PIGMENT BIOSYNTHESIS GENES IN THE BLUE CHEESE FUNGUS *PENICILLIUM ROQUEFORTI*

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ABSTRACT

The fungus *Penicillium roqueforti* is used in the production of blue cheese worldwide. It confers a distinct flavour and texture due to a wide range of volatiles and enzymes produced. Blue veins that develop during the culturing process add a uniquely recognizable characteristic to the final product. The resulting colour comes from the pigments that make up melanin covering the spore surface. The process by which *P. roqueforti* produces these melanin pigments has yet to be identified. New strains have been produced using UV mutagenesis to generate spore colour mutants whose conidia range from brown, pink, green, intense blue, and white in appearance. This research may have commercial potential because it might facilitate the production of novel cheeses with a range of vein colours.

In this study, the genetic controls of pigment biosynthesis in *P. roqueforti* were investigated using a combination of bioinformatic, biochemical, and gene manipulation approaches. Initially, bioinformatics analysis was performed to determine whether a DHN-Melanin pathway, involved with pigment biosynthesis in the phylogenetically related *Aspergillus fumigatus*, was present in *P. roqueforti.* This identified a set of six genes, some of which were clustered in the genome. The genes identified were homologues of *alb1* (Albino 1), *ayg1* (*Aspergillus* yellow-green 1), *arp2* (*Aspergillus* reddish-pink 2), *arp1* (*Aspergillus* reddish-pink 1), *abr1* (*Aspergillus* brownish-red 1), and *abr2* (*Aspergillus* brownish-red 2). To determine whether the DHN-Melanin pathway was functional in *P. roqueforti*, first biochemical inhibitor work was performed. Second, gene sequencing of a series of UV colour mutants was undertaken to assess any correlation between change in spore colour and mutation in genes of the DHN-Melanin pathway. Third, deletion strains were produced targeting each of the six genes believed to be involved in pigment biosynthesis. Finally, experimental work was conducted to gain insights into the ecological importance of spore pigment formation in *P. roqueforti.*

As a result of the combined bioinformatic, biochemical and gene manipulation work, a definitive DHN-Melanin pathway for the fungus *P. roqueforti* is proposed. This

information will be of importance to both the scientific community who study *Penicillium roqueforti* and related organisms and the industrial cheese producers who use them.

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ABBREVIATIONS

ACM	*Aspergillus* complete media
ACMB	*Aspergillus* complete media broth
BLAST	Basic Local Alignment Search Tool
cDNA	Complimentary deoxyribonucleic acid
DEPC	Diethylpyrocarbonate
DHN	Dihydroxynaphthalene
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanine
EDTA	Ethylenediaminetetraacetic acid
gDNA	Genomic DNA
HKG	House-keeping gene
*hsd*R	Host restriction; endonuclease R
ITS	Internal transcribed spacer
LB	Lysogeny broth
LBA	Lysogeny broth agar
mRNA	Messenger RNA
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEG	Polyethylene glycol
PKS	Polyketide synthase
QRT-PCR	Quick real time PCR
RNA	Ribonucleic acid
SDW	Sterile distilled water
TAE	Tris base, acetic acid, and EDTA
TBE	Tris base, boric acid, and EDTA
THN	Tetrahydroxynaphtalene
UV	Ultraviolet

1. GENERAL INTRODUCTION

1.1 The Genus *Penicillium*

Penicillium is a genus of fungi containing over three hundred identified species within the phylum Ascomycota (Kirk *et al*. 2008). Commonly referred to as moulds, these filamentous organisms form a thallus of fungal hyphae upon which conidiophores are borne. Each conidiophore bears individually constricted condiospores (conidia) forming the shape of a "painter's brush" (English translation of the Latin root word penicillium) (Haubrich, 2003). The genus includes a number of economically important species such as beneficial ones responsible for antibiotic production and cheese making, but also detrimental taxa that can be opportunistic human pathogens and cause food spoilage. For example, *Penicillium chrysogenum* was responsible for the antibiotic revolution after it was shown to inhibit the growth of staphylococci due to its production of penicillin (Fleming, 1929). *Penicillium marneffei* is an opportunistic pathogen of immuno-compromised patients such as those suffering from the HIV-AIDS virus (Wong and Wong 2011). While various *Penicillium* species such as *P. camembertii* and *P. roqueforti* can be used to produce a wide array of fine cheeses (section 1.1.3), they and other species such as *P. expansum* can also be a major source of food spoilage. *Penicillium* moulds seem particularly well adapted to the indoor environment giving them a competitive advantage over other species for colonizing food. Whereas most fungal spores are in higher volumes outside the home and only find their way in through passive movement, indoor *Penicillium* type spores can easily match or even exceed outdoor levels, regardless of seasonal changes (Fairs *et al.* 2010).

1.1.1 *Penicillium roqueforti* Overview

Work in the present study will be focused on the species *Penicillium roqueforti*. This is a filamentous fungus that has undergone multiple taxonomic changes in its over 100-year history. Originally described by Charles Thom in 1906, its reclassification has been documented over the years with a major shift after the advent of DNA sequencing (Thom, 1906; Dattilo-Rubbo, 1938; Samson *et al.* 1977a; Boyson *et al.*

1996; Cheeseman *et al.* 2014). Recently, the entire genome has been published allowing in-depth genetic investigations (Cheeseman *et al.* 2014).

Pencillium roqueforti grows radially beginning as a white fluff. As it matures, the colony changes from white to greenish-blue in appearance due to the development of a dense felt of erect conidiophores. These conidiophores are two to three-stage branched, 100-200 μm tall and 4.0-6.5 μm wide, with a stipe typically ornamented with conspicuous warts. The conidia form in loose columns, globose to subglobose, mostly 4-6 μm in length (Samson *et al.* 1977a). Unlike other species in the genus such as *P. chrysogenum*, whose odor can be fruity and aromatic, *P. roqueforti* is odorless (Samson *et al.* 1977b).

1.1.2 Sexual Recombination

For more than 100 years the vast majority of species from the genus *Penicillium* have been considered asexual (Dyer and O'Gorman, 2011). This has prevented the use of the sexual cycle as a method for improving strains of economic value, with other methods such as UV mutagenesis used instead. However, scientists have recently identified a heterothallic sexual cycle in the medically important fungus *Penicillium chrysogenum* (Böhm *et al.* 2012). The highly specific conditions required to induce sex in *P. chrysogenum* could be the reason why it was so long before this cycle was discovered. It was only after plates of oatmeal agar supplemented with biotin were used as the growth medium that the full ascospore production and recombination was achieved (Böhm *et al.* 2012). Different *MAT1-1* and *MAT1-2* mating types of the fungus were needed to complete the sexual cycle, consistent with the heterothallic lifecycle required to produce cleistothecia in other related filamentous fungi such as *Aspergillus* species (Figure 1.1). This breakthrough was soon followed by the discovery of sexual recombination in *P. roqueforti*, which underwent sexual reproduction when isolates of compatible mating type were grown under similar conditions (Swilaiman, 2013; Ropars *et al.* 2014).

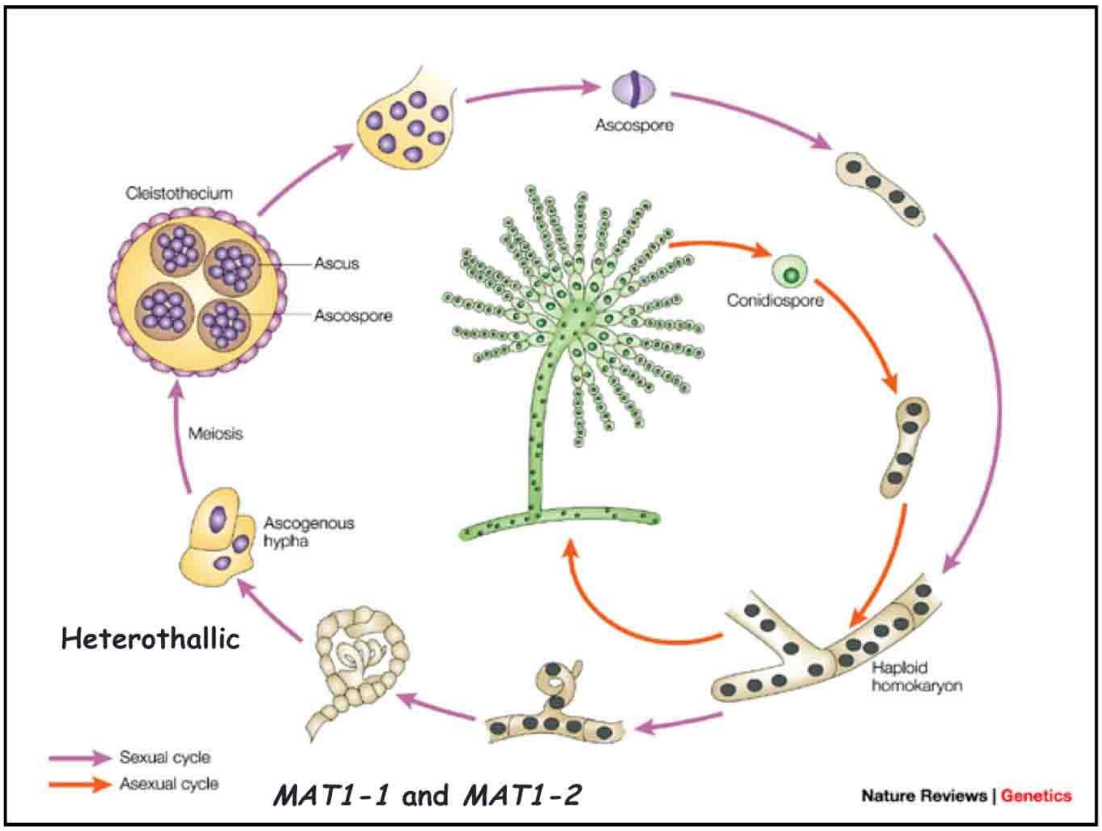


Figure 1.1 Sexual and asexual reproductive cycle in filamentous fungi (*Aspergillus nidulans* depicted). Adapted from Casselton and Zolan, 2002.

1.1.3 Cheese Production and Industry

Penicillium roqueforti is an industrially important species that has been used extensively as a starter culture for the production of blue-veined cheese (Engel *et al*. 1989). The ability to grow in acidic conditions, high ethanol concentrations, and most importantly, low oxygen environments, makes it ideally suited for growth in cheese (Engel and Teuber, 1978; Frisvad *et al.* 1991). The three major varieties of blue cheese currently available on the market are Roquefort, Stilton, and Danish Blue. *Penicillium roqueforti* produces several secondary metabolites that have been classified as toxic. However, theses toxins are present in low enough concentrations that they represent a low public health risk, even when blue cheese is consumed in large quantities (Teuber and Engel, 1983). This could be due to some of the known mycotoxins, such as PR toxin, being unstable in cheese and transforming into the non-toxic PR imine and PR amide (Chang et al, 1993).

What makes this fungus such a good candidate for growth in dairy environments also makes it, unfortunately, highly adapted to growth on wheat products and silage (Pitt and Hocking, 2009). Besides the spoilage of food consumed by humans, *P. roqueforti* can find its way into animal farm feed and cause serious illness. Dairy cows that have consumed maize silage inoculated with *P. roqueforti* suffer from loss of appetite, stoppage of rumen activity, and gastrointestinal inflammation (Veselý and Veselá, 1981).

The discovery of the sexual cycle of *P. roqueforti* in 2013 has raised the exciting possibility of using the sexual reproduction to generate ascospore offspring with new flavour profiles (Swilaiman, 2013; Ropars *et al.* 2014). Previously, commercial strain suppliers have been limited to using the same clonal cultures without the ability to use the sexual cycle for strain improvement. For the first time in their history, breeding of individuals can be used to create new products never before tasted e.g. a hybrid Roquefort-Stilton flavour. This had led to the present interest in understanding how the production of colour in *P. roqueforti* through pigmentation is achieved. If the colour of *P. roqueforti* strains used in blue cheese can be manipulated along with the creation of novel flavours, it could open a new niche in the market for novel mould-ripened cheeses. Being able to choose both taste and colour offers an attractive marketing opportunity for cheese producers.

1.1.4 UV Colour Mutants of *P. roqueforti*

Ultraviolet (UV) light is constructed of different components occupying varying wavelengths such as UVA (320-400 nm), UVB (280-320 nm), and UVC (200-280nm). This radiation has a distinct mutagenic property that can cause transitions, transversions, additions, or deletions in the genetic structure of an organism (Pfeifer *et al.* 2005). Within the field of microbiology, exposure to UV light under controlled conditions has been used to induce mutations with the hopes of isolating improved strains through random chance. For example, UV mutagenesis was successfully used to produce thermo-stable strains of the bakers yeast *Saccharomyces cerevisiae* (Demchenko and Kobrina, 1979) and enhance cellulase production in filamentous *Aspergillus* species (Kaur *et al.* 2014).

Prior to this project, multiple mutants of a *P. roqueforti* blue cheese commercial production strain (74-88) were successfully produced using UV radiation (Figure 1.2: H Darbyshir and PS Dyer, unpublished results). Individuals were selected based on colour to provide a working body of strains here termed colour mutants. These colour mutants are believed to possess mutations somewhere in the genome resulting in the defective production of pigments, giving rise to the alteration in their appearance.

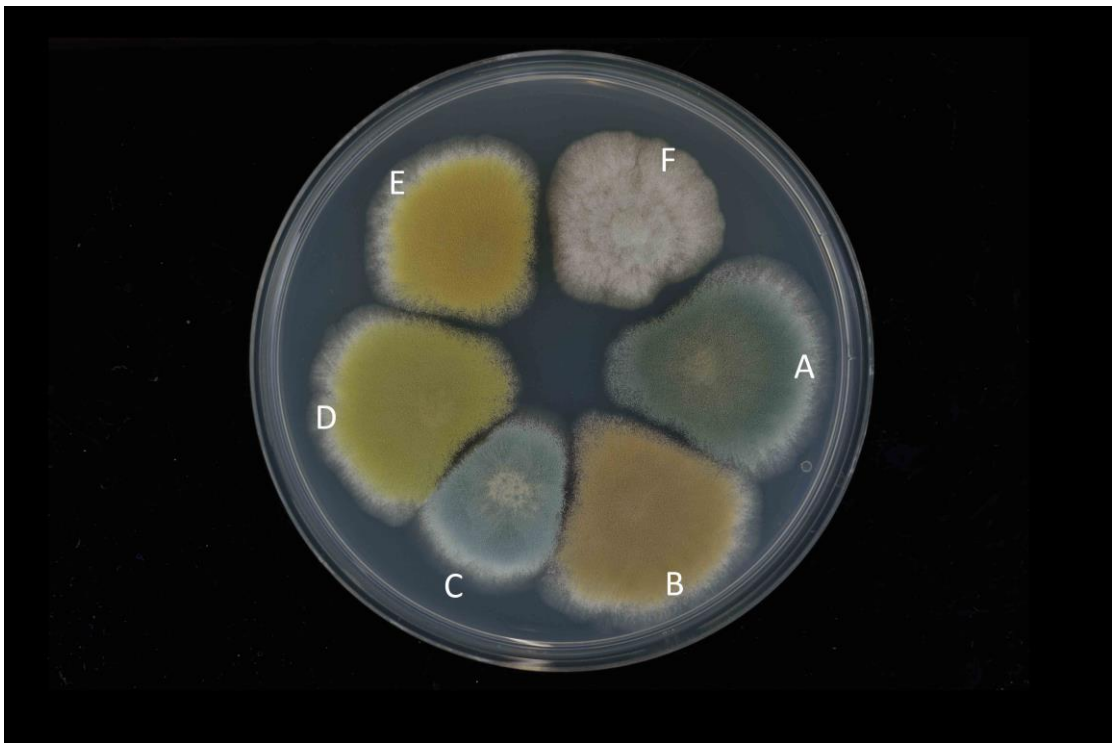


Figure 1.2 Wild type parent and UV colour mutants of *Penicillium roqueforti*. (A) Parental 74-88, (B) 74-88-1, (C) 74-88-10, (D) 74-88-5, (E) 74-88-12, and (F) 74-88-6.

