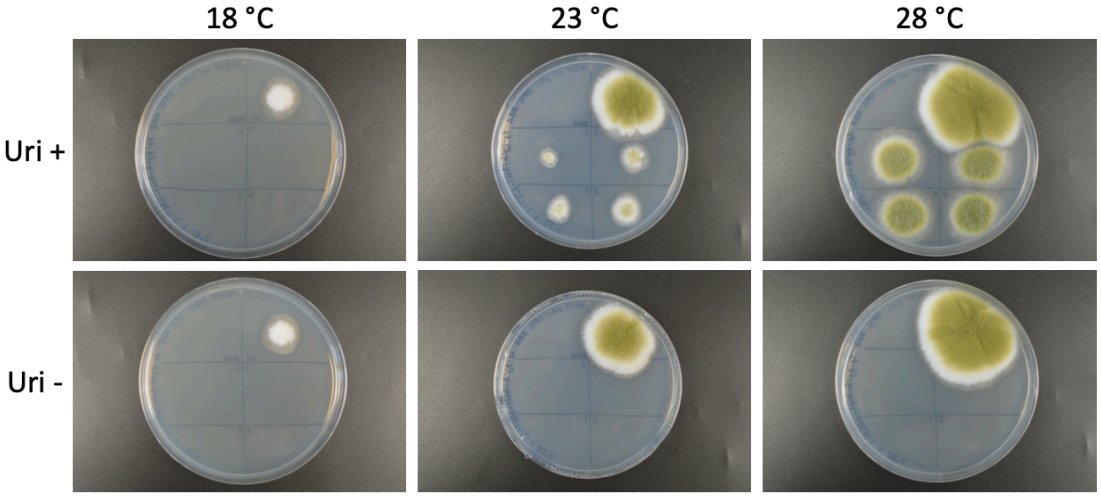


Figure 6.9. Growth of *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1* *Xp-pyrG_his1*_pJET1.2 transformants. The OP12 wild-type positive control is located on the right of the top row, and the OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1* negative control is located on the left of the top row. The bottom two rows correspond to transformant strains.

The heterologous expression of *X. parietina pabaA* in *A. niger* ATNT16 resulted in the complementation of the para-aminobenzoic acid auxotrophy in all four of the selected transformants (Figure 6.10). However, growth of all four transformants was observed on plates without para-aminobenzoic acid supplementation that were incubated at 23 °C and 28 °C, whereas growth of only one transformant was apparent at 18 °C. This appeared to be mainly due to the severely reduced growth rate of the transformants, which was also observed on plates with para-aminobenzoic acid supplementation. The significantly reduced growth rate of the transformants suggested that the remaining three transformants not seen at 18 °C were perhaps growing so slowly that colonies were simply not visible after 7 days. This slow growth rate of transformants was also observed for *A. oryzae* OP12 strains expressing *X. parietina pabaA*, and was again seen on plates with and without para-aminobenzoic acid supplementation (Figure 6.11). On plates without para-aminobenzoic acid supplementation, growth of three transformants was seen at 28 °C and one transformant was able to grow at 23 °C, however these colonies were barely visible. Growth was not seen for any of the transformants at 18 °C on plates without para-aminobenzoic acid supplementation.

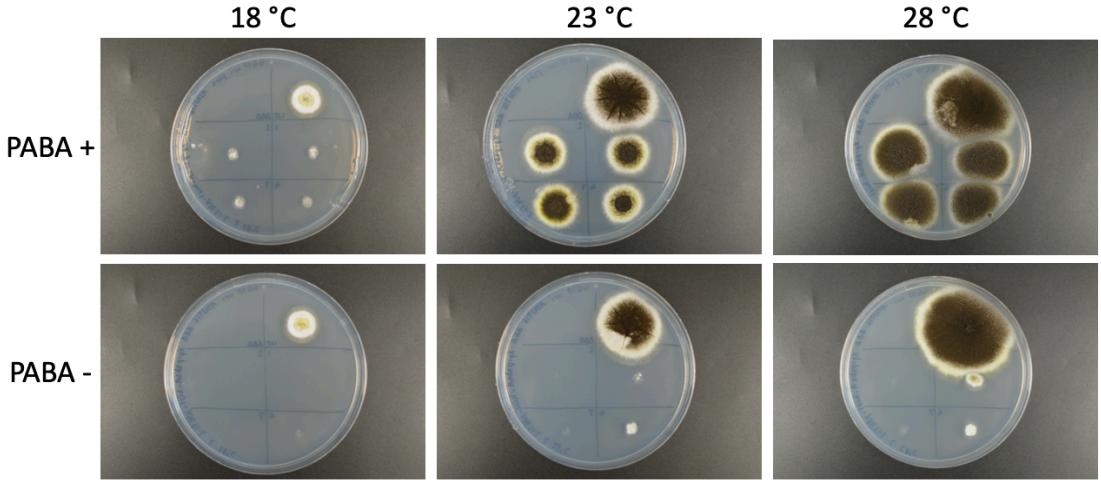


Figure 6.10. Growth of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1 Xp-pabaA_his1*_pJET1.2 transformants. The ATNT16 wild-type positive control is located on the right of the top row, and the ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* negative control is located on the left of the top row. The bottom two rows correspond to transformant strains.

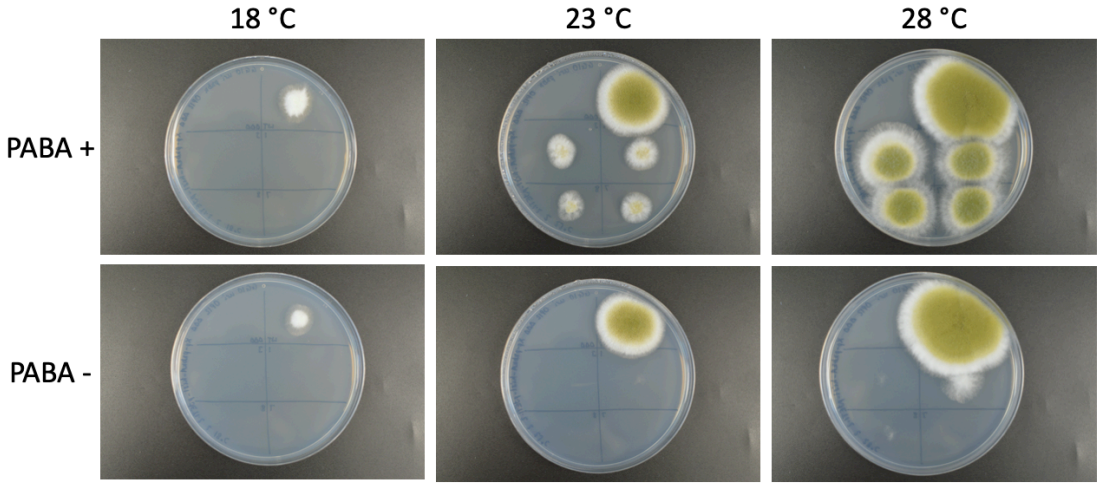


Figure 6.11. Growth of *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1 Xp-pabaA_his1*_pJET1.2 transformants. The OP12 wild-type positive control is located on the right of the top row, and the OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1* negative control is located on the left of the top row. The bottom two rows correspond to transformant strains.

Complementation of the histidine auxotrophy was not achieved for any of the four *A. niger* ATNT16 transformants expressing *X. parietina his1*, as growth was not observed for any of the transformants on plates without histidine supplementation (Figure 6.12). This was the case for plates incubated at all three assay temperatures. *A. oryzae* OP12 transformant strains expressing *X. parietina his1* were also unable to grow in the absence of histidine at all three temperatures (Figure 6.13). Again, this suggests that complementation of the histidine auxotrophy was not successful in the *A. oryzae* OP12 transformants.

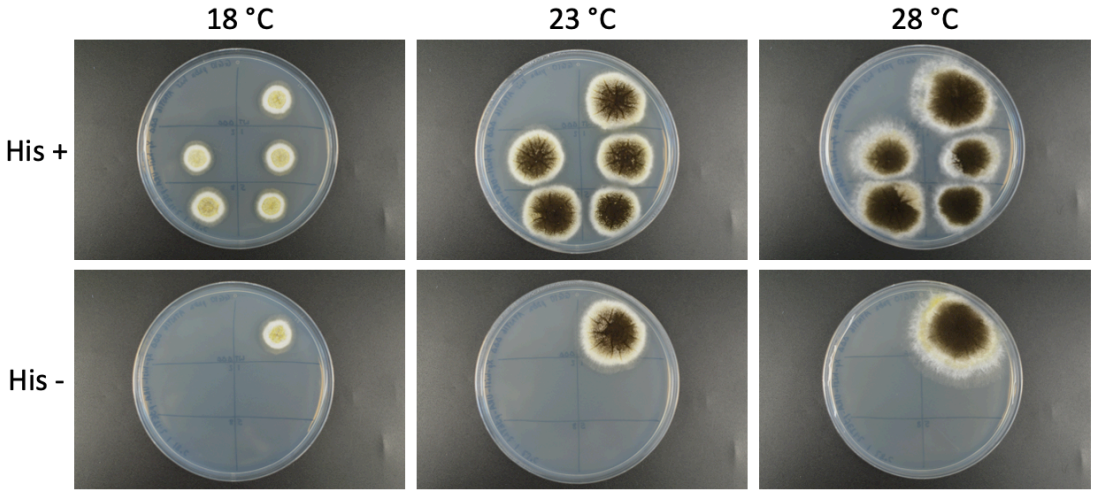


Figure 6.12. Growth of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1 Xp-his1*_URA_pJET1.2 transformants. The ATNT16 wild-type positive control is located on the right of the top row, and the ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* negative control is located on the left of the top row. The bottom two rows correspond to transformant strains.

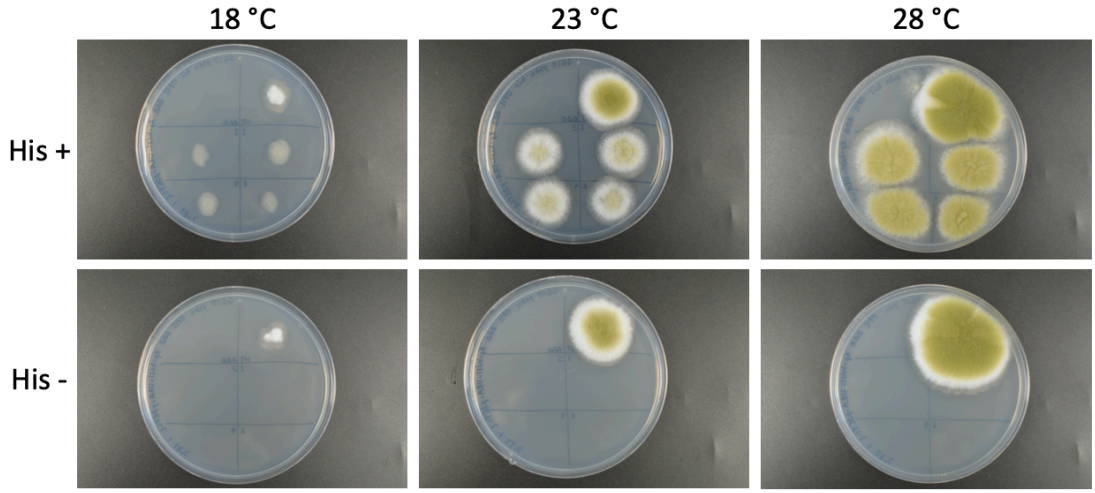


Figure 6.13. Growth of *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1 Xp-his1*_URA_pJET1.2 transformants. The OP12 wild-type positive control is located on the right of the top row, and the OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1* negative control is located on the left of the top row. The bottom two rows correspond to transformant strains.

6.3.2.1.3 Temperature Dependency of *Cladonia grayi pyrG* and *pabaA*

Complementation of the uridine auxotrophy did not appear to be successful in any of the four *A. niger* ATNT16 transformants selected (Figure 6.14) or in the four *A. oryzae* transformants selected expressing *C. grayi pyrG* (Figure 6.15). This was illustrated by the fact that none of the transformants were capable of growing in the absence of uridine at any of the three temperatures.

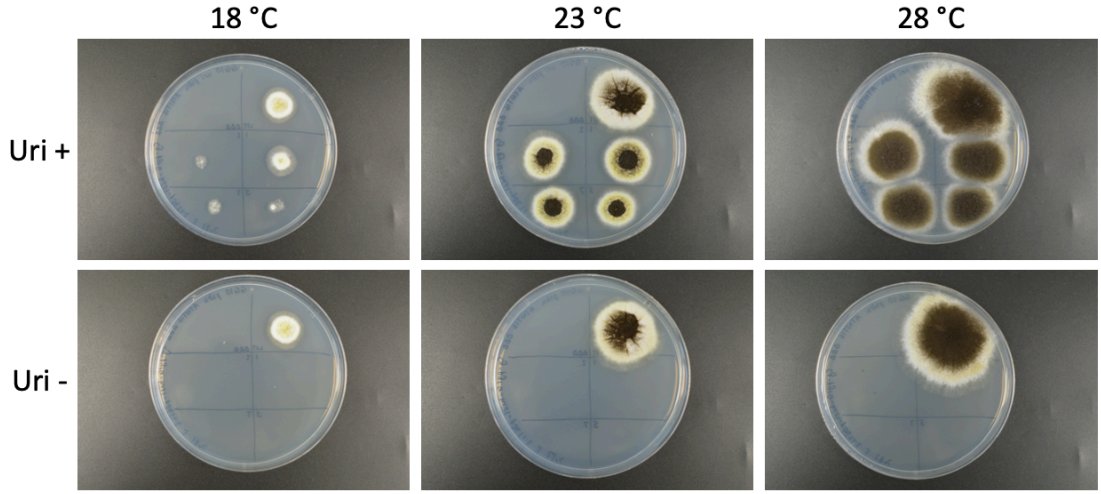


Figure 6.14. Growth of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1 Cg-pyrG_his1*_pJET1.2 transformants. The ATNT16 wild-type positive control is located on the right of the top row, and the ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* negative control is located on the left of the top row. The bottom two rows correspond to transformant strains.

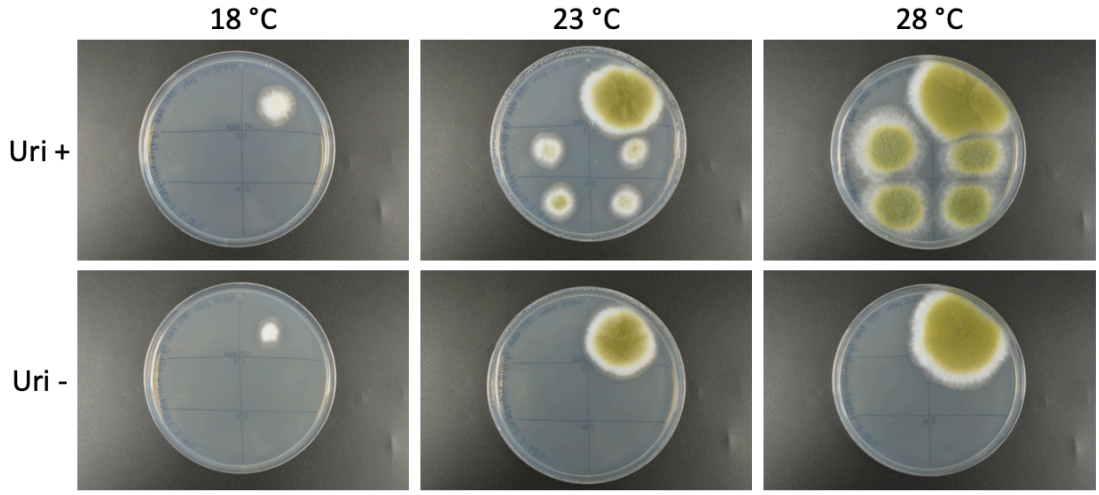


Figure 6.15. Growth of *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1 Cg-pyrG_his1*_pJET1.2 transformants. The OP12 wild-type positive control is located on the right of the top row, and the OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1* negative control is located on the left of the top row. The bottom two rows correspond to transformant strains.

Similar to the strains expressing *C. grayi pyrG*, complementation was also not successful in any of the *A. niger* ATNT16 transformants expressing *C. grayi pabaA* (Figure 6.16). Again, this was illustrated by the fact that growth of the transformants was not observed on plates without uridine supplementation at 18 °C, 23 °C or 28 °C. For *A. oryzae* OP12 transformants expressing *C. grayi pabaA*, complementation was only successful for two of the transformants, with growth only observed at 28 °C (Figure 6.17). As suggested for *A. niger* ATNT16 and *A. oryzae* OP12 transformants expressing *X. parietina pabaA*, the lack of colony growth observed on the 18 °C and 23 °C plates could be due to an extremely slow growth rate of the transformants, again meaning that colonies were not visible after 7 days.

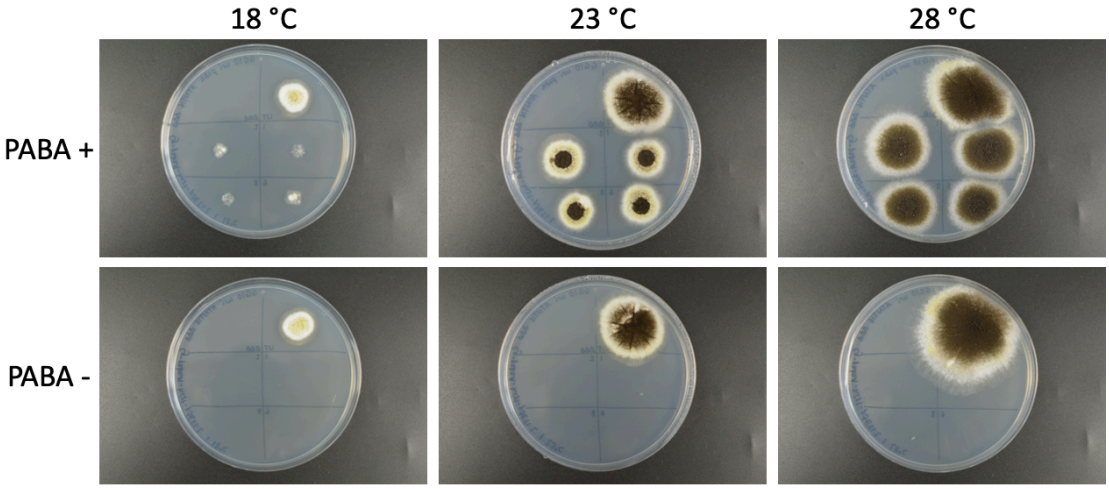


Figure 6.16. Growth of *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1 Cg-pabaA_his1*_pJET1.2 transformants. The ATNT16 wild-type positive control is located on the right of the top row, and the ATNT16 Δ*pyrG* Δ*pabaA* Δ*his1* negative control is located on the left of the top row. The bottom two rows correspond to transformant strains.

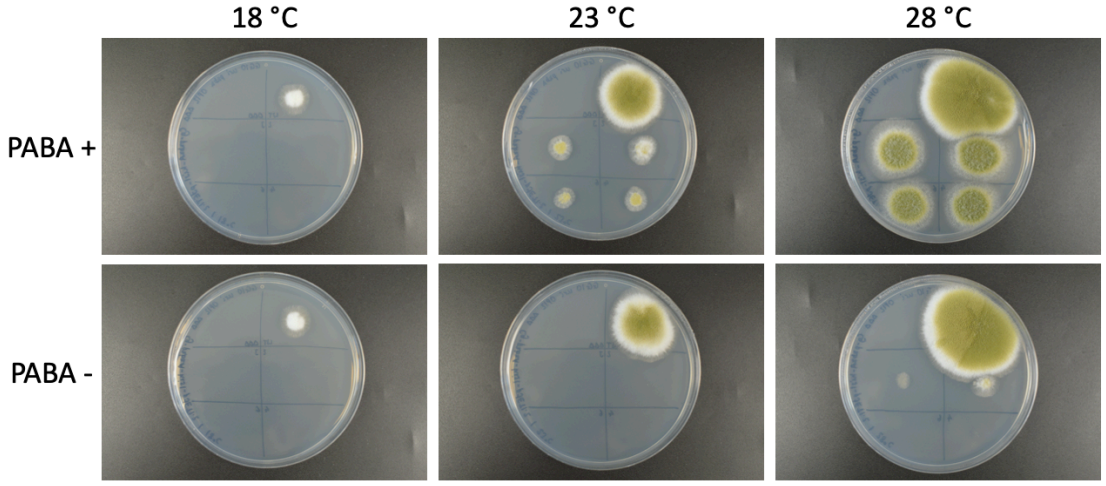


Figure 6.17. Growth of *A. oryzae* OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1 Cg-pabaA_his1*_pJET1.2 transformants. The OP12 wild-type positive control is located on the right of the top row, and the OP12 *pyrG*$^{-}$ Δ*pabaA* Δ*his1* negative control is located on the left of the top row. The bottom two rows correspond to transformant strains.

6.3.2.2 Heat Sensitivity of Lichen-Derived Secondary Metabolism Proteins

In order to determine the heat sensitivity of the Clagr3.11 and Clagr3.30 proteins from *C. grayi*, as well as the Clagr3.11:T-TE$_{abrA}$ fusion protein, *A. oryzae* OP12 strains expressing each of the three proteins were grown in parallel under inducing conditions at 23 °C or 28 °C. Cell-free extracts of the resulting mycelia were adjusted to various total protein concentrations, and samples of each were spotted onto a nitrocellulose membrane. A Strep-Tactin AP conjugate was used bind to the Strep-tagged Clagr3.11, Clagr3.30 and Clagr3.11:T-TE$_{abrA}$ proteins, and subsequent staining

of the nitrocellulose membrane was used to indicate levels of Strep-tagged protein production.

When considering all three proteins, it was observed from the dot blot that the intensity of the dots at 23 °C were higher than those at 28 °C (Figure 6.18). This showed that protein production of Clagr3.11, Clagr3.30 and Clagr3.11:T-TE$_{abrA}$ was higher at 23 °C compared to 28 °C, especially for Clagr3.30. These results are in agreement with the HPLC results obtained in the previous chapter, in which metabolite production appeared to be higher at 23 °C than at 28 °C for strains expressing *Clagr3.11* or *Clagr3.11*:T-TE$_{abrA}$. Furthermore, the very low protein production observed on the dot blot for the *A. oryzae* OP12 strain expressing *Clagr3.30* at 28 °C correlates with the lack of metabolite production for this strain at 28 °C observed in the previous chapter. This, therefore, indicates that the Clagr3.30 protein is very unstable at 28 °C. When comparing the protein production of the Clagr3.11 protein to the Clagr3.11:T-TE$_{abrA}$ fusion protein, it was apparent that protein production was higher for the fusion protein at both 23 °C and 28 °C compared to that of Clagr3.11 (Figure 6.18). This suggests that the hybrid enzyme was more thermostable than the Clagr3.11 enzyme, therefore meaning that higher levels of proteins can be produced.

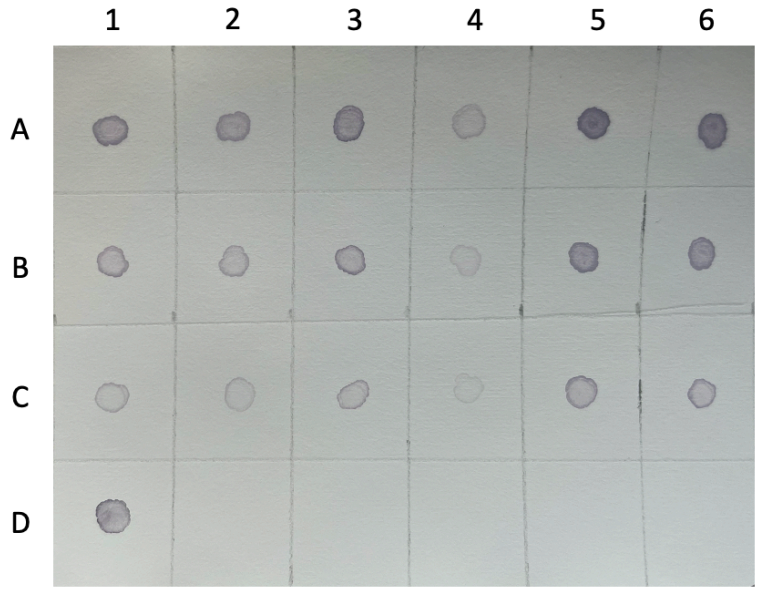


Figure 6.18. Heat sensitivity of the Clagr3.11, Clagr3.30 and Clagr3.11:T-TE$_{abrA}$ proteins as illustrated by dot blot analysis. Columns 1 and 2 correspond to Clagr3.11 at 23 °C and 28 °C, respectively, columns 3 and 4 correspond to Clagr3.30 at 23 °C and 28 °C, respectively, and columns 5 and 6 correspond to Clagr3.11:T-TE$_{abrA}$ at 23 °C and 28 °C, respectively. Row A corresponds to 2 mg/ml total protein, row B corresponds to 1 mg/ml total protein, and row C corresponds to 0.5 mg/ml total protein. Cell D1 corresponds to the positive control of 0.025 mg/ml purified Strep-tagged Clagr3.11, and cell D2 corresponds to the negative control of 0.025 mg/ml purified His-tagged MelA.