1.2 Melanin and the Pigment Pathway

The colouration of many colonies of filamentous fungi is due to the presence of the pigment melanin, which is normally produced specifically in the spore coat wall and typically confers a dark colouration. The detailed chemical structure of the pigment melanin remains unknown in the kingdom fungi due to difficulties in biochemical extraction and characterisation. Melanins are insoluble in organic and aqueous solvents and are therefore problematic when approached by traditional biochemical

and biophysical techniques. Formed by oxidative polymerization, they are by definition negatively charged hydrophobic pigments with a high molecular weight (Casadevall *et al.* 2000). 'Melanin' is a broad term used to encompass different final polymeric molecules. Predominately they are assembled through either the dihydroxynaphthalene (DHN)-melanin (in ascomycete fungi) or dihydroxyphenylalanine (DOPA)-melanin (in some basidiomycete fungi) pathways (Langfelder *et al.* 2003). Melanins have several possible functions. For example, they have been documented as a contributing factor in the survival of fungi in harsh environments. Fungal melanins allow survival of exposure to ultraviolet light, ionizing radiation, and oxidizing agents through their ability to absorb radiation and act as reducing agents, respectively (Eisenman and Casadevall, 2012). Furthermore, melanins are required as structural components to allow correct assembly of the conidial cell wall (Pihet *et al.* 2009). The other major function of melanin in fungi is to act as a virulence factor (Casadevall *et al.* 2000). The biosynthesis of fungal pigments has been studied mainly in phytopathogenic species. *Colletotrichum lagenarium*, *Verticillium dahlia* and *Cochliobolus miyabeanus* are just a few species of major economic importance, due to their effects on food crops, in which melanins have shown to be required for plant infection (Wheeler and Bell, 1988). In humans, the fungus *Cryptococcus neoformans* causes life-threatening meningoencephalitis in patients with compromised immune systems (Casadevall *et al.* 2000). Animal studies using this fungus as a model organism show that melanization is a contributory factor to host death (Salas *et al.* 1996).

1.2.1 Secondary Metabolism and Melanin Production

Primary, or central, metabolites are the key components of an organism in maintaining the normal physiological processes. They are involved in reproduction, growth, and development. Nucleotides, amino acids, and organic acids are all examples of primary metabolites. Secondary metabolites are organic compounds produced in the stationary phase of growth. They play an important role in ecological function and defense and have been harnessed by people for everyday use. Natural products such as dyes, fibers, polymers, waxes, glues, oils, and drugs are all the result of secondary metabolism (Buchanan *et al.* 2000).

A class of secondary metabolites called polyketides is responsible for the production of mycotoxins (aflatoxins and ochratoxins), antibiotics, and DHN-melanin (Hoffmeister and Keller, 2007). Polyketide synthases (PKS) catalyze carbon chain building with the successive addition of carboxylic acid units. Each unit contributes two carbon atoms with a keto group always attached to the β-carbon. The name "polyketide" comes from the many keto groups at alternating carbon atoms along the chain (Hopwood and Sherman, 1990).

The genetic basis of melanin biosynthesis has been determined for a number of different ascomycete species (Langfelder *et al.* 2003), and a review of findings from species most closely phylogenetically related to *P. roqueforti* will now be presented.

1.2.2 DHN-Melanin Pathway in the Genus *Aspergillus*

Aspergillus fumigatus is an opportunistic pathogen that targets mostly immunocompromised patients. It can also cause aspergillosis in animals, both through the colonization of airways and disseminating infections (Latgé, 1999). The genetic basis of melanin pigment production has been studied in *A. fumigatus* due to the fact that melanins are thought to be contributory virulence factors in fungal infection. So far, six genes clustered within a 19 kb region of the genome have been identified to be involved in conidial pigment biosynthesis in this species (Tsai *et al.* 1999) (Figure 1.3). These genes have been designated *alb1* (*albino*), *ayg1* (*aspergillus yellow-green*), *arp1* (*aspergillus reddish-pink 1*), *arp2* (*aspergillus reddish-pink 2*), *abr1* (*aspergillus brown 1*), and *abr2* (*aspergillus brown 2*) (full gene name and colours resulting from gene deletion in parentheses) (Tsai *et al.* 1999). The largest and arguably most important gene in the cluster is *alb1* (albino). BLAST searches have shown high similarity to the *wA* (*white*) gene in *Aspergillus nidulans*, which encodes a polyketide synthase. This gene is at the start of the pigment biosynthetic pathway and is necessary for conidial pigmentation (Tsai *et al.* 1998). *Alb1* catalyses the formation of a heptaketide monomer which then undergoes chemical modification by other members of the biosynthetic pathway before a final

polymerization step (Figure 1.3). *Arp1* is similar to an enzyme involved in 1,8-dihydroxynapthalene-melanin synthesis in other fungi known as scytalone dehydratase (Tsai *et al.* 1997). The other genes are considered to encode for a hydrolase (*ayg1*), HN-reductase (*arp2*), cupredoxin (*abr1*), and a copper oxidase laccase (*abr2*) (Tsai *et al.* 1999). It is thought that gene clusters are beneficial for coordinated gene regulation and may therefore offer an evolutionary advantage (Keller and Hohn, 1997).

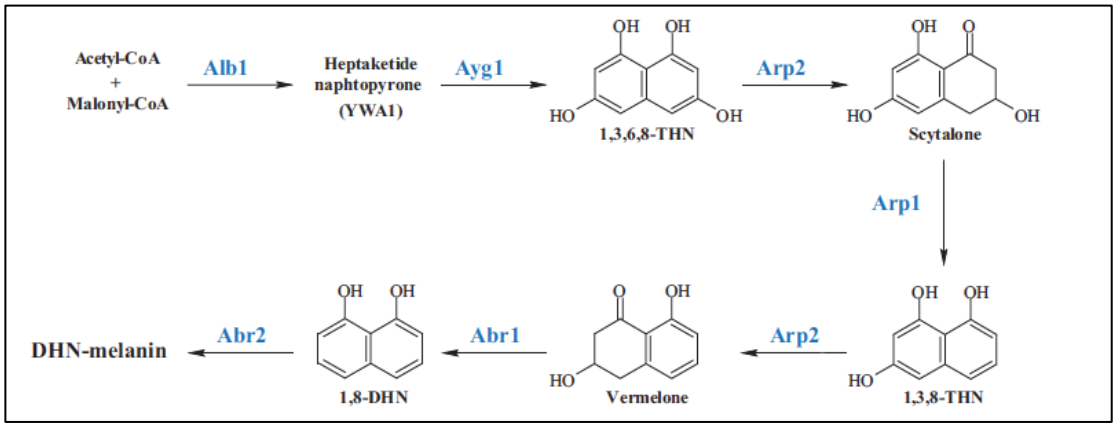


Figure 1.3 DHN-Melanin pathway in *Aspergillus fumigatus* (edited from Pihet *et al.* 2009)

1.2.3 DHN-Melanin Pathway in the Genus *Penicillium*

Less is known about the pigment biosynthetic pathway that *Penicillium* species use to produce melanin. One species of medical importance is *Penicillium marneffei*, which is the leading thermal dimorphic fungus causing skin, respiratory and systemic mycosis in China and Southeast Asia (Hsueh *et al.* 2000). Research was conducted into the genetic basis of melanin production in *P. marneffei* in order to elucidate whether it might act as a virulence factor for fungal infections. BLAST searching using known members of the *Aspergillus* DHN-melanin biosynthetic pathway revealed a 25.3 kb melanin-biosynthesis gene cluster in *P. marneffei*. It contains the same six genes that make up the DHN-Melanin pathway used in *Aspergillus* species to produce pigments (Woo *et al.* 2010). Knowing that the DHN-Melanin pathway is present in at least one *Penicillium* species that is also genetically similar to *Aspergillus* provided a strong backbone of information for the present study of *P. roqueforti*.

1.3 Aims

Previous research into the genetic basis of pigment biosynthesis in *Penicillium marneffei* and various *Aspergillus* species has shown the existence of a DHN-Melanin pathway. The close genetic relationship that *Penicillium roqueforti* shares with these species implies that a similar pathway is likely to be involved in melanin production in *P. roqueforti*. However, there has been recent evidence that fungal pigments can be produced by alternative non-DHN melanin pathways in species such as *A. terreus* (Geib et al. 2016). **Therefore the overall aim of the present study was to determine the genetic basis of pigment biosynthesis in *Penicillium roqueforti*.** This was to be achieved through the following work, testing the hypothesis that the production of melanin in *P. roqueforti* is achieved through a DHN-Melanin pathway composed of genes similar to those found in the genus *Aspergillus* and *P. marneffei*.

- Identification of whether a canonical DHN melanin pathway is present in *P. roqueforti* by genome analysis using bioinformatic approaches.
- If present, to test whether the DHN melanin pathway is functional by use of biochemical enzyme inhibitors to study their impact on colour biosynthesis.
- If present, to test whether mutations of genes of the DHN melanin pathway were present in the previously produced UV colour mutants.
- Establishing of a transformation system for *P. roqueforti* and use of this to confirm or not the role of any DHN melanin pathway genes in pigment production by gene deletion and complementation work.
- Finally, evaluation of the role of pigment production in the ecological survival of spores of *P. roqueforti* i.e. why be blue?

It was hoped that results of the present study would provide insights into the genetic controls of pigment production in *P. roqueforti*. This is important to both the understanding of the biological importance of pigment production, but might also allow the commercial exploitation of the pigment pathway to allow the directed production of *P. roqueforti* cheese production strains with novel colours.

2. MATERIALS AND METHODS

General materials and methods used throughout the present study are listed in the following chapter, whereas materials and methods specific to certain chapters are listed within the relevant chapters. Where appropriate sterilized equipment and consumables were used throughout the following study and work was performed in a class 2-biosafety cabinet.

2.1 Strains and Materials

2.1.1 *Penicillium roqueforti* Strains

The majority of work in this study was performed using a commercial blue cheese production strain 74-88 (wild type; gift from DANISCO, isolated in 2011). A series of UV colour mutant strains had previously been derived from this parent (H Darbyshir and PS Dyer, unpublished work), namely 74-88-1 (Brown), 74-88-2 (Olive Brown), 74-88-4 (Greyish Green), 74-88-5 (Green), 74-88-6 (White), 74-88-10 (Intense Blue), and 74-88-12 (Fawn) (colony colours in parentheses) (Figure 2.1). Other strains used in the present study were obtained from the BDUN (University of Nottingham) culture collection. 74-182 was cultured from a soft Beuvale blue cheese and 74-130 from a blue Stilton cheese, both from the Cropwell Bishop Creamery, UK. 74-133 comes from a Gorgonzola Piccante grown in the Caves of Valassina, Italy. 74-144 was cultured from a French Roquefort cheese. Finally, B20-1 is an albino laboratory strain obtained by UV mutagenesis of an ascospore-derived line (B20) from the progeny of a sexual cross between isolates 74-133 and 74-146 (French Roquefort) (Figure 2.1).

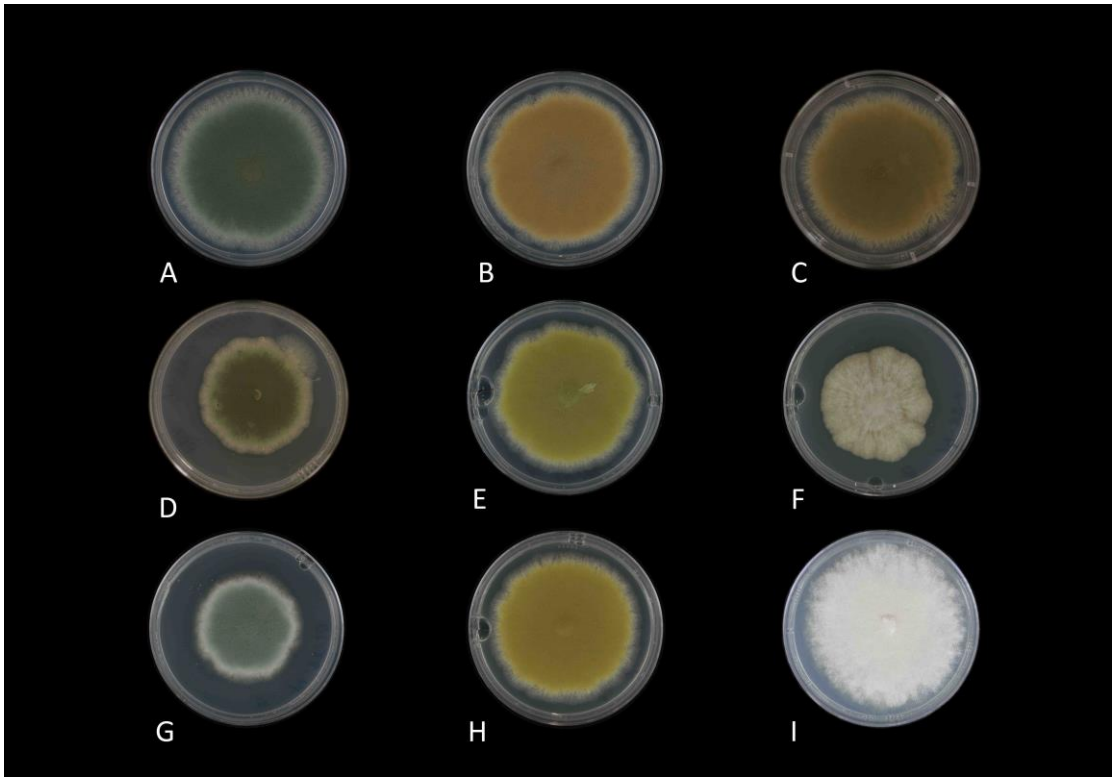


Figure 2.1 Wild type and UV colour mutant strains of *Pencillium roqueforti* used in the present study. (A) Parental (wild type) 74-88, (B) 74-88-1, (C) 74-88-2, (D) 74-88-4, (E) 74-88-5, (F) 74-88-6, (G) 74-88-10, (H) 74-88-12, and (I) B20-1.

2.1.2 *E. coli* Strain

The *Escherichia coli* strain used for genetic transformation was XL1-Blue, which is endonuclease (*end*A) deficient to improve the quality of miniprep DNA. This strain is also recombination (*rec*A) deficient, which improves insert stability. An *hsd*R mutation prevents the cleavage of cloned DNA by the *Eco*K endonuclease system (Stratagene, 2004).

2.1.3 Media for Strain Cultivation

Potato dextrose agar (PDA) (39 g Oxoid Potato Dextrose Agar per liter, sterilized by autoclaving at 117 °C for 15 mins) was the principal medium used for strain cultivation. Liquid cultures were cultivated using *Aspergillus* complete medium broth (ACMB) (20 ml *Aspergillus* salt solution [26 g magnesium sulphate, 76 g potassium dihydrogen phosphate and 26 g potassium chloride per liter], 10 ml *Aspergillus* vitamin solution [400 mg p-aminobenzoic acid, 2 mg d-biotin, 1.4 g choline chloride, 100 mg nicotinic acid, 250 mg pyridoxine hydrochloride, 100 mg riboflavin and 50 mg

thiamine hydrochloride acid per liter], 0.075 g adenine, 1 g casmino acids, 10 g glucose, 2 g peptone and 1 g yeast extract per liter, adjusted to pH 6.5 with NaOH, then sterilized by autoclaving at 121 °C/15 mins). *Escherichia coli* was grown either in Lysogeny Broth (LB) (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter, adjusted to pH 7 with NaOH, then sterilized by autoclaving at 121 °C/15 mins) or on Lysogeny Broth Agar (LBA) (as previous with 15 g agar per liter). Sexual crosses were performed on Oatmeal Agar (80 g oatmeal, 40 g agar, and 2 liters of tap water) prepared from Odlums Irish Pinhead Oatmeal as described by Swilaiman (2013).

2.1.4 Maintenance of *P. roqueforti* Strains

All working stocks of strains were cultured at 28ºC in diffuse light and kept on 10 ml slopes of PDA in 30 ml universal tubes. Multiple stocks were created over the months and stored either in a refrigerator or cold room maintained at a constant 4 °C. The original UV mutant strains were kept in 30% glycerol stock at a constant -80 °C. Glycerol stocks of 74-88, 74-130, 74-133, 74-144, 74-182, and B-20 were kept under liquid nitrogen for long term storage.

2.1.5 Decontamination and Stabilization

All stocks of 74-88-12 were contaminated and needed purification. A sample from the edge of a colony was taken where there was no contamination and spread over a new 9 cm Petri dish containing PDA. This was grown overnight and then single colonies were plucked with a sterile pick and sub-cultured onto PDA. Those that remained uncontaminated were then re-cultured into working stocks.



Figure 2.2 Sectoring Plate (74-88-6).

For 74-88-5 and 74-88-6 in particular, sectoring became a reoccurring issue (Figure 2.2). A similar technique to decontamination was used to repeatedly sub-culture un-sectored growth onto new plates of PDA. This was a manual process to select for stability and phase out phenotypes prone to sectoring. This was repeated as many as ten times before complete stabilization of the phenotype.

2.1.6 Preparation of Spore Suspensions

Spore suspensions of *P. roqueforti* were prepared as follows. 1 ml of 0.1% tween 80 (1 ml tween 80 stock made up to 1 L with dH_2O) was placed into a sterile 1.5 ml Eppendorf tube. A sterile plastic loop, dipped in tween 80, was used to lightly pick up some spores off a cultured slope and swirled in the Eppendorf tube. After vortexing, 10 µl of solution was pipetted under the coverslip on a haemocytometer. Using light microscopy at 40x magnification every spore was counted in each square of the four 4x4 grids (64 squares total). The total was divided by four and multiplied by 10,000 to get the number of spores per ml. Spore suspensions were then diluted as required (usually to 100,000 per ml) using sterile 0.1% tween 80 or DI water to create the required spore concentration.

2.1.7 Plug Growth Method

For certain experiments plugs of mycelium were used as inocula. To produce these, 9 cm Petri dishes were filled with approximately 25 ml of PDA. These were inoculated with a strain of *P. roqueforti* by taking up spores from a slope using a sterile Q-tip and gently dabbing once on the centre of the plate. Plates were incubated at a constant 28 °C until growth almost reached the edge of the plate. Once grown, a sterilized number 2 plug borer (ca. 5 mm width) was used to bore plugs evenly near the periphery of the plate growth (this was to ensure a similar number and age of spores). A sterilized needle pick was used to gently remove the plug, invert it, and then place the plug directly on a new medium for growth.

2.2 Extractions, PCR, and Gel Electrophoresis

2.2.1 Inoculating Liquid Cultures

Using a class 2-biosafety cabinet, Erlenmeyer flasks were filled with 100 ml of ACMB. Flasks were inoculated with a few drops of *P. roqueforti* spore suspension (obtained by spreading 0.1% tween 80 over the surface of an inoculated PDA slope and then removing the crude spore suspension by sucking up with a sterile 2 ml pipette) and the Erlenmeyer flasks sealed with a sterile breathable foam bung. Flasks were kept

at 28 °C and constantly shaken at 150 rpm to prevent conidiation. Cultures were grown for 2-3 days depending on rate of growth.

2.2.2 Solid Media Extraction Cultures

To allow harvest of fungal mycelium from solid agar media without contamination of the underlying agar, Whatman 0.2 µm nylon membrane filters were placed in dH_2O and sterilized (autoclaved at 121 °C/15 min.). Membranes were removed, placed on PDA plates to prevent mycelial growth into the medium and inoculated with 100 µl of spore suspension (100,000 spores/ml) spread over the surface using a sterile plastic spreader. These plates were grown at 28 °C for 4-6 days dependent on growth assays.

2.2.3 DNA Extraction

Liquid cultures were removed from incubation and filtered using a funnel lined with Miracloth. The sample was kept in the Miracloth and pressed dry using a paper towel. Once dry, the sample was removed from the Miracloth and placed in a falcon tube and flash frozen in liquid nitrogen.

The frozen mycelium was ground under liquid nitrogen using a sterilized pestle and mortar (autoclaved dry run at 121 °C for 15 min.). Approximately 500 mg of sample was added to 1 ml of DNA extraction buffer (50 mM Tris adjusted to pH 6.5 with HCl, 10 mM EDTA, 50 mM NaCl and 1% [w/v] SDS) in a 2 ml tube. After inverting several times, the sample was incubated at 65 °C in a heat block for 30 min, followed by centrifugation (13,000 rpm for 10 min). 600 µl of supernatant was removed, placed in a 2 ml phase-lock tube with the addition of 600 µl of phenol chloroform isoamyl alcohol, inverted several times, and centrifuged at 13,000 rpm for 10 min. The upper aqueous phase was removed and placed in a new 1.5 ml Eppendorf tube, mixed with 70% volume of cold propan-2-ol, inverted several times, and incubated at -20 °C for at least 30 min. The resultant sample was centrifuged at 13,000 rpm for 10 min, the supernatant removed, and the pellet washed with 700 µl of 70% cold ethanol. After centrifuging again at 13,000 rpm for 10 min, the ethanol was removed and the pellet left to dry in a laminar flow hood with the cap open. The pellet was re-suspended in

100 µl of TE buffer (10 mM Tris, 1 mM EDTA, adjusted to pH 8.0 with HCl) and stored overnight at 4 °C. DNA concentrations were measured the next day using a P330 Geneflow NanoPhotometer.

2.2.4 RNA Extraction

For liquid cultures, Miracloth was autoclaved (121 °C for 15 min) and used to filter mycelium, which was placed on a paper towel (not pressed dry). A 2 ml Eppendorf tube was filled with mycelium and flash frozen in liquid nitrogen. For solid media cultures, a sterile (prepackaged) knife was used to scrape the mycelium off the membrane, trying not to gather too many spores, placed in a 2 Eppendorf ml tube and flash frozen in liquid nitrogen.

Contents of the 2 ml tube were ground under liquid nitrogen in a sterile pestle and mortar (autoclaved dry run at 121 °C for 15 min). In a 2 ml tube containing 1 ml of Trizol (Invitrogen), ground mycelia was added up to 1.5 ml, inverted several times and left to incubate at room temperature for 10 min. 200 µl of chloroform was added, vortexed, left for 2-3 min, vortexed, then centrifuged at 13,000 rpm for 10 min. 750 µl of the upper aqueous phase was removed, placed in a 1.5 ml Eppendorf tube, mixed with 750 µl cold propan-2-ol, inverted several times, and incubated at -20 °C for 20 min. After centrifugation (13,000 rpm for 10 min) the supernatant was removed, the pellet washed with 700 µl of 70% ethanol (diluted with DEPC [Diethylpyrocarbonate] water), and then centrifuged again at 13,000 rpm for 10 min. The ethanol was removed and the pellet was left to dry in a laminar flow hood with tubes open. 100 µl of DEPC water was added to sample, stored at 4 °C for 30 min, and then pellet was re-suspended by pipetting up and down.

The Macherey-Nagel RNA isolation cleanup kit was used to purify the RNA and digest any remaining DNA. This was achieved by following the user manual protocol steps 5.6.1-5.6.2 then 5.1.5-5.1.9. Samples were checked for RNA concentration using a P330 Geneflow NanoPhotometer and then stored at -80 °C.

2.2.5 Genomic DNA PCR

Sequence amplification of DNA was performed through PCR reactions using Biolabs Phusion High Fidelity DNA Polymerase in either 50 µl reactions (35.5 µl SDW [Sterile Distilled Water], 10 µl 5x buffer, 1 µl 10 mM dNTP mix, 1 µl 10 µM forward primer, 1 µl 10 µM reverse primer, 1 µl ~40 ng/µl gDNA template and 0.5 µl 2,000 U/ml Phusion Polymerase) or 20 µl reactions (14.2 µl SDW, 4 µl 5x buffer, 0.4 µl 10 mM dNTP mix, 0.4 µl 10 µM forward primer, 0.4 µl 10 µM reverse primer, 0.4 µl ~40 ng/µl gDNA template and 0.2 µl 2,000 U/ml Phusion Polymerase). These reactions were placed in a thermo cycler with PCR conditions set to: 105 °C heated lid, initial denaturation at 95 °C for 5 min., 30-35 cycles of 20 sec. at 98 °C for denaturation, 20 sec. at a primer specific temperature for annealing, and 20-240 sec. at 72 °C for elongation, followed by a 72 °C final extension for 5 min. and a hold of 4-10 °C.

2.2.6 Gel Electrophoresis

Agarose gels (0.7% - 1.5% w/v) were made up with either TAE Buffer (242 g Tris, 57.1 ml Glacial Acetic Acid, 100 ml of 0.5 M stock EDTA solution, made up to 10 L with dH_2O) for DNA samples or TBE Buffer (108 g Tris, 55 g Boric Acid, 20 ml of 0.5M stock EDTA solution, made up to 10 L with dH_2O) for RNA samples (or sharper bands for DNA samples). This was supplemented with 100 µl/L of 1 mg/ml EtBr (ethidium bromide) for visualization by fluorescence under ultraviolet light. Gel electrophoresis was performed at 100 V for small gels (40 ml), 120 V for medium gels (100 ml), and 150 V for large gels (250 ml) in either TAE or TBE buffer to match what was used to make up the agarose gel. Biolab ladders of 100 or 1000 bp were used to determine the size of the PCR products while being examined under UV light in the transilluminator.

3. BIOINFORMATIC ANALYSIS OF *PENICILLIUM ROQUEFORTI*

3.1 Introduction

Much like the Internet, it is hard to imagine a world now without the molecular technique of polymerase chain reaction to genetically examine organisms. For most of the 20th century though, the main form of identification and investigation into an organism was through phenotypic experimentation (Bartlett and Stirling, 2003). Late into the century a method was produced to exponentially amplify a nucleic acid sequence *in vitro* (Mullis and Faloona, 1987). This method started by amplification of only 110 base pairs and has evolved today into a process by which the entire genome of an organism can be sequenced. Phenotypic taxonomy was simultaneously being overhauled by the genomics revolution and its higher accuracy for differentiation. From a mycological standpoint, a 600-800 bp sequence known as the ITS-region is routinely used to identify taxonomically challenging species such as mycorrhizae and rusts (Bruns and Gardes, 1993; Schoch *et al.* 2012). When this region was amplified from representative isolates of *Penicillium roqueforti* it revealed that what had been classified as *P. roqueforti* up to that point was actually three separate species (Boysen *et al.* 1996). Almost 110 years after its first description by Charles Thom, through the advent of genetic sequencing technology and due to its economical importance, the full genome of *P. roqueforti* was finally published in 2014 providing an unprecedented resource for genetic analysis (Cheeseman *et al.* 2014), which could now be exploited in the present study.

Currently, the pigment pathway that *P. roqueforti* uses to produce fungal melanin has not been identified. However, as described in the introduction (sections 1.2.2 and 1.2.3) a DHN-melanin biosynthesis pathway has been identified from two phylogenetically closely related species, *Aspergillus fumigatus* and *Penicillium marneffei.* A genome cluster of approximately 19 kb in size has been described in *A. fumigatus* containing six genes designated *alb1, ayg1, arp1, arp2, abr1,* and *abr2* involved in the production of DHN-Melanin (Tsai *et al.* 1999). BLAST searching using

these known genes against the genome sequence of *P. marneffei* revealed that *P. marneffei* contains homologues of the same six genes that make up the DHN-melanin pathway in *A. fumigatus* (Woo *et al.* 2010). The availability of the recently published genome of *P. roqueforti* therefore offered the opportunity to use bioinformatic analysis to investigate whether a similar DHN-melanin pathway was present in *P. roqueforti*, as will now be described.

3.2 Materials and Methods

3.2.1 Genome Sequences of *A. fumigatus* and *P. roqueforti*

The entire *Aspergillus fumigatus* genome was published in 2005 as a 29.4 Mb sequence from the clinical isolate Af293 (Nierman *et al.* 2005). This is available online at the NCBI (http://www.ncbi.nlm.nih.gov/pubmed) and *Aspergillus* Genome Database (http://www.aspgd.org/) websites (Arnaud *et al.* 2012). In 2014 the first full *Penicillium roqueforti* genome was published by a French consortium and deposited on the NCBI website (Cheeseman *et al.* 2014). This included both chromosomal and mitochondrial DNA sequences.

3.2.2 BLAST Analysis

BLAST is an acronym for basic local alignment search tool and is used for rapid sequence searching and comparison (Altschul *et al.* 1990). Currently available through the *Aspergillus* Genome Database (http://www.aspgd.org/) and NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) websites, this program in its current form allows for both nucleotide and protein alignment searches. Sequences of the Alb1, Ayg1, Arp1, Arp2, Abr1, and Abr2 proteins involved in the conidial pigmentation of *A. fumigatus* (Tsai *et al.* 1999) were extracted from the Af293 genome via the *Aspergillus* Genome Database. All sequences were stored and manipulated as necessary using MacVector 12 (MacVector, Inc.). Sequences were then used in protein BLASTP searches against the newly published *P. roqueforti* genome. Sequences with E-values less than 0.01 were recorded as possible matches.

3.2.3 Amplification and Sequencing of Pathway Genes

In order to verify whether the same DNH-melanin genes were present in the production strain 74-88, used in the current laboratory work, as were present in the French genome sequenced isolate of *P. roqueforti*, PCR and gene sequencing experiments were performed. Sequences from the French *P. roqueforti* genes believed to produce fungal melanin were used to design sets of oligonucleotide primers in order to amplify and sequence the same gene from the isolate 74-88. PCR primers were designed using either the primer design utility of MacVector 12, or were manually designed. Genomic DNA was extracted from isolate 74-88 as described in section 2.2.3.

PCR and gel electrophoresis was performed as described in sections 2.2.5 and 2.2.6 using a high-fidelity proof-reading enzyme with the following modifications. 50 µl reactions were used in a standard PCR run with the number of cycles changed to 35 in order to produce a larger concentration of product. Primers used for gene amplification and sequencing are listed in Appendix 9.5. Samples (4 µl) of the PCR were analysed by gel electrophoresis (40 ml TAE gel, 1.0%, 100 V, 100 bp ladder) to see if amplification was successful. PCR product cleanup was then performed with a Macherey-Nagel PCR cleanup kit using steps 5.1.1-5.1.5 in the user manual protocols. DNA concentrations were tested on a P330 Geneflow NanoPhotometer.. Samples were made up to a specific concentration based on size (500 – 1000 bp [5 ng/µl – 20 ng/µl] or 1000-2000 bp [10 ng/µl – 40 ng/µl]) as required for DNA sequencing. Each sample was sent at the required concentration with accompanying forward and reverse primers (5 µl at 10 µM) to the Queen's Medical Centre sequencing lab. Double strand sequencing was performed to increase accuracy.

3.2.4 DNA Sequence Analysis and Alignment of Pathway Genes

Sequence data was returned via e-mail containing both a DNA chromatogram and raw data file. Sequences were sorted, trimmed, assembled, and compiled into genes using MacVector 12 to join the PCR gene fragments. These genes were labeled for the presence of start codons, stop codons, introns and exons. Sequences were aligned using Multalign (http://multalin.toulouse.inra.fr/multalin/) multiple

sequence alignment and examined for variances (Corpet, 1998). Alignments of the base pair sequences of the French genome isolate and 74-88 were made in MultiAlign, and checked for any variation in DNA sequence. Those sequences that were genetically identical to the wild-type parent were disregarded. Any regions showing a polymorphism were separated for further analysis. Known introns were removed. With the introns cut, the resulting sequences were translated into amino acid sequences (using MacVector 12) to see if the base pair polymorphism(s) resulted in any amino acid variation between the French genome isolate and that present in 74-88.

3.3 Results

3.3.1 BLAST Searching of *P. roqueforti* genome

BLASTP searching using the six target proteins from *A. fumigatus* (*alb1*, *ayg1*, *arp1*, *arp2*, *abr1*, and *abr2*) against the published genome of *P. roqueforti* led to the identification of homologues, with E-values matches less than 0.01, of all six proteins encoded by genes in *P. roqueforti* (Appendix Figures A9.1.1-A9.1.6). For the purpose of this study, the same gene names were adopted for the *P. roqueforti* genes as those used for the homologous genes from *A. fumigatus.* Five of these genes sequences (*alb1, ayg1, arp1, arp2,* and *abr1*) were found on super-contig. HG792015.1 spanning a total of 124,471 bp. Four of these genes (*ayg1, arp1, arp2*, and *abr1*) were found clustered together in a region totaling 11,345 bp, a distance of 106,241 bp upstream of *alb1*. The *abr2* gene was found on super-contig. sHG792016.1 spanning a region of 2,865 bp (Figure 3.1). The *alb1* gene is a predicted polyketide synthase (PKS or “wA”) 6,652 bp in length that runs 3’ to 5’ relative to the genome deposited contig sequence. The *ayg1* gene is a hydrolase 1,453 bp in length that runs 3’ to 5’. The *arp2* gene is an HN reductase 860 bp in length that runs 3’ to 5’. The *arp1* gene is a scytalone dehydratase 621 bp in length that runs from 5’ to 3’. The *abr1* gene is a cupredoxin 1,691 bp in length that runs 5’ to 3’. The *abr2* gene is a laccase (“yA”) 2,010 bp in length that runs 5’ to 3’.