6.3.2.3 Co-Expression of Native *hacA* and Lichen Secondary Metabolism Genes

An expression construct containing the native *A. niger hacA* gene in the SM-X_PABA plasmid was created (Figure 6.19A) for the co-expression of the active form of the transcription factor in *A. niger* ATNT16 Δ*pyrG* Δ*pabaA* strains in which the uridine auxotrophy had already been complemented in a previous transformation. The *A. niger* ATNT16 Δ*pyrG* Δ*pabaA Clagr3.6*_SM-X_URA 2.7 and *A. niger* ATNT16 Δ*pyrG* Δ*pabaA Clagr3.30*_SM_S-tag_X_URA 2.5 strains were chosen for co-expression of *A. niger hacA* in order to see if this transcription factor could increase protein production of a PKS and an NRPS-like enzyme, respectively, at 23 °C and lead to metabolite production at 28 °C. The *A. niger* ATNT16 Δ*pyrG* Δ*pabaA Clagr3.26*_SM-X_URA 1.2 strain was chosen for co-expression of *A. niger hacA* in order to see if the transcription factor could lead to metabolite production at either 23 °C or 28 °C, which had not been observed at either temperature previously. Positive transformants for each strain were identified by screening via PCR for full-length integration of the *hacA* construct and were then grown under inducing conditions at 23 °C and 28 °C in parallel. Culture filtrates were subsequently analysed by HPLC for metabolite production.

As shown in Figures 6.19B and 6.19C, no metabolites were produced by transformant strains co-expressing *Clagr3.6* and *hacA* or *Clagr3.30* and *hacA* at 28 °C, therefore suggesting that overexpression of *A. niger hacA* does not increase the levels of correctly-folded proteins being produced at 28 °C and thereby lead to metabolite production. Furthermore, the amount of metabolite being produced at 23 °C for the *A. niger* ATNT16 Δ*pyrG* Δ*pabaA Clagr3.30*_SM_S-tag_X_URA *An-hacA*_SM-X_PABA strain appeared to be lower than that of the *A. niger* ATNT16 Δ*pyrG* Δ*pabaA Clagr3.30*_SM_S-tag_X_URA strain, and there did not appear to be any metabolite production at 23 °C for the *A. niger* ATNT16 Δ*pyrG* Δ*pabaA Clagr3.6*_SM-X_URA *An-hacA*_SM-X_PABA strain. This suggests that, rather than increasing metabolite production, co-expression of *A. niger hacA* with either *Clagr3.6* or *Clagr3.30* was in fact detrimental to these enzymes and, thus, resulted in reduced or no metabolite production. Furthermore, metabolite production was not observed in the transformant strain co-expressing *Clagr3.26* and *hacA* at either 23 °C or 28 °C (Figure

6.19D). Again, this suggests that overexpression of *A. niger hacA* does not increase the amount of correctly-folded protein being produced at either temperature in order to achieve metabolite production.

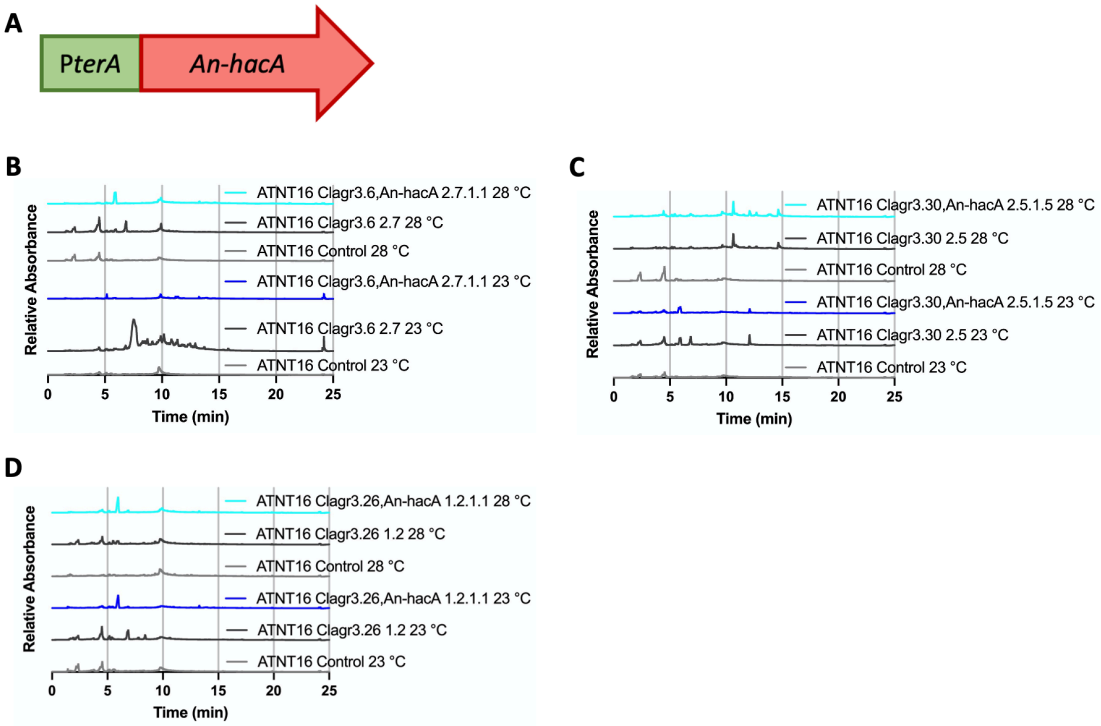


Figure 6.19. Metabolite production from *Clagr3.6*, *Clagr3.30* and *Clagr3.26* co-expressed with *hacA*. (A) Schematic representation of the expression construct containing the *hacA* gene. **(B)** HPLC analysis of culture filtrates of ATNT16 Δ*pyrG* Δ*pabaA Clagr3.6*_SM-X_URA *An-hacA*_SM-X_PABA 2.7.1.1 grown at 23 °C and 28 °C compared to ATNT16 Δ*pyrG* Δ*pabaA Clagr3.6*_SM-X_URA 2.7 and ATNT16 control. **(C)** HPLC analysis of culture filtrates of ATNT16 Δ*pyrG* Δ*pabaA Clagr3.30*_SM_S-tag_X_URA *An-hacA*_SM-X_PABA 2.5.1.5 grown at 23 °C and 28 °C compared to ATNT16 Δ*pyrG* Δ*pabaA Clagr3.30*_SM_S-tag_X_URA 2.5 and ATNT16 control. **(D)** HPLC analysis of culture filtrates of ATNT16 Δ*pyrG* Δ*pabaA Clagr3.26*_SM-X_URA *An-hacA*_SM-X_PABA 1.2.1.1 grown at 23 °C and 28 °C compared to ATNT16 Δ*pyrG* Δ*pabaA Clagr3.26*_SM-X_URA 1.2 and ATNT16 control.

6.4 Discussion

When attempting the heterologous expression of PKSs and NRPS-like enzymes from various lichen species in the previous two chapters, the results obtained suggested that the lichen-derived proteins exhibited a higher sensitivity to an increase in temperature relative to that seen in many other filamentous fungi. Therefore, it was decided that the heat sensitivity of lichens would be further investigated by focussing on the heat sensitivity of lichen sexual propagules, as well as on the heat sensitivity of proteins deriving from lichen primary metabolism genes. In addition, the

idea of co-expressing lichen secondary metabolism genes with a transcription factor known to assist in the correct folding of proteins was explored, in order to determine if this would be a suitable strategy to increase metabolite production from lichen SM genes in heterologous expression platforms, in addition to potentially help induce metabolite production from lichen SM genes for which attempts in previous chapters had been unsuccessful.

6.4.1 Heat Sensitivity of Lichen Ascospores

High temperature and desiccation both have effects on the cell at the level of interactions between water and macromolecules, and lichens are known to be desiccation-tolerant organisms (Crowe *et al.*, 1984; Crittenden and Porter, 1991; Prestrelski *et al.*, 1993; Wyatt *et al.*, 2015; Dijksterhuis, 2019). This could, therefore, imply that their ascospores will accumulate high concentrations of compatible solutes, such as trehalose or mannitol (Dijksterhuis, 2019), when in the desiccated state in order to withstand this type of stress. The high concentration of compatible solutes already present within the ascospores as a result of desiccation could also potentially confer resistance to high temperature. Indeed, temperature stress experiments presented in this chapter revealed that when lichen ascospores were exposed to dry heat stress for 30 min, for all three test species of lichen ascospore survival was possible up to between 70-80 °C. However, much lower resistance to elevated temperature was seen in the wet heat stress, with ascospores of all three test species failing to germinate when exposed to 40 °C and above for 30 min. This increased resistance of lichen ascospores to a dry heat treatment might be explained by the presence of desiccation-resistant solutes and could be advantageous in the natural environment once dispersal of the ascospores has occurred, as the ascospores are more likely to be exposed to higher temperatures when they are no longer protected by the lichen thallus. Therefore, the dry heat resistance of the ascospores will enable the survival of the ascospores prior to germination and association with the photobiont.

Lichens are generally regarded as extremely stress-tolerant organisms (Beckett *et al.*, 2008). This would, therefore, suggest that lichen ascospores would also display high levels of stress tolerance, and would be more resistant to heat than the

ascospores of non-lichenised species. Therefore, comparisons were made between ascospore survival in the lab model organism *A. nidulans* and representative lichen species as a pilot study. However, the results obtained in this study showed, perhaps surprisingly, that *A. nidulans* ascospores were capable of survival at higher temperatures than the ascospores of *X. parietina*, *L. chlarotera* and *A. punctata* when exposed to both wet heat and dry heat treatments. This suggests that the ascospores of *A. nidulans* are in fact more resistant to heat stress than ascospores of the test lichen species. This unexpected finding may in part be explained by previous reports that the lichen symbiosis increases the stress tolerance of each partner in comparison to each partner on its own (de Vera *et al*., 2008). Therefore, the fact that the lichen ascospores used in this study were not in association with the photobiont partner when subjected to wet or dry heat treatments may mean that the ascospores are not as tolerant to heat as would be expected, and display a reduced stress tolerance compared to that of the lichen as a whole. Furthermore, *A. nidulans* can be described as a tropical species (Raper and Fennell, 1965), meaning that the ascospores of this species are likely to encounter higher temperatures in its natural habitat. Therefore, it might in retrospect be expected that the ascospores of *A. nidulans* would be tolerant of higher temperatures. It was planned to make further comparisons with ascospores from additional non-lichenised ascomycete species, but there was insufficient time available.

Regardless of the unexpected results concerning the higher tolerance of *A. nidulans* ascospores to wet and dry heat compared to the ascospores of the lichen species used in this study, when considering the results of the lichen species alone it appears as though these results support the idea that lichens are extremely tolerant to environmental stresses. This is due to the fact that, when subjected to dry heat, the lichen ascospores are able to withstand temperatures much in excess of what it would feasibly be exposed to in its natural habitat. All three lichen species used in this study were collected locally in the UK, and are therefore found in temperate climates. It can be assumed that the lichen specimens collected for this study would be adapted to the lower temperatures found in temperate climates, and that they would not have routinely been exposed to extremely high temperatures. Thus, the fact that the ascospores of *X. parietina* and *L. chlarotera* were able to withstand a dry heat

treatment of 80 °C, and *A. punctata* ascospores were able to tolerate a dry heat treatment of 70 °C is remarkable. Furthermore, the results obtained here seem to correlate with previous studies carried out on lichen thalli, in which it has been observed that hydrated lichen thalli die when temperatures exceed 35 °C, whereas dry lichen thalli were able to survive up to 60 °C (Beckett *et al.*, 2008; Gasulla *et al.*, 2021). When comparing these findings to the results obtained here, it appears that whole lichen thalli and lichen ascospores show similar tolerances to wet heat, as both were able to survive up to 35 °C. However, it appears that lichen ascospores are more tolerant to dry heat than whole thalli, as the results from this study show that lichen ascospores can withstand temperatures in excess of 60 °C. One incidental benefit of these findings is that lichen ascospores might be purified of the presence of contaminating species (e.g. yeast and bacteria), which might not survive the 70-80 °C temperature treatment for 30 min, which will assist in the isolation and axenic culture of lichen-forming fungi.

6.4.2 Heat Sensitivity of Lichen Primary Metabolism Proteins

When assessing the growth of transformants expressing lichen primary metabolism genes, it was hard to draw general conclusions because the precise results varied according to the gene of interest and also the heterologous host organism (i.e. *A. niger* or *A. oryzae*). There was also a lack of successful complementation for the majority of assay genes. Nevertheless a few results were noteworthy. For example, it appeared that some of the lichen-derived proteins were sensitive to an increase in temperature. This was particularly apparent for the His1 protein from *E. prunastri*, which was not able to complement a heterologous host above 18-23 °C. This apparent heat sensitivity of proteins encoded by primary metabolism proteins, which are essential for survival of the lichen, suggests that certain proteins produced by lichen species are adapted to the lower temperatures found in their natural habitats. Thus, it is important to consider the natural habitat of the lichen species being studied in order to ensure that a suitable cultivation temperature is used when attempting to achieve the production of lichen-derived metabolites. However, the cultivation temperature of the expression platform should also be considered, as the optimum cultivation temperature of *A. niger* and *A. oryzae*, for example, is higher than that of

the optimum temperature for the lichen proteins. It is also noted that the *E. prunastri* PyrG protein did not show such a clear temperature effect, and that the *X. parietina* PabaA might even have shown better activity at higher temperature so it is difficult to generalise.

The lack of successful complementation observed in *A. niger* ATNT16 and *A. oryzae* OP12 strains expressing *Xp-pyrG*, *Xp-his1*, *Cg-pyrG* and *Cg-pabaA* is in agreement with the results from Chapter 4 for lichen PKSs. The results obtained here again suggest that the choice of expression platform is critical for the successful heterologous expression of lichen genes, including those involved in primary metabolism. Further to this, it appears that the successful heterologous expression of primary metabolism genes from a particular lichen species does not guarantee the successful heterologous expression of SM genes from the same species. This is evidenced by that fact that even though the functional expression of both *pyrG* and *his1* from *E. prunastri* was achieved in this study, attempts to heterologously express genes encoding both PKSs and NRPS-like enzymes from the same species was unsuccessful, as described in previous chapters. Equally, the successful heterologous expression of SM genes from a lichen species does not guarantee that the heterologous expression of primary metabolism genes from the same species will also be successful, as was found to be the case here for *C. grayi*.

6.4.3 Heat Sensitivity of Lichen Secondary Metabolism Proteins

Results obtained in the previous two chapters suggested that the proteins encoded by SM genes in *C. grayi* were more sensitive to increase in temperature than that seen for filamentous ascomycetes in general. This apparent heat sensitivity was confirmed by the dot blot experiments carried out in this chapter, with strains expressing either *Clagr3.11* or *Clagr3.30* being shown to have lower levels of protein production for both the Clagr3.11 and Clagr3.30 enzymes at 28 °C compared to 23 °C. Further to this, a strain expressing the hybrid *Clagr3.11*:T-TE$_{abrA}$ showed higher levels of production of the Clagr3.11:T-TE$_{abrA}$ hybrid enzyme at both 23 °C and 28 °C compared to the Clagr3.11 enzyme. This demonstrates that fusion of the Clagr3.11 A domain with the T and TE domains of the more thermostable AbrA resulted in a hybrid

enzyme with an increased thermostability and, therefore, increased amounts of protein production at both temperatures.

6.4.4 Co-Expression of Lichen Genes with an *A. niger* Native Transcription Factor

Studies involving the co-expression of the *A. niger* transcription factor *hacA* with lichen SM genes did not result in metabolite production at 28 °C. This implies that over-expression of the HacA transcription factor in the *A. niger* ATNT16 expression platform was not able to increase the levels of correctly-folded lichen-derived proteins within the heterologous host. Although UPR and HSR have been found to be linked, the *hacA* transcription factor is primarily involved in activation of UPR in fungal species (Cox and Walter, 1996; Mulder and Nikolaev, 2009; Weindling and Bar-Nun, 2015). However, the UPR is specific to the ER, and it can therefore be expected that increasing levels of HacA within *A. niger* ATNT16 would lead to the correct folding of proteins located in the ER rather than those found in the cytosol. This could perhaps explain why co-expression of *hacA* with the lichen SM genes had a negative impact on metabolite production at 23 °C, as cell resources would have been pushed towards production of HacA and the resulting upregulation of UPR-related proteins, rather than towards the production of the lichen-derived proteins.

6.5 Conclusions

The results obtained in this chapter regarding the heat sensitivity of lichen ascospores support the idea that lichens are highly stress-tolerant organisms which possess a range of mechanisms in order to allow the lichen to survive extreme environmental stresses. However, this resistance to high temperatures was only observed when the ascospores were in a desiccated state. The fact that protein production only occurs in lichens when they are in a hydrated state suggests that lichen proteins will not be resistant to the extremely high temperatures that lichen thalli are exposed to in the desiccated state. The results obtained in this chapter in relation to the heat sensitivity of lichen proteins deriving from both primary and secondary metabolism suggest that certain lichen proteins are adapted to the temperatures found in the natural habitat of the lichen, and may not be functional at elevated temperatures.

Future work following on from the results obtained in this chapter could focus on attempting the co-expression of other transcription factors or chaperones with the lichen SM genes studied in the previous chapters. Focussing on transcription factors or chaperones involved in HSR instead may result in the correct folding of the lichen-derived protein, and therefore lead to metabolite production at higher temperatures.

Chapter 7: General Discussion

The overall aim of the research undertaken in this study was to achieve the production and characterisation of SMs derived from lichen species. Lichens are known to produce a myriad of SMs, many of which are unique to lichens and have not been detected in other non-lichen fungal species (Crittenden and Porter, 1991; Stocker-Wörgötter, 2008; Elix and Stocker-Wörgötter, 2008; Calcott *et al.*, 2018). These natural products produced by lichens display a wide range of biological activities with potential for pharmaceutical applications (Boustie and Grube, 2005; Verma and Behera, 2015). However, it is not currently feasible to use lichens for the production of SMs on an industrial scale due to the fact that they possess an extremely slow growth rate and are not amenable to genetic manipulation (Sinnemann *et al.*, 2000; Miao *et al.*, 2001; Boustie and Grube, 2005; Verma and Behera, 2015). Therefore, alternative methods are required to achieve the production of lichen-derived natural products on an industrial scale.

Two approaches were used to achieve the production and characterisation of lichen-derived natural products. One approach focussed on the growth rate of the lichen mycobiont, with the view that increasing the growth rate of the mycobiont could lead to the eventual use of lichen species in biotechnology in the production of SMs. The generation of mutants of various lichen mycobionts possessing a faster growth rate would not only enable the biotechnological use of lichens to produce SMs, but would also help to increase our general understanding of growth regulation in lichens. Three species of lichens, *X. parietina*, *L. chlarotera* and *A. punctata*, were chosen for the creation of faster-growing mutants due to the fact that they are locally abundant, thus meaning that ample lichen material was available. The second approach focussed on using non-lichen expression platforms for the heterologous production and characterisation of lichen-derived SMs. Fungal heterologous expression platforms produce large amounts of metabolites, in addition to having low background metabolic profiles, meaning that they are well-suited to be used for the heterologous production of lichen natural products (Miao *et al.*, 2001; Geib and Brock, 2017). Further to this, a previous study by Sinnemann *et al.* (2000), in which the

successful heterologous expression of *S. crocea pyrG* was achieved, demonstrated that *Ascomycota* hosts show the potential of the heterologous expression of lichen-derived genes. Here, two types of lichen SM genes were used, that is genes encoding PKSs and NRPS-like enzymes. *A. niger* and *A. oryzae* expression platforms formed the basis in this study, as the *Aspergillus* genus is phylogenetically relatively closely related to the majority of lichens. Therefore, it was assumed that *Aspergillus* expression platforms are a suitable choice for the heterologous expression of lichen genes (Stenroos and DePriest, 1998).

7.1 Generation of Faster-Growing Lichen Mycobionts

Before carrying out the UV mutagenesis of lichen ascospores for generating faster-growing mutants of the mycobiont, the amount of time for which the ascospores of each species should be exposed to UV-C needed to be established. This involved exposing the spores of *X. parietina*, *L. chlarotera* and *A. punctata* to UV-C for different lengths of time to determine the exposure time which led to a 5-10% survival rate of the ascospores. The results of these initial studies revealed that the exposure times for *X. parietina*, *L. chlarotera* and *A. punctata* ascospores were 300 s, 216 s and 280 s, respectively. As mentioned in Chapter 3, the shorter exposure time required to achieve a 5% survival rate for *L. chlarotera* ascospores suggests that the spores of this species are less tolerant to UV-C exposure, but the molecular basis for this difference has not been determined yet.

Once the exposure times had been determined for each of the lichen species, ascospores of *X. parietina* were exposed to UV-C for 300 s in an attempt to generate mutants of the mycobiont which possessed a relatively faster growth rate than that of *X. parietina* mycobiont colonies growing on control plates. It was decided that a faster-growing colony would be defined as those which showed an area 3x greater than that of the control. Of the 400 surviving *X. parietina* ascospores that were exposed to UV-C, only six colonies were identified as having a colony area more than three times that of the average area of colonies growing on the control plates, and therefore potentially possess a faster growth rate. Attempts to produce faster-growing mutants of the mycobionts of *L. chlarotera* and *A. punctata* were not able to

progress due to the fact that insufficient numbers of ascospores were collected from the available lichen material.

The combination of time constraints and the inability to collect enough spores for *L. chlarotera* and *A. punctata* meant that only preliminary work was carried out in this study. Lichen growth is known to be a multifactorial process which is affected by a multitude of factors and is controlled by complex regulatory mechanisms (Benedict, 1990; Honegger, 1993; Ahmadjian, 1993; Armstrong and Bradwell, 2010; Armstrong and Bradwell, 2011). Thus, it can be assumed that multiple mutations in different genes would be needed in order to produce a lichen mycobiont with an appreciably faster growth rate. It is unlikely, however, that UV mutagenesis would induce all the mutations needed in a single mycobiont colony, meaning that multiple rounds of UV mutagenesis would be required in order to achieve all the mutations that are required for faster growth. Further to this, the randomised nature of UV mutagenesis means that mutations which are detrimental to lichen growth could also be introduced into the lichen mycobiont, thus perhaps resulting in mutants with a slower growth rate. The likelihood of introduction of these unwanted mutations into the genome of the mycobiont would increase with each successive round of UV mutagenesis. In addition, if genes supporting a faster growth as present in saprophytic species have been lost in symbiotic mycobionts, a UV mutagenesis approach can most likely not compensate for this gene loss.

These observations are important in the sense that they suggest that a method which introduces mutations into the mycobiont genome in a random manner is unsuitable for the production of mycobiont strains with an increased growth rate. Instead it would perhaps be better to use a targeted method in which mutations are introduced into specific genes within the mycobiont genome. This approach would ensure that any changes in growth rate could be attributed to a particular mutation and would also prevent the introduction of any additional unwanted mutations into the mycobiont genome. Furthermore, studies focussing on genera which contain both obligate and facultative lichen species could allow the comparison of genomes to perhaps elucidate genes that are specific for the symbiosis. These genes could, therefore, be targeted in an attempt to increase the growth rate of the mycobiont.

7.2 Heterologous Expression of Lichen Polyketide Synthases and Non-Ribosomal Peptide Synthetase-Like Enzymes

At the onset of this study, there had not been a successful example of the functional expression of a lichen SM gene in a heterologous host. Although the successful heterologous expression of the *S. crocea pyrG* gene in *A. nidulans* by Sinnemann *et al.* (2000) led to the suggestion that lichen genes were suitable for heterologous expression, this remained the only successful example for some time. This was despite numerous attempts by various different research groups, in which they attempted to heterologously express different PKS genes from various lichen species in a range of *Aspergillus* expression platforms (Chooi *et al.*, 2008; Gagunashvili *et al.*, 2009; Armaleo *et al.*, 2011; Wang *et al.*, 2016; Bertrand and Sorensen, 2019a; Bertrand and Sorensen, 2019b). This lack of success led to the assumption that there was a systematic problem associated with the heterologous expression of lichen-derived SM genes in *Ascomycota* hosts (Abdel-Hameed *et al.*, 2018). Abdel-Hameed *et al.* (2018) and Bertrand and Sorensen (2019a) tested numerous different hypotheses as to why the heterologous expression of lichen SM genes remained unsuccessful, but no definitive answer was found. It was not until 2021 that the first example of the successful heterologous expression of a lichen SM gene was achieved, that of the *S. alpinum atr1* gene (Kim *et al.*, 2021). However, Kim *et al.* (2021) needed to create a new expression platform in the dothideomycete *A. rabiei*, a non-lichenised relative of lichen-forming fungi, as their attempts to express *S. alpinum atr1* in *Aspergillus* expression platforms were unsuccessful. These results were, therefore, still in agreement with Abdel-Hameed *et al.* (2018) in that a systematic problem appeared to exist with the heterologous expression of lichen SM genes in *Ascomycota*, or at least in *Aspergillus*, hosts.

The results obtained in this study show that the successful expression of two PKSs and two NRPS-like enzymes, all from the lichen *C. grayi*, was achieved in *Aspergillus* expression platforms. These results are significant due to the fact that they directly contradict the suggestion made previously by others that there is a systematic problem with the functional expression of lichen-derived SM genes in *Ascomycota* hosts. However, currently, we can only speculate on the reasons for the successful heterologous expression of especially these genes. Although we achieved success with

multiple lichen SM genes, our attempts to heterologously express some PKSs (from *C. grayi*, *E. prunastri* and *U. longissima*) and NRPS-like enzymes (from *E. prunastri*) were unsuccessful. As mentioned previously, this implies that the choice of expression platform in combination with the choice of lichen SM gene to be expressed is crucial if heterologous expression is to be successful. This hypothesis was further supported by the results obtained in Chapter 6 when attempting the heterologous expression of various lichen primary metabolism genes, as not all attempts were successful. Again, it is not clear what makes a particular combination of expression platform and lichen gene successful and another unsuccessful. However, it is noteworthy that all successful approaches for secondary metabolite gene expression resulted from *C. grayi*, and future studies should focus on related species such as *Cladonia uncialis* or *Cladonia rangiferina* to analyse whether genes from these mycobionts show a high success rate in heterologous metabolite production.