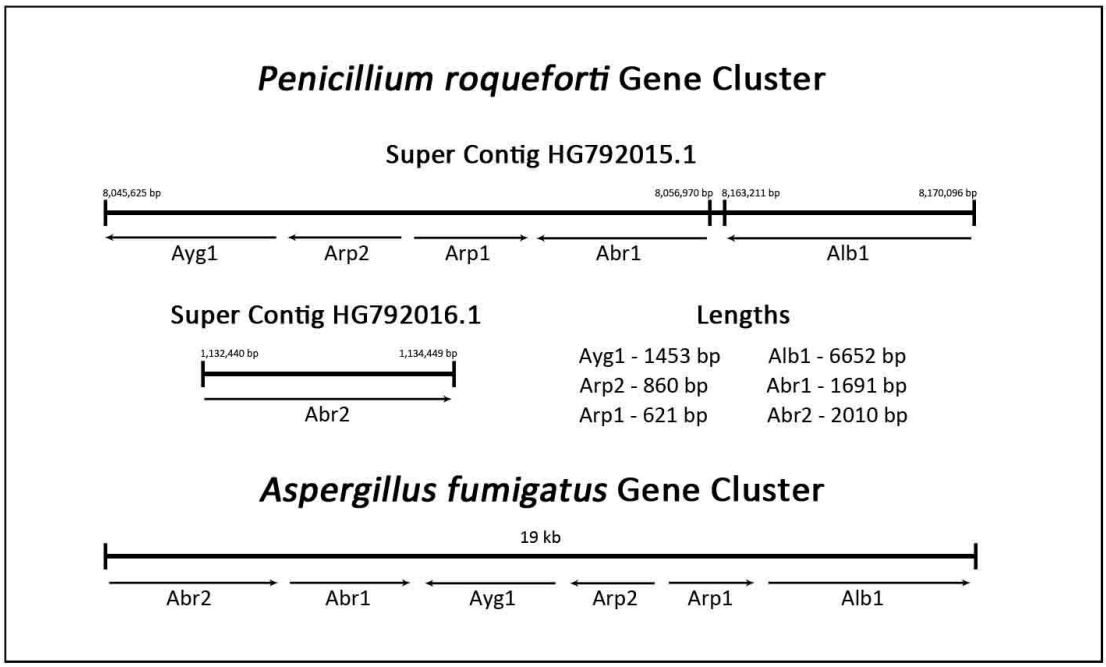


Figure 3.1 Gene clusters involved in the DHN-melanin pathway in *Penicillium roqueforti* and *Aspergillus fumigatus*.

3.3.2 Sequencing and Variation in Wild Type Strains

18 sets of primers (36 total) were required to completely sequence all six genes in the 74-88 wild type strain (2 sets for *ayg1* and *abr1*, 1 set for *arp1* and *arp2*, 3 sets for *abr2*, and 9 sets for *alb1*; Appendix 9.5). For the most part the DNA sequences between the French genome sequenced isolate and that of 74-88 were identical. The only change directly affecting an exon was in the *abr1* gene but this did not result in any variation in the amino acid sequence i.e. this was a silent mutation. For *ayg1, arp1, arp2,* and *abr2,* only a single base pair change was observed in the non-coding intron region of the genome.

3.4 Discussion

One of the most abundant and universal building blocks of life is coenzyme A. This is notable for its role in the synthesis and oxidation of fatty acids and all genomes sequenced so far encode enzymes that use coenzyme A as a substrate (Hillis, 2014). The fungal DHN-Melanin pathway utilises either acetyl-Coenzyme A and/or malonyl-Coenzyme A as an initial building block (Figure 1.3) (Krijgsheld *et al.* 2013). The PKS gene in fungal pigment biosynthesis is used to convert these coenzymes into a

heptaketide naphtopyrone (Watanabe *et al*. 2000; Krijgsheld *et al.* 2013). This is followed by a hydrolase that cleaves chemical bonds by the addition of water and produces 1,3,6,8-Tetrahydroxynaphtalene (1,3,6,8-THN) from the heptaketide naphtopyrone (Fujii *et al*. 2004). The 1,3,6,8-THN undergoes a redox reaction by the HN reductase producing scytalone. In a reverse of a hydrolase, a scytalone dehydratase produces 1,3,8-THN through an enzyme that catalyzes the removal of oxygen and hydrogen in the form of water. The product subsequently re-enters the HN reductase to produce vermelone from 1,3,8-THN in another redox reaction (Tsai *et al*. 1999). Next, cupredoxin takes this vermelone and produces 1,8-Dihydroxynaphaline (1,8-DHN) (Pihet *et al*. 2009). In the DHN-Melanin pathway, cupredoxin uses a type 1 copper centre (T1Cu) that is a copper protein characterised by a single copper atom in a trigonal planar structure. It is due to the S → Cu charge transfer that a strong absorption near 600 nm gives a blue colour. This can also be absorbed around 460 nm giving a greenish tinge that would explain the current pigmentation of a wild type *P. roqueforti* strain (Lippard and Berg, 1994; Holm *et al*. 1996; Lu *et al.* 1993). Finally, a laccase initiates the oxidative polymerization of 1,8-DHN to produce the polymer melanin (Sugareva *et al.* 2006).

3.4.1 Evidence of a Gene Cluster in *P. roqueforti*

A DHN-melanin pathway has been shown to be present and functional in the filamentous fungi *A. fumigatus* (Tsai *et al*. 1999). The same pathway has since been identified in the phylogenetically related *P. marneffei* (Woo *et al.* 2010). Elements of the same pathway have also previously been shown to be present in other ascomycete fungi (Langfelder *et al.* 2003, Woo *et al.* 2010). Using a similar bioinformatic approach, the recently published genome of *P. roqueforti* was interrogated for the presence of a DHN-melanin pathway. Protein BLAST searches confirmed the presence of all six genes characteristic of the DHN-melanin pathway in related *Aspergillus* and *Pencillium* species. The same set of genes was also identified from the blue cheese production strain 74-88, which has been used for previous experimental work at the University of Nottingham. Genome analysis revealed that four of the six genes are clustered in an area of approximately 11 kb, five on the same super-contig, and all six stretched over adjacent super-contigs. Therefore

similarities and differences in the genome arrangement were present compared to the other *Aspergillus* and *Penicillium* species. All species had regions where 4 or more genes of the pathway were clustered together. However, whereas all of the DHN-melanin pathway genes were clustered together in the same region in *A. fumigatus* and *P. marneffei*, in the case of *P. roqueforti* two of the genes of the pathway (*alb1* and *abr2*) were separated from the other members of the gene cluster. The *alb1* gene is also physically separated in the DHN-melanin genome cluster in *Penicillium chrysogenum* (Woo et al. 2010). The functional significance of the lack of a complete genome cluster is unclear. That caveat aside, the data gained in the current chapter strongly suggests that *P. roqueforti* uses six of the genes found in the canonical DHN-melanin pathway in order to produce the fungal pigment melanin.

4. EXPERIMENTAL ANALYSIS OF PIGMENT PRODUCTION IN *PENICILLIUM ROQUEFORTI* (1) TESTING THE LIMITATIONS

4.1 Introduction

Bioinformatic analysis in the previous chapter strongly suggested that a canonical DNH-melanin biosynthesis pathway was present in *Penicillium roqueforti.* However, there was as yet no experimental evidence that this pathway was functionally active in *P. roqueforti*, and it was not known whether other cellular activities might also contribute to pigment production and therefore colouration in *P. roqueforti*. However, some colour mutants of *P. roqueforti* were available from previous UV mutagenesis work (H Darbyshir and PS Dyer, unpublished work), which offered ideal materials for investigation. A programme of experimental work was therefore initiated to test the hypothesis that the DNH-melanin biosynthesis pathway contributes to pigment formation in *P. roqueforti*. The initial stage of experimental work involved investigation into the following four areas.

4.1.1 Optimisation of Growth Media and Use of White Stilton

In order to undertake investigations of pigment production and colouration in *P. roqueforti*, it was first necessary to establish a laboratory model system which would repeatedly yield consistent and reliable spore colours, ideally representative of that seen when *P. roqueforti* is used as a starter culture and grows in cheese. The first step to understanding the colouration of *P. roqueforti* was to observe the organism's ability to grow under varying conditions. Colour mutants and wild type strains were therefore grown on both PDA and ACM to see which medium produced the most intensely coloured spores. Spores are the progeny of fungi that are produced to ensure the continued spread and survival of the species. It requires a lot of energy to produce a spore so in the face of dwindling resources a fungus will produce larger quantities of spores to ensure survival (Dahlberg and Etten, 1982). PDA is a rich medium that offers a more abundant supply of resources *in vitro* than an individual would likely come across in the wild. It was hypothesized that this may have an

effect on the production of spores. Growth was therefore tested on a range of different concentrations of PDA.

In addition growth of both wild-type and the UV colour mutants was examined when inoculated onto White Stilton cheese as a growth medium. This cheese is produced in a similar fashion to blue-veined Stilton, but simply excluding the addition of *P. roqueforti* as a starter culture (Cropwell Bishop Creamery, pers. comm.). Growth on White Stilton was assessed to check that growth colour seen on agar media would be representative of that seen when *P. roqueforti* grows in cheese.

4.1.2 Enzyme Inhibition

Enzymes are biological molecules that act as a catalyst and facilitate complex reactions occurring in all life forms. The DHN-melanin pathway described earlier (sections 1.2.2 and 1.2.3) is composed of enzymes that catalyse the process of turning acetyl-Coenzyme A and/or malonyl-Coenzyme A into fungal melanin. In order to determine whether this pathway might be functional in *P. roqueforti*, the use of enzyme inhibitors was tested. Previous work with other ascomycete fungi has established that certain chemicals inhibit certain steps of the DHN-melanin biosynthetic pathway, in particular the four enzyme inhibitors tricyclazole, pyroquilon, phthalide, and fenoxanil (Wheeler and Klich, 1995). The *arp2* gene in the pigment pathway is an HN-reductase that produces both scytalone and vermelone. Tricyclazole, pyroquilon, and phthalide have been shown to inhibit both reductase reactions in the DHN-melanin pathway (Wheeler and Klich, 1995). The *arp1* gene in the pigment pathway is a scytalone dehydratase that produces 1,3,8-THN. Fenoxanil has been shown to inhibit the *arp1* gene (Pihet *et al.* 2009). The effects of these various enzyme inhibitors was therefore tested on wild type and colour mutant strains of *P. roqueforti*, with the hypothesis that if the DHN-melanin pathway was indeed active, then treatment with the enzyme inhibitors would be expected to lead to change in colony colouration.

4.1.3 DNA Sequence Analysis of UV Colour Mutants

A series of UV colour mutants ranging in colour from white (albino) through to reddish-brown through to green and bright blue were available from previous experimental work at the University of Nottingham, as described in section 2.1.1 (Figure 2.1). These offered ideal experimental materials to evaluate a possible functional role of the DHN-melanin pathway because it was hypothesized that such colour mutants might arise due to mutations of genes in the DHN-melanin pathway. Therefore a complete set of DHN-melanin pathway genes was characterised from each colour mutant to determine any correlation between gene mutation and colour change.

4.1.3 Effect of Temperature on Growth of UV Colour Mutants

Finally, the effect of growth temperature on colouration was examined as it was speculated that some of the UV colour mutants might exhibit temperature-sensitive mutations. When an organism is exposed to UV radiation, mutations in the genetic code can occur causing a change in gene function (Pfeifer *et al.* 2005). The result can be the direct termination of gene function, or in some cases, an alteration in the optimal conditions for the gene to work properly. For example, *E. coli* that was exposed to UV radiation produced mutants that could no longer replicate their DNA at 42 °C. But when these mutant strains were grown at 30 °C they were able to replicate their DNA at the same efficiency as their wild-type counterparts (Wechsler and Gross, 1971). Some of these strains of *E. coli* also contain a mutation preventing the induction of error-prone repair making them less susceptible to further UV mutagenesis at 30 °C (Mount and Kosel, 1974).

Due to an observed instability in growth of some of the UV colour mutants, 74-88-5 and 74-88-6 in particular, it was proposed that they might have some form of temperature-sensitive mutation. Originally this problem of culture instability was dealt with as described in section 2.1.6, but some subcultures continued to show unstable and altered growth. For example, 74-88-5 was observed to frequently grow with the same pigmentation as 74-88-12 and it may be that a semi-functional protein creates green conidia which when rendered non-functional creates fawn

conidia. Meanwhile, 74-88-6 was no longer sectoring but started to produce colonies resembling the wild type. It may be that the change is due to instability at the routine temperature of growth, 28ºC (section 2.1.4). Furthermore, in small-scale cheese production trials 74-88-6 and 74-88-12 were found to produce different coloured veins during the cheese making process (Highland Fine Cheeses and PS Dyer, pers. comm.). For example, 74-88-6 (a supposed albino mutant) when used as a starter culture yielded a blue-veined cheese, while 74-88-12 (a supposed fawn mutant) yielded a green-veined green cheese (although other colour mutants retained their colours in the cheese veins). In order to investigate whether the colour instability was due to temperature changes, tests were conducted where each strain was grown at varying growth temperatures.

4.2 Materials and Methods

4.2.1 PDA Concentration Experiment

750 ml batches of PDA were made up in 1 L bottles at a concentration of 100%, 50%, 20%, and 10% of the manufacturer's recommendation (100% PDA contained 24 g of potato dextrose; all media contained an additional 15 g of agar). 24 Petri dishes (9 cm) containing approximately 25 ml of PDA per concentration were used totaling 96 plates. The 8 strains tested in this experiment were 74-88, 74-88-1, 74-88-5, 74-88-10, 74-88-12, 74-130, 74-133, and 74-144. Each strain was used to inoculate 3 plates per concentration via the plug method (section 2.1.7) totaling 12 plates per strain tested. All plates were incubated at 28 °C and their growth monitored over the next 5 days.

4.2.2 White Stilton Experiment

A half wheel of Cropwell Bishop Creamery White Stilton was cut up into slices (approximately 3-4 mm thick) and wide enough to fit in a 9 cm Petri dish. The 9 strains tested in this experiment were 74-88, 74-88-1, 74-88-5, 74-88-6, 74-88-10, 74-88-12, 74-130, 74-133, and 74-144. One slice of White Stilton was placed onto each of 18 plates containing water agar (15 g agar/L). Each strain was used to

inoculate 2 plates of White Stilton via the plug method (section 2.1.7) totaling 2 plates per strain tested. All plates were incubated at 28 °C and their growth monitored over the next 7 days.

4.2.3 Enzyme Inhibitors

The enzyme inhibitors used in these experiments were tricyclazole, pyroquilon, phthalide, and fenoxanil. They were dissolved in ethanol and made up to a stock concentration of 3 mg/ml. Dimethyl sulfoxide (DMSO), another solvent, was not used to dissolve any enzyme inhibitors but to test if other solvents had an effect on enzyme inhibition.

4.2.3.1 Enzyme Inhibitor Experiment 1

Sterile 250 ml plastic beakers were used to make up 120 ml volumes of PDA for all concentrations of enzyme inhibitors, solvents, and controls. Each 120 ml volume was distributed in 10 ml aliquots into 5 cm Petri dishes totaling 12 plates per container. Tricyclazole, pyroquilon, and phthalide were made up to 120 ml volumes with PDA at concentrations of 30 µg/ml, 15 µg/ml, 8 µg/ml, and 4 µg/ml. For an ethanol control, 1.2 ml of ethanol was added to 120 ml PDA. 600 µl of DMSO was made up to 120 ml with PDA for a concentration of 0.5%.

Spore suspensions of 100,000 spores/ml were made for strains 74-88, 74-130, 74-133, and 74-144 (section 2.1.7). 45 plates were used per strain (3 per concentration/per enzyme [36 plates], 3 PDA controls, 3 ethanol controls, and 3 DMSO [0.5%]) for a total of 180, each inoculated with 5 µl of spore suspension (500 spores). Plates were incubated at 28 °C for 7 days.

4.2.3.2 Enzyme Inhibitor Experiment 2

Sterile 250 ml plastic beakers were used to make up 120 ml volumes of PDA for all concentrations of enzyme inhibitors, solvents, and controls. Each 120 ml volume was distributed in 10 ml aliquots into 5 cm petri dishes totaling 12 plates per container. Tricyclazole, pyroquilon, phthalide, and fenoxanil were made up to 120 ml volumes with PDA at a concentration of 30 µg/ml. 1.2 ml of ethanol was made up to 120 ml

with PDA for an ethanol control. 600 µl of DMSO was made up to 120 ml with PDA for a concentration of 0.5%.

Spore suspensions of 100,000 spores/ml were made for strains 74-88, 74-88-1, 74-88-5, 74-88-6, 74-88-10, and 74-88-12. 21 plates were used per strain (3 per enzyme [12 plates], 3 PDA controls, 3 ethanol controls, and 3 DMSO [0.5%]) for a total of 126, each inoculated with 5 µl of spore suspension (500 spores). Plates were incubated at 28 °C for 7 days.

4.2.3.3 Fenoxanil Experiment

Sterile 100 ml plastic beakers were used to make up 30 ml volumes of PDA for the enzyme inhibitor, solvent, and control. Each 30 ml volume was distributed in 10 ml aliquots into 5 cm petri dishes totaling 3 plates per container. Fenoxanil was made up to 30 ml volumes with PDA at a concentration of 60 µg/ml and 90 µg/ml. 900 µl of ethanol was made up to 30 ml with PDA for an ethanol control. A spore suspension of 100,000 spores/ml was made for the strain 74-88. 12 plates (3 per fenoxanil concentration, 3 PDA controls, and 3 ethanol controls) were inoculated with 5 µl of spore suspension (500 spores) and incubated at 28 °C for 7 days.

4.2.4 Characterisation of UV Colour Mutants

Genomic DNA was extracted from the UV colour mutants 74-88-1, 74-88-5, 74-88-6, 74-88-10, and 74-88-12 (section 2.1.1 and Figure 2.1) as described in section 2.2.3. All six genes of the putative DHN-melanin pathway were then characterised from all of the 74-88- series colour mutants using the same experimental methods used to amplify, sequence and analyse the pathway genes as described for the wild-type parental isolate 74-88 (sections 3.2.3 and 3.2.4). Genomic DNA was also extracted from isolate B20 and an albino UV colour mutant, B20-1 (section 2.1.1), derived from this isolate (N Salih and PS Dyer, unpublished work). In this case only the *alb1* gene was amplified and sequenced from these latter two isolates.

4.2.4.1 Effect of Temperature Experiment on UV Colour Mutants

Five strains (74-88-1, 74-88-5, 74-88-6, 74-88-10, and 74-88-12) were tested with wild-type 74-88 as a control. All 6 strains were tested in triplicate at growth temperatures of 10 °C, 15 °C, 25 °C, 28 °C, and 37 °C for a total of 90 plates (in 5 cm Petri dishes containing 10 ml of PDA). 10 ml slopes of PDA were inoculated with each strain and grown at 37 °C for 7 days. Tween 80 was added to each slope (a few drops from a pipette) and then shaken. A loop was dipped into the spore filled tween solution and dabbed once onto the centre of the PDA plate. Plates were then placed at each growth temperature (3 plates per strain/per temp.) and grown in darkness. Growth was monitored over the next two weeks with plates being removed if fully grown for analysis. This experiment was repeated for just the 15 °C setting, grown in darkness.

4.3 Results

4.3.1 Growth Media Experiments

Strains grown on 100% PDA were found to produce the largest amount of spores and most intense depth of colour. It was observed that as the PDA concentration decreased, so did both radial growth and sporulation (Figure 4.1). Meanwhile, after seven days growth on White Stilton approximately the same colony colour was given by all strains as on 100% PDA i.e. there was no significant variation in spore colour from the agar medium (Figure 4.2).

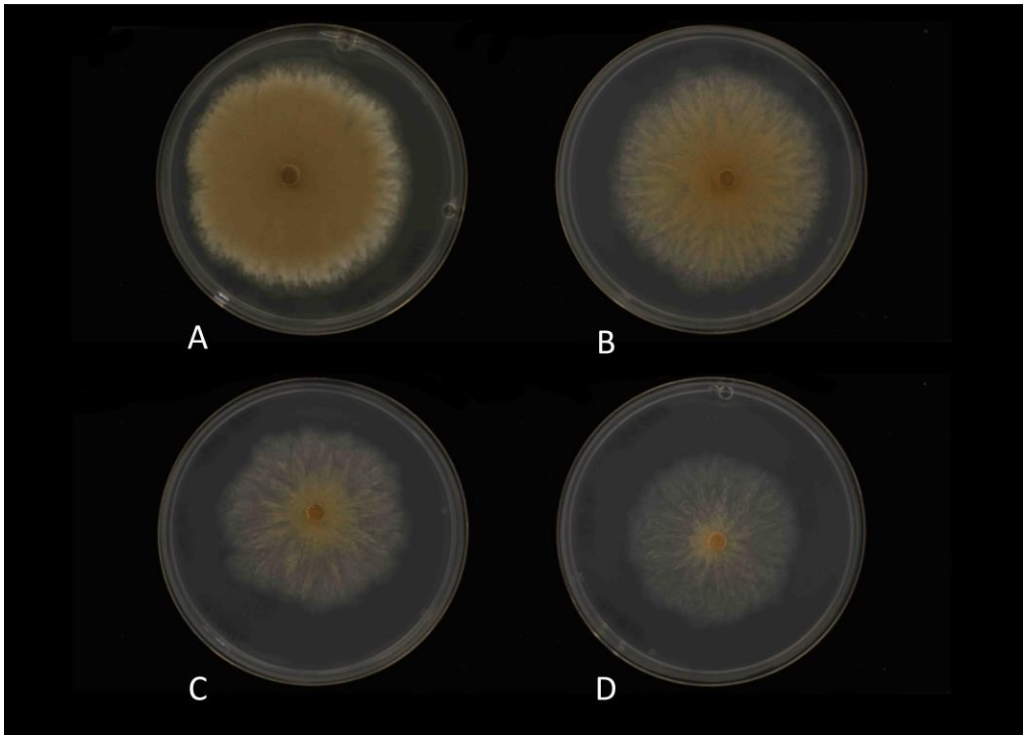


Figure 4.1 Effect of PDA concentration on colony colour of *Pencillium roqueforti.* Representative results from isolate 74-88-1 on varying PDA concentrations. (A) 100%, (B) 50%, (C) 20%, (D) 10%.

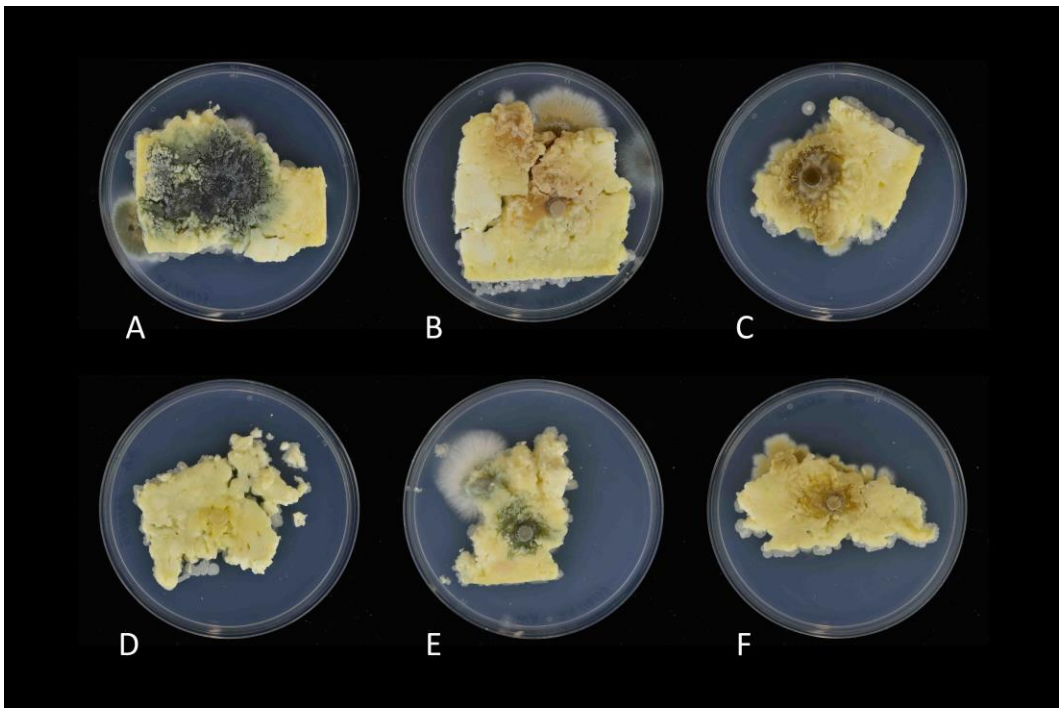


Figure 4.2 Growth of wild-type parent and UV mutants of *Penicillium roqueforti* on White Stilton. (A) 74-88, (B) 74-88-1, (C) 74-88-5, (D) 74-88-6, (E) 74-88-10, (F) 74-88-12.

4.3.2 Enzyme Inhibitor Experiments

In a first experiment, tricyclazole, pyroquilon, and phthalide were tested for activity at concentrations of 30 μg/ml, 15 μg/ml, 8 μg/ml, and 4 μg/ml on four wild-type strains. These concentrations were chosen based on successful performances observed in a previous publication (Wheeler and Klich, 1995). All four inhibitors were subsequently tested on 74-88 and all the colour mutant strains at a concentration of 30 μg/ml in a second experiment. Fenoxanil was subsequently tested for activity on 74-88 at concentrations of 60 μg/ml and 90 μg/ml.

The first enzyme inhibitor experiment showed that all wild-type strains (74-88, 74-130, 74-133, and 74-182) reacted similarly to each condition. Tricyclazole and pyroquilon, inhibitors of HN-reductase, successfully produced brown colonies of *P. roqueforti*. Treatment with phthalide on the other hand did not produce any noticeable change in colouration, unlike previously published results observed on *Penicillium* species (Wheeler and Klich, 1995). The effects of phthalide were nearly identical to that of the ethanol control used. The highest concentrations of enzyme inhibitors were the most successful at inhibiting the pathway with effectiveness decreasing as concentrations fell. Even at a low concentration of 4 μg/ml, tricyclazole and pyroquilon were still able to inhibit the HN-reductase to the point of producing brown colonies.

The second enzyme inhibitor experiment was conducted on 74-88 and the UV mutants. 74-88 reacted the same as the previous experiment when it came into contact with tricyclazole, pyroquilon, and phthalide. Fenoxanil was added to the experiment because previous research indicated that it is supposed to inhibit the scytalone dehydratase. What was observed was a reduction in radial growth of each strain but no significant variation in colour. The change observed on the inner colouration of the colony was congruent with the ethanol controls (Figure 4.3). 74-88-1, 74-88-5, 74-88-6, and 74-88-12 showed either negligible or no change in colouration under any of the enzyme inhibitors. Fenoxanil did however reduce the radial growth of each strain. 74-88-10 reacted the same as the wild type parent 74-88 with the HN-reductase inhibitors tricyclazole and pyroquilon producing brown

colonies. Enzyme inhibitor experiment three was to test if the fenoxanil results were only due to 30 µg/ml being too low a concentration. The retest on 74-88 with concentrations of 60 µg/ml and 90 µg/ml showed no change in colouration. They did show as concentration increases, radial growth decreases.

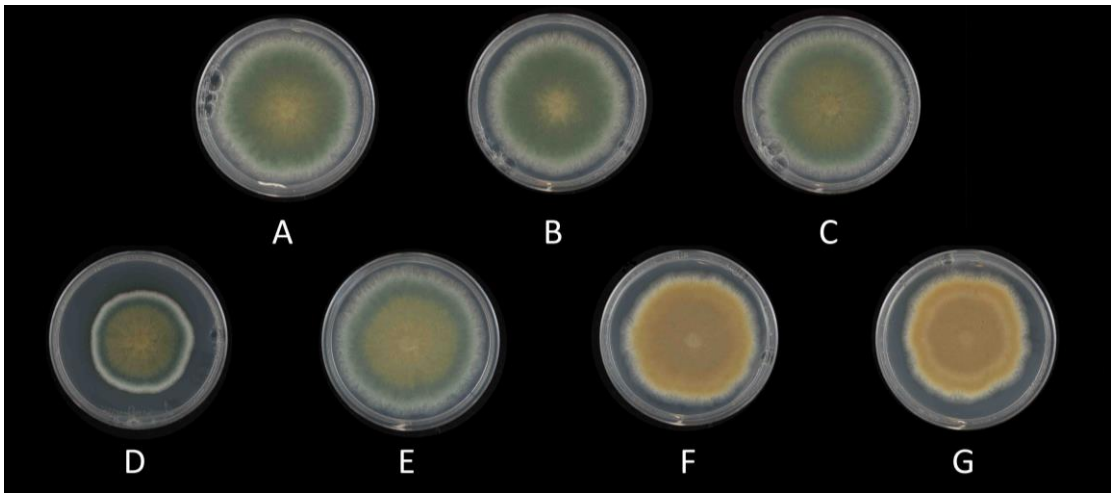


Figure 4.3 Effect of enzyme inhibitors on colony colour *of Penicillium roqueforti*. Representative results from isolate 74-88. (A) Control, (B) DMSO, (C) Ethanol Control, (D) Fenoxanil, (E) Phthalide (F) Pyroquilon, (G) Tricyclazole.

4.3.3 DNA Sequence Analysis of UV Colour Mutants

The DNA sequences of all six putative DHN-melanin pathway genes of all of the 74-88-derived UV colour mutants were compared to those present in the wild-type parent. The *alb1* DNA sequence from the B20-1 albino mutant was also compared to the B20 wild type parent sequence. Alignments of the base pair sequences were made in MultiAlign. A series of specific mutations were indeed found in certain genes of the putative DHN-melanin pathway (Table 4.1).

74-88-1 (a brown mutant) was found to have a point mutation from thymine to adenine causing an amino acid change from valine to aspartic acid within the *arp2* gene (Table 4.1 and Figure 4.4). This has caused an amino acid change from a nonpolar, aliphatic R group to a negatively charge R group. 74-88-2 (a silver/brown mutant) was found to have three adjacent point mutations changing an ATG to GAA causing an amino acid change from tyrosine-aspartic acid to a stop codon-aspargine

in the *abr1* gene (Table 4.1 and Figure 4.5). This has caused an amino acid change from an aromatic R group to a stop codon and a negatively charged R group to a polar, uncharged R group. 74-88-5 (a green mutant) was found to have a point mutation from thymine to cytosine causing an amino acid change from leucine to proline in the *ayg1* gene (Table 4.1 and Figure 4.6). This has caused an amino acid change from a nonpolar, aliphatic R group to a polar, uncharged R group. 74-88-12 (a fawn mutant) has a point mutation from thymine to cytosine causing an amino acid change from cysteine to arginine in the *ayg1* gene (Table 4.1 and Figure 4.7). This has caused an amino acid change from a polar, uncharged R group to a positively charged R group. The albino mutant B20-1 was found to have a point mutation from thymine to cytosine causing an amino acid change from leucine to serine in the *alb1* gene (Table 4.1 and Figure 4.8). This has caused an amino acid change from a nonpolar, aliphatic R group to a polar, uncharged R group. Intriguingly though, all other colour mutant strains (74-88-4, 74-88-6, 74-88-10) showed no mutation in any of the six genes and were in those cases, genetically identical in the DHN-melanin pathway genes as the parental 74-88 strain (Table 4.1).

Strain	Color	Gene	Mutation (bp)	Mutation (aa)	Amino Acid Change	Group Change
(B)74-88-1	Brown	Arp2	From T to A	From V to D	Valine to Aspartic Acid	From Nonpolar, aliphatic R group to Negatively charged R group
(C)74-88-2	Silver/Brown	Abr1	From ATG to GAA	From YD to *N	Tyrosine-Aspartic Acid to Stop-Aspargine	2 group change
(D)74-88-4	Blue	None	None	None	None	None
(E)74-88-5	Green	Ayg1	From T to C	From L to P	Leucine to Proline	From Nonpolar, aliphatic R group to Polar, uncharged R group
(F)74-88-6	White	None	None	None	None	None
(G)74-88-10	Bright Blue	None	None	None	None	None
(H)74-88-12	Fawn	Ayg1	From T to C	From C to R	Cysteine to Arginine	From Polar, uncharged R group to Positively charged R group
(J)B20-1	Albino	Alb1	From T to C	From L to S	Leucine to Serine	From Nonpolar, aliphatic R group to Polar, uncharged R group

Table 4.1 UV induced mutations present in different colour mutants of *Penicillium roqueforti.*
Strain A is the 74-88 wild type.

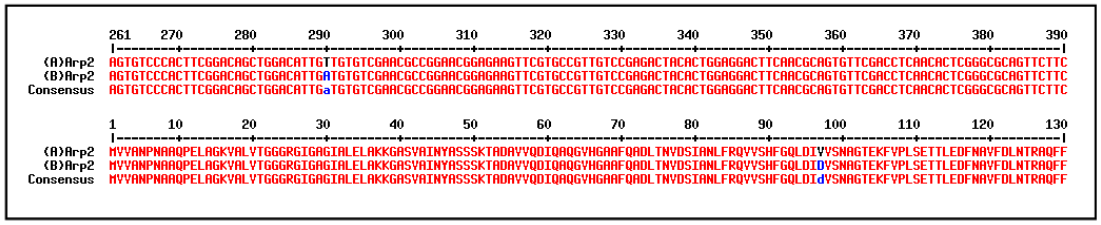


Figure 4.4 Base pair and amino acid UV induced mutation in the *arp2* gene. (A) 74-88, (B) 74-88-1.