7.3 Discovery of a Novel Lichen Non-Ribosomal Peptide Synthetase-Like Enzyme

The metabolites atromentin, ascocorynin and polyporic acid are compounds with very similar structures, each of which possesses a quinone core. Polyporic acid is a compound with non-hydroxylated side chains and a quinone core, whereas atromentin is a compound where the quinone core has two hydroxyphenyl-side chains. The monohydroxylated ascocorynin can be described as an intermediate between polyporic acid and atromentin, as its quinone core has one hydroxyphenyl-side chain and one phenyl-side chain (Quack *et al.*, 1982). Thus, ascocorynin, and potentially atromentin, could theoretically be produced by different biosynthetic pathways. One pathway involves a single-enzyme approach in which an NRPS-like enzyme is solely responsible for the production of the metabolite. This would involve the condensation of two *p*-hydroxyphenylpyruvate molecules by the NRPS-like enzyme for the production of atromentin, two phenylpyruvate molecules for the production of polyporic acid, or the condensation of one *p*-hydroxyphenylpyruvate molecule and one phenylpyruvate molecule for the production of ascocorynin (Quack *et al.*, 1982). The alternative approach would involve the use of tailoring enzymes for the production of ascocorynin or atromentin, with polyporic acid acting as starter molecule that gets hydroxylated once to form ascocorynin, and a subsequent

hydroxylation of ascocorynin to produce atromentin (Quack *et al.*, 1982). Of NRPS-like enzymes from ascomycetes studied so far, all appear to show a high substrate specificity, and are only able to carry out the condensation of two identical α-keto acids to form a single product (Schneider *et al.*, 2007; Yeh *et al.*, 2012; Geib *et al.*, 2016; Geib *et al.*, 2019). These findings suggest that the biosynthesis of ascocorynin would, therefore, involve the use of tailoring enzymes to produce a compound with one hydroxylated side chain and one unhydroxylated side chain. *A. sarcoides* is an ascomycete that produces pink to purple fruiting bodies and the main metabolite responsible for this colouration is ascocorynin (Quack *et al.*, 1982). In an attempt to characterise its biosynthesis, the NRPS-like enzyme AcyN had been identified and our study showed that it is highly specific for the production of polyporic acid. However, the monooxygenase MO6277 from *A. sarcoides* specifically performs a monohydroxylation of polyporic acid in *A. sarcoides*, resulting in the production of ascocorynin (Wieder *et al.*, 2022, in press). This shows that in *A. sarcoides*, the "two enzyme" pathway is used for the production of ascocorynin.

However, our results from the heterologous expression of *C. grayi Clagr3.11* show that the production of three metabolites (atromentin, ascocorynin and polyporic acid) can also be performed by a single enzyme. Thus, the production of each of these metabolites is carried out in a one-enzyme process in which no tailoring enzymes are necessary. Therefore, the Clagr3.11 enzyme is capable of using either solely *p*-hydroxyphenylpyruvate, solely phenylpyruvate or a combination of the two as substrate to produce atromentin, polyporic acid or ascocorynin, respectively. These results are remarkable as they provide the first example of an NRPS-like enzyme with a flexible substrate specificity that results in a mixed product portfolio. The relaxed substrate specificity of the enzyme was subsequently confirmed in *in vitro* assays using the purified Clagr3.11 enzyme, as Clagr3.11 was able to produce a mixture of atromentin, ascocorynin and polyporic acid in reactions containing a combination of *p*-hydroxyphenylpyruvate and phenylpyruvate. Furthermore, we carried out domain-swapping experiments with the Clagr3.11 enzyme and the AbrA atromentin synthetase from *A. brasiliensis*, which confirms that the A domain of Clagr3.11 is responsible for the relaxed substrate specificity of the enzyme.

The observed flexible substrate specificity of the Clagr3.11 enzyme is particularly significant as it provides the scope to use alternative substrates with this enzyme. This would enable the production of novel compounds not found in nature, and was confirmed as feasible in our studies when using an *A. oryzae* OP12 strain expressing *Clagr3.11* in feeding experiments. Analysis of the metabolites produced by the *A. oryzae* OP12 strain when supplied with 4-chloro-phenylalanine showed that chlorinated products were present in the extracts, therefore demonstrating that Clagr3.11 is able to accept the α-keto acid derivative of 4-chloro-phenylalanine as a substrate in addition to *p*-hydroxyphenylpyruvate and phenylpyruvate. As discussed previously in Chapter 5, this provides the opportunity to use a range of alternative substrates with the Clagr3.11 enzyme in order to produce analogous metabolites with as-yet-unknown biological activities that could have potential pharmaceutical applications. It is known that biosynthetic modifications of natural products leads to the production of non-natural analogues which possess improved or modified biological activities (Le *et al.*, 2014; Chu *et al.*, 2016; Pandey *et al.*, 2016; Nguyen *et al.*, 2020).

7.4 Heat Sensitivity of Lichens

When attempting the heterologous expression of various lichen-derived SM genes, we found that the lichen proteins appeared to demonstrate a sensitivity to higher temperatures, as metabolite production was not observed at 28 °C for all lichen enzymes with the exception of the Clagr3.11 enzyme. As discussed previously, this suggests that lichen proteins are adapted to the temperatures found in the natural habitat of each lichen species. As a result of this, careful consideration of the temperature that a lichen species would be expected to be exposed to in its natural environment should be taken when determining the incubation temperature for a heterologous host expressing a lichen gene. The use of an unsuitable temperature could lead to a rapid denaturation of the produced enzyme, resulting in the lack of metabolite production in a heterologous host in which expression of the lichen gene has actually been successful.

In response to this observation, we decided to investigate the heat sensitivity of lichen proteins involved in primary metabolism. *A. niger* ATNT16 and *A. oryzae*

OP12 transformants expressing *E. prunastri pyrG*, *E. prunastri his1* and *X. parietina pabaA* were grown at a range of temperatures and displayed reduced growth at higher temperatures. This suggests that these lichen proteins are also sensitive to higher temperatures. Our results are in agreement with that of Sinnemann *et al.* (2000), and mean that the idea of lichen proteins being adapted to the temperatures found in the natural habitat of the lichen encompasses lichen primary metabolism proteins as well as secondary metabolism proteins. In addition to investigating the heat sensitivity of lichen primary metabolism proteins, we also attempted to use the co-expression of *A. niger hacA* with various lichen SM genes in order to induce metabolite production at 28 °C. However, this approach was not successful, with *hacA* co-expression in fact appearing to be detrimental to metabolite production. As mentioned previously, this suggests that HSR machinery should be targeted to increase levels of correctly-folded lichen proteins, rather than machinery involved in the UPR.

Upon further investigation of the heat sensitivity of lichens, our studies involving the heat sensitivity of lichen ascospores demonstrated that the ascospores of *X. parietina*, *L. chlarotera* and *A. punctata* appear to be extremely tolerant to dry heat but are much more sensitive to wet heat, with ascospores failing to germinate after being exposed to temperatures above 35 °C during wet heat treatments. As discussed in Chapter 6, the resistance of lichen ascospores to high temperatures during dry heat treatments supports the idea that lichens are extremely stress-tolerant organisms, especially when desiccated. Furthermore, the observed sensitivity of lichen ascospores to more moderate temperatures during wet heat treatments suggests that that the extremophile nature of lichens can be attributed to their ability to undergo desiccation. This is due to the fact that lichens enter a state of anhydrobiosis when in the desiccated state. Although this lack of metabolic activity allows lichens to survive extreme stresses, it appears that their stress tolerance is significantly reduced when metabolically active, as would be the case when the lichen is in a hydrated state. Further to this, the apparent heat sensitivity of lichen proteins involved in both primary metabolism and secondary metabolism further supports the idea that lichens are inherently sensitive to heat when they are hydrated and metabolically active. If lichens were resistant to higher temperatures when metabolically active, it would be expected that the lichen proteins would not show

the heat sensitivity observed here. It would be interesting to widen this study to lichen species from tropical regions to investigate whether they show a different pattern in terms of heat sensitivity of their proteins.

7.5 Future Work

The work undertaken in this study demonstrates the suitability of using heterologous expression of lichen genes as a method to produce lichen-derived natural products. However, further work following on from the results established here needs to be undertaken in order to realise the full potential of lichen SMs and their applications. As such, suggestions for areas of further investigation are described below.

(1) Heterologous expression of additional lichen SM genes. The results obtained in this study have demonstrated that lichen genes encoding both PKSs and NRPS-like enzymes can be heterologously expressed in *Aspergillus* expression platforms. However, not all lichen SM genes are guaranteed to be functionally expressed. Therefore, attempting the heterologous expression of lichen PKSs and NRPS-like enzymes from a wide range of lichen species may result in the successful heterologous production and characterisation of additional lichen-derived SMs.

(2) Use of Clagr3.11 enzyme to produce novel natural products. The discovery of an NRPS-like enzyme which possesses a flexible substrate specificity provides the opportunity to create novel and non-natural metabolites analogous to those naturally produced by the Clagr3.11 enzyme. This could be achieved through the use of Clagr3.11 with alternative substrates, as well as through domain-swapping experiments to create novel NRPS-like enzymes which incorporate domains from the Clagr3.11 enzyme.

(3) Investigation of heterologous expression of lichen SM genes in *Aspergillus*. The reasons behind why the heterologous expression of some lichen SM genes in *Aspergillus* expression platforms is successful, but is unsuccessful for others is currently not well understood. Further research into the possible reasons as to why

this is the case should be carried out in order to increase our understanding, in addition to potentially being able to circumvent these obstacles to increase the number of lichen SM genes that can be functionally expressed in *Aspergillus* heterologous hosts. Investigations into the supplementation of growth media with polyols could be carried out in order to determine if this leads to metabolite production.

7.6 Concluding Remarks

The results of this study are significant, as we have demonstrated the first successful examples of the functional expression of lichen-derived PKSs and NRPS-like enzymes within *Aspergillus* heterologous hosts. This confirms that the heterologous expression of lichen SM genes is a suitable method for the production of lichen-derived natural products on an industrial scale, thus meaning that the potential of lichen SMs and their pharmaceutical applications can be investigated and exploited. Further to this, the results obtained in this study associated with that of the *C. grayi* Clagr3.11 enzyme are particularly significant due to the fact that we believe that this is the first example of an NRPS-like enzyme with an unprecedented flexible substrate specificity. This discovery provides enormous scope for the use of this enzyme in the production of novel natural products not found in nature. Initial studies carried out confirm that this is possible, with the successful production of chlorinated compounds using the Clagr3.11 enzyme.

Appendices

Appendix 1

The table below lists the oligonucleotides used for the generation of expression constructs and for the screening of transformants. Sequences shown are in 5' to 3' orientation.

#	Name	Sequence	Purpose
1	PterA_orsA_for	CATCACAGCACCATGGCTCCAAATCACGTTCTT	Amplification of *orsA* from *A. nidulans*
2	TE_Nco_IF_P2A_r	TAGCGCCGCTTCTCCATGGTGCACCGATCAAGCGATCTAG	Amplification of *orsA*:*TE(terA)*$_L$
3	orsA_Nco_P2A_r	TAGCGCCGCTTCTCCATGGTTCCACCGCCCGAGTCAC	Amplification of *orsA* from *A. nidulans*
4	TE_P2A_r	TCGCCAGCCTGCTTCAACAGGCTGAAATTGGTAGCGCCGCTTGCACCGATCAAGCGATCTAG	Amplification of *orsA*:*TE(terA)*$_L$
5	P2A_Me473_f	TGTTGAAGCAGGCTGGCGACGTGGAGGAAAACCCTGGCCCTATGCCCTCTTCACTCAAACTCC	Amplification of *Me473* from *A. mellea*
6	Me473_Nco_P2A_r	CGCCGCTTCTCCATGGCACTGGCACCGCGACC	Amplification of *Me473* from *A. mellea*
7	orsA_P2A_r	TCGCCAGCCTGCTTCAACAGGCTGAAATTGGTAGCGCCGCTTTCCACCGCCCGAGTCAC	Amplification of *orsA* from *A. nidulans*
8	TE00145_cont_f	CTGCAACCTCGATCGTACTG	Cloning control primer for *orsA*:*TE*$_L$

			and *orsA*:*TE$_L$*_*Me473*
9	lucOPTmi_up	GATGTCGACCTCGATGTGG	Cloning control primer for *orsA:TE$_L$* and *orsA*
10	orsAcode_f1	GTACCTTTGAGGGCATTAACC	Cloning control primer for *orsA* and *orsA_Me473*
11	Me473_cont_r	GCACCCCAGCTGACGAAC	Cloning control primer for *orsA:TE$_L$_Me473* and *orsA_Me473*
12	Me473_Nsi_f	CATCACAGCACCATGCATCCCTCT TCACTCAAACTCC	Amplification of *Me473* from *A. mellea*
13	Me473_Strep_r	GGGTGGGACCAATGCATCACTGG CACCGCGACC	Amplification of *Me473* from *A. mellea*
14	Me473_cont_f	CGTCACTCATCAACACGTTCG	Cloning control primer for *Me473*
15	Seq_AttrpC_rv	GAATTTTACCAGTGGCCTAGG	Cloning control primer in T*trpC*
16	O-MeT40_Strep_f	GTTCGAGAAGCCATGGATGGCTG CCTCACGCATC	Amplification of *O-MeT-c40* from *E. prunastri*
17	O-MeT40_Nco_r	CTGCTGTTACCATGGGATACGCCT ATGACGGTCTC	Amplification of *O-MeT-c40* from *E. prunastri*
18	O-MeT41_Strep_f	GTTCGAGAAGCCATGGATGATGG AAACAGCGACAACTG	Amplification of *O-MeT-c41* from *E. prunastri*

19	O-MeT41_Nco_r	CTGCTGTTACCATGGGCTCAACCA TGACTGATTGG	Amplification of *O-MeT-c41* from *E. prunastri*
20	O-MeT40_f2	GTTACTCCTGGGCCTCAATC	Cloning control primer for *O-MeT-c40*
21	O-MeT41_f2	GGATTTGATTGGGCTTCTCTTG	Cloning control primer for *O-MeT-c41*
22	Ep_PKSc40_f	CATCACAGCACCATGGGCTCTACC AAGACATCCGTCC	Amplification of *PKS-c40* from *E. prunastri*
23	Ep_PKSc40frag1_r	CTGTCGCATTTGTTGCAAGC	Amplification of *PKS-c40* from *E. prunastri*
24	Ep_PKSc40frag2_f	GCTTGCAACAAATGCGACAG	Amplification of *PKS-c40* from *E. prunastri*
25	Ep_PKS-c40_r	ATCACTGCTGCCATGGACATCTCC CTTCTCTTCTCC	Amplification of *PKS-c40* from *E. prunastri*
26	Ep_PKSc41_f	CATCACAGCACCATGGCTGCTTCA CTATCATTATACATC	Amplification of *PKS-c41* from *E. prunastri*
27	Ep_PKSc41frag1_r	GAGTGTTGAGAGATCAATGTCG	Amplification of *PKS-c41* from *E. prunastri*
28	Ep_PKSc41frag2_f	CGACATTGATCTCTCAACACTC	Amplification of *PKS-c41* from *E. prunastri*

29	Ep_PKS-c41_r	ATCACTGCTGCCATGGGAAGCGTGTTCCAGCTACC	Amplification of *PKS-c41* from *E. prunastri*
30	Ep_PKSc40_cont_f	GAGCGGGTCGATGTCATATG	Cloning control primer for *PKS-c40*
31	Ep_PKSc41_cont_f	ACAGGCATCTGCTGACTCC	Cloning control primer for *PKS-c41*
32	UlPKS6_SM-X_f	CATCACAGCACCATGGCTTCCCAAGTTCTACTCC	Amplification of *UlPKS6* from *U. longissima*
33	Ul_PKS6_frag1_r	CAGCAAGCATTCCAGAAGAG	Amplification of *UlPKS6* from *U. longissima*
34	Ul_PKS6_frag2_f	CTCTTCTGGAATGCTTGCTG	Amplification of *UlPKS6* from *U. longissima*
35	Ul_PKS6_r	ATCACTGCTGCCATGGTCAATTTTCATCCGCGATAG	Amplification of *UlPKS6* from *U. longissima*
36	Ulon_PKS6_f	TGCTCTGTCGAGGAAGTCTG	Cloning control primer for *UlPKS6*
37	Clagr3.6_Nco_f	CATCACAGCACCATGGGCGCTGGGATGCAGTTCCTC	Amplification of *Clagr3.6* from *C. grayi*
38	Clagr3.6_frag1_r	CAATATCGCTGAAGGTACCAC	Amplification of *Clagr3.6* from *C. grayi*
39	Clagr3.6_frag2_f	GTGGTACCTTCAGCGATATTG	Amplification of *Clagr3.6* from *C. grayi*

40	Clagr3.6_Nco_r	TCACTGCTGCCCATGGCCATTCTTCACCATCTCCTC	Amplification of *Clagr3.6* from *C. grayi*
41	Clagr3.21_Nco_f	CATCACAGCACCATGGGCTCAACTGTACTGTTCTCACGC	Amplification of *Clagr3.21* from *C. grayi*
42	Clagr321_frag1_r	GACTCCTTCCGTGTTAATGAC	Amplification of *Clagr3.21* from *C. grayi*
43	Clagr321_frag2_f	GTCATTAACACGGAAGGAGTC	Amplification of *Clagr3.21* from *C. grayi*
44	Clagr3.21_Nco_r	TCACTGCTGCCCATGGGCCAAAATCCATCATCATTGAG	Amplification of *Clagr3.21* from *C. grayi*
45	Clagr3.26_Nco_f	CATCACAGCACCATGGGCGCTGAAGAACTTCAACTTCTTC	Amplification of *Clagr3.26* from *C. grayi*
46	Clagr326_frag1_r	CTGACTCCATGAGCGGTTC	Amplification of *Clagr3.26* from *C. grayi*
47	Clagr326_frag2_f	GAACCGCTCATGGAGTCAG	Amplification of *Clagr3.26* from *C. grayi*
48	Clagr3.26_Nco_r	TCACTGCTGCCCATGGCACTGCATTGGACTTGAGTC	Amplification of *Clagr3.26* from *C. grayi*
49	Clagr3.31_Nco_f	CATCACAGCACCATGGGCATGGAGATATTGCTCTTCGGTG	Amplification of *Clagr3.31* from *C. grayi*

50	Clagr331_frag1_r	CTTTCCGGAGACAGCTTCG	Amplification of *Clagr3.31* from *C. grayi*
51	Clagr331_frag2_f	CGAAGCTGTCTCCGGAAAG	Amplification of *Clagr3.31* from *C. grayi*
52	Clagr3.31_Nco_r	TCACTGCTGCCCATGGGATCAATTTGGCATCTGCAGC	Amplification of *Clagr3.31* from *C. grayi*
53	Clagr3.6_cont_f	CTGATCATCGAGCCTCTTCC	Cloning control primer for *Clagr3.6*
54	Clagr3.21_cont_f	CGATTCCCATTAAAGATAAGCTG	Cloning control primer for *Clagr3.21*
55	Clagr3.26_cont_f	CAGGCTACGATGAAGAATCTC	Cloning control primer for *Clagr3.26*
56	Clagr3.31_cont_f	GCTTCTATCGATGCTCTCAAAC	Cloning control primer for *Clagr3.31*
57	PterAend_fw	CCTCCAAGAGAGATCCAGAC	Cloning control primer in P*terA*
58	EpNRPSL1_Strep_f	GTTCGAGAAGCCATGGATGGCTAGCACGGATCTCC	Amplification of *EpNRPSL1* from *E. prunastri*
59	EpNRPSL1_Nco_r	ACTGCTGTTACCATGGCCTGGTTATCCGACGATACC	Amplification of *EpNRPSL1* from *E. prunastri*
60	EpNRPSL2_Strep_f	GTTCGAGAAGCCATGGATGGCACGATCCATTAGTCTAAC	Amplification of *EpNRPSL2* from *E. prunastri*

61	EpNRPSL2_Nco_r	ACTGCTGTTACCATGGCTATACATCCTCAAATGCGTCTC	Amplification of *EpNRPSL2* from *E. prunastri*
62	EpNRPSL3_Strep_f	GTTCGAGAAGCCATGGATGAAGAACCAAAGAAAAAGAGC	Amplification of *EpNRPSL3* from *E. prunastri*
63	EpNRPSL3_Nco_r	ACTGCTGTTACCATGGGACAAATATGGTTAGGATGGTG	Amplification of *EpNRPSL3* from *E. prunastri*
64	EpNRPSL1_cont_f	GCGAAATATCCGTGGCCATG	Cloning control primer for *EpNRPSL1*
65	EpNRPSL2_cont_f	CTGCCATGTACGACTCACC	Cloning control primer for *EpNRPSL2*
66	EpNRPSL3_cont_f	GGCTTGAGGATGAATACAGAG	Cloning control primer for *EpNRPSL3*
67	Clagr311_Strep_f	GTTCGAGAAGCCATGGATGGCGGCACAGAACCTC	Amplification of *Clagr3.11* from *C. grayi*
68	Clagr3.11_Nco_r	ACTGCTGTTACCATGGCGCATTCAACCTGGCCATC	Amplification of *Clagr3.11* from *C. grayi*
69	Clagr3.30_Strep_f	GTTCGAGAAGCCATGGATGTGTTTTCGGCATCTTACAG	Amplification of *Clagr3.30* from *C. grayi*
70	Clagr3.30_Nco_r	ACTGCTGTTACCATGGCAAAGCAGGACAGGGTACC	Amplification of *Clagr3.30* from *C. grayi*

71	Clagr3.11_cont_f	CTCAACCTCGCATATTTCCTC	Cloning control primer for *Clagr3.11*
72	Clagr3.30_cont_f	CTTCATGCAGACCCAGCAC	Cloning control primer for *Clagr3.30*
73	Clag311A_abrAT_r	GGTGCGGTAGTCGCCAGCCTCGAATGCCGTTCG	Amplification of *Clagr3.11* A domain from *C. grayi*
74	Clag311A_abrAT_f	ACGGCATTCGAGGCTGGCGACTACCGCACCTATC	Amplification of *abrA* T+TE domains from *A. brasiliensis*
75	abrATE_NcoI_r	ACTGCTGTTACCATGGTCAAATTCCTCTTGCATCTAGTG	Amplification of *abrA* T+TE domains from *A. brasiliensis*
76	Clag311TabrATE_r	GGACTGCAGCGTCACGACGGGATTGTAGCTGGTG	Amplification of *Clagr3.11* A+T domains from *C. grayi*
77	Clag311TabrATE_f	AGCTACAATCCCGTCGTGACGCTGCAGTCCCAG	Amplification of *abrA* TE domain from *A. brasiliensis*
78	Clagr311A_cont_f	CATATGCGCCTGACGATGATG	Cloning control primer for *Clagr3.11* hybrid enzymes
79	Ep_pyrG_prom_f1	GTGCATGGAAGACAAGATCC	Amplification of putative *pyrG* from *E. prunastri*

80	Ep_pyrG_down_r1	GATGAAGTTTATGAGAACAGCG	Amplification of putative *pyrG* from *E. prunastri*
81	Ep_his1_prom_f2	CATCCTCAGAGTCACCAAAAC	Amplification of putative *his1* from *E. prunastri*
82	Ep_his1_down_r2	GGATTCGGTGGGATACTGTC	Amplification of putative *his1* from *E. prunastri*
83	Ep_pyrG_cont_f	GAGTCTGACCACGGCTAC	Cloning control primer for putative *pyrG* from *E. prunastri*
84	pJET1.2_rev	AAGAACATCGATTTTCCATGGCAG	Cloning control primer in pJET1.2
85	Ep_his1_cont_f	AGACGGGATTGTGGATCTTG	Cloning control primer for putative *his1* from *E. prunastri*
86	HisAnCont_f	GCTTCACTGCGCTGAGCG	Control primer for *his1* selection marker
87	HisAnNotI_r	GCGGCCGCGGATTCCAGAGTGTG AAGCTG	Control primer for *his1* selection marker
88	pJET1.2_for	CGACTCACTATAGGGAGAGCGGC	Cloning control primer in pJET1.2
89	URAblast_Cont_r	CTGCCTCTGGGTACTTGAC	Control primer for *pyrG* selection marker

90	Xp_pyrG_prom_f1	GCTTGTCAAAGGAGTGGTCTC	Amplification of putative *pyrG* from *X. parietina*
91	Xp_pyrG_down_r1	GTTCTAGTCGATGTCGAAAGG	Amplification of putative *pyrG* from *X. parietina*
92	Xp_pabaA_prom_f1	GAAGAAGTGGCAAATGCAAAAG	Amplification of putative *pabaA* from *X. parietina*
93	Xp_pabaA_down_r1	CTTCCGAGATCTCGCTAATC	Amplification of putative *pabaA* from *X. parietina*
94	Xp_his1_prom_f1	CGTCGGTACTGACCAGTTAC	Amplification of putative *his1* from *X. parietina*
95	Xp_his1_down_r1	GGCCTGAAATGGTGGATAGTG	Amplification of putative *his1* from *X. parietina*
96	Xp_pyrG_cont_f	GTCCTCATCAGGCGATACAC	Cloning control primer for putative *pyrG* from *X. parietina*
97	Xp_pabaA_cont_f	GATGTTCTCGCTGCCTCTC	Cloning control primer for putative *pabaA* from *X. parietina*
98	Xp_his1_cont_f	CTCGAGGCTATTGACACAGTG	Cloning control primer for putative *his1* from *X. parietina*

99	Cg_pyrG_prom_f2	GGTCATATCCACGGGACAAG	Amplification of putative *pyrG* from *C. grayi*
100	Cg_pyrG_down_r2	GTCAGACCCGTCTCGAATC	Amplification of putative *pyrG* from *C. grayi*
101	Cg_pabaA_prom_f2	CTCCACCGCCTCAGCAAC	Amplification of putative *pabaA* from *C. grayi*
102	Cg_pabaA_down_r2	GCATGACTCACTCACTTTACTG	Amplification of putative *pabaA* from *C. grayi*
103	Cg_pyrG_cont_f	GCGTCAAGGCTGCGAGAG	Cloning control primer for putative *pyrG* from *C. grayi*
104	Cg_pabaA_cont_f	CAGCTCCTTCGCTCCCTTG	Cloning control primer for putative *pabaA* from *C. grayi*
105	HacAAn_5'_f	CAAACTTCTCATCACAGCACCATG GAAGAAGCATTCTCTCCAG	Amplification of *hacA* from *A. niger*
106	HacAAn_5'_r	CGGACACTGCAGGATGTTGTGTC ACGTC	Amplification of *hacA* from *A. niger*
107	HacAAn_3'_f	CAACATCCTGCAGTGTCCGTCGCT GG	Amplification of *hacA* from *A. niger*
108	HacAAn_3'_r	ATTGAAATCACTGCTGCCATGGCT AACAGCCAGCTGCAATGC	Amplification of *hacA* from *A. niger*
109	HacAAn_cont_f	GTATGTACACGATGCTAATTCGAC	Cloning control primer for *hacA*

Appendix 2

The table below gives details of the collection sites for the lichen species studied.