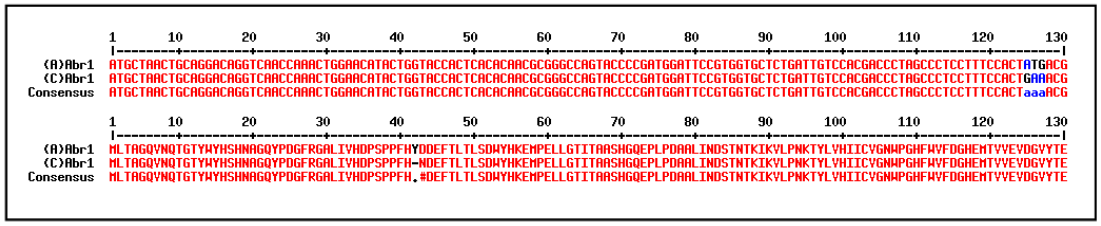


Figure 4.5 Base pair and amino acid UV induced mutation in the *abr1* gene. (A) 74-88, (C) 74-88-2.

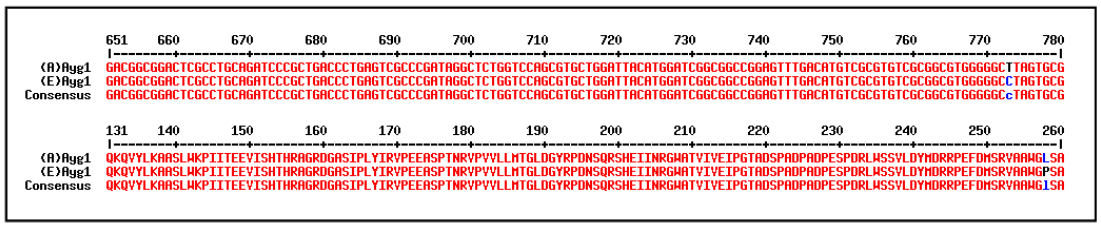


Figure 4.6 Base pair and amino acid UV induced mutation in the *ayg1* gene. (A) 74-88, (E) 74-88-5.

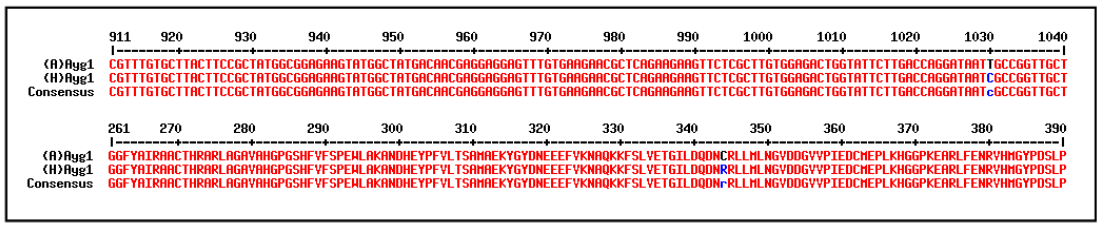


Figure 4.7 Base pair and amino acid UV induced mutation in the *ayg1* gene. (A) 74-88, (H) 74-88-12.

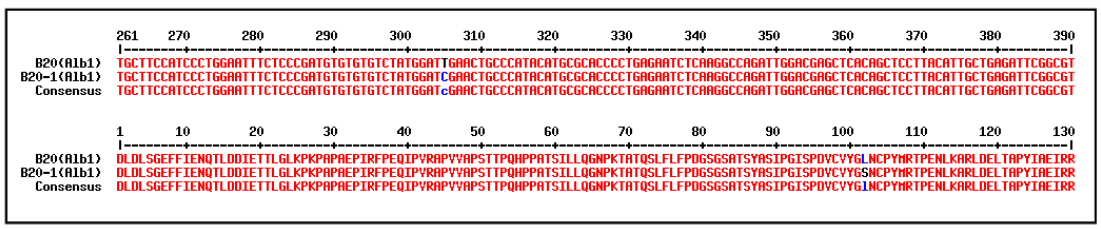


Figure 4.8 Base pair and amino acid UV induced mutation in the *alb1* gene. (B20) Wild type, (B20-1).

4.3.4 Effect of Temperature on Colony Colour

All strains showed no growth when they were incubated at 37 °C. Colonies of all strains appeared the same at 25 °C as they did for the growth conditions of PDA at 28 °C i.e. growth at 28 °C was similar to that at 25 °C. All colonies at 15 °C took longer to grow than at 28 °C. At 15 °C pigmentation for 74-88, 74-88-1, and 74-88-10 was similar to the pigmentation at 25 °C and 28 °C in the dark. There was a slight bleaching effect from the colder environment but everything else was consistent. 74-88-6 saw a strong reversion to the original colony growth and pigmentation seen in the earlier samples. 74-88-12 was a much darker fawn/brown than the cultures grown at 28 °C (Figure 4.9). 74-88-5 showed its original green pigmentation on the outer fanning of the colony (Figure 4.9 and 4.10). At 10 °C all of the colonies took approximately twice as long to grow versus the cultures grown at 28 °C. 74-88-5 and 74-88-12 were both the same colour, a pale light yellow that grew semi-translucent. All the colonies incubated at 10 °C did not have the appearance of healthy growth or abundant pigmentation.

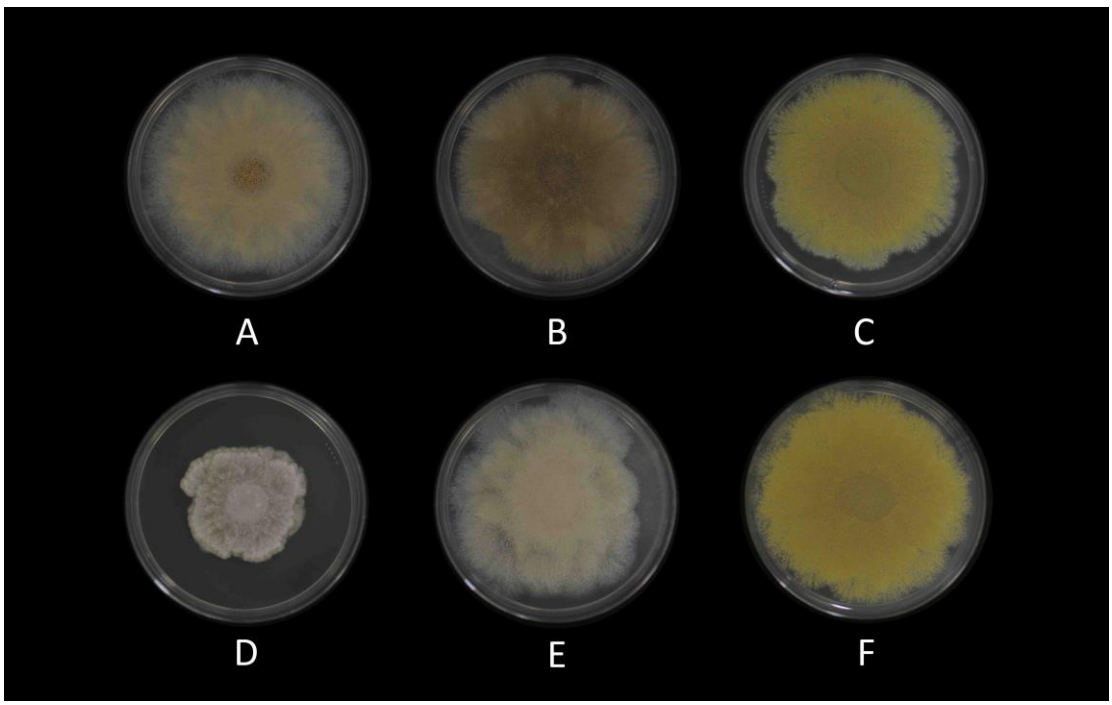


Figure 4.9 Effect of temperature on growth and colony colour of *Penicillium roqueforti.* Wild-type and UV colour mutant strains grown in darkness at 15 °C. (A) 74-88 (B) 74-88-1, (C) 74-88-5, (D) 74-88-6, (E) 74-88-10, (F) 74-88-12.

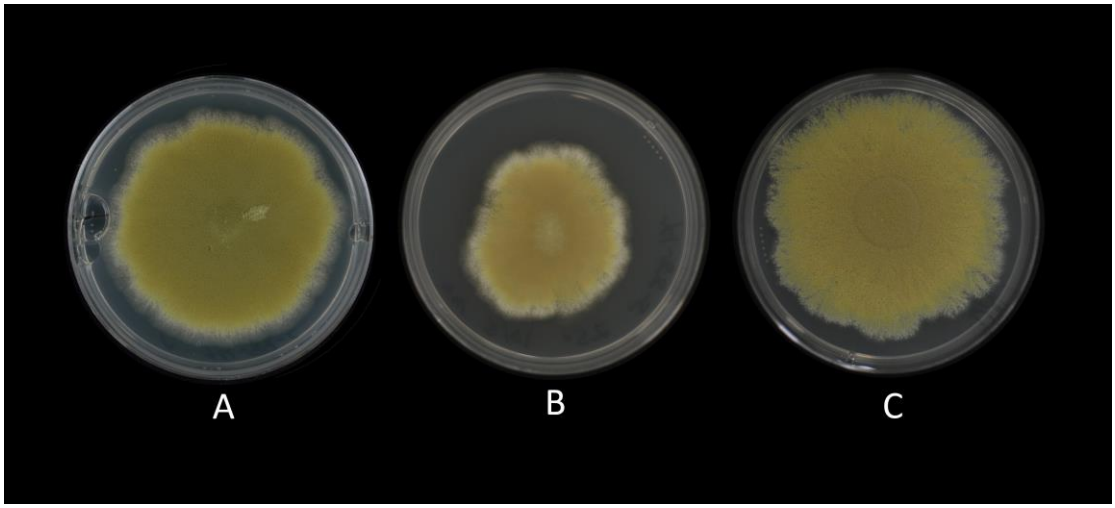


Figure 4.10 Comparison of temperature on growth and colony colour for UV colour mutant 74-88-5. (A) Original strain (green) at 28 °C (B) Current strain (browning) at 25 °C, (C) Current strain (green fanning) at 15 °C.

4.4 Discussion

The bioinformatic analysis of *P. roqueforti* described in chapter 3 had identified genes of a putative DHN-melanin pathway within the genome. In order to examine whether this pathway was functional an initial set of experiments were conducted. Overall these gave a strong indication that the DHN-melanin pathway is indeed involved in pigment production in *P. roqueforti* as will now be described.

4.4.1 Establishing Conditions for Colony Colour Assays

It was first important to find conditions under which the colour of colonies of *P. roqueforti* could reliably be assayed to show differences between different colour mutants. Previous work had shown that growth on certain agar media failed to distinguish between the different colour mutants (H Darbyshir and PS Dyer, pers. comm.). Once the optimal environment is achieved to demonstrate differences in colours then further work can begin on testing its limitations. Tests were performed on different media, nutrient concentrations, and substrates. It was concluded that the optimal environment for *P. roqueforti* in regards to growth and pigmentation is growth on PDA at 100% concentration at 28ºC. Most intense colouration and good rates of growth were achieved under these conditions. Given the importance of this research to the industry of cheese production it was then important to examine whether varying colour mutants still exhibited the same colour on the cheese

substrate. Results of inoculations with White Stilton showed that once externally inoculated with a colour mutant strain, there was no alteration in pigmentation or growth.

4.4.2 Inhibitors Effects on Pathway

Secondly, the effects were assayed of known enzyme inhibitors of the DNH-melanin pathway. The effects of tricyclazole and pyroquilon on all *P. roqueforti* wild-type strains were the same as observed in previous studies of other ascomycete fungi (Wheeler and Klich, 1995; Pihet *et al.* 2009). The observed change in colony colour in *P. roqueforti* (Figure 4.3) strongly indicated that these chemicals were inhibiting the reductase reactions of the DHN-melanin pathway (Wheeler and Klich, 1995) supporting that the DHN-melanin pathway is necessary for pigment production in *P. roqueforti.* It is noted that there were no colour changes observed in the ethanol control, so it can be concluded that the effects are due to the inhibitor rather than that of the solvent. In contrast, phthalide treatment had no clear effect on colony colour, although there is a previous study in which this chemical was said to inhibit the *arp2* HN-reductase in *P. roqueforti* (Wheeler and Klich 1995). This contradiction could be due to a difference in wild-type strains used, such that the one used in the previous study is more sensitive to this particular inhibitor.

The enzyme inhibitors of the DNH-melanin pathway were then assayed against a series of UV colour mutants. 74-88-1, 74-88-5, 74-88-6, and 74-88-12 showed negligible changes in colour in response to treatment with tricyclazole, pyroquilon, phthalide and fenoxanil (Appendix Figure A9.2.1). Before this test was conducted, the colour of 74-88-1 was compared to those of the brown colonies observed in previous studies (Wheeler and Klich 1995; Pihet *et al.* 2009). 74-88-1 is believed to have a mutation in the *arp2* HN-reductase gene producing a brown colour mutant. The fact that this mutant is the same colour as 74-88 in response to tricyclazole and pyroquilon, and itself has no change in colour when treated with these inhibitors, gives evidence to the claim that a mutation has occurred in the *arp2* gene (Figure 4.11).

Based upon their colour, 74-88-5 and 74-88-12 are believed to have mutations in the hydrolase *ayg1* gene that occurs before the HN-reductase *arp2* gene. If this is true, tricyclazole and pyroquilon would be inhibiting a portion of the pathway that is never reached due to an inactivation of the previous *ayg1* step and therefore no change in colour would be observed. This was indeed the case for both 74-88-5 and 74-88-12 as they retained their colouration throughout the entire experiment despite tricyclazole and pyroquilon treatment (Figure 4.12). This gives evidence to the claim that 74-88-5 and 74-88-12 are a result of mutations in the hydrolase *ayg1* gene. 74-88-6 is believed to have a mutation in the polyketide synthase *alb1* gene that also occurs before the HN-reductase *arp2* gene. The same concept as described for 74-88-5 and 74-88-12 also applies to 74-88-6 to explain why there was no change observed in colouration throughout the experiment.

Fenoxanil has been shown to inhibit the *arp1* scytalone dehydratase in the DHN-melanin pathway of some filamentous fungi (Pihet *et al.* 2009). This was not observed in the first test using fenoxanil where the only change that occurred was in radial growth. After increasing the concentrations it was shown that fenoxanil does not appear to inhibit effectively the scytalone dehydratase in the *P. roqueforti* strain 74-88.

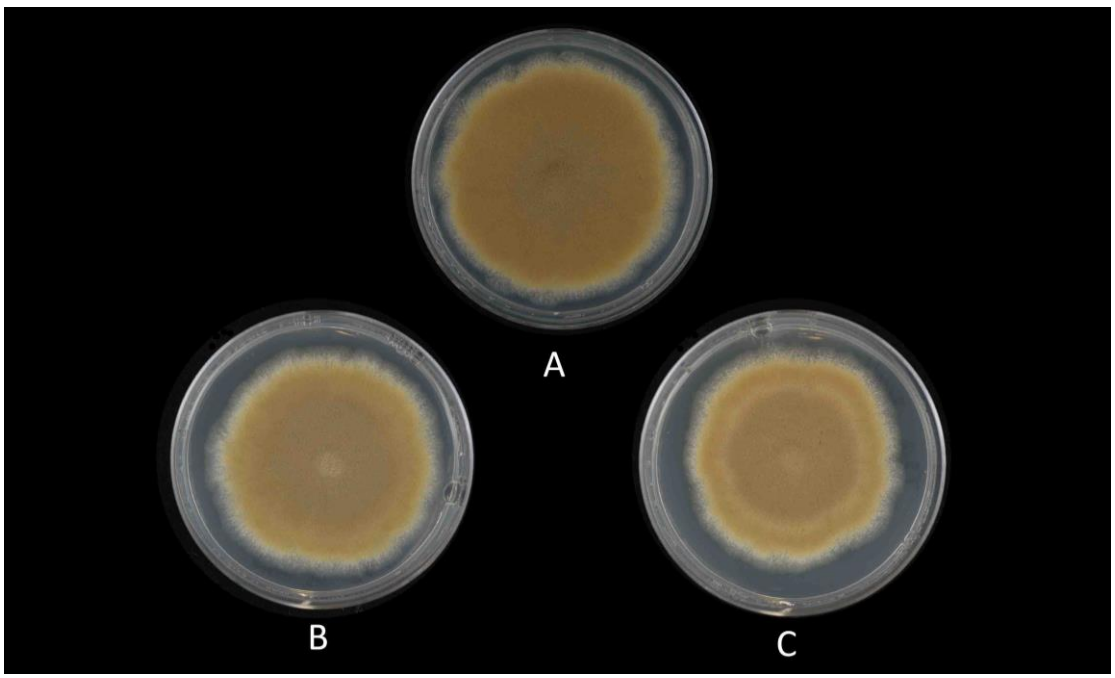


Figure 4.11. Effects on growth of isolate 74-88-1 of enzyme inhibitors targeting the *arp2* gene. (A) 74-88-1 control, (B) Pyroquilon treatment, (C) Tricyclazole treatment.

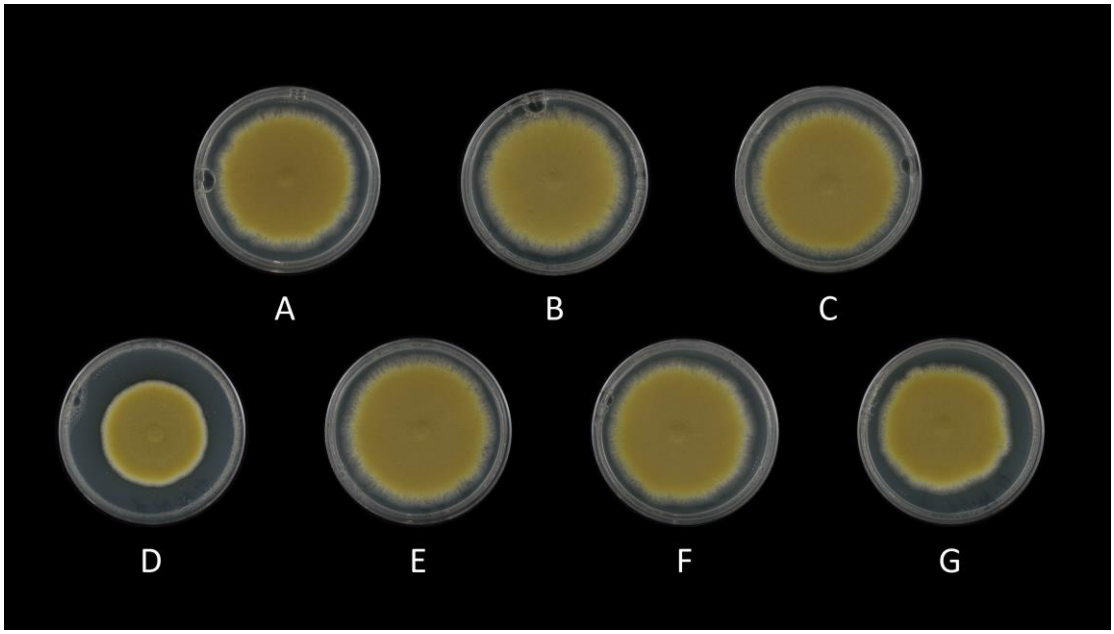


Figure 4.12. Effects on growth of isolate 74-88-12 of enzyme inhibitors targeting the DHN-melanin pathway. (A) Control, (B) DMSO, (C) Ethanol Control, (D) Fenoxanil, (E) Phthalide, (F) Pyroquilon, (G) Tricyclazole.

4.4.3 DNA Analysis of UV Colour Mutants

Thirdly, a set of UV colour mutants of *P. roqueforti* were characterised by molecular means. It was predicted that if the DHN-melanin pathway was functional in *P. roqueforti* then it would be expected that mutations would be detected in the pathway genes present in these UV colour mutants. This is because when a point mutation is introduced into a genetic sequence that alters the amino acid sequence, the structure of the protein can change. This can either make a protein inactive or in some instances, change its function (Liu *et al.* 2001; National Library of Medicine, 2016). And indeed, very strong evidence was obtained of a correlation between gene mutation and colour change for certain of the UV mutants. In the case of 74-88-2 (a silver brown mutant), a stop codon was detected in the middle of the *abr1* gene sequence due to a point mutation. It is safe to say that this protein is no longer functional because as the sequence is translated, half way through it is designated to stop. On the other hand, in a situation like 74-88-5 (green colour mutant) and 74-88-12 (fawn colour mutant), there are two different mutations in the *ayg1* gene resulting in two different colours. Now given that there are colour mutants (74-88-4, 74-88-6, and 74-88-10) where no mutation in any of the six DHN-melanin pathway genes has occurred (see below), it is possible that the variation in colour is due to a

mutation somewhere else in the genome. However, it is more likely that one of the two strains with a mutation in the *ayg1* gene is still producing a partially functional protein while the other represents the true colour produced from total gene inactivation. 74-88-12 is the fawn "yellowish-green" to be expected from the inactivation of the *ayg1* gene but the rich green of 74-88-5 is not. The change in amino acid from leuicine to proline in 74-88-5 changes dramatically the structure of the amino acid and may have caused a unique folding of the protein whilst retaining some activity (Figure 4.13).

Intriguingly, three of the UV colour mutants, 74-88-4 (blue), 74-88-6 (white), and 74-88-10 (bright blue), were shown to be genetically identical to 74-88 in all six genes of the DHN-melanin pathway. When using UV radiation, the entire genome of an organism is under pressure and can be subject to change in any or multiple locations. In these strains mutation has not occurred in what is believed to be the key pigmentation biosynthesis pathway in *P. roqueforti*. It can therefore be inferred that a genetic mutation somewhere else in the genome is responsible for the changes observed. This result is significant because it demonstrates that the DHN-melanin pathway is not the sole mechanism determining pigmentation in *P. roqueforti.* Without sequencing the entire genome, the cause of these changes will remain unknown.

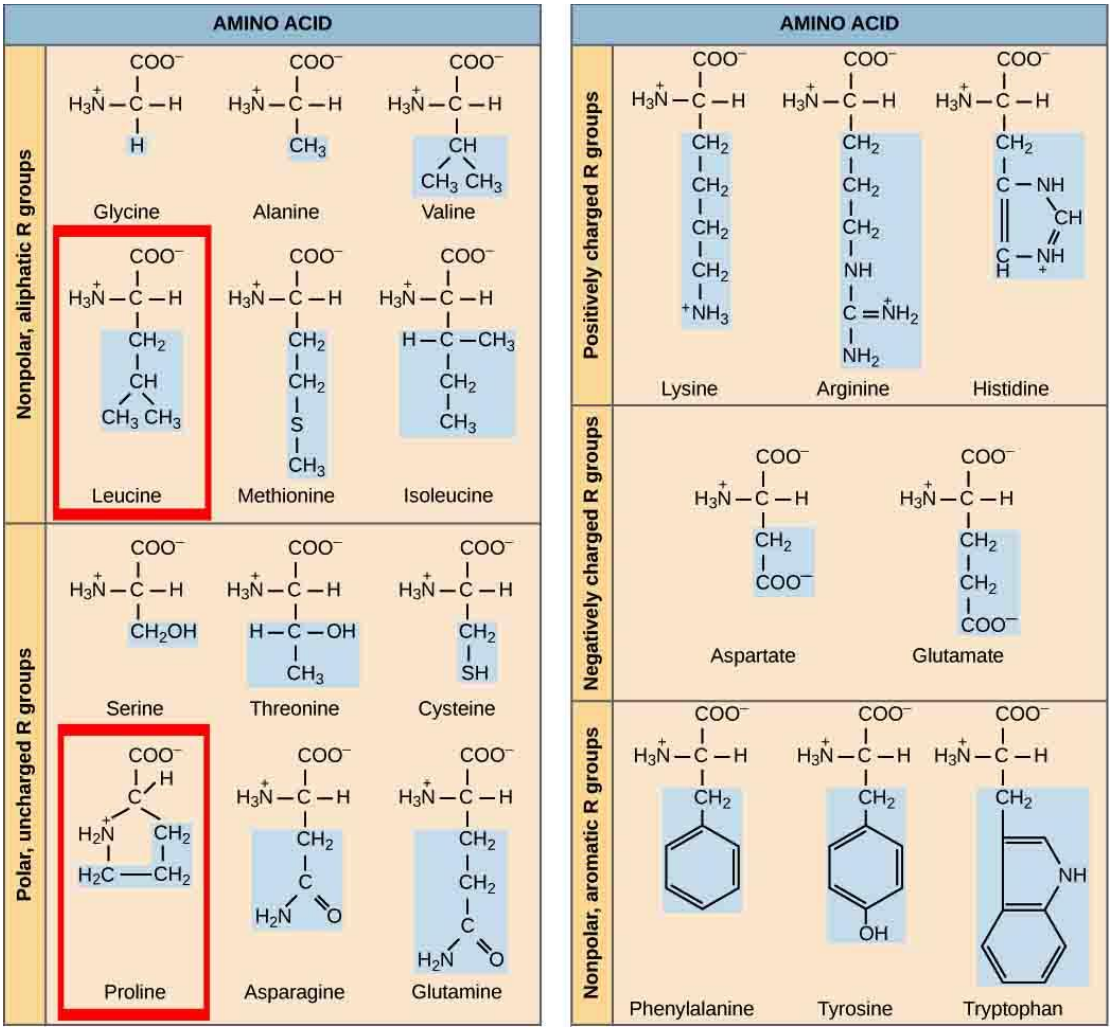


Figure 4.13 Amino acid chart with structures. Leucine and Proline are labeled in red. (Edited from https://ka-perseus-images.s3.amazonaws.com/ef725413ac787262fb1f9a80d7923e4b94710cf6.png)

4.4.4 Effects of Temperature on Pigmentation and Growth

Finally, the effect of temperature on growth and colony colour was examined both on wild-type and UV colour mutants of *P. roqueforti*. At 37 °C growth was found to be inhibited. This temperature is therefore too high for the fungus to replicate successfully. Some spores may die at this temperature but it is most likely they are remaining dormant until more optimal conditions occur. Meanwhile, no clear difference in growth was observed between colonies grown at 28 °C or at 25 °C in the dark. It will be worth investigating within the optimal range of *P. roqueforti*'s growth if a change can be seen at slightly lower or higher temperatures. At 25 °C however, the variation in growth and pigmentation is negligible.

There was a change in colony formation and pigmentation when the temperature was brought down to 15 °C. The overall bleaching effect of the colder environment could be due to a decreased number of spores per colony instead of an actual change in pigmentation. For 74-88-5, only at the very edge of colony growth was there a reversion to the brighter green colour that originally characterised this isolate. Given that the youngest growth of the fungus is at its colony edge, there may be a connection between, age, temperature, and pigmentation for 74-88-5. If fawn is the true colour of an *ayg1* gene knockout, then possibly for 74-88-5 there is an alteration in protein structure that remains active when the temperature is low enough. It is possible that as the conidia reach maturity they change protein activity and alter in colour. For 74-88-6 there was a clear reversion back to the colour that originally characterised this isolate. The off white conidia, gnarled growth, and smaller colony diameter is good evidence that this is a temperature-sensitive mutant (Figure 4.14).

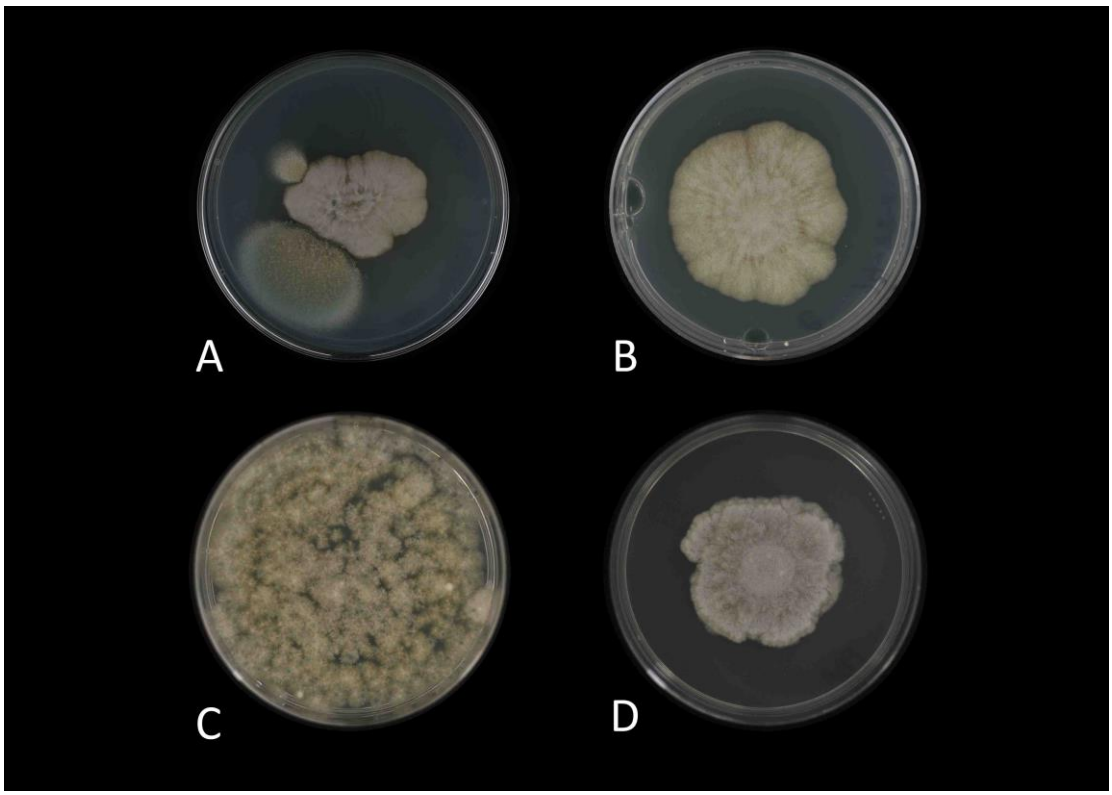


Figure 4.14. Change of growth in 74-88-6 over 11 months. (A) early sectoring, (B) stabilization through selection, (C) temperature instability, (D) return to original phenotype following growth at 15 °C

Thus, there is evidence that 74-88-5 is a temperature-sensitive mutant and strong evidence that 74-88-6 is also a temperature-sensitive mutant. However, it is very interesting to note that results of small-scale cheese trials with the Highland Fine Cheese Company do not align with the observations seen in the lab. 15 °C is the temperature at which the cheese is cultured yet when they used 74-88-6 it produced blue conidia. When 74-88-5 and 74-88-12 were used as a starter culture they produced rich green conidia instead of a fawn colour. Given that some of these strains are temperature sensitive, these results most likely mean there are other variables at work due to the cheese making process. Both temperature and light were controlled for in this experiment. These strains may also react differently at varying pH and this combined with a colder temperature could create the results observed at the Highland Fine Cheese Company. The cheese itself may be less nutrient rich as potato dextrose and have a different consistency than agar. Further investigation would need to be done to test for these variables to see if it were possible to replicate what they have observed.

5. EXPERIMENTAL ANALYSIS OF PIGMENT PRODUCTION IN *PENCILLIUM ROQUEFORTI* (2) RNA GENE EXPRESSION

5.1 Introduction

5.1.1 Secondary Metabolite Gene Expression

For this research, the main focus has been on secondary metabolites that are classified as non-essential for normal growth, development, and reproduction of an organism. These kinds of compounds are expressed only at the times they are needed and are shut off when their function is complete (Hoopes, 2008). All six genes believed to be involved with the pigment pathway for *P. roqueforti* are used to produce fungal melanin in each spore. Starting with the polyketide synthase, it can be inferred that each gene is activated and expressed in the process of producing spore colour. When the colony has reached maturation, the fungal melanin has been assembled in full leaving a mat of bluish-green colonies. These genes are then believed to shut off and no longer produce the proteins involved. In work described in Chapter 3, bioinformatic work had identified a putative DHN-melanin pathway in *P. roqueforti.* However, it was not determined whether the genes identified in this pathway were functionally active. To test this, RNA expression studies were next undertaken. Primers were designed either side of introns in each gene and used to amplify cDNA from varying growth conditions. It was hypothesized that if the pathway genes were indeed functional that gene expression should be detected in sporulating colonies, and that greater gene expression should be observed under sporulating than non-sporulating growth conditions.

5.2 Materials and Methods

5.2.1 DHN-melanin Pathway and HKG gene Expression

Sequences of the putative DHN-melanin pathway genes *alb1, ayg1, arp1, arp2, abr1* and *abr2* had previously been identified as described in section 3.3.1. In addition the tubulin housekeeping gene (HKG) was identified from the genome of *P. roqueforti* (available online at the NCBI [http://www.ncbi.nlm.nih.gov/pubmed] website) by BLASTP searching with the homologous gene from *Aspergillus nidulans.* Primer sets for all six genes plus the HKG were designed using MacVector 12 to yield products between 590 bp – 627 bp, each spanning a region of the respective gene containing 1-2 introns (Appendix 9.5). Thus, if mRNA were present it would be predicted that the cDNA product from amplification would be smaller in size than the gDNA control due to splicing of the intron(s) present.