Lichen Species	Habitat	Collection Site	Collection Date
Xanthoria parietina	Metal railings	University Park Campus, Nottingham	17/1/18
	Epiphytic	Quorn, Leicestershire	9/2/18
Lecanora chlarotera	Epiphytic	Via Gellia, Derbyshire	11/3/18
		Via Gellia, Derbyshire	30/10/18
		Monsal Head, Derbyshire	29/7/19
Amandinea punctata	Epiphytic	Via Gellia, Derbyshire	11/3/18
		Monsal Head, Derbyshire	29/7/19

Appendix 3

The table below shows the areas of *X. parietina* colonies growing on the control plates.

Plate Number	**Colony Number**	**Area of Colony (pixels)**
Control i	1	692
	2	8939
	3	11,561
	4	2804
	5	10,441
	6	2640
	7	2108
	8	4163
	9	2924
	10	615
	11	1096
	12	7894
	13	6580
	14	4801
	15	3298
	16	7823
	17	579
	18	2435
Control ii	1	3733
	2	21,324
	3	9874
	4	1316
	5	357
	6	883
	7	9869
	8	14,148
	9	8586

	10	9662
	11	168
	12	9786
	13	11,041
	14	9893
Control iii	1	1346
	2	273
	3	3873
	4	8396
	5	7110
	6	15,698
	7	7635
	8	5694
	9	3732
	10	1719
	11	10,706
	12	5770
	13	200
	14	1713
	15	4962
	16	10,091
	17	12,895

The table below shows the areas of *X. parietina* colonies growing on the treatment plates.

Plate Number	**Colony Number**	**Area of Colony (pixels)**
A-1	1	5695
	2	5768
	3	2198
	4	980
	5	242

	6	136
	7	1844
	8	1265
	9	178
	10	117
	11	2238
	12	483
	13	1199
	14	1382
	15	1704
	16	3659
	17	312
	18	6593
	19	1944
A-2	1	5456
	2	413
	3	4852
	4	415
	5	434
	6	528
	7	46
A-3	1	3213
	2	5220
	3	130
A-5	1	168
	2	146
	3	3806
	4	2381
	5	6178
	6	4770
	7	1045

A-6	1	3956
	2	134
	3	17,625
	4	591
	5	9269
	6	348
	7	6225
	8	299
	9	1270
	10	1906
	11	19
A-7	1	147
	2	141
	3	7449
	4	142
	5	5513
	6	135
	7	7621
A-9	1	2970
	2	3837
	3	620
	4	241
	5	105
A-10	1	131
	2	2224
	3	25,697
	4	1891
	5	3667
	6	9959
	7	555
	8	5577

A-12	1	20,510
	2	11,654
	3	160
	4	315
	5	176
	6	10,467
	7	2166
	8	342
	9	3239
	10	928
	11	272
	12	9219
	13	2134
	14	246
	15	1632
	16	5647
	17	792
	18	1677
	19	112
	20	5795
	21	3125
	22	417
	23	2178
	24	5775
	25	63
	26	1100
	27	1006
A-13	1	132
	2	2752
	3	1702
	4	208

	5	5808
	6	6721
	7	108
	8	4625
	9	71
	10	5482
	11	791
A-15	1	6905
	2	6520
	3	11,094
	4	273
	5	66
A-17	1	15,567
	2	2579
	3	16,325
	4	217
	5	275
	6	7201
	7	2582
A-19	1	329
	2	1518
	3	359
	4	7167
	5	5099
	6	261
	7	9533
	8	103
	9	91
A-20	1	248
	2	8411
	3	3368

	4	14,364
	5	2331
	6	10,854
	7	13,733
	8	3231
	9	3711
	10	79
	11	82
A-21	1	449
	2	3871
	3	178
	4	1095
	5	2745
	6	116
	7	1427
	8	1429
	9	745
	10	241
	11	247
	12	5053
	13	9627
	14	5522
	15	10,030
	16	349
	17	587
	18	6275
	19	4110
	20	1206
A-22	1	23,733
	2	300
	3	1814

	4	124
	5	912
	6	129
	7	6991
	8	1205
	9	66
A-23	1	9603
	2	330
	3	49
A-24	1	145
	2	13,917
	3	4019
	4	11,314
	5	742
	6	160
	7	7331
	8	128
	9	1424
	10	872
	11	15,870
A-26	1	1080
	2	398
	3	340
	4	1202
	5	9672
	6	3590
	7	233
	8	639
	9	10,522
	10	2729
	11	4222

A-27	1	371
	2	2045
	3	108
	4	44
A-28	1	6722
	2	2258
	3	1005
	4	114
	5	830
	6	4848
	7	154
	8	12,161
	9	6494
	10	1195
	11	4606
	12	251
	13	74
A-29	1	4719
	2	1387
	3	3446
	4	1163
	5	9811
	6	1566
	7	225
	8	990
	9	2905
A-30	1	2962
	2	236
	3	6391
	4	102
	5	1799

	6	1845
A-31	1	942
	2	5900
	3	7996
	4	192
	5	8132
	6	12513
	7	4538
	8	9905
A-32	1	1834
	2	7477
	3	4315
	4	838
	5	10,983
	6	9778
	7	15,576
	8	169
	9	268
	10	834
	11	847
	12	643
	13	195
	14	4904
	15	1144
	16	393
	17	85
A-33	1	123
	2	14,835
	3	11,771
	4	3475
	5	600

	6	648
	7	946
	8	822
	9	7396
	10	3365
	11	10,525
	12	7397
	13	13,630
	14	2699
	15	64
A-34	1	11,521
	2	1381
A-35	1	127
	2	8883
	3	145
	4	8988
	5	6460
	6	4035
	7	344
A-36	1	14,831
	2	8571
	3	7119
	4	237
	5	658
	6	4112
	7	8221
	8	1956
	9	187
	10	7370
	11	1289
A-37	1	2051

	2	15,148
	3	667
	4	219
	5	4139
	6	2247
	7	5686
	8	7312
	9	19,573
	10	9047
	11	625
	12	166
	13	2393
	14	1649
	15	2711
	16	666
	17	3914
A-39	1	2232
	2	9397
	3	6465
A-40	1	4601
	2	616
	3	3259
	4	1590
	5	559
	6	725
	7	17,614
	8	2869
	9	3761
	10	1617
	11	97
	12	4985

A-41	1	115
	2	1012
	3	8564
	4	166
	5	3947
	6	1064
	7	655
	8	3359
	9	3669
	10	2821
	11	225
	12	2645
	13	425
	14	7268
	15	7074
A-42	1	19,767
	2	6677
	3	185
	4	5272
	5	17,940
A-43	1	2216
	2	1518
	3	2585
	4	2844
	5	1755
	6	5431
	7	14,886
	8	1852
	9	5110
	10	92
	11	22,842

	12	3523
A-44	1	152
	2	5003
	3	151
	4	660
	5	7270
	6	245
	7	6221
	8	264
	9	3398
	10	349
	11	1677
	12	1642
	13	88
	14	70
	15	92
A-45	1	8940
	2	6176
	3	506
	4	7612
	5	4004
	6	1872
	7	411
	8	505
	9	587
	10	250
	11	6008
	12	1319
	13	970
	14	3319
	15	615

	16	3127
	17	885
A-46	1	483
	2	223
A-47	1	164
	2	406
	3	4737
A-48	1	1635
	2	4316
	3	272
	4	8886
	5	1526
A-49	1	2821
	2	719
	3	514
	4	2726
	5	3080
	6	448
	7	10,244
A-50	1	369
	2	2596
	3	277
	4	12,525

Appendix 4

Plasmid maps of the principle plasmids used in this study are shown below.

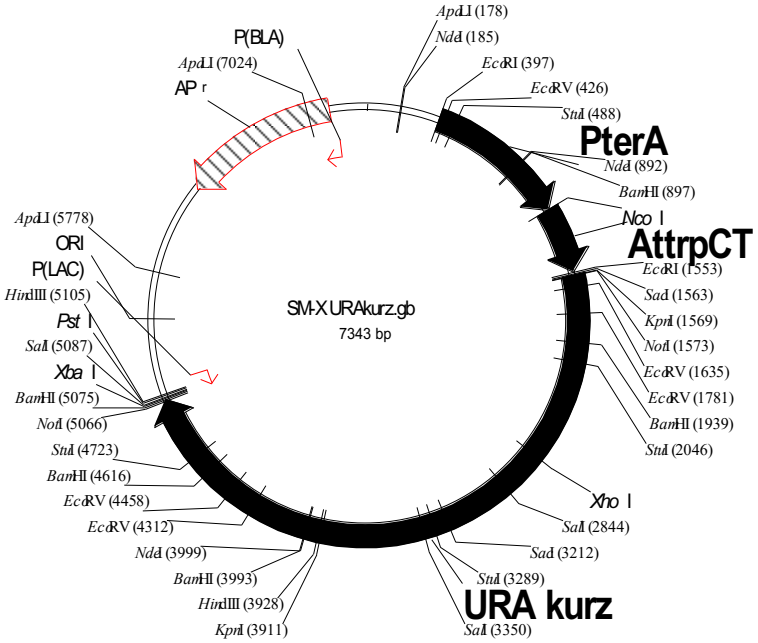


Figure A4.1. Plasmid map of SM-X_URA (Geib *et al.*, 2019).

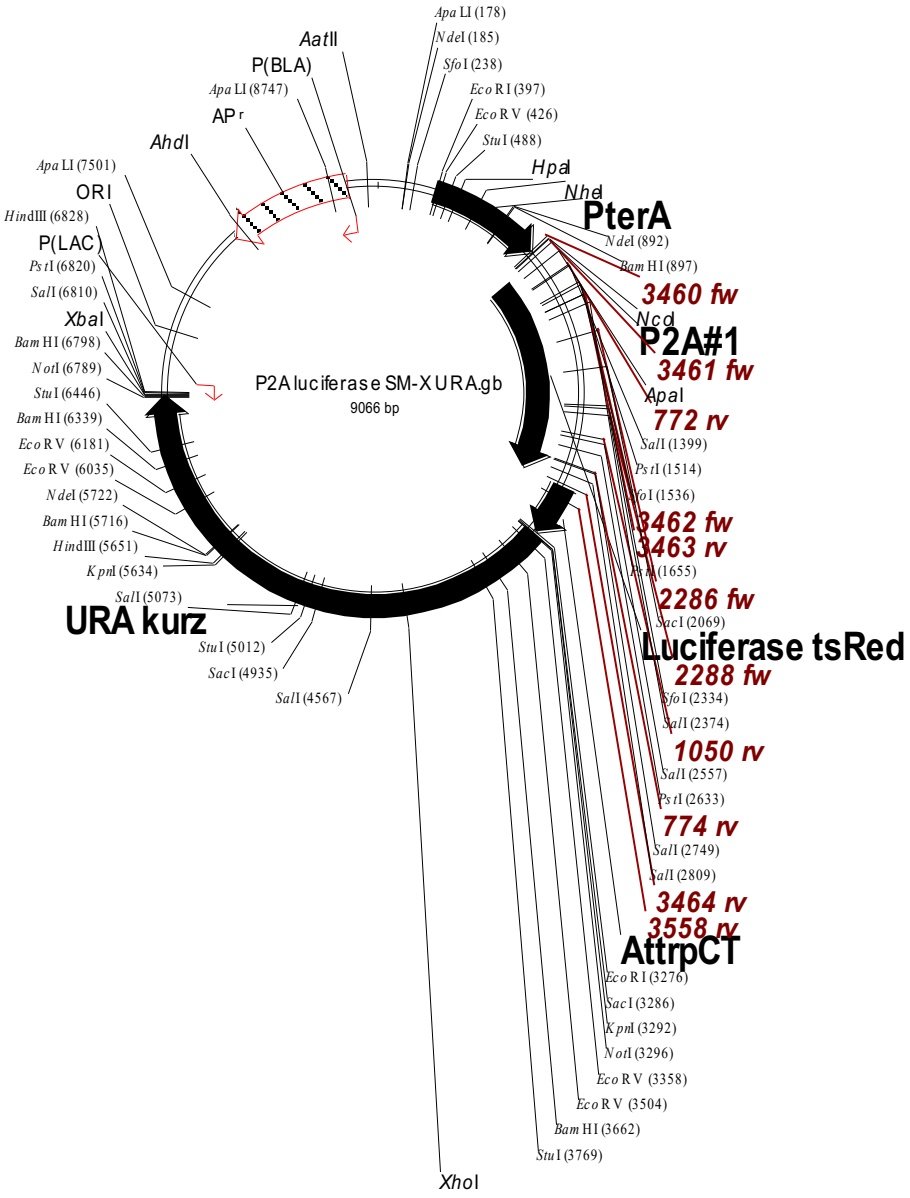


Figure A4.2. Plasmid map of P2A_*luciferase*_SM-X_URA (Geib and Brock, unpublished).

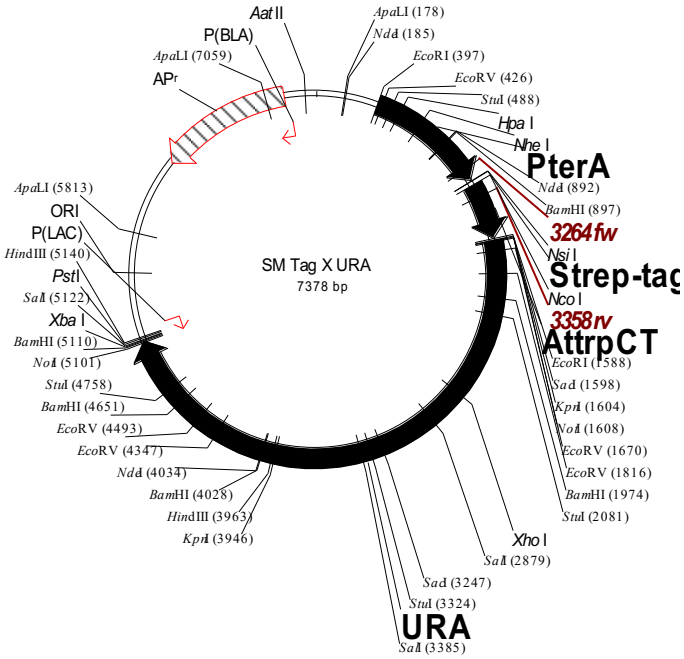


Figure A4.3. Plasmid map of SM_S-tag_X_URA (Wieder *et al.*, 2022, in press).

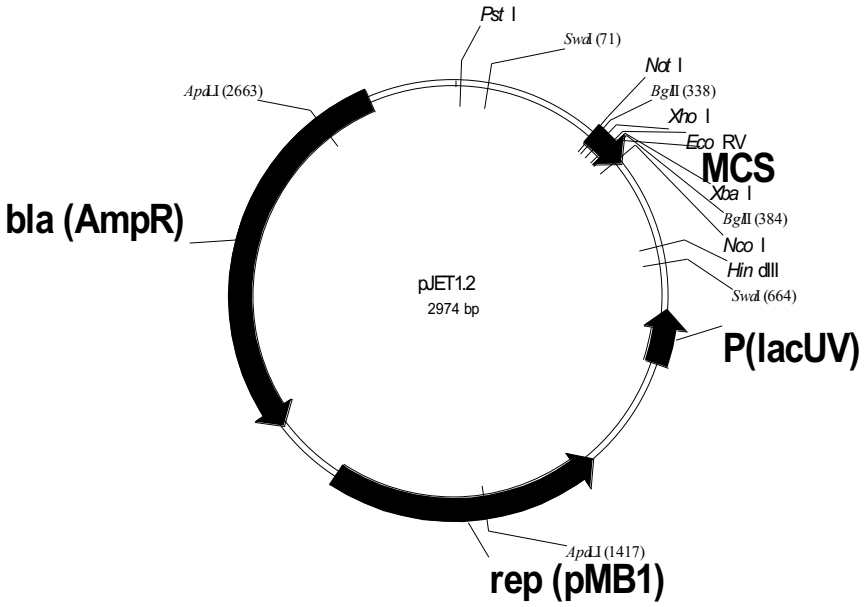


Figure A4.4. Plasmid map of pJET1.2 (Thermo Fisher Scientific, UK).

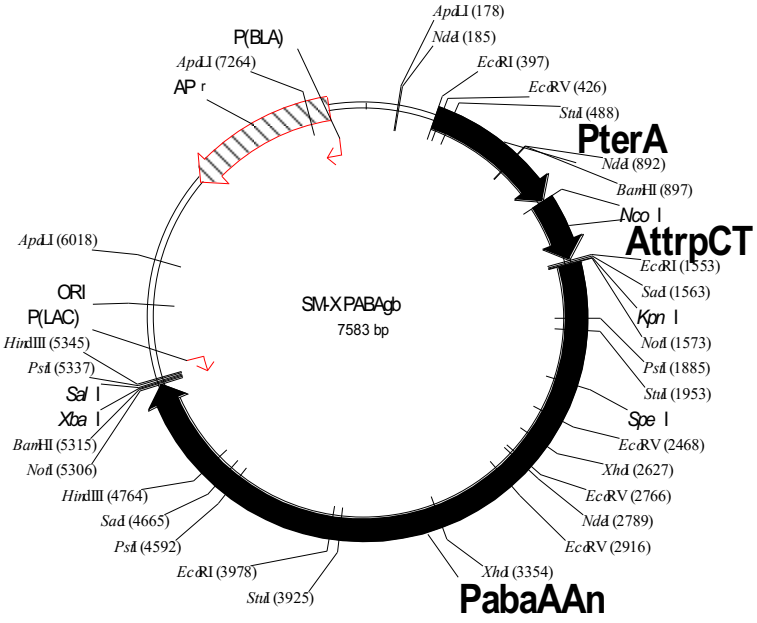


Figure A4.5. Plasmid map of SM-X_PABA (Peres da Silva and Brock, unpublished).

Appendix 5

Note to examiners:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

PIPS Reflective Statement

I conducted my PIPs placement at Biotechnology and Biological Sciences Research Council (BBSRC), within the Global Challenges Research Fund (GCRF) Hub. I was based at their office located in Swindon, UK.

The work undertaken during my placement contributed to the development and delivery of the impact and communication strategy for BBSRC GCRF. One part of my project consisted of conducting an analysis of the GCRF portfolio, in terms of identifying and creating visual representations of the research being supported by BBSRC GCRF funding. The work produced as a result of this analysis will be used in the future by BBSRC for spending reviews, as well as for additional reporting purposes.

The other part of my project involved developing a strategy for the production of case studies that would showcase the research that is funded by GCRF and highlight the real-world impacts of this research. This work involved collating information from various sources, as well as engaging with different groups of people in order to obtain the relevant information needed. During the placement, I was given the opportunity to take ownership of the project and implement my own ideas with regards to the direction that was taken when developing the communication strategy for GCRF. The chance to contribute my own ideas to the GCRF impact and communication strategy was something that I found challenging at times, but also rewarding. The outcome of this part of my project was the creation of a database of information relating to the GCRF-funded grants identified by myself as showing evidence of excellent impact or

potential for impact. This database provides easily-accessible and readily-available information required to produce case studies in the future which will subsequently be used as evidence for the impact of BBSRC GCRF-funded research and to support applications for further funding from government.

This placement has allowed me to develop and improve upon various skills. The main skills that I have been able to work on during my time at BBSRC are my communication skills, both written and verbal forms. During the placement, I was required to engage in discussions with various groups of people (specialists and non-specialists) and also to present different types of information effectively both in written and oral formats. Further to this, the work undertaken during the placement included the gathering and collation of large amounts of information that had been obtained from various sources. This allowed to me develop my skills in terms of concise and effective presentation of data. The placement gave me the chance to apply the skills that I have developed as part of my PhD to different types of work to ensure that I was working effectively and to a high standard. In addition to this, the various transferrable skills developed during the placement can be utilised during the remainder of my PhD studies.

In terms of impacting upon future career plans, I have found that the placement has encouraged me to consider a wider range of areas when considering future career plans. Specifically considering my placement at BBSRC, I was surprised by how much I enjoyed working in a sector that was vastly different to that of research and academia. Further to this, completing the placement has demonstrated that the skills I am developing as part of my PhD are not just specific to a research-based setting, but can be applied to a wide range of careers. I feel that the placement has provided an excellent opportunity to experience a completely different type of work to that conducted in an academic research lab. Further to this, the placement has highlighted the fact that undertaking a PhD has provided me with valuable skills which can be applied to a wide range of careers.

References

Abdel-Hameed, M., Bertrand, R. L., Piercey-Normore, M. D. and Sorensen, J. L. (2016a) Putative identification of the usnic acid biosynthetic gene cluster by *de novo* whole-genome sequencing of a lichen-forming fungus. *Fungal Biology* **120** (3), 306-316.

Abdel-Hameed, M., Bertrand, R. L., Piercey-Normore, M. D. and Sorensen, J. L. (2016b) Identification of 6-Hydroxymellein Synthase and Accessory Genes in the Lichen *Cladonia uncialis*. *Journal of Natural Products* **79** (6), 1645-1650.

Abdel-Hameed, M. E., Bertrand, R. L., Donald, L. J. and Sorensen, J. L. (2018) Lichen ketosynthase domains are not responsible for inoperative polyketide synthases in *Ascomycota* hosts. *Biochemical and Biophysical Research Communications* **503** (3), 1228-1234.

Adl, S. M., Bass, D., Lane, C. E., Lukeš, J., Schoch, C. L., Smirnov, A., Agatha, S., Berney, C., Brown, M. W., Burki, F., Cárdenas, P., Čepička, I., Chistyakova, L., del Campo, J., Dunthorn, M., Edvardsen, B., Eglit, Y., Guillou, L., Hampl, V., Heiss, A. A., Hoppenrath, M., James, T. Y., Karnkowska, A., Karpov, S., Kim, E., Kolisko, M., Kudryavtsev, A., Lahr, D. J. G., Lara, E., Le Gall, L., Lynn, D. H., Mann, D. G., Massana, R., Mitchell, E. A. D., Morrow, C., Park, J. S., Pawlowski, J. W., Powell, M. J., Richter, D. J., Rueckert, S., Shadwick, L., Shimano, S., Spiegel, F. W., Torruella, G., Youssef, N., Zlatogursky, V. and Zhang, Q. (2019) Revisions to the Classification, Nomenclature, and Diversity of Eukaryotes. *Journal of Eukaryotic Microbiology* **66** (1), 4-119.

Ahmadjian, V. (1993) *The Lichen Symbiosis*, John Wiley & Sons, Inc.: New York, USA.

Ahmadjian, V., Russell, L. A. and Hildreth, K. C. (1980) Artificial Reestablishment of Lichens. I. Morphological Interactions between the Phycobionts of Different Lichens and the Mycobionts *Cladonia cristatella* and *Lecanora chrysoleuca*. *Mycologia* **72** (1), 73-89.

Al-Rubeai, M. A. and El-Hassi, M. F. (1986) Inactivation of wild type and mutant *Aspergillus nidulans* conidia by far-UV, near-UV, visible and sun lights. *Environmental and Experimental Botany* **26** (3), 243-252.

Aplin, P. S. and Hill, D. J. (1979) Growth analysis of circular lichen thalli. *Journal of Theoretical Biology* **78** (3), 347-363.

Armaleo, D. (1991) Experimental Microbiology of Lichens: Mycelia Fragmentation, A Novel Growth Chamber, and the Origins of Thallus Differentiation. *Symbiosis* **11** (2-3), 163-177.

Armaleo, D. and Clerc, P. (1991) Lichen chimeras: DNA analysis suggests that one fungus forms two morphotypes. *Experimental Mycology* **15** (1), 1-10.

Armaleo, D. and Miao, V. (1999) Symbiosis and DNA Methylation in the *Cladonia* Lichen Fungus. *Symbiosis* **26** (2), 143-163.

Armaleo, D., Sun, X. and Culberson, C. (2011) Insights from the first putative biosynthetic gene cluster for a lichen depside and depsidone. *Mycologia* **103** (4), 741-754.

Armaleo, D., Müller, O., Lutzoni, F., Andrésson, Ó. S., Blanc, G., Bode, H. B., Collart, F. R., Dal Grande, F., Dietrich, F., Grigoriev, I. V., Joneson, S., Kuo, A., Larsen, P. E., Logsdon Jr, J. M., Lopez, D., Martin, F., May, S. P., McDonald, T. R., Merchant, S. S., Miao, V., Morin, E., Oono, R., Pellegrini, M., Rubinstein, N., Sanchez-Puerta, M. V., Savelkoul, E., Schmitt, I., Slot, J. C., Soanes, D., Szövényi, P., Talbot, N. J., Veneault-Fourrey, C. and Xavier, B. B. (2019) The lichen symbiosis re-viewed through the genomes of *Cladonia grayi* and its algal partner *Asterochloris glomerata*. *BMC Genomics* **20**, 605.

Armstrong, E. E., Prost, S., Ertz, D., Westberg, M., Frisch, A. and Bendiksby, M. (2018) Draft Genome Sequence and Annotation of the Lichen-Forming Fungus *Arthonia radiata*. *Genome Announcements* **6** (14), e00281-18.

Armstrong, R. A. (1974) Growth phases in the life of a lichen thallus. *New Phytologist* **73** (5), 913-918.

Armstrong, R. A. (1976) The influence of the frequency of wetting and drying on the radial growth of three saxicolous lichens in the field. *New Phytologist* **77** (3), 719-724.

Armstrong, R. A. (1979) Growth and regeneration of lichen thalli with the central portions artificially removed. *Environmental and Experimental Botany* **19** (3), 175-178.

Armstrong, R. A. (1984) Growth of Experimentally Reconstructed Thalli of the Lichen *Parmelia conspersa*. *New Phytologist* **98** (3), 497-502.

Armstrong, R. A. (1985) Field experiments on competition between three foliose lichens using the de wit experimental design. *Environmental and Experimental Botany* **25** (4), 369-374.

Armstrong, R. A. (2006) Seasonal growth of the crustose lichen *Rhizocarpon geographicum* (L.) DC. in South Gwynedd, Wales. *Symbiosis* **41** (2), 97-102.

Armstrong, R. A. and Smith, S. N. (1987) Development and Growth of the Lichen *Rhizocarpon geographicum*. *Symbiosis* **3** (3), 287-300.

Armstrong, R. and Bradwell, T. (2010) Growth of crustose lichens: a review. *Geografiska Annaler: Series A, Physical Geography* **92A** (1), 3-17.

Armstrong, R. A. and Bradwell, T. (2011) Growth of foliose lichens: a review. *Symbiosis* **53** (1), 1-16.

Arnone, A., Cardillo, R. and Nasini, G. (1986) Structures of melleolides B-D, three antibacterial sesquiterpenoids from *Armillaria mellea*. *Phytochemistry* **25** (2), 471-474.

Arvas, M., Kivioja, T., Mitchell, A., Saloheimo, M., Ussery, D., Penttila, M. and Oliver, S. (2007) Comparison of protein coding gene contents of the fungal phyla Pezizomycotina and Saccharomycotina. *BMC Genomics* **8**, 325.

Aschenbrenner, I. A., Cernava, T., Berg, G. and Grube, M. (2016) Understanding Microbial Multi-Species Symbioses. *Frontiers in Microbiology* **7**, 180.

Asplund, J. and Wardle, D. A. (2017) How lichens impact on terrestrial community and ecosystem properties. *Biological Reviews* **92** (3), 1720-1738.

Avalos, J. and Carmen Limón, M. (2015) Biological roles of fungal carotenoids. *Current Genetics* **61** (3), 309-324.

Bačkor, M., Hudák, J., Repčák, M., Ziegler, W. and Bačkorová, M. (1998) The influence of pH and lichen metabolites (vulpinic acid and (+) usnic acid) on the growth of the lichen photobiont *Trebouxia irregularis*. *The Lichenologist* **30** (6), 577-582.

Bačkor, M., Klemová, K., Bačkorová, M. and Ivanova, V. (2010) Comparison of the Phytotoxic Effects of Usnic Acid on Cultures of Free-Living Alga *Scenedesmus quadricauda* and Aposymbiotically Grown Lichen Photobiont *Trebouxia erici*. *Journal of Chemical Ecology* **36** (4), 405-411.

Balibar, C. J., Howard-Jones, A. R. and Walsh, C. T. (2007) Terrequinone A biosynthesis through L-tryptophan oxidation, dimerization and bisprenylation. *Nature Chemical Biology* **3** (9), 584-592.

Bayram, Ö. and Braus, G. H. (2012) Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. *FEMS Microbiology Reviews* **36** (1), 1-24.

Bayram, Ö., Krappmann, S., Ni, M., Bok, J. W., Helmstaedt, K., Valerius, O., Braus-Stromeyer, S., Kwon, N.-J., Keller, N. P., Yu, J.-H. and Braus, G. H. (2008) VelB/VeA/LaeA Complex Coordinates Light Signal with Fungal Development and Secondary Metabolism. *Science* **320** (5882), 1504-1506.

Beckett, R. P., Kranner, I. and Minibayeva, F. V. (2008) Stress physiology and the symbiosis. In *Lichen Biology* (T. H. Nash III, ed.), pp. 134-151, Cambridge University Press: Cambridge, UK.

BeGora, M. D. and Fahselt, D. (2001) Usnic Acid and Atranorin Concentrations in Lichens in Relation to Bands of UV Irradiance. *The Bryologist* **104** (1), 134-140.

Benedict, J. B. (1990) Experiments on Lichen Growth. I. Seasonal Patterns and Environmental Controls. *Arctic and Alpine Research* **22** (3), 244-254.

Benedict, J. B. (2008) Experiments on Lichen Growth, III. The Shape of the Age-Size Curve. *Arctic, Antarctic, and Alpine Research* **40** (1), 15-26.

Bergmann, S., Funk, A. N., Scherlach, K., Schroeckh, V., Shelest, E., Horn, U., Hertweck, C. and Brakhage, A. A. (2010) Activation of a Silent Fungal Polyketide Biosynthesis Pathway through Regulatory Cross Talk with a Cryptic Nonribosomal Peptide Synthetase Gene Cluster. *Applied and Environmental Microbiology* **76** (24), 8143-8149.

Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P. and Ron, D. (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nature Cell Biology* **2** (6), 326-332.

Bertrand, R. L. and Sorensen, J. L. (2019a) Lost in Translation: Challenges with Heterologous Expression of Lichen Polyketide Synthases. *ChemistrySelect* **4** (21), 6473-6483.

Bertrand, R. L. and Sorensen, J. L. (2019b) Transcriptional heterologous expression of two type III PKS from the lichen *Cladonia uncialis*. *Mycological Progress* **18** (12), 1437-1447.

Bertrand, R. L., Abdel-Hameed, M. and Sorensen, J. L. (2018) Lichen Biosynthetic Gene Clusters. Part I. Genome Sequencing Reveals a Rich Biosynthetic Potential. *Journal of Natural Products* **81** (4), 723-731.

Bertrand, S., Bohni, N., Schnee, S., Schumpp, O., Gindro, K. and Wolfender, J.-L. (2014) Metabolite induction via microorganism co-culture: A potential way to enhance chemical diversity for drug discovery. *Biotechnology Advances* **32** (6), 1180-1204.

Białońska, D. and Dayan, F. E. (2005) Chemistry of the Lichen *Hypogymnia physodes* Transplanted to an Industrial Region. *Journal of Chemical Ecology* **31** (12), 2975-2991.

Bidochka, M. J., Kamp, A. M., Lavender, T. M., Dekoning, J. and De Croos, J. N. A. (2001) Habitat Association in Two Genetic Groups of the Insect-Pathogenic Fungus *Metarhizium anisopliae*: Uncovering Cryptic Species? *Applied and Environmental Microbiology* **67** (3), 1335-1342.

Bilger, W., Rimke, S., Schreiber, U. and Lange, O. L. (1989) Inhibition of Energy-Transfer to Photosystem II in Lichens by Dehydration: Different Properties of Reversibility with Green and Blue-green Phycobionts. *Journal of Plant Physiology* **134** (3), 261-268.

Bills, G. F. and Gloer, J. B. (2016) Biologically Active Secondary Metabolites from the Fungi. *Microbiology Spectrum* **4** (6).

Bingle, L. E. H., Simpson, T. J. and Lazarus, C. M. (1999) Ketosynthase Domain Probes Identify Two Subclasses of Fungal Polyketide Synthase Genes. *Fungal Genetics and Biology* **26** (3), 209-223.

Birkeland, P. W. (1981) Soil data and the shape of the lichen growth-rate curve for the Mt Cook area (Note). *New Zealand Journal of Geology and Geophysics* **24** (3), 443-445.

Bjerke, J. W., Gwynn-Jones, D. and Callaghan, T. V. (2005a) Effects of enhanced UV-B radiation in the field on the concentration of phenolics and chlorophyll fluorescence in two boreal and arctic-alpine lichens. *Environmental and Experimental Botany* **53** (2), 139-149.

Bjerke, J. W., Joly, D., Nilsen, L. and Brossard, T. (2004) Spatial trends in usnic acid concentrations of the lichen *Flavocetraria nivalis* along local climatic gradients in the Arctic (Kongsfjorden, Svalbard). *Polar Biology* **27** (7), 409-417.

Bjerke, J. W., Elvebakk, A., Domínguez, B. and Dahlback, A. (2005b) Seasonal trends in usnic acid concentrations of Arctic, alpine and Patagonian populations of the lichen *Flavocetraria nivalis*. *Phytochemistry* **66** (3), 337-344.

Blaha, J., Baloch, E. and Grube, M. (2006) High photobiont diversity associated with the euryoecious lichen-forming ascomycete *Lecanora rupicola* (Lecanoraceae, Ascomycota). *Biological Journal of the Linnean Society* **88** (2), 283-293.

Blin, K., Wolf, T., Chevrette, M. G., Lu, X., Schwalen, C. J., Kautsar, S. A., Duran, H. G. S., de los Santos, E. L. C., Kim, H. U., Nave, M., Dickschat, J. S., Mitchell, D. A., Shelest, E., Breitling, R., Takano, E., Lee, S. Y., Weber, T. and Medema, M. H. (2017) antiSMASH 4.0 – improvements in chemistry prediction and gene cluster boundary identification. *Nucleic Acids Research* **45** (W1), W36-W41.

Bok, J. W. and Keller, N. P. (2004) LaeA, a Regulator of Secondary Metabolism in *Aspergillus* spp. *Eukaryotic Cell* **3** (2), 527-535.

Bok, J. W., Noordermeer, D., Kale, S. P. and Keller, N. P. (2006a) Secondary metabolic gene cluster silencing in *Aspergillus nidulans*. *Molecular Microbiology* **61** (6), 1636-1645.

Bok, J. W., Hoffmeister, D., Maggio-Hall, L. A., Murillo, R., Glasner, J. D. and Keller, N. P. (2006b) Genomic Mining for *Aspergillus* Natural Products. *Chemistry & Biology* **13** (1), 31-37.

Bok, J. W., Balajee, S. A., Marr, K. A., Andes, D., Nielsen, K. F., Frisvad, J. C. and Keller, N. P. (2005) LaeA, a Regulator of Morphogenetic Fungal Virulence Factors. *Eukaryotic Cell* **4** (9), 1574-1582.

Boustie, J. and Grube, M. (2005) Lichens – a promising source of bioactive secondary metabolites. *Plant Genetic Resources* **3** (2), 273-287.

Boustie, J., Tomasi, S. and Grube, M. (2011) Bioactive lichen metabolites: alpine habitats as an untapped source. *Phytochemistry Reviews* **10** (3), 287-307.

Braesel, J., Götze, S., Shah, F., Heine, D., Tauber, J., Hertweck, C., Tunlid, A., Stallforth, P. and Hoffmeister, D. (2015) Three Redundant Synthetases Secure Redox-Active Pigment Production in the Basidiomycete *Paxillus involutus*. *Chemistry & Biology* **22** (10), 1325-1334.

Braga, G. U. L., Flint, S. D., Miller, C. D., Anderson, A. J. and Roberts, D. W. (2001) Variability in Response to UV-B among Species and Strains of *Metarhizium* Isolated from Sites at Latitudes from 61°N to 54°S. *Journal of Invertebrate Pathology* **78** (2), 98-108.

Braga, G. U. L., Rangel, D. E. N., Fernandes, É. K. K., Flint, S. D. and Roberts, D. W. (2015) Molecular and physiological effects of environmental UV radiation on fungal conidia. *Current Genetics* **61** (3), 405-425.

Brakhage, A. A. (2013) Regulation of fungal secondary metabolism. *Nature Reviews Microbiology* **11** (1), 21-32.

Brakhage, A. A. and Schroeckh, V. (2011) Fungal secondary metabolites – Strategies to activate silent gene clusters. *Fungal Genetics and Biology* **48** (1), 15-22.

Brodo, I. M. and Richardson, D. H. S. (1978) Chimeroid associations in the genus *Peltigera*. *The Lichenologist* **10**, 157-170.

Brown, D. W. and Salvo, J. J. (1994) Isolation and Characterization of Sexual Spore Pigments from *Aspergillus nidulans*. *Applied and Environmental Microbiology* **60** (3), 979-983.

Brunauer, G., Hager, A., Krautgartner, W. D., Türk, R. and Stocker-Wörgötter, E. (2006) Experimental studies on *Lecanora rupicola* (L.) Zahlbr.: chemical and microscopical investigations of the mycobiont and re-synthesis stages. *The Lichenologist* **38** (6), 577-585.

Brunauer, G., Hager, A., Grube, M., Türk, R. and Stocker-Wörgötter, E. (2007) Alterations in secondary metabolism of aposymbiotically grown mycobionts of *Xanthoria elegans* and cultured resynthesis stages. *Plant Physiology and Biochemistry* **45** (2), 146-151.

Büdel, B. and Rhiel, E. (1987) Studies on the Ultrastructure of Some Cyanolichen Haustoria. *Protoplasma* **139** (2-3), 145-152.

Büdel, B. and Scheidegger, C. (2008) Thallus morphology and anatomy. In *Lichen Biology* (T. H. Nash III, ed.), pp. 40-68, Cambridge University Press: Cambridge, UK.

Budʼová, J., Bačkor, M., Bačkorová, M. and Židzik, J. (2006) Usnic acid and copper toxicity in aposymbiotically grown lichen photobiont *Trebouxia erici*. *Symbiosis* **42** (3), 169-174.

Bull, W. B. and Brandon, M. T. (1998) Lichen dating of earthquake-generated regional rockfall events, Southern Alps, New Zealand. *Geological Society of America Bulletin* **110** (1), 60-84.

Cairns, T. C., Zheng, X., Zheng, P., Sun, J. and Meyer, V. (2019) Moulding the mould: understanding and reprogramming filamentous fungal growth and morphogenesis for next generation cell factories. *Biotechnology for Biofuels* **12**, 77.

Calchera, A., Dal Grande, F., Bode, H. B. and Schmitt, I. (2019) Biosynthetic Gene Content of the 'Perfume Lichens' *Evernia prunastri* and *Pseudevernia furfuracea*. *Molecules* **24** (1), 203.

Calcott, M. J., Ackerley, D. F., Knight, A., Keyzers, R. A. and Owen, J. G. (2018) Secondary metabolism in the lichen symbiosis. *Chemical Society Reviews* **47** (5), 1730-1760.

Caten, C. E. (1979) Genetic determination of conidial colour in *Aspergillus heterocaryoticus* and relationship of this species to *Aspergillus amstelodami*. *Transactions of the British Mycological Society* **73**, 65-74.

Černajová, I. and Škaloud, P. (2019) The first survey of Cystobasidiomycete yeasts in the lichen genus *Cladonia*; with the description of *Lichenozyma pisutiana* gen. nov., sp. nov. *Fungal Biology* **123** (9), 625-637.

Cernava, T., Aschenbrenner, I. A., Soh, J., Sensen, C. W., Grube, M. and Berg, G. (2019) Plasticity of a holobiont: desiccation induces fasting-like metabolism within the lichen microbiota. *The ISME Journal* **13** (2), 547-556.

Cernava, T., Erlacher, A., Aschenbrenner, I. A., Krug, L., Lassek, C., Riedel, K., Grube, M. and Berg, G. (2017) Deciphering functional diversification within the lichen microbiota by meta-omics. *Microbiome* **5**, 82.

Childress, S. and Keller, J. B. (1980) Lichen growth. *Journal of Theoretical Biology* **82** (1), 157-165.

Chooi, Y.-H. and Tang, Y. (2012) Navigating the Fungal Polyketide Chemical Space: From Genes to Molecules. *Journal of Organic Chemistry* **77** (22), 9933-9953.

Chooi, Y.-H., Stalker, D. M., Davis, M. A., Fujii, I., Elix, J. A., Louwhoff, S. H. J. J. and Lawrie, A. C. (2008) Cloning and sequence characterization of a non-reducing polyketide synthase gene from the lichen *Xanthoparmelia semiviridis*. *Mycological Research* **112** (2), 147-161.

Chu, L. L., Pandey, R. P., Jung, N., Jung, H. J., Kim, E.-H. and Sohng, J. K. (2016) Hydroxylation of diverse flavonoids by CYP450 BM3 variants: biosynthesis of eriodictyol from naringenin in whole cells and its biological activities. *Microbial Cell Factories* **15**, 135.

Cichewicz, R. H. (2010) Epigenome manipulation as a pathway to new natural product scaffolds and their congeners. *Natural Product Reports* **27** (1), 11-22.

Clayden, S. R., Pentecost, A. and Dawson, R. J. M. (2004) Growth of the lichen *Rhizocarpon lecanorinum*, with comments on Aplin-Hill and lichenometric curves. *Symbiosis* **37** (1-3), 379-393.

Close, T. J. (1997) Dehydrins: A commonality in the response of plants to dehydration and low temperature. *Physiologia Plantarum* **100** (2), 291-296.

Collemare, J. and Seidl, M. F. (2019) Chromatin-dependent regulation of secondary metabolite biosynthesis in fungi: is the picture complete? *FEMS Microbiology Reviews* **43** (6), 591-607.

Conti, E., Stachelhaus, T., Marahiel, M. A. and Brick, P. (1997) Structural basis for the activation of phenylalanine in the non-ribosomal biosynthesis of gramicidin S. *The EMBO Journal* **16** (14), 4174-4183.

Cordero, R. J. B. and Casadevall, A. (2017) Functions of fungal melanin beyond virulence. *Fungal Biology Reviews* **31** (2), 99-112.

Cortesão, M., de Haas, A., Unterbusch, R., Fujimori, A., Schütze, T., Meyer, V. and Moeller, R. (2020) *Aspergillus niger* Spores Are Highly Resistant to Space Radiation. *Frontiers in Microbiology* **11**, 560.

Cox, J. S. and Walter, P. (1996) A Novel Mechanism for Regulating Activity of a Transcription Factor That Controls the Unfolded Protein Response. *Cell* **87** (3), 391-404.

Crittenden, P. D. and Porter, N. (1991) Lichen-forming fungi: potential sources of novel metabolites. *Trends in Biotechnology* **9** (12), 409-414.

Crittenden, P. D., David, J. C., Hawksworth, D. L. and Campbell, F. S. (1995) Attempted isolation and success in the culturing of a broad spectrum of lichen-forming and lichenicolous fungi. *New Phytologist* **130** (2), 267-297.

Crowe, J. H., Crowe, L. M. and Chapman, D. (1984) Preservation of Membranes in Anhydrobiotic Organisms: The Role of Trehalose. *Science* **223** (4637), 701-703.

Cruz-Leite, V. R. M., Tomazett, M. V., Santana de Curcio, J., Sbaraini, N., Bailão, A. M., Gonçales, R. A., Moraes D., Pereira M., Vainstein, M. H., Schrank, A., Peres da Silva, R., Brock, M. and Soares, C. M. A. (2022) Bioluminescence imaging in *Paracoccidioides* spp.: A tool to monitor the infectious processes. *Microbes and Infection*, in press.

Culberson, C. F. (1964) Joint Occurrence of a Lichen Depsidone and Its Probable Depside Precursor. *Science* **143** (3603), 255-256.

Culberson, C. F. (1969) The lichen substances, their biogenesis, and their relationship to the products of free-living fungi. In *Chemical and Botanical Guide to Lichen Products*, pp. 17-72, The University of North Carolina Press: USA.

Culberson, C. F. and Elix, J. A. (1989) Lichen Substances. In *Methods in Plant Biochemistry, Volume 1: Plant Phenolics* (P. M. Dey and J. B. Harborne, eds.), pp. 509-535, Academic Press Limited: London, UK.

da Silva, I. P., Brissow, E., Kellner Filho, L. C., Senabio, J., de Siqueira, K. A., Filho, S. V., Damasceno, J. L., Mendes, S. A., Tavares, D. C., Magalhães, L. G., Sales Junior, P. A., Januário, A. H. and Soares, M. A. (2017) Bioactive compounds of *Aspergillus terreus*-F7, an endophytic fungus from *Hyptis suaveolens* (L.) Poit. *World Journal of Microbiology & Biotechnology* **33** (3), 62.

Dahlman, L., Persson, J., Palmqvist, K. and Näsholm, T. (2004) Organic and inorganic nitrogen uptake in lichens. *Planta* **219** (3), 459-467.

Dal Grande, F., Meiser, A., Greshake Tzovaras, B., Otte, J., Ebersberger, I. and Schmitt, I. (2018) The draft genome of the lichen-forming fungus *Lasallia hispanica* (Frey) Sancho & A. Crespo. *The Lichenologist* **50** (3), 329-340.

Davé, A., Jeenes, D. J., Mackenzie, D. A. and Archer, D. B. (2006) HacA-Independent Induction of Chaperone-Encoding Gene *bipA* in *Aspergillus niger* Strains Overproducing Membrane Proteins. *Applied and Environmental Microbiology* **72** (1), 953-955.

de Bary, A. (1879) *Die Erscheinung der Symbiose*, Verlag von Karl J. Trübner: Strassburg, Austria.

de Vera, J.-P., Rettberg, P. and Ott, S. (2008) Life at the Limits: Capacities of Isolated and Cultured Lichen Symbionts to Resist Extreme Environmental Stresses. *Origins of Life and Evolution of Biospheres* **38** (5), 457-468.

del Carmen Molina, M. and Crespo, A. (2000) Comparison of development of axenic cultures of five species of lichen-forming fungi. *Mycological Research* **104** (5), 595-602.

Dellaporta, S. L., Wood, J. and Hicks, J. B. (1983) A plant DNA minipreparation: Version II. *Plant Molecular Biology Reporter* **1**, 19-21.

Dewi, R. T., Tachibana, S., Fajriah, S. and Hanafi, M. (2015) α-Glucosidase inhibitor compounds from *Aspergillus terreus* RCC1 and their antioxidant activity. *Medicinal Chemistry Research* **24** (2), 737-743.

Diederich, P., Lawrey, T. D. and Ertz, D. (2018) The 2018 classification and checklist of lichenicolous fungi, with 2000 non-lichenized, obligately lichenicolous taxa. *The Bryologist* **121** (3), 340-425.

Dijksterhuis, J. (2019) Fungal spores: Highly variable and stress-resistant vehicles for distribution and spoilage. *Food Microbiology* **81**, 2-11.

Dowzer, C. E. A. and Kelly, J. M. (1991) Analysis of the *creA* Gene, a Regulator of Carbon Catabolite Repression in *Aspergillus nidulans*. *Molecular and Cellular Biology* **11** (11), 5701-5709.

Drew, E. A. and Smith, D. C. (1966) The physiology of the symbiosis in *Peltigera polydactyla* (Neck.) Hoffm. *The Lichenologist* **3** (2), 197-201.

Dyer, P. S. (2002) Hydrophobins in the lichen symbiosis. *New Phytologist* **154** (1), 1-4.

Dyer, P. S. (2008) Evolutionary Biology: Genomic Clues to Original Sex in Fungi. *Current Biology* **18** (5), R207-R209.

Eastmond, D. L. and Nelson, H. C. M. (2006) Genome-wide Analysis Reveals New Roles for the Activation Domains of the *Saccharomyces cerevisiae* Heat Shock Transcription Factor (Hsf1) during the Transient Heat Shock Response. *Journal of Biological Chemistry* **281** (43), 32909-32921.

Elix, J. A. and Gaul, K. L. (1986) The Interconversion of the Lichen Depsides *Para*- and *Meta*-Scrobiculin, and the Biosynthetic Implications. *Australian Journal of Chemistry* **39** (4), 613-624.

Elix, J. A. and Stocker-Wörgötter, E. (2008) Biochemistry and secondary metabolites. In *Lichen Biology* (T. H. Nash III, ed.), pp. 104-133, Cambridge University Press: Cambridge, UK.

Elix, J. A., Jenie, U. A. and Parker, J. L. (1987) A Novel Synthesis of the Lichen Depsidones Divaronic Acid and Stenosporonic Acid, and the Biosynthetic Implications. *Australian Journal of Chemistry* **40** (8), 1451-1464.

Ellgaard, L., Molinari, M. and Helenius, A. (1999) Setting the standards: Quality control in the secretory pathway. *Science* **286** (5446), 1882-1888.

Elshobary, M. E., Osman, M. E., Abo-Shady, A. M., Komatsu, E., Perreault, H., Sorensen, J. and Piercey-Normore, M. D. (2016) Algal carbohydrates affect polyketide synthesis of the lichen-forming fungus *Cladonia rangiferina*. *Mycologia* **108** (4), 646-656.

Engels, B., Heinig, U., McElroy, C., Meusinger, R., Grothe, T., Stadler, M. and Jennewein, S. (2021) Isolation of a gene cluster from *Armillaria gallica* for the synthesis of armillyl orsellinate-type sesquiterpenoids. *Applied Microbiology and Biotechnology* **105** (1), 211-224.

Eymann, C., Lassek, C., Wegner, U., Bernhardt, J., Fritsch, O. A., Fuchs, S., Otto, A., Albrecht, D., Schiefelbein, U., Cernava, T., Aschenbrenner, I., Berg, G., Grube, M. and Riedel, K. (2017) Symbiotic Interplay of Fungi, Algae, and Bacteria within the Lung Lichen *Lobaria pulmonaria* L. Hoffm. as Assessed by State-of-the-Art Metaproteomics. *Journal of Proteome Research* **16** (6), 2160-2173.

Fazio, A. T., Adler, M. T., Bertoni, M. D. and Maier, M. S. (2012) Culture studies on the mycobiont of *Caloplaca erythrantha* (Tuck.) Zahlbr. (*Teloschistaceae*): high production of major lichen secondary metabolites. *The Lichenologist* **44** (4), 533-542.

Fazio, A. T., Bertoni, M. D., Adler, M. T., Ruiz, L. B., Rosso, M. L., Muggia, L., Hager, A., Stocker-Wörgötter, E. and Maier, M. S. (2009) Culture studies on the mycobiont isolated from *Parmotrema reticulatum* (Taylor) Choisy: metabolite production under different conditions. *Mycological Progress* **8** (4), 359-365.

Fernández-Mendoza, F., Domaschke, S., García, M. A., Jordan, P., Martín, M. P. and Printzen, C. (2011) Population structure of mycobionts and photobionts of the widespread lichen *Cetraria aculeata*. *Molecular Ecology* **20** (6), 1208-1232.

Fischbach, M. A. and Walsh, C. T. (2006) Assembly-Line Enzymology for Polyketide and Nonribosomal Peptide Antibiotics: Logic, Machinery, and Mechanisms. *Chemical Reviews* **106** (8), 3468-3496.

Fleißner, A. and Dersch, P. (2010) Expression and export: recombinant protein production systems for *Aspergillus*. *Applied Microbiology and Biotechnology* **87** (4), 1255-1270.

Fox, E. M. and Howlett, B. J. (2008) Secondary metabolism: regulation and role in fungal biology. *Current Opinion in Microbiology* **11** (6), 481-487.

Foyer, C. H. and Halliwell, B. (1976) The Presence of Glutathione and Glutathione Reductase in Chloroplasts: A Proposed Role in Ascorbic Acid Metabolism. *Planta* **133** (1), 21-25.

Frisvad, J. C. (2012) Media and Growth Conditions for Induction of Secondary Metabolite Production. In *Fungal Secondary Metabolism. Methods in Molecular Biology (Methods and Protocols), Vol. 944* (N. P. Keller and G. Turner, eds.), pp. 47-58, Humana Press: Totowa, New Jersey, USA.

Gacek, A. and Strauss, J. (2012) The chromatin code of fungal secondary metabolite gene clusters. *Applied Microbiology and Biotechnology* **95** (6), 1389-1404.

Gagunashvili, A. N., Daviðsson, S. P., Jónsson, Z. O. and Andrésson, Ó. S. (2009) Cloning and heterologous transcription of a polyketide synthase gene from the lichen *Solorina crocea*. *Mycological Research* **113** (3), 354-363.

Galun, M. (1988) Carbon metabolism. In *CRC Handbook of Lichenology Vol. I* (M. Galun, ed.), pp. 147-156, CRC Press: Boca Raton, Florida, USA.

Gargas, A., DePriest, P. T., Grube, M. and Tehler, A. (1995) Multiple origins of lichen symbioses in fungi suggested by SSU rDNA phylogeny. *Science* **268** (5216), 1492-1495.

Garrido, C., Paul, C., Seigneuric, R. and Kampinga, H. H. (2012) The small heat shock proteins family: The long forgotten chaperones. *The International Journal of Biochemistry & Cell Biology* **44** (10), 1588-1592.

Gasulla, F., del Campo, E. M., Casano, L. M. and Guéra, A. (2021) Advances in Understanding of Desiccation Tolerance of Lichens and Lichen-Forming Algae. *Plants* **10** (4), 807.

Gauslaa, Y., Bidussi, M., Solhaug, K. A., Asplund, J. and Larsson, P. (2013) Seasonal and spatial variation in carbon based secondary compounds in green algal and cyanobacterial members of the epiphytic lichen genus *Lobaria*. *Phytochemistry* **94**, 91-98.

Geib, E. and Brock, M. (2017) ATNT: an enhanced system for expression of polycistronic secondary metabolite gene clusters in *Aspergillus niger*. *Fungal Biology and Biotechnology* **4**, 13.

Geib, E., Baldeweg, F., Doerfer, M., Nett, M. and Brock, M. (2019) Cross-Chemistry Leads to Product Diversity from Atromentin Synthetases in Aspergilli from Section *Nigri*. *Cell Chemical Biology* **26** (2), 223-234.

Geib, E., Gressler, M., Viediernikova, I., Hillmann, F., Jacobsen, I. D., Nietzsche, S., Hertweck, C. and Brock, M. (2016) A Non-canonical Melanin Biosynthesis Pathway Protects *Aspergillus terreus* Conidia from Environmental Stress. *Cell Chemical Biology* **23** (5), 587-597.

Gething, M.-J. and Sambrook, J. (1992) Protein folding in the cell. *Nature* **355** (6355), 33-45.

Goldman, G. H. and Kafer, E. (2004) *Aspergillus nidulans* as a model system to characterize the DNA damage response in eukaryotes. *Fungal Genetics and Biology* **41** (4), 428-442.

Gonzalez, T. N., Sidrauski, C., Dörfler, S. and Walter, P. (1999) Mechanism of non-spliceosomal mRNA splicing in the unfolded protein response pathway. *The EMBO Journal* **18** (11), 3119-3132.

Green, T. G. A., Sancho, L. G. and Pintado, A. (2011) Ecophysiology of Desiccation/Rehydration Cycles in Mosses and Lichens. In *Plant Desiccation Tolerance. Ecological Studies (Analysis and Synthesis), Vol. 215* (U. Lüttge, E. Beck and D. Bartels, eds.), pp. 89-120, Springer: Berlin, Heidelberg, Germany.

Gressler, M., Hortschansky, P., Geib, E. and Brock, M. (2015) A new high-performance heterologous fungal expression system based on regulatory elements from the *Aspergillus terreus* terrein gene cluster. *Frontiers in Microbiology* **6**, 184.

Grube, M. (2010) Die Hard: Lichens. In *Symbioses and Stress: Joint Ventures in Biology, Cellular Origin, Life in Extreme Habitats and Astrobiology Vol. 17* (J. Seckbach and M. Grube, eds.), pp. 509-523, Springer: Berlin, Heidelberg, Germany.

Grube, M. and Hawksworth, D. L. (2007) Trouble with lichen: the re-evaluation and re-interpretation of thallus form and fruit body types in the molecular era. *Mycological Research* **111** (9), 1116-1132.

Grube, M. and Wedin, M. (2016) Lichenized Fungi and the Evolution of Symbiotic Organization. *Microbiology Spectrum* **4** (6).

Grube, M., Cardinale, M., De Castro, J. V., Müller, H. and Berg, G. (2009) Species-specific structural and functional diversity of bacterial communities in lichen symbioses. *The ISME Journal* **3** (9), 1105-1115.

Grube, M., Cernava, T., Soh, J., Fuchs, S., Aschenbrenner, I., Lassek, C., Wegner, U., Becher, D., Riedel, K., Sensen, C. W. and Berg, G. (2015) Exploring functional contexts of symbiotic sustain within lichen-associated bacteria by comparative omics. *The ISME Journal* **9** (2), 412-424.

Gulluce, M., Aslan, A., Sokmen, M., Sahin, F., Adiguzel, A., Agar, G. and Sokmen, A. (2006) Screening the antioxidant and antimicrobial properties of the lichens *Parmelia saxatilis*, *Platismatia glauca*, *Ramalina pollinaria*, *Ramalina polymorpha* and *Umbilicaria nylanderiana*. *Phytomedicine* **13** (7), 515-521.

Guo, C.-J., Knox, B. P., Sanchez, J. F., Chiang, Y.-M., Bruno, K. S. and Wang, C. C. C. (2013) Application of an Efficient Gene Targeting System Linking Secondary Metabolites to their Biosynthetic Genes in *Aspergillus terreus*. *Organic Letters* **15** (14), 3562-3565.

Guo, F., Li, Z., Xu, X., Wang, K., Shao, M., Zhao, F., Wang, H., Hua, H., Pei, Y. and Bai, J. (2016) Butenolide derivatives from the plant endophytic fungus *Aspergillus terreus*. *Fitoterapia* **113**, 44-50.

Hahn, J.-S., Hu, Z., Thiele, D. J. and Iyer, V. R. (2004) Genome-Wide Analysis of the Biology of Stress Responses through Heat Shock Transcription Factor. *Molecular and Cellular Biology* **24** (12), 5249-5256.

Halama, P. and Van Haluwin, C. (2004) Antifungal activity of lichen extracts and lichenic acids. *BioControl* **49** (1), 95-107.

Harris, S. D., Turner, G., Meyer, V., Espeso, E. A., Specht, T., Takeshita, N. and Helmstedt, K. (2009) Morphology and development in *Aspergillus nidulans*: A complex puzzle. *Fungal Genetics and Biology* **46**, S82-S92.

Hashimoto, M., Nonaka, T. and Fujii, I. (2014) Fungal type III polyketide synthases. *Natural Product Reports* **31** (10), 1306-1317.

Hauck, M. (2008) Metal homeostasis in *Hypogymnia physodes* is controlled by lichen substances. *Environmental Pollution* **153** (2), 304-308.

Hauck, M. and Huneck, S. (2007) Lichen Substances Affect Metal Adsorption in *Hypogymnia physodes*. *Journal of Chemical Ecology* **33** (1), 219-223.

Hauck, M., Willenbruch, K. and Leuschner, C. (2009) Lichen Substances Prevent Lichens from Nutrient Deficiency. *Journal of Chemical Ecology* **35** (1), 71-73.

Hawksworth, D. L. and Lücking, R. (2017) Fungal Diversity Revisited: 2.2 to 3.8 Million Species. *Microbiology Spectrum* **5** (4).

Henrikson, J. C., Hoover, A. R., Joyner, P. M. and Cichewicz, R. H. (2009) A chemical epigenetics approach for engineering the *in situ* biosynthesis of a cryptic natural product from *Aspergillus niger*. *Organic & Biomolecular Chemistry* **7** (3), 435-438.

Herbst, D. A., Townsend, C. A. and Maier, T. (2018) The architectures of iterative type I PKS and FAS. *Natural Product Reports* **35** (10), 1046-1069.

Hertweck, C. (2009) Hidden biosynthetic treasures brought to light. *Nature Chemical Biology* **5** (7), 450-452.

Hidalgo, M. E., Fernández, E., Quilhot, W. and Lissi, E. (1994) Antioxidant activity of depsides and depsidones. *Phytochemistry* **37** (6), 1585-1587.

Hill, D. J. (1985) Changes in Photobiont Dimensions and Numbers During Co-Development of Lichen Symbionts. In *Lichen Physiology and Cell Biology* (D. H. Brown, ed.), pp. 303-317, Springer: Boston, Massachusetts, USA.

Hill, D. J. (1989) The control of the cell cycle in microbial symbionts. *New Phytologist* **112** (2), 175-184.

Hill, D. J. (1992) The coordination of development of symbionts in mutualistic symbiosis with reference to the cell-cycle of the photobiont in lichens. *Symbiosis* **14** (1-3), 325-333.

Hitch, C. J. B. and Millbank, J. W. (1975) Nitrogen Metabolism in lichens. VI. The Blue-Green Phycobiont Content, Heterocyst Frequency and Nitrogenase Activity in *Peltigera* Species. *New Phytologist* **74** (3), 473-476.

Hodkinson, B. P. and Lutzoni, F. (2009) A microbiotic survey of lichen-associated bacteria reveals a new lineage from the Rhizobiales. *Symbiosis* **49** (3), 163-180.

Hoffmeister, D. and Keller, N. P. (2007) Natural products of filamentous fungi: enzymes, genes, and their regulation. *Natural Product Reports* **24** (2), 393-416.

Honegger, R. (1984) Cytological Aspects of the Mycobiont-Phycobiont Relationship in Lichens. *The Lichenologist* **16** (2), 111-127.

Honegger, R. (1986a) Ultrastructural Studies in Lichens. II. Mycobiont and Photobiont Cell Wall Surface Layers and Adhering Crystalline Lichen Products in Four Parmeliaceae. *New Phytologist* **103** (4), 797-808.

Honegger, R. (1986b) Ultrastructural Studies in Lichens. I. Haustorial Types and Their Frequencies in a Range of Lichens with Trebouxioid Photobionts. *New Phytologist* **103** (4), 785-795.

Honegger, R. (1991a) Haustoria-Like Structures and Hydrophobic Cell Wall Surface Layers in Lichens. In *Electron Microscopy of Plant Pathogens* (K. Mendgen and D-E. Lesemann, eds.), pp. 277-290, Springer-Verlag: Berlin, Heidelberg, Germany.

Honegger, R. (1991b) Functional Aspects of the Lichen Symbiosis. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**, 553-578.

Honegger, R. (1993) Tansley Review No. 60. Developmental biology of lichens. *New Phytologist* **125** (4), 659-677.

Honegger, R. (1997) Metabolic Interactions at the Mycobiont-Photobiont Interface in Lichens. In *The Mycota V Plant Relationships* (G. C. Carroll and P. Tudzynski, eds.), pp. 209-221, Springer-Verlag: Berlin, Heidelberg, Germany.

Honegger, R. (2001) The Symbiotic Phenotype of Lichen-Forming Ascomycetes. In *The Mycota IX Fungal Associations* (B. Hock, ed.), pp. 165-188, Springer-Verlag: Berlin, Heidelberg, Germany.

Honegger, R. (2008a) Mycobionts. In *Lichen Biology* (T. H. Nash III, ed.), pp. 25-39, Cambridge University Press: Cambridge, UK.

Honegger, R. (2008b) Morphogenesis. In *Lichen Biology* (T. H. Nash III, ed.), pp. 69-93, Cambridge University Press: Cambridge, UK.

Honegger, R. (2009) Lichen-Forming Fungi and Their Photobionts. In *The Mycota V Plant Relationships* (H. B. Deising, ed.), pp. 307-333, Springer-Verlag: Berlin, Heidelberg, Germany.

Honegger, R., Kutasi, V. and Ruffner, H. P. (1993) Polyol patterns in eleven species of aposymbiotically cultured lichen mycobionts. *Mycological Research* **97** (1), 35-39.

Honegger, R., Edwards, D. and Axe, L. (2013) The earliest records of internally stratified cyanobacterial and algal lichens from the Lower Devonian of the Welsh Borderland. *New Phytologist* **197** (1), 264-275.

Honegger, R., Zippler, U., Gansner, H. and Scherrer, S. (2004) Mating systems in the genus *Xanthoria* (lichen-forming ascomycetes). *Mycological Research* **108** (5), 480-488.

Hopwood, D. A. (1997) Genetic contributions to understanding polyketide synthases. *Chemical Reviews* **97** (7), 2465-2497.

Hopwood, D. A. and Sherman, D. H. (1990) Molecular genetics of polyketides and its comparison to fatty-acid biosynthesis. *Annual Review of Genetics* **24**, 37-66.

Hühner, E., Öqvist, K. and Li, S.-M. (2019) Design of α-Keto Carboxylic Acid Dimers by Domain Recombination of Nonribosomal Peptide Synthetase (NRPS)-Like Enzymes. *Organic Letters* **21** (2), 498-502.

Hühner, E., Backhaus, K., Kraut, R. and Li, S.-M. (2018) Production of α-keto carboxylic acid dimers in yeast by overexpression of NRPS-like genes from *Aspergillus terreus*. *Applied Microbiology and Biotechnology* **102** (4), 1663-1672.

Huneck, S. and Yoshimura, I. (1996) *Identification of Lichen Substances*, Springer-Verlag: Berlin, Heidelberg, Germany.

Hutner, S. H., Provasoli, L., Schatz, A. and Haskins, C. P. (1950) Some approaches to the study of the role of metals in the metabolism of microorganisms. *Proceedings of the American Philosophical Society* **94** (2), 152-170.

Hyvärinen, M. and Crittenden, P. D. (1998) Growth of the cushion-forming lichen, *Cladonia portentosa*, at nitrogen-polluted and unpolluted heathland sites. *Environmental and Experimental Botany* **40** (1), 67-76.

Insarova, I. D. and Blagoveshchenskaya, E. Y. (2016) Lichen symbiosis: Search and recognition of partners. *Biology Bulletin* **43** (5), 408-418.

Itoh, T., Tokunaga, K., Matsuda, Y., Fujii, I., Abe, I., Ebizuka, Y. and Kushiro, T. (2010) Reconstitution of a fungal meroterpenoid biosynthesis reveals the involvement of a novel family of terpene cyclases. *Nature Chemistry* **2** (10), 858-864.

Jahns, H. M. (1973) Anatomy, Morphology, and Development. In *The Lichens* (V. Ahmadjian and M. E. Hale, eds.), pp. 3-58, Academic Press, Inc. (London) Ltd.: London, UK.

Jahns, H. M. (1988) The establishment, individuality and growth of lichen thalli. *Botanical Journal of the Linnean Society* **96** (1), 21-29.

Jahns, H. M. (1993) Culture experiments with lichens. *Plant Systematics and Evolution* **187** (1-4), 145-174.

Jain, S. and Keller, N. (2013) Insights to fungal biology through LaeA sleuthing. *Fungal Biology Reviews* **27** (2), 51-59.

James, T. Y., Kauff, F., Schoch, C. L., Matheny, P. B., Hofstetter, V., Cox, C. J., Celio, G., Gueidan, C., Fraker, E., Miadlikowska, J., Lumbsch, H. T., Rauhut, A., Reeb, V., Arnold, A. E., Amtoft, A., Stajich, J. E., Hosaka, K., Sung, G.-H., Johnson, D., O'Rourke, B., Crockett, M., Binder, M., Curtis, J. M., Slot, J. C., Wang, Z., Wilson, A. W., Schüßler, A., Longcore, J. E., O'Donnell, K., Mozley-Standridge, S., Porter, D., Letcher, P. M., Powell, M. J., Taylor, J. W., White, M. M., Griffith, G. W., Davies, D. R., Humber, R. A., Morton, J. B., Sugiyama, J., Rossman, A. Y., Rogers, J. D., Pfister, D. H., Hewitt, D., Hansen, K., Hambleton, S., Shoemaker, R. A., Kohlmeyer, J., Volkmann-Kohlmeyer, B., Spotts, R. A., Serdani, M., Crous, P. W., Hughes, K. W., Matsuura, K., Langer, E., Langer, G., Untereiner, W. A., Lücking, R., Büdel, B., Geiser, D. M., Aptroot, A., Diederich, P., Schmitt, I., Schultz, M., Yahr, R., Hibbett, D. S., Lutzoni, F., McLaughlin, D. J., Spatafora, J. W. and Vilgalys, R. (2006) Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature* **443** (7113), 818-822.

Jenkins, B. and Richards, T. A. (2019) Symbiosis: Wolf Lichens Harbour a Choir of Fungi. *Current Biology* **29** (3), R86-R103.

Joneson, S. and Lutzoni, F. (2009) Compatibility and thigmotropism in the lichen symbiosis: A reappraisal. *Symbiosis* **47** (2), 109-115.

Joneson, S., Armaleo, D. and Lutzoni, F. (2011) Fungal and algal gene expression in early developmental stages of lichen-symbiosis. *Mycologia* **103** (2), 291-306.

Jung, C. and Till, B. (2021) Mutagenesis and genome editing in crop improvement: perspectives for the global regulatory landscape. *Trends in Plant Science* **26** (12), 1258-1269.

Kale, S. P., Milde, L., Trapp, M. K., Frisvad, J. C., Keller, N. P. and Bok, J. W. (2008) Requirement of LaeA for secondary metabolism and sclerotial production in *Aspergillus flavus*. *Fungal Genetics and Biology* **45** (10), 1422-1429.

Kappen, L. (1988) Ecophysiological relationships in different climatic regions. In *CRC Handbook of Lichenology, Volume 2* (M. Galun, ed.), pp. 37-99, CRC Press, Inc.: Boca Raton, Florida, USA.

Karakus, B., Odabasoglu, F., Cakir, A., Halici, Z., Bayir, Y., Halici, M., Aslan, A. and Suleyman, H. (2009) The effects of methanol extract of *Lobaria pulmonaria*, a lichen species, on indometacin-induced gastric mucosal damage, oxidative stress and neutrophil infiltration. *Phytotherapy Research* **23** (5), 635-639.

Kawahara, T., Yanagi, H., Yura, T. and Mori, K. (1997) Endoplasmic Reticulum Stress-induced mRNA Splicing Permits Synthesis of Transcription Factor Hac1p/Ern4p That Activates the Unfolded Protein Response. *Molecular Biology of the Cell* **8** (10), 1845-1862.

Keller, N. P. (2015) Translating biosynthetic gene clusters into fungal armor and weaponry. *Nature Chemical Biology* **11** (9), 671-677.

Keller, N. P. (2019) Fungal secondary metabolism: regulation, function and drug discovery. *Nature Reviews Microbiology* **17** (3), 167-180.

Keller, N. P. and Hohn, T. M. (1997) Metabolic Pathway Gene Clusters in Filamentous Fungi. *Fungal Genetics and Biology* **21** (1), 17-29.

Keller, N. P., Turner, G. and Bennett, J. W. (2005) Fungal secondary metabolism - from biochemistry to genomics. *Nature Reviews Microbiology* **3** (12), 937-947.

Kershaw, K. A. (1985) *Physiological Ecology of Lichens*, Cambridge University Press: Cambridge, UK.

Kershaw, K. A. and Millbank, J. W. (1970) Nitrogen Metabolism in Lichens II. The Partition of Cephalodial-Fixed Nitrogen Between the Mycobiont and Phycobionts of *Peltigera aphthosa*. *New Phytologist* **69** (1), 75-79.

Kim, H., Deng, L., Xiong, X., Hunter, W. D., Long, M. C. and Pirrung, M. C. (2007) Glyceraldehyde 3-Phosphate Dehydrogenase Is a Cellular Target of the Insulin Mimic Demethylasterriquinone B1. *Journal of Medicinal Chemistry* **50** (15), 3423-3426.

Kim, W., Liu, R., Woo, S., Kang, K. B., Park, H., Yu, Y. H., Ha, H.-H., Oh, S.-Y., Yang, J. H., Kim, H., Yun, S.-H. and Hur, J.-S. (2021) Linking a Gene Cluster to Atranorin, a Major Cortical Substance of Lichens, through Genetic Dereplication and Heterologous Expression. *mBio* **12** (3), e01111-21.

Knops, J. M. H., Nash III, T. H., Boucher, V. L. and Schlesinger, W. H. (1991) Mineral Cycling and Epiphytic Lichens: Implications at the Ecosystem Level. *The Lichenologist* **23** (3), 309-321.

Kofler, L. (1970) A Method to Use Lichen Spores in Quantitative Studies on Germination. *The Bryologist* **73** (3), 602-606.

Kraft, J., Bauer, S., Keilhoff, G., Miersch, J., Wend, D., Riemann, D., Hirschelmann, R., Holzhausen, H.-J. and Langner, J. (1998) Biological effects of the dihydroorotate dehydrogenase inhibitor polyporic acid, a toxic constituent of the mushroom *Hapalopilus rutilans*, in rats and humans. *Archives of Toxicology* **72** (11), 711-721.

Kranner, I., Beckett, R., Hochman, A. and Nash III, T. H. (2008) Desiccation-tolerance in lichens: a review. *The Bryologist* **111** (4), 576-593.

Kranner, I., Cram, W. J., Zorn, M., Wornik, S., Yoshimura, I., Stabentheiner, E. and Pfeifhofer, H. W. (2005) Antioxidants and photoprotection in a lichen as compared with its isolated symbiotic partners. *Proceedings of the National Academy of Sciences of the United States of America* **102** (8), 3141-3146.

Larson, D. W. (1984) Habitat overlap/niche segregation in two *Umbilicaria* lichens: a possible mechanism. *Oecologia* **62** (1), 118-125.

Lawrey, J. D. (1986) Biological Role of Lichen Substances. *The Bryologist* **89** (2), 111-122.

Lawrey, J. D. and Diederich, P. (2003) Lichenicolous Fungi: Interactions, Evolution, and Biodiversity. *The Bryologist* **106** (1), 80-120.

Le, T. T., Pandey, R. P., Gurung, R. B., Dhakal, D. and Sohng, J. K. (2014) Efficient enzymatic systems for synthesis of novel α-mangostin glycosides exhibiting antibacterial activity against Gram-positive bacteria. *Applied Microbiology and Biotechnology* **98** (20), 8527-8538.

Lendemer, J. C., Keepers, K. G., Tripp, E. A., Pogoda, C. S., McCain, C. M. and Kane, N. C. (2019) A taxonomically broad metagenomic survey of 339 species spanning 57 families suggests cystobasidiomycete yeasts are not ubiquitous across all lichens. *American Journal of Botany* **106** (8), 1090-1095.

Leuckert, C., Ahmadjian, V., Culberson, C. F. and Johnson, A. (1990) Xanthones and Depsidones of the Lichen *Lecanora dispersa* in Nature and of Its Mycobiont in Culture. *Mycologia* **82** (3), 370-378.

Li, X., Zheng, S.-L., Li, X., Li, J.-L., Qiang, O., Liu, R. and He, L. (2012) Synthesis and anti-breast cancer activity of new indolylquinone derivatives. *European Journal of Medicinal Chemistry* **54**, 42-48.

Lim, F. Y., Sanchez, J. F., Wang, C. C. C. and Keller, N. P. (2012) Toward Awakening Cryptic Secondary Metabolite Gene Clusters in Filamentous Fungi. In *Methods in Enzymology Volume 517: Natural Product Biosynthesis by Microorganisms and Plants, Part C* (D. A. Hopwood, ed.), pp. 303-324, Academic Press, Inc. (London) Ltd.: London, UK.

Lines, C. E. M., Ratcliffe, R. G., Rees, T. A. V. and Southon, T. E. (1989) A ^{13}C NMR study of photosynthate transport and metabolism in the lichen *Xanthoria calcicole* Oxner. *New Phytologist* **111** (3), 447-456.

Liu, Y. and Chang, A. (2008) Heat shock response relieves ER stress. *The EMBO Journal* **27** (7), 1049-1059.

Lokajová, V., Bačkorová, M. and Bačkor, M. (2014) Allelopathic effects of lichen secondary metabolites and their naturally occurring mixtures on cultures of aposymbiotically grown lichen photobiont *Trebouxia erici* (Chlorophyta). *South African Journal of Botany* **93**, 86-91.

Lücking, R., Hodkinson, B. P. and Leavitt, S. D. (2016) The 2016 classification of lichenized fungi in the Ascomycota and Basidiomycota - Approaching one thousand genera. *The Bryologist* **119** (4), 361-416.

Luo, H., Yamamoto, Y., Kim, J. A., Jung, J. S., Koh, Y. J. and Hur, J.-S. (2009) Lecanoric acid, a secondary lichen substance with antioxidant properties from *Umbilicaria antarctica* in maritime Antarctica (King George Island). *Polar Biology* **32** (7), 1033-1040.

Luo, H., Yamamoto, Y., Liu, Y., Jung, J. S., Kahng, H.-Y., Koh, Y. J. and Hur, J.-S. (2010) The *In Vitro* Antioxidant Properties of Chinese Highland Lichens. *Journal of Microbiology and Biotechnology* **20** (11), 1524-1528.

Luque, E. M., Gutiérrez, G., Navarro-Sampedro, L., Olmedo, M., Rodrígez-Romero, J., Ruger-Herreros, C., Tagua, V. G. and Corrochano, L. M. (2012) A Relationship between Carotenoid Accumulation and the Distribution of Species of the Fungus *Neurospora* in Spain. *PLoS ONE* **7** (3), e33658.

Lutzoni, F., Pagel, M. and Reeb, V. (2001) Major fungal lineages are derived from lichen symbiotic ancestors. *Nature* **411** (6840), 937-940.

Lutzoni, F., Kauff, F., Cox, C. J., McLaughlin, D., Celio, G., Dentinger, B., Padamsee, M., Hibbett, D., James, T. Y., Baloch, E., Grube, M., Reeb, V., Hofstetter, V., Schoch, C., Arnold, A. E., Miadlikowska, J., Spatafora, J., Johnson, D., Hambleton, S., Crockett, M., Shoemaker, R., Sung, G.-H., Lücking, R., Lumbsch, T., O'Donnell, K., Binder, M., Diederich, P., Ertz, D., Gueidan, C., Hansen, K., Harris, R. C., Hosaka, K., Lim, Y.-W., Matheny, B., Nishida, H., Pfister, D., Rogers, J., Rossman, A., Schmitt, I., Sipman, H., Stone, J., Sugiyama, J., Yahr, R. and Vilgalys, R. (2004) Assembling the Fungal Tree of Life: Progress, Classification and Evolution of Subcellular Traits. *American Journal of Botany* **91** (10), 1446-1480.

MacFarlane, J. D. and Kershaw, K. A. (1980) Physiological-Environmental Interactions in Lichens. IX. Thermal Stress and Lichen Ecology. *New Phytologist* **84** (4), 669-685.

Macheleidt, J., Mattern, D. J., Fischer, J., Netzker, T., Weber, J., Schroeckh, V., Valiante, V. and Brakhage, A. A. (2016) Regulation and Role of Fungal Secondary Metabolites. *Annual Review of Genetics* **50**, 371-392.

Maiya, S., Grundmann, A., Li, S.-M. and Turner, G. (2006) The Fumitremorgin Gene Cluster of *Aspergillus fumigatus*: Identification of a Gene Encoding Brevianamide F Synthetase. *ChemBioChem* **7** (7), 1062-1069.

Manjunatha, U. H., Rao, S. P. S., Kondreddi, R. R., Noble, C. G., Camacho, L. R., Tan, B. H., Ng, S. H., Ng, P. S., Ma, N. L., Lakshminarayana, S. B., Herve, M., Barnes, S. W., Yu, W., Kuhen, K., Blasco, F., Beer, D., Walker, J. R., Tonge, P. J., Glynne, R., Smith, P. W. and Diagana, T. T. (2015) Direct inhibitors of InhA active against *Mycobacterium tuberculosis*. *Science Translational Medicine* **7** (269).

Marahiel, M. A., Stachelhaus, T. and Mootz, H. D. (1997) Modular Peptide Synthetases Involved in Nonribosomal Peptide Synthesis. *Chemical Reviews* **97** (7), 2651-2673.

Marante, F. J. T., Castellano, A. G. I., Rosas, F. E., Aguiar, J. Q. and Barrera, J. B. (2003) Identification and Quantitation of Allelochemicals from the Lichen *Lethariella canariensis*: Phytotoxicity and Antioxidative Activity. *Journal of Chemical Ecology* **29** (9), 2049-2071.

Mark, K., Laanisto, L., Bueno, C. G., Niinemets, Ü., Keller, C. and Scheidegger, C. (2020) Contrasting co-occurrence patterns of photobiont and cystobasidiomycete yeast associated with common epiphytic lichen species. *New Phytologist* **227** (5), 1362-1375.

Marmann, A., Aly, A. H., Lin, W., Wang, B. and Proksch, P. (2014) Co-Cultivation – A Powerful Emerging Tool for Enhancing the Chemical Diversity of Microorganisms. *Marine Drugs* **12** (2), 1043-1065.

McEvoy, M., Gauslaa, Y. and Solhaug, K. A. (2007) Changes in pools of depsidones and melanins, and their function, during growth and acclimation under contrasting natural light in the lichen *Lobaria pulmonaria*. *New Phytologist* **175** (2), 271-282.

McEvoy, M., Nybakken, L., Solhaug, K. A. and Gauslaa, Y. (2006) UV triggers the synthesis of the widely distributed secondary lichen compound usnic acid. *Mycological Progress* **5** (4), 221-229.

McKenzie, S. K., Walston, R. F. and Allen, J. L. (2020) Complete, high-quality genomes from long-read metagenomic sequencing of two wolf lichen thalli reveals enigmatic genome architecture. *Genomics* **112** (5), 3150-3156.

Medina, M., Collins, A. G., Taylor, J. W., Valentine, J. W., Lipps, J. H., Amaral-Zettler, L. and Sogin, M. L. (2003) Phylogeny of Opisthokonta and the evolution of multicellularity and complexity in Fungi and Metazoa. *International Journal of Astrobiology* **2** (3), 203-211.

Meeßen, J. and Ott, S. (2013) Recognition mechanisms during the pre-contact state of lichens: I. Mycobiont-photobiont interactions of the mycobiont of *Fulgensia bracteata*. *Symbiosis* **59** (3), 121-130.

Meiser, A., Otte, J., Schmitt, I. and Dal Grande, F. (2017) Sequencing genomes from mixed DNA samples - evaluating the metagenome skimming approach in lichenized fungi. *Scientific Reports* **7**, 14881.

Meyer, V., Arentshorst, M., Flitter, S. J., Nitsche, B. M., Kwon, M. J., Reynaga-Peña, C. G., Bartnicki-Garcia, S., van den Hondel, C. A. M. J. J. and Ram, A. F. J. (2009) Reconstruction of Signaling Networks Regulating Fungal Morphogenesis by Transcriptomics. *Eukaryotic Cell* **8** (11), 1677-1691.

Miao, V., Coëffet-LeGal, M.-F., Brown, D., Sinnemann, S., Donaldson, G. and Davies, J. (2001) Genetic approaches to harvesting lichen products. *Trends in Biotechnology* **19** (9), 349-355.

Millbank, J. W. and Kershaw, K. A. (1969) Nitrogen Metabolism in Lichens. I. Nitrogen Fixation in the Cephalodia of *Peltigera aphthosa*. *New Phytologist* **68** (3), 721-729.

Millbank, J. W. and Kershaw, K. A. (1973) Nitrogen Metabolism. In *The Lichens* (V. Ahmadjian and M. E. Hale, eds.), pp. 289-309, Academic Press, Inc. (London) Ltd.: London, UK.

Millot, M., Dieu, A. and Tomasi, S. (2016) Dibenzofurans and derivatives from lichens and ascomycetes. *Natural Product Reports* **33** (6), 801-811.

Millot, M., Di Meo, F., Tomasi, S., Boustie, J. and Trouillas, P. (2012) Photoprotective capacities of lichen metabolites: A joint theoretical and experimental study. *Journal of Photochemistry and Photobiology B: Biology* **111**, 17-26.

Molnár, K. and Farkas, E. (2010) Current Results on Biological Activities of Lichen Secondary Metabolites: a Review. *Zeitschrift für Naturforschung Section C* **65** (3-4), 157-173.

Moore, D., Robson, G. D. and Trinci, A. P. J (2011) *21st Century Guidebook to Fungi*, Cambridge University Press: Cambridge, UK.

Mosbach, K. (1969) Biosynthesis of Lichen Substances, Products of a Symbiotic Association *Angewandte Chemie – International Edition* **8** (4), 240-250.

Muggia, L. and Grube, M. (2018) Fungal Diversity in Lichens: From Extremotolerance to Interactions with Algae. *Life* **8** (2), 15.

Muir, P. S., Shirazi, A. M. and Patrie, J. (1997) Seasonal Growth Dynamics in the Lichen *Lobaria pulmonaria*. *The Bryologist* **100** (4), 458-464.

Mulder, H. J. and Nikolaev, I. (2009) HacA-Dependent Transcriptional Switch Releases *hacA* mRNA from a Translational Block upon Endoplasmic Reticulum Stress. *Eukaryotic Cell* **8** (4), 665-675.

Mulder, H. J., Saloheimo, M., Penttilä, M. and Madrid, S. M. (2004) The transcription factor HACA mediates the unfolded protein response in *Aspergillus niger*, and up-regulates its own transcription. *Molecular Genetics and Genomics* **271** (2), 130-140.

Müller, K. (2001) Pharmaceutically relevant metabolites from lichens. *Applied Microbiology and Biotechnology* **56** (1-2), 9-16.

Murtagh, G. J., Dyer, P. S. and Crittenden, P. D. (2000) Reproductive systems: Sex and the single lichen. *Nature* **404** (6778), 564.

Murtagh, G. J., Dyer, P. S., Furneaux, P. A. and Crittenden, P. D. (2002) Molecular and physiological diversity in the bipolar lichen-forming fungus *Xanthoria elegans*. *Mycological Research* **106** (11), 1277-1286.

Naranjo-Ortiz, M. A. and Gabaldón, T. (2019a) Fungal evolution: diversity, taxonomy and phylogeny of the Fungi. *Biological Reviews* **94** (6), 2101-2137.
Naranjo-Ortiz, M. A. and Gabaldón, T. (2019b) Fungal evolution: major ecological adaptations and evolutionary transitions. *Biological Reviews* **94** (4), 1443-1476.

Nascimento, É., Da Silva, S. H., Marques, E. D. R., Roberts, D. W. and Braga, G. U. L. (2010) Quantification of Cyclobutane Pyrimidine Dimers Induced by UVB Radiation in Conidia of the Fungi *Aspergillus fumigatus*, *Aspergillus nidulans*, *Metarhizium acridum* and *Metarhizium robertsii*. *Photochemistry and Photobiology* **86** (6), 1259-1266.

Nash III, T. H. (2008a) Introduction. In *Lichen Biology* (T. H. Nash III, ed.), pp. 1-8, Cambridge University Press: Cambridge, UK.

Nash III, T. H. (2008b) Nitrogen, its metabolism and potential contribution to ecosystems. In *Lichen Biology* (T. H. Nash III, ed.), pp. 216-233, Cambridge University Press: Cambridge, UK.

Nash III, T. H. (2008c) Nutrients, elemental accumulation, and mineral cycling. In *Lichen Biology* (T. H. Nash III, ed.), pp. 234-251, Cambridge University Press: Cambridge, UK.

Netzker, T., Fischer, J., Weber, J., Mattern, D. J., König, C. C., Valiante, V., Schroeckh, V. and Brakhage, A. A. (2015) Microbial communication leading to the activation of silent fungal secondary metabolite gene clusters. *Frontiers in Microbiology* **6**, 299.

Nguyen, H. T., Pokhrel, A. R., Nguyen, C. T., Pham, V. T. T., Dhakal, D., Lim, H. N., Jung, H. J., Kim, T.-S., Yamaguchi, T. and Sohng, J. K. (2020) *Streptomyces* sp. VN1, a producer of diverse metabolites including non-natural furan-type anticancer compound. *Scientific Reports* **10** (1), 1756.

Nguyen, K.-H., Chollet-Krugler, M., Gouault, N. and Tomasi, S. (2013) UV-protectant metabolites from lichens and their symbiotic partners. *Natural Product Reports* **30** (12), 1490-1508.

Nieboer, E., Richardson, D. H. S. and Tomassini, F. D. (1978) Mineral Uptake and Release by Lichens: An Overview. *The Bryologist* **81** (2), 226-246.

Nützmann, H.-W., Scazzocchio, C. and Osbourn, A. (2018) Metabolic Gene Clusters in Eukaryotes. *Annual Review of Genetics* **52**, 159-183.

Nybakken, L. and Gauslaa, Y. (2007) Difference in secondary compounds and chlorophylls between fibrils and main stems in the lichen *Usnea longissima* suggests different functional roles. *The Lichenologist* **39** (5), 491-494.

O'Neal, M. A. and Schoenenberger, K. R. (2003) A *Rhizocarpon geographicum* growth curve for the Cascade Range of Washington and northern Oregon, USA. *Quaternary Research* **60** (2), 233-241.

Oberwinkler, F. (2017) Yeasts in Pucciniomycotina. *Mycological Progress* **16** (9), 831-856.

Odabasoglu, F., Aslan, A., Cakir, A., Suleyman, H., Karagoz, Y., Halici, M. and Bayir, Y. (2004) Comparison of antioxidant activity and phenolic content of three lichen species. *Phytotherapy Research* **18** (11), 938-941.

Ostrofsky, A. and Denison, W. C. (1980) Ascospore Discharge and Germination in *Xanthoria polycarpa*. *Mycologia* **72** (6), 1171-1179.

Palmer, J. M., Wiemann, P., Greco, C., Chiang, Y. M., Wang, C. C. C., Lindner, D. L. and Keller, N. P. (2021) The sexual spore pigment asperthecin is required for normal ascospore production and protection from UV light in *Aspergillus nidulans*. *Journal of Industrial Microbiology and Biotechnology* **48** (9-10), kuab055.

Palmqvist, K. (2000) Tansley Review No. 117. Carbon economy in lichens. *New Phytologist* **148** (1), 11-36.

Palmqvist, K. and Dahlman, L. (2006) Responses of the green algal foliose lichen *Platismatia glauca* to increased nitrogen supply. *New Phytologist* **171** (2), 343-356.

Palmqvist, K., Dahlman, L., Jonsson, A. and Nash III, T. H. (2008) The carbon economy of lichens. In *Lichen Biology* (T. H. Nash III, ed.), pp. 182-215, Cambridge University Press: Cambridge, UK.

Pandey, R. P., Parajuli, P., Koffas, M. A. G. and Sohng, J. K. (2016) Microbial production of natural and non-natural flavonoids: Pathway engineering, directed evolution and systems/synthetic biology. *Biotechnology Advances* **34** (5), 634-662.

Pankratov, T. A., Kachalkin, A. V., Korchikov, E. S. and Dobrovol'skaya, T. G. (2017) Microbial communities of lichens. *Microbiology* **86** (3), 293-309.

Paoletti, M., Rydholm, C., Schwier, E. U., Anderson, M. J., Szakacs, G., Lutzoni, F., Debeaupuis, J.-P., Latgé, J.-P., Denning, D. W. and Dyer, P. S. (2005) Evidence for Sexuality in the Opportunistic Fungal Pathogen *Aspergillus fumigatus*. *Current Biology* **15** (13), 1242-1248.

Park, S.-Y., Choi, J., Kim, J. A., Jeong, M.-H., Kim, S., Lee, Y.-H. and Hur, J.-S. (2013a) Draft Genome Sequence of *Cladonia macilenta* KoLRI003786, a Lichen-Forming Fungus Producing Biruloquinone. *Genome Announcements* **1** (5), e00695-13.

Park, S.-Y., Choi, J., Kim, J. A., Yu, N.-H., Kim, S., Kondratyuk, S. Y., Lee, Y.-H. and Hur, J.-S. (2013b) Draft Genome Sequence of Lichen-Forming Fungus *Caloplaca flavorubescens* Strain KoLRI002931. *Genome Announcements* **1** (4), e00678-13.

Park, S.-Y., Choi, J., Lee, G.-W., Jeong, M.-H., Kim, J. A., Oh, S.-O., Lee, Y.-H. and Hur, J.-S. (2014a) Draft Genome Sequence of *Umbilicaria muehlenbergii* KoLRILF000956, a Lichen-Forming Fungus Amenable to Genetic Manipulation. *Genome Announcements* **2** (2), e00357-14.

Park, S.-Y., Choi, J., Lee, G.-W., Park, C.-H., Kim, J. A., Oh, S.-O., Lee, Y.-H. and Hur, J.-S. (2014b) Draft Genome Sequence of *Endocarpon pusillum* Strain KoLRILF000583. *Genome Announcements* **2** (3), e00452-14.

Park, S.-Y., Choi, J., Lee, G.-W., Kim, J. A., Oh, S.-O., Jeong, M.-H., Yu, N.-H., Kim, S., Lee, Y.-H. and Hur, J.-S. (2014c) Draft Genome Sequence of Lichen-Forming Fungus *Cladonia metacorallifera* Strain KoLRI002260. *Genome Announcements* **2** (1), e01065-13.

Paterson, D. R., Paterson, E. W. and Kenworthy, J. B. (1983) Physiological Studies on Temperate Lichen Species. I. A Mathematical Model to Predict Assimilation in the Field, Based on Laboratory Responses. *New Phytologist* **94** (4), 605-618.

Paudel, B., Bhattarai, H. D., Lee, J. S., Hong, S. G., Shin, H. W. and Yim, J. H. (2008) Antibacterial potential of Antarctic lichens against human pathogenic Gram-positive bacteria. *Phytotherapy Research* **22** (9), 1269-1271.

Pauly, J., Nett, M. and Hoffmeister, D. (2014) Ralfuranone Is Produced by an Alternative Aryl-Substituted γ-Lactone Biosynthetic Route in *Ralstonia solanacearum*. *Journal of Natural Products* **77** (8), 1967-1971.

Payne, J. A. E., Schoppet, M., Hansen, M. H. and Cryle, M. J. (2017) Diversity of nature's assembly lines – recent discoveries in non-ribosomal peptide synthesis. *Molecular BioSystems* **13** (1), 9-22.

Pentecost, A. (1980) Aspects of Competition in Saxicolous Lichen Communities. *The Lichenologist* **12** (1), 135-144.

Perrin, R. M., Fedorova, N. D., Bok, J. W., Cramer Jr., R. A., Wortman, J. R., Kim, H. S., Nierman, W. C. and Keller, N. P. (2007) Transcriptional Regulation of Chemical Diversity in *Aspergillus fumigatus* by LaeA. *PLoS Pathogens* **3** (4), 508-517.

Pfannenstiel, B. T. and Keller, N. P. (2019) On top of biosynthetic gene clusters: How epigenetic machinery influences secondary metabolism in fungi. *Biotechnology Advances* **37** (6), 107345.

Pizarro, D., Dal Grande, F., Leavitt, S. D., Dyer, P. S., Schmitt, I., Crespo, A., Lumbsch, H. T. and Divakar, P. K. (2019) Whole-Genome Sequence Data Uncover Widespread Heterothallism in the Largest Group of Lichen-Forming Fungi. *Genome Biology and Evolution* **11** (3), 721-730.

Prestrelski, S. J., Tedeschi, N., Arakawa, T. and Carpenter, J. F. (1993) Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. *Biophysical Journal* **65** (2), 661-671.

Quack, W., Scholl, H. and Budzikiewicz, H. (1982) Ascocorynin, a terphenylquinone from *Ascocoryne sarcoides*. *Phytochemistry* **21** (12), 2921-2923.

Rai, A. N. (1990) Cyanobacterial-fungal symbioses: the cyanolichens. In *CRC Handbook of Symbiotic Cyanobacteria* (A. N. Rai, ed.), pp. 9-41, CRC Press: Boca Raton, Florida, USA.

Rai, A. N. and Bergman, B. (2002) Cyanolichens. *Biology and Environment: Proceedings of the Royal Irish Academy* **102B** (1), 19-22.

Ranković, B., Mišić, M. and Sukdolak, S. (2008) The antimicrobial activity of substances derived from the lichens *Physcia aipolia*, *Umbilicaria polyphylla*, *Parmelia caperata* and *Hypogymnia physodes*. *World Journal of Microbiology & Biotechnology* **24** (7), 1239-1242.

Raper, K. B. and Fennell, D. I. (1965) *The genus Aspergillus*, Williams and Wilkins: Baltimore, USA.

Renhorn, K.-E., Esseen, P.-A., Palmqvist, K. and Sundberg, B. (1997) Growth and vitality of epiphytic lichens. I. Responses to microclimate along a forest edge-interior gradient. *Oecologia* **109** (1), 1-9.

Reutimann, P. and Scheidegger, C. (1987) Importance of Lichen Secondary Products in Food Choice of Two Oribatid Mites (Acari) in an Alpine Meadow Ecosystem. *Journal of Chemical Ecology* **13** (2), 363-369.

Reyes-Dominguez, Y., Bok, J. W., Berger, H., Shwab, E. K., Basheer, A., Gallmetzer, A., Scazzocchio, C., Keller, N. and Strauss, J. (2010) Heterochromatic marks are associated with the repression of secondary metabolism clusters in *Aspergillus nidulans*. *Molecular Microbiology* **76** (6), 1376-1386.

Richardson, D. H. S. and Smith, D. C. (1966) The physiology of the symbiosis in *Xanthoria aureola* (Ach.) Erichs. *The Lichenologist* **3** (2), 202-206.

Richardson, D. H. S. and Smith, D. C. (1968) Lichen Physiology. IX. Carbohydrate Movement from the *Trebouxia* Symbiont of *Xanthoria aureola* to the Fungus. *New Phytologist* **67** (1), 61-68.

Richardson, D. H. S., Hill, D. J. and Smith, D. C. (1968) Lichen Physiology. XI. The Role of the Alga in Determining the Pattern of Carbohydrate Movement Between Lichen Symbionts. *New Phytologist* **67** (3), 469-486.

Rikkinen, J. (1995) What's behind the pretty colours? A study on the photobiology of lichens. *Bryobrothera* **4**, 1-239.

Rikkinen, J. (2002) Cyanolichens: An Evolutionary Overview. In *Cyanobacteria in Symbiosis* (A. N. Rai, B. Bergman and U. Rasmussen, eds.), pp. 31-72, Kluwer Academic Publishers: Amsterdam, The Netherlands.

Rikkinen, J. (2015) Cyanolichens. *Biodiversity and Conservation* **24** (4), 973-993.

Rodriguez, R. J., Henson, J., Van Volkenburgh, E., Hoy, M., Wright, L., Beckwith, F., Kim, Y.-O. and Redman, R. S. (2008) Stress tolerance in plants via habitat-adapted symbiosis. The *ISME Journal* **2** (4), 404-416.

Romagni, J. G., Rosell, R. C., Nanayakkara, N. P. D. and Dayan, F. E. (2004) Ecophysiology and Potential Modes of Action for Selected Lichen Secondary Metabolites. In *Allelopathy: Chemistry and Mode of Action of Allelochemicals* (F. A. Macías, J. C. G. Galindo, J. M. G. Molinillo and H. G. Cutler, eds.), pp. 13-33, CRC Press: Boca Raton, Florida, USA.

Ron, D. and Walter, P. (2007) Signal integration in the endoplasmic reticulum unfolded protein response. *Nature Reviews Molecular Cell Biology* **8** (7), 519-529.

Rudolf, J. D., Chang, C.-Y., Ma, M. and Shen, B. (2017) Cytochromes P450 for natural product biosynthesis in *Streptomyces*: sequence, structure, and function. *Natural Product Reports* **34** (9), 1141-1172.

Rüegsegger, U., Leber, J. H. and Walter, P. (2001) Block of *HAC1* mRNA Translation by Long-Range Base Pairing is Released by Cytoplasmic Splicing upon Induction of the Unfolded Protein Response. *Cell* **107** (1), 103-114.

Rundel, P. W. (1978) The ecological role of secondary lichen substances. *Biochemical Systematics and Ecology* **6** (3), 157-170.

Rutkowski, D. T. and Kaufman, R. J. (2004) A trip to the ER: coping with stress. *Trends in Cell Biology* **14** (1), 20-28.

Rutledge, P. J. and Challis, G. L. (2015) Discovery of microbial natural products by activation of silent biosynthetic gene clusters. *Nature Reviews Microbiology* **13** (8), 509-523.

Saloheimo, M., Valkonen, M. and Penttilä, M. (2003) Activation mechanisms of the HAC1-mediated unfolded protein response in filamentous fungi. *Molecular Microbiology* **47** (4), 1149-1161.

Sanchez, J. F., Somoza, A. D., Keller, N. P. and Wang, C. C. C. (2012) Advances in *Aspergillus* secondary metabolite research in the post-genomic era. *Natural Product Reports* **29** (3), 351-371.

Scharf, D. H., Heinekamp, T. and Brakhage, A. A. (2014) Human and Plant Fungal Pathogens: The Role of Secondary Metabolites. *PLoS Pathogens* **10** (1), e1003859.

Schimek, C. (2011) Evolution of Special Metabolism in Fungi: Concepts, Mechanisms, and Pathways. In *The Mycota XIV Evolution of Fungi and Fungal-Like Organisms* (S. Pöggeler and J. Wöstemeyer, eds.), pp. 293-329, Springer-Verlag: Berlin, Heidelberg, Germany.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P. and Cardona, A. (2012) Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9** (7), 676-682.

Schmitt, I., Martín, M. P., Kautz, S. and Lumbsch, H. T. (2005) Diversity of non-reducing polyketide synthase genes in the Pertusariales (lichenized Ascomycota): A phylogenetic perspective. *Phytochemistry* **66** (11), 1241-1253.

Schneider, A. (1897) *A text-book of general lichenology, with descriptions and figures of the genera occurring in the northeastern United States*, Willard N. Clute & Company: Binghamton, New York, USA.

Schneider, P., Bouhired, S. and Hoffmeister, D. (2008) Characterization of the atromentin biosynthesis genes and enzymes in the homobasidiomycete *Tapinella panuoides*. *Fungal Genetics and Biology* **45** (11), 1487-1496.

Schneider, P., Weber, M., Rosenberger, K. and Hoffmeister, D. (2007) A One-Pot Chemoenzymatic Synthesis for the Universal Precursor of Antidiabetes and Antiviral Bis-Indolylquinones. *Chemistry & Biology* **14** (6), 635-644.

Schoch, C. L., Sung, G.-H., López-Giráldez, F., Townsend, J. P., Miadlikowska, J., Hofstetter, V., Robbertse, B., Matheny, P. B., Kauff, F., Wang, Z., Gueidan, C., Andrie, R. M., Trippe, K., Ciufetti, L. M., Wynns, A., Fraker, E., Hodkinson, B. P., Bonito, G., Groenewald, J. Z., Arzanlou, M., de Hoog, G. S., Crous, P. W., Hewitt, D., Pfister, D. H., Peterson, K., Gryzenhout, M., Wingfield, M. J., Aptroot, A., Suh, S.-O., Blackwell, M., Hillis, D. M., Griffith, G. W., Castlebury, L. A., Rossman, A. Y., Lumbsch, H. T., Lücking, R., Büdel, B., Rauhut, A., Diederich, P., Ertz, D., Geiser, D. M., Hosaka, K., Inderbitzin, P., Kohlmeyer, J., Volkmann-Kohlmeyer, B., Mostert, L., O'Donnell, K., Sipman, H., Rogers, J. D., Shoemaker, R. A., Sugiyama, J., Summerbell, R. C., Untereiner, W., Johnston, P. R., Stenroos, S., Zuccaro, A., Dyer, P. S., Crittenden, P. D., Cole, M. S., Hansen, K., Trappe, J. M., Yahr, R., Lutzoni, F. and Spatafora, J. W. (2009) The Ascomycota Tree of Life: A Phylum-wide Phylogeny Clarifies the Origin and Evolution of Fundamental Reproductive and Ecological Traits. *Systematic Biology* **58** (2), 224-239.

Schroeckh, V., Scherlach, K., Nützmann, H.-W., Shelest, E., Schmidt-Heck, W., Schuemann, J., Martin, K., Hertweck, C. and Brakhage, A. A. (2009) Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. *Proceedings of the National Academy of Sciences of the United States of America* **106** (34), 14558-14563.

Schüffler, A., Liermann, J. C., Opatz, T. and Anke, T. (2011) Elucidation of the Biosynthesis and Degradation of Allantofuranone by Isotopic Labelling and Fermentation of Modified Precursors. *ChemBioChem* **12** (1), 148-154.

Schwendener, S. (1869) *Die Algentypen der Flechtengonidien. Programm für die Rectoratsfeier der Universitæt*, Universitætsbuchdruckerei von C. Schultze: Basel, Switzerland.

Seaward, M. R. D. (2008) Environmental role of lichens. In *Lichen Biology* (T. H. Nash III, ed.), pp. 274-298, Cambridge University Press: Cambridge, UK.

Seshadri, T. R. (1944) A theory of biogenesis of lichen depsides and depsidones. *Proceedings of the Indian Academy of Sciences Section A* **20** (1), 1-14.

Seymour, F. A., Crittenden, P. D. and Dyer, P. S. (2005a) Sex in the extremes: lichen-forming fungi. *Mycologist* **19** (2), 51-58.

Seymour, F. A., Crittenden, P. D., Dickinson, M. J., Paoletti, M., Montiel, D., Cho, L. and Dyer, P. S. (2005b) Breeding systems in the lichen-forming fungal genus *Cladonia*. *Fungal Genetics and Biology* **42** (6), 554-563.

Shanmugam, K., Srinivasan, M. and Hariharan, G. N. (2016) Developmental stages and secondary compound biosynthesis of mycobiont and whole thallus cultures of *Buellia subsororioides*. *Mycological Progress* **15** (4), 41.

Shelest, E. (2017) Transcription Factors in Fungi: TFome Dynamics, Three Major Families, and Dual-Specificity TFs. *Frontiers in Genetics* **8**, 53.

Shwab, E. K. and Keller, N. P. (2008) Regulation of secondary metabolite production in filamentous ascomycetes. *Mycological Research* **112** (2), 225-230.

Shwab, E. K., Bok, J. W., Tribus, M., Galehr, J., Graessle, S. and Keller, N. P. (2007) Histone Deacetylase Activity Regulates Chemical Diversity in *Aspergillus*. *Eukaryotic Cell* **6** (9), 1656-1664.

Sidrauski, C. and Walter, P. (1997) The Transmembrane Kinase Ire1p Is a Site-Specific Endonuclease That Initiates mRNA Splicing in the Unfolded Protein Response. *Cell* **90** (6), 1031-1039.

Sidrauski, C., Cox, J. S. and Walter, P. (1996) tRNA Ligase Is Required for Regulated mRNA Splicing in the Unfolded Protein Response. *Cell* **87** (3), 405-413.

Singaravelan, N., Grishkan, I., Beharav, A., Wakamatsu, K., Ito, S. and Nevo, E. (2008) Adaptive Melanin Response of the Soil Fungus *Aspergillus niger* to UV Radiation Stress at "Evolution Canyon", Mount Carmel, Israel. *PLoS ONE* **3** (8), e2993.

Singh, R. S. and Walia, A. K. (2014) Characteristics of lichen lectins and their role in symbiosis. *Symbiosis* **62** (3), 123-134.

Sinha, R. P. and Häder, D.-P. (2002) UV-induced DNA damage and repair: a review. *Photochemical & Photobiological Sciences* **1** (4), 225-236.

Sinnemann, S. J., Andrésson, Ó. S., Brown, D. W. and Miao, V. P. W. (2000) Cloning and heterologous expression of *Solorina crocea pyrG*. *Current Genetics* **37** (5), 333-338.

Smith, C. W., Aptroot, A., Coppins, B. J., Fletcher, A., Gilbert, O. L., James, P. W. and Wolseley, P. A., eds. (2009) *The Lichens of Great Britain and Ireland*, British Lichen Society: London, UK.

Smith, D. C. (1978) What Can Lichens Tell Us about Real Fungi? *Mycologia* **70** (5), 915-934.

Smith, D. C. and Molesworth, S. (1973) Lichen Physiology. XIII. Effects of Rewetting Dry Lichens. *New Phytologist* **72** (3), 525-533.

Solhaug, K. A. and Gauslaa, Y. (2004) Photosynthates stimulate the UV-B induced fungal anthraquinone synthesis in the foliose lichen *Xanthoria parietina*. *Plant, Cell and Environment* **27** (2), 167-176.

Solhaug, K. A., Gauslaa, Y., Nybakken, L. and Bilger, W. (2003) UV-induction of sun-screening pigments in lichens. *New Phytologist* **158** (1), 91-100.

Sorger, P. K. (1991) Heat shock factor and the heat shock response. *Cell* **65** (3), 363-366.

Sorger, P. K., Lewis, M. J. and Pelham, H. R. B. (1987) Heat shock factor is regulated differently in yeast and HeLa cells. *Nature* **329** (6134), 81-84.

Spatafora, J. W., Aime, M. C., Grigoriev, I. V., Martin, F., Stajich, J. E. and Blackwell, M. (2017) The Fungal Tree of Life: from Molecular Systematics to Genome-Scale Phylogenies. *Microbiology Spectrum* **5** (5), FUNK-0053-2016.

Spribille, T., Tuovinen, V., Resl, P., Vanderpool, D., Wolinski, H., Aime, M. C., Schneider, K., Stabentheiner, E., Toome-Heller, M., Thor, G., Mayrhofer, H., Johannesson, H. and McCutcheon, J. P. (2016) Basidiomycete yeasts in the cortex of ascomycete macrolichens. *Science* **353** (6298), 488-492.

Spribille, T., Tagirdzhanova, G., Goyette, S., Tuovinen, V., Case, R. and Zandberg, W. F. (2020) 3D biofilms: in search of the polysaccharides holding together lichen symbioses. *FEMS Microbiology Letters* **367** (5).

St Clair, S. B., St Clair, L. L., Weber, D. J., Mangelson, N. F. and Eggett, D. L. (2002) Element Accumulation Patterns in Foliose and Fruticose Lichens from Rock and Bark Substrates in Arizona. *The Bryologist* **105** (3), 415-421.

Stachelhaus, T., Hüser, A. and Marahiel, M. A. (1996) Biochemical characterization of peptidyl carrier protein (PCP), the thiolation domain of multifunctional peptide synthetases. *Chemistry & Biology* **3** (11), 913-921.

Stachelhaus, T., Mootz, H. D. and Marahiel, M. A. (1999) The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. *Chemistry & Biology* **6** (8), 493-505.

Stachelhaus, T., Mootz, H. D., Bergendahl, V. and Marahiel, M. A. (1998) Peptide Bond Formation in Nonribosomal Peptide Biosynthesis - Catalytic Role of the Condensation Domain. *Journal of Biological Chemistry* **273** (35), 22773-22781.

Stajich, J. E., Berbee, M. L., Blackwell, M., Hibbett, D. S., James, T. Y., Spatafora, J. W. and Taylor, J. W. (2009) The Fungi. *Current Biology* **19** (18), R840-R845.

Steenkamp, E. T., Wright, J. and Baldauf, S. L. (2006) The Protistan Origins of Animals and Fungi. *Molecular Biology and Evolution* **23** (1), 93-106.

Stenroos, S. K. and DePriest, P. T. (1998) SSU rDNA phylogeny of cladoniiform lichens. *American Journal of Botany* **85** (11), 1548-1559.

Stocker-Wörgötter, E. (2001) Experimental Lichenology and Microbiology of Lichens: Culture Experiments, Secondary Chemistry of Cultured Mycobionts, Resynthesis, and Thallus Morphogenesis. *The Bryologist* **104** (4), 576-581.

Stocker-Wörgötter, E. (2008) Metabolic diversity of lichen-forming ascomycetous fungi: culturing, polyketide and shikimate metabolite production, and PKS genes. *Natural Product Reports* **25** (1), 188-200.

Stocker-Wörgötter, E. (2015) Biochemical Diversity and Ecology of Lichen-Forming Fungi: Lichen Substances, Chemosyndromic Variation and Origin of Polyketide-Type Metabolites (Biosynthetic Pathways). In *Recent Advances in Lichenology: Modern Methods and Approaches in Lichen Systematics and Culture Techniques, Volume 2* (D. K. Upreti, P. K. Divakar, V. Shukla and R. Bajpai, eds.), pp. 161-179, Springer India: New Delhi, India.

Stocker-Wörgötter, E. and Elix, J. A. (2002) Secondary chemistry of cultured mycobionts: formation of a complete chemosyndrome by the lichen fungus of *Lobaria spathulata*. *The Lichenologist* **34** (4), 351-359.

Stocker-Wörgötter, E., Cordeiro, L. M. C. and Iacomini, M. (2013) Accumulation of Potential Pharmaceutically Relevant Lichen Metabolites in Lichens and Cultured Lichen Symbionts. In *Studies in Natural Products Chemistry Volume 39* (A. Rahman, ed.), pp. 337-380, Elsevier: Oxford, UK.

Strauss, J. and Reyes-Dominguez, Y. (2011) Regulation of secondary metabolism by chromatin structure and epigenetic codes. *Fungal Genetics and Biology* **48** (1), 62-69.

Sun, W.-W., Guo, C.-J. and Wang, C. C. C. (2016) Characterization of the product of a nonribosomal peptide synthetase-like (NRPS-like) gene using the doxycycline dependent Tet-on system in *Aspergillus terreus*. *Fungal Genetics and Biology* **89**, 84-88.

Sundberg, B., Näsholm, T. and Palmqvist, K. (2001) The effect of nitrogen on growth and key thallus components in the two tripartite lichens, *Nephroma arcticum* and *Peltigera aphthosa*. *Plant, Cell and Environment* **24** (5), 517-527.

Syers, J. K. and Iskandar, I. K. (1973) Pedogenetic Significance of Lichens. In *The Lichens* (V. Ahmadjian and M. E. Hale, eds.), pp. 225-248, Academic Press, Inc. (London) Ltd.: London, UK.

Tang, M.-C., Zou, Y., Watanabe, K., Walsh, C. T. and Tang, Y. (2017) Oxidative Cyclization in Natural Product Biosynthesis. *Chemical Reviews* **117** (8), 5226-5333.

Taylor, J. W. and Berbee, M. L. (2006) Dating divergences in the Fungal Tree of Life: review and new analyses. *Mycologia* **98** (6), 838-849.

Tehler, A. and Wedin, M. (2008) Systematics of lichenized fungi. In *Lichen Biology* (T. H. Nash III, ed.), pp. 336-352, Cambridge University Press: Cambridge, UK.

Tilburn, J., Sarkar, S., Widdick, D. A., Espeso, E. A., Orejas, M., Mungroo, J., Peñalva, M. A. and Arst Jr, H. N. (1995) The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. *The EMBO Journal* **14** (4), 779-790.

Traut, T. W. and Temple, B. R. S. (2000) The Chemistry of the Reaction Determines the Invariant Amino Acids during the Evolution and Divergence of Orotidine 5'-Monophosphate Decarboxylase. *Journal of Biological Chemistry* **275** (37), 28675-28681.

Trembley, M. L., Ringli, C. and Honegger, R. (2002) Morphological and molecular analysis of early stages in the resynthesis of the lichen *Baeomyces rufus*. *Mycological Research* **106** (7), 768-776.

van Dijk, J. W. A., Guo, C.-J. and Wang, C. C. C. (2016) Engineering Fungal Nonribosomal Peptide Synthetase-like Enzymes by Heterologous Expression and Domain Swapping. *Organic Letters* **18** (24), 6236-6239.

Van Lanen, S. G. and Shen, B. (2006) Microbial genomics for the improvement of natural product discovery. *Current Opinion in Microbiology* **9** (3), 252-260.

Verdoes, J. C., Punt, P. J., Schrickx, J. M., van Verseveld, H. W., Stouthamer, A. H. and van den Hondel, C. A. M. J. J. (1993) Glucoamylase overexpression in *Aspergillus niger*: molecular genetic analysis of strains containing multiple copies of the *glaA* gene. *Transgenic Research* **2** (2), 84-92.

Verma, N. and Behera, B. C. (2015) In Vitro Culture of Lichen Partners: Need and Implications. In *Recent Advances in Lichenology: Modern Methods and Approaches in Lichen Systematics and Culture Techniques, Volume 2* (D. K. Upreti, P. K. Divakar, V. Shukla and R. Bajpai, eds.), pp. 147-159, Springer India: New Delhi, India.

Vining, L. C. (1990) Functions of secondary metabolites. *Annual Review of Microbiology* **44**, 395-427.

Vivas, M., Sacristán, M., Legaz, M. E. and Vicente, C. (2010) The cell recognition model in chlorolichens involving a fungal lectin binding to an algal ligand can be extended to cyanolichens. *Plant Biology* **12** (4), 615-621.

Wackler, B., Lackner, G., Chooi, Y. H. and Hoffmeister, D. (2012) Characterization of the *Suillus grevillei* Quinone Synthetase GreA Supports a Nonribosomal Code for Aromatic α-Keto Acids. *ChemBioChem* **13** (12), 1798-1804.

Walton, J. D. (2000) Horizontal Gene Transfer and the Evolution of Secondary Metabolite Gene Clusters in Fungi: An Hypothesis. *Fungal Genetics and Biology* **30** (3), 167-171.

Wang, M., Beissner, M. and Zhao, H. (2014a) Aryl-aldehyde Formation in Fungal Polyketides: Discovery and Characterization of a Distinct Biosynthetic Mechanism. *Chemistry & Biology* **21** (2), 257-263.

Wang, Y., Wang, J., Cheong, Y. H. and Hur, J. S. (2014b) Three New Non-reducing Polyketide Synthase Genes from the Lichen-Forming Fungus *Usnea longissima*. *Mycobiology* **42** (1), 34-40.

Wang, Y., Yuan, X., Chen, L., Wang, X. and Li, C. (2018) Draft Genome Sequence of the Lichen-Forming Fungus *Ramalina intermedia* Strain YAF0013. *Genome Announcements* **6** (23), e00478-18.

Wang, Y., Zhao, N., Yuan, X., Hua, M., Hur, J.-S., Yang, Y. and Wang, J. (2016) Heterologous transcription of a polyketide synthase gene from the lichen forming fungi *Usnea longissima*. *Research Journal of Biotechnology* **11** (10), 16-21.

Wang, Y.-Y., Liu, B., Zhang, X.-Y., Zhou, Q.-M., Zhang, T., Li, H., Yu, Y.-F., Zhang, X.-L., Hao, X.-Y., Wang, M., Wang, L. and Wei, J.-C. (2014c) Genome characteristics reveal the impact of lichenization on lichen-forming fungus *Endocarpon pusillum* Hedwig (Verrucariales, Ascomycota). *BMC Genomics* **15**, 34.

Weber, T., Baumgartner, R., Renner, C., Marahiel, M. A. and Holak, T. A. (2000) Solution structure of PCP, a prototype for the peptidyl carrier domains of modular peptide synthetases. *Structure* **8** (4), 407-418.

Weidner, G., d'Enfert, C., Koch, A., Mol, P. C. and Brakhage, A. A. (1998) Development of a homologous transformation system for the human pathogenic fungus *Aspergillus fumigatus* based on the *pyrG* gene encoding orotidine 5'-monophosphate decarboxylase. *Current Genetics* **33** (5), 378-385.

Weindling, E. and Bar-Nun, S. (2015) Sir2 links the unfolded protein response and the heat shock response in a stress response network. *Biochemical and Biophysical Research Communications* **457** (3), 473-478.

Weissman, L., Garty, J. and Hochman, A. (2005) Rehydration of the Lichen *Ramalina lacera* Results in Production of Reactive Oxygen Species and Nitric Oxide and a Decrease in Antioxidants. *Applied and Environmental Microbiology* **71** (4), 2121-2129.

Wick, J., Heine, D., Lackner, G., Misiek, M., Tauber, J., Jagusch, H., Hertweck, C. and Hoffmeister, D. (2016) A Fivefold Parallelized Biosynthetic Process Secures Chlorination of *Armillaria mellea* (Honey Mushroom) Toxins. *Applied and Environmental Microbiology* **82** (4), 1196-1204.

Wieder, C., Peres da Silva, R., Witts, J., Jäger, C. M., Geib, E. and Brock, M. (2022) Characterisation of ascocorynin biosynthesis in the purple jellydisc fungus *Ascocoryne sarcoides*. In press.

Williams, R. B., Henrikson, J. C., Hoover, A. R., Lee, A. E. and Cichewicz, R. H. (2008) Epigenetic remodeling of the fungal secondary metabolome. *Organic & Biomolecular Chemistry* **6** (11), 1895-1897.

Winkelblech, J., Fan, A. and Li, S.-M. (2015) Prenyltransferases as key enzymes in primary and secondary metabolism. *Applied Microbiology and Biotechnology* **99** (18), 7379-7397.

Wisecaver, J. H., Slot, J. C. and Rokas, A. (2014) The Evolution of Fungal Metabolic Pathways. *PLoS Genetics* **10** (12), e1004816.

Wyatt, T. T., van Leeuwen, M. R., Golovina, E. A., Hoekstra, F. A., Kuenstner, E. J., Palumbo, E. A., Snyder, N. L., Visagie, C., Verkennis, A., Hallsworth, J. E., Wösten, H. A. B. and Dijksterhuis, J. (2015) Functionality and prevalence of trehalose-based oligosaccharides as novel compatible solutes in ascospores of *Neosartorya fischeri* (*Aspergillus fischeri*) and other fungi. *Environmental Microbiology* **17** (2), 395-411.

Yahr, R., Vilgalys, R. and DePriest, P. T. (2006) Geographic variation in algal partners of *Cladonia subtenuis* (Cladoniaceae) highlights the dynamic nature of a lichen symbiosis. *New Phytologist* **171** (4), 847-860.

Yamamoto, A., Mizukami, Y. and Sakurai, H. (2005) Identification of a Novel Class of Target Genes and a Novel Type of Binding Sequence of Heat Shock Transcription Factor in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **280** (12), 11911-11919.

Yamamoto, Y., Miura, Y., Higuchi, M., Kinoshita, Y. and Yoshimura, I. (1993) Using Lichen Tissue Cultures in Modern Biology. *The Bryologist* **96** (3), 384-393.

Yeh, H.-H., Chiang, Y.-M., Entwistle, R., Ahuja, M., Lee, K.-H., Bruno, K. S., Wu, T.-K., Oakley, B. R. and Wang, C. C. C. (2012) Molecular genetic analysis reveals that a nonribosomal peptide synthetase-like (NRPS-like) gene in *Aspergillus nidulans* is responsible for microperfuranone biosynthesis. *Applied Microbiology and Biotechnology* **96** (3), 739-748.

Yim, G., Wang, H. H. and Davies, J. (2007) Antibiotics as signalling molecules. *Philosophical Transactions of the Royal Society B: Biological Sciences* **362** (1483), 1195-1200.

Yin, W. and Keller, N. P. (2011) Transcriptional regulatory elements in fungal secondary metabolism. *Journal of Microbiology* **49** (3), 329-339.

Yin, X., Shin, H.-D., Li, J., Du, G., Liu, L. and Chen, J. (2017) Comparative genomics and transcriptome analysis of *Aspergillus niger* and metabolic engineering for citrate production. *Scientific Reports* **7**, 41040.

Yoshino, K., Yamamoto, K., Hara, K., Sonoda, M., Yamamoto, Y. and Sakamoto, K. (2019) The conservation of polyol transporter proteins and their involvement in lichenized Ascomycota. *Fungal Biology* **123** (4), 318-329.

Yu, J.-H. and Keller, N. (2005) Regulation of Secondary Metabolism in Filamentous Fungi. *Annual Review of Phytopathology* **43**, 437-458.

Zapun, A., Jakob, C. A., Thomas, D. Y. and Bergeron, J. J. M. (1999) Protein folding in a specialized compartment: the endoplasmic reticulum. *Structure* **7** (8), R173-R182.

Zhang, H., Boghigian, B. A., Armando, J. and Pfeifer, B. A. (2011) Methods and options for the heterologous production of complex natural products. *Natural Product Reports* **28** (1), 125-151.

Zotz, G., Büdel, B., Meyer, A., Zellner, H. and Lange, O. L. (1998) *In situ* studies of water relations and CO_2 exchange of the tropical macrolichen, *Sticta tomentosa*. *New Phytologist* **139** (3), 525-535.

Zuo, D., Subjeck, J. and Wang, X.-Y. (2016) Unfolding the Role of Large Heat Shock Proteins: New insights and Therapeutic Implications. *Frontiers in Immunology* **7**, 75.