5.2.2 RNA Extraction and One-Step PCR

RNA was extracted from colonies of *P. roqueforti* 74-88 grown under conditions favoring sporulation (solid media, section 2.2.2) or in liquid cultures in which sporulation was repressed (section 2.2.1). RNA was then extracted as described in section 2.2.4. A Qiagen One-Step PCR kit was used to reverse transcript RNA into cDNA and then allow amplification through PCR. 25 µl reactions (14.6 µl RNase free H_2O, 5 µl 5x buffer, 1 µl 10 mM dNTP mix, 1.5 µl 10 µM forward primer, 1.5 µl 10 µM reverse primer, 0.4 µl 250 ng/µl RNA template and 1 µl Taq DNA Polymerase) were prepared on ice, then transferred into the thermo cycler at 50 °C with PCR conditions set to: 105 °C heated lid, reverse transcription at 50 °C for 30 min., initial PCR activation at 95 °C for 15 min., 30 cycles of 30 sec. at 94 °C for denaturation, 30 sec. at 60 °C annealing, and 1 min. at 72 °C for elongation, followed by a 72 °C final extension for 10 min. and a hold of 5-10 °C.

One-Step PCR was performed on RNA extracted from 74-88 and run with a number of cycles ranging from 20-30 to allow semi-quantitative end-point RT-PCR. A standard PCR was run on gDNA of 74-88 along with a water control. Samples were mixed with loading dye at a ratio of 1:3 (dye/sample), loaded into a gel, and ran

under gel electrophoresis. Gels were examined under UV light and photographed (section 2.2.6).

5.3 Results

The tubulin HKG produced a band of 350 bp from the genomic DNA and a 300 bp band from the solid and liquid media cDNA samples, consistent with mRNA presence and splicing of an intron (Figure 5.1A). All six genes of the putative DHN-melanin pathway were amplified using a 30 cycle PCR. gDNA from each gene produced a band of 600 bp. For both solid and liquid media extractions, the cDNA of *alb1, abr1,* and *arp2* produced bands of 550 bp while the cDNA of *ayg1,* and *arp1* produced bands of 500 bp. *abr2* produced a liquid media band of 500 bp. These band sizes were all consistent with mRNA presence and splicing of an intron. For *alb1* and *arp2* the cDNA band derived from the solid media extraction was brighter and more pronounced than that of the liquid media cDNA extraction (Figure 5.1B). For *arp1* and *abr1* both bands were the same intensity and for *ayg1* and *abr2* the liquid medium band was more pronounced that the solid medium band (Appendix Figures A9.3.1 and A9.3.2).

When the number of PCR cycles was reduced to 25 for *alb1* and *ayg1*, the difference in intensity of the solid and liquid media cDNA bands grew more pronounced, favoring the solid medium band (Figure 5.2A and Appendix A9.3.3). When the number of PCR cycles for *alb1* was reduced to 24, the liquid medium cDNA band was almost gone while the solid medium cDNA band was still pronounced (Figure 5.2B). At 23 cycles, no bands were visible for cDNA from either solid or liquid media.

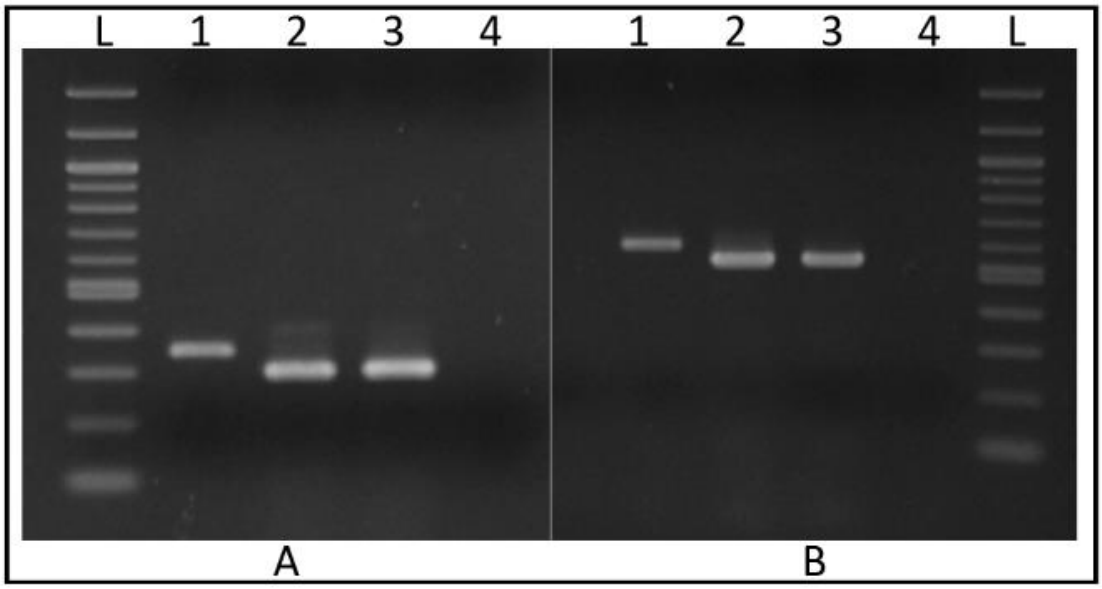


Figure 5.1 (A) Tubulin HKG. (B) Alb1 example of band intensity; L – 100 bp ladder, 1 – genomic DNA, 2 – cDNA from solid media RNA extraction, 3 – cDNA from liquid media RNA extraction, 4 – water control.

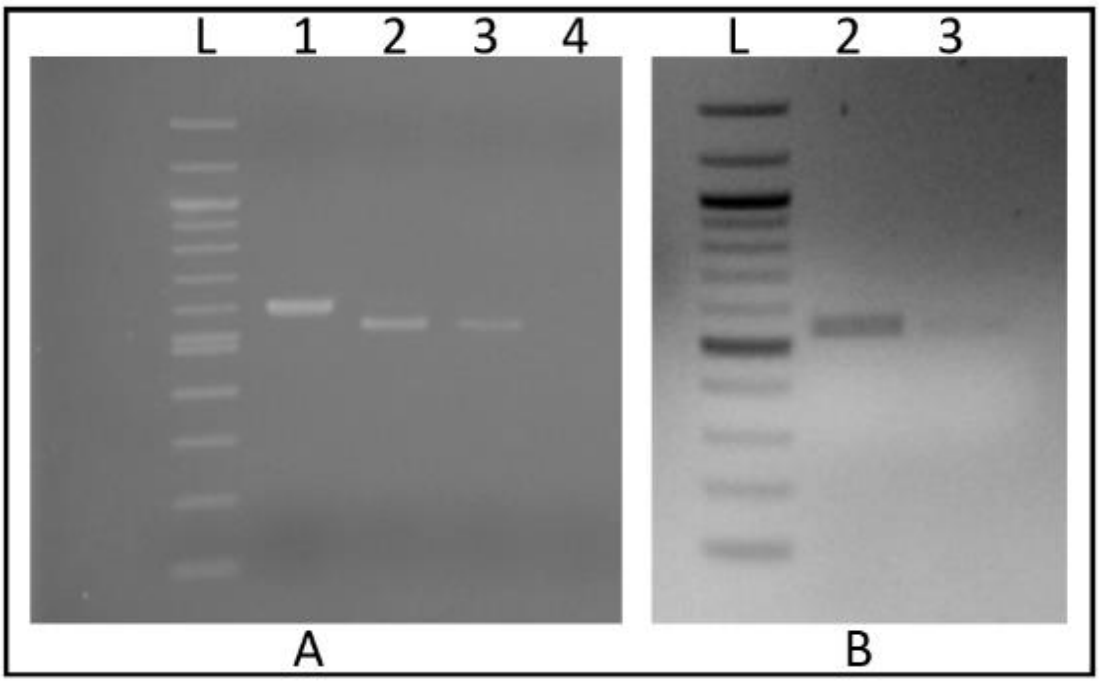


Figure 5.2 (A) Alb1 PCR 25 cycles. (B) Alb1 PCR 24 cycles; L – 100 bp ladder, 1 – genomic DNA, 2 – cDNA from solid media RNA extraction, 3 – cDNA from liquid media RNA extraction, 4 – water control.

5.4 Discussion

A key finding of the current chapter was that all six putative DHN-melanin pathway genes were expressed at an mRNA level in sporulating cultures of *P. roqueforti* (representative results shown in Figures 5.1 and 5.2). This is consistent with these genes having a functional role in *P. roqueforti.* There was success in production of the desired set of bands from the tubulin HKG as a control in this experiment. The genomic DNA produces a band including the intron of 350 bp while cDNA from RNA extractions produce a band without the intron of 300 bp. This gene is constantly active in all phases of the fungi's life cycle so a pronounced band would be expected to be produced from both solid and liquid media extractions. In parallel work Geib et al. (2016) were similarly able to demonstrate expression of putative pigment biosynthesis genes during sporulation of *Aspergillus terreus.*

In contrast, the definitive result of each pigment gene remaining inactive and not being expressed under non-sporulating conditions and becoming active during sporulation was not achieved. If a band was only produced from the solid media cDNA while no band was formed from liquid media cDNA, the result would have been conclusive in correlating gene expression with pigment production. Indeed, Geib et al. (2016) were able to demonstrate a lack of expression of pigment biosynthesis genes during growth in liquid cultures of *A. terreus.* However, a noticeable difference in band intensity was observed between the sporulating and non-sporulating conditions with *P. roqueforti*. This could be due to a constant low-level background activity of each gene during the entire life cycle of the fungus. This was investigated by adjusting the number of cycles run in each PCR focusing on *alb1* and *ayg1.* Being the first two genes in the pathway it was thought they would be the first to show background expression. An activated gene will hit the log phase in a PCR cycle earlier than a small amount of background activity, due to a larger amount of initial RNA present. If the number of cycles is too high, the small amount of cDNA produced from background activity will be amplified beyond the log phase and produce a similarly bright band. When the cycles were reduced to 23, no bands were present in either case. However, when the cycles were increased to 24, a bright band

was produced from sporulating conditions but only a very faint band was produced from non-sporulating conditions. This could be due to an increase in gene activity during sporulation compared to the low concentrations being amplified from slight background activity.

One further possibility is that even in shaken liquid culture there might still be a low level of sporulation occurring. This could be located in any mycelium that makes it way onto the side of the flask and is no longer in circulation. Another possibility is that the spheres of mycelium in the flask could have a small number of spores at the centre that are no longer in contact with the outer surface. These could in theory produce a small amount of gene activity, which would account for these observations. Further tests were conducted to cut open grown mycelial balls from liquid culture to see if any sporulation was occurring but none was observed.

In future work QRT-PCR could be used to monitor gene expression in real time. This might provide evidence of gene function if it could be established that the DHN-melanin pathway genes are under low activation during the entire life cycle of the fungus, but that expression increases during sporulation linked to pigment production. If any of the genes are found to be active at all times, further investigation would be needed to see if the gene performs multiple functions outside of what is being investigated by this research.

EXPERIMENTAL ANALYSIS OF PIGMENT PRODUCTION IN *PENICILLIUM ROQUEFORTI* (3) *P. ROQUEFORTI* TRANSFORMATIONS

6.1 Introduction

Genetic transformations can be used to either add or remove DNA to an organism's genome through targeted interruption of a specified sequence. There have been multiple approaches in the past that have garnered positive results in the disruption of fungal genomic DNA. Electroporation uses a pulse of electricity to temporarily open the pores of a cell in order to let the vector in (Margolin *et al.* 1997). When the bacteria *Agrobacterium tumefaciens* infects a plant, the T-DNA region of its Ti plasmid is transferred to the infected host. This process has been used successfully through the production of spheroplasts to disrupt genes in *Aspergillus fumigatus* (Sugui *et al.* 2005). The most widely used method however is the production of fungal protoplasts through lytic enzymes in order to facilitate the cellular uptake of foreign DNA. This process is superior to the previous two methods because of its ease of reproduction in most laboratory environments (Goarin *et al.* 2015).

Efficient production of protoplasts is a key factor in the transformation of fungi. The complex structure of the fungal cell wall is composed mainly of chitin, 1,3-β-glucans, 1,6-β-glucans, proteins, and mannins. The lysing enzymes used to degrade this tightly bound cell wall are therefore made up of chitinases, β-glucanases, proteases, and cellulases. Once the cell wall is removed, these vulnerable protoplasts become osmotically sensitive and require an osmotic stabilizer. The most common mediator used in protoplast transformations is a highly viscous solution of polyethylene glycol. Once the selection vector has been taken up by the protoplast it is then reconstituted in a regeneration media and placed under some sort of selection pressure (Berg and Maruthachalam, 2015).

Initially, early successes in fungal transformations focused on the *pyr4* gene in *Neurospora crassa* to complement uracil auxotrophs (Buxton and Radford, 1983).

Use of uracil as a selection marker through auxotrophic mutants was less efficient though due to the difficulty of producing adequate auxotrophs that did not affect growth. The use of dominant resistance markers soon became the preferred method focusing on the antifungals hygromycin-B and phleomycin. The use of hygromycin-B as a resistance marker reproduced through *E. coli* can be seen in early transformations of *Aspergillus nidulans* (Punt *et al.* 1987).

Escherichia coli can be used to replicate in large volumes a plasmid containing the selection vector used for fungal transformations. An antifungal (hygromycin-B) resistance gene forms the deletion cassette with a vector backbone coding for antibiotic (ampicillin) resistance in *E. coli* (Goarin *et al.* 2015; Casali and Preston, 2003). The regions flanking the antifungal resistance gene act as guides to find the target gene along the genome. When the DNA attempts to replicate, a double homologous recombination event occurs where the new resistance cassette takes the place of the targeted gene removing it completely from the genome (Hall *et al.* 2009).

6.1.1 Individual Deletion of Genes

All of the previous research and experiments in the present study have been leading up to work involving the targeted deletion of each putative gene of the DHN-melanin pigment biosynthesis pathway in *Penicillium roqueforti*. Work described in the previous chapters has firstly identified six genes believed to be involved in the production of conidial pigmentation, namely *alb1, ayg1, arp1, arp2, abr1,* and *abr2*. Experimental work involving the use of enzyme inhibitors, analysis of the DNA sequence of UV induced colour mutants, and RNA expression work have produced strong data to support the functionality of this pathway. But gene deletion work is required to produce definitive evidence of gene functionality. Therefore each gene of the putative DHN-Melanin pathway were individually targeted for deletion in work described in the present chapter. Methods for the genetic transformation of *P. roqueforti* have recently been described by Goarin et al. (2015), providing a starting point for these studies.

6.2 Materials and Methods

6.2.1 Overview of Gene Deletion Strategy

A separate gene deletion transformation of the *P. roqueforti* wild type strain 74-88 was performed for each of the six genes believed to be involved in the production of fungal melanin. For each gene, a deletion cassette was constructed containing the hygromycin-B antifungal resistance marker. An upstream region of the target gene on the 5' end and a downstream region of the target gene on the 3' end were added as flanks to increase the efficiency of gene targeting. For *alb1, arp1*, and *abr1*, both the upstream and downstream regions are 1kb in length. For *ayg*1 the upstream region is 946 bp and the downstream region is 1kb in length. For *arp*2 the upstream region is 967 bp and the downstream region is 786 bp in length. For *abr2* the upstream region is 856 bp and the downstream region is 668 bp in length. The hygromycin-B resistance gene was 2,981 bp in length making each cassette between 4505 bp and 4981 bp in size.

Each cassette was assembled to include the pUC19 vector backbone (2,692 bp long) coding for ampicillin antibiotic resistance. The combination of the deletion cassette and the pUC19 backbone produced a fully formed plasmid. Each plasmid was integrated into chemically competent cells of *E. coli* and grown in the presence of ampicillin as a selection pressure. Plasmids were removed from the *E. coli* after growth and the deletion cassette was removed from the vector backbone via restriction enzyme digest. Specific details of the methodology used in the plasmid construction and transformation will now be described.

6.2.2 NEB Builder Vector Design and Assembly

All transformations of *P. roqueforti* were performed using a linear transformation method. Resistance cassettes for all six genes were assembled using hygromycin as a selection marker. The vector backbone used was pUC19 containing an ampicillin marker for *E. coli* cloning. The NEB Builder website was used to design 4 of the 6 plasmid vectors (for deletion of genes *alb1, ayg1, arp1*, and *abr1*). Vectors for the

deletion of *arp2* and *abr2* were designed manually in MacVector 12. The 2.7 kb PUC19 backbone was inserted followed by the 1 kb upstream sequence, both by PCR. Repeat restriction enzyme sites that were not located in any of the 74-88 gene tails were added (*Hind*III for *alb1*, *Kpn*I for all others). The 2.9 kb hygromycin resistance gene was inserted followed by the 1 kb downstream region, both by PCR (Figure 6.1). A common set of primers was designed for hygromycin to act as a universal pair for all six plasmid vectors.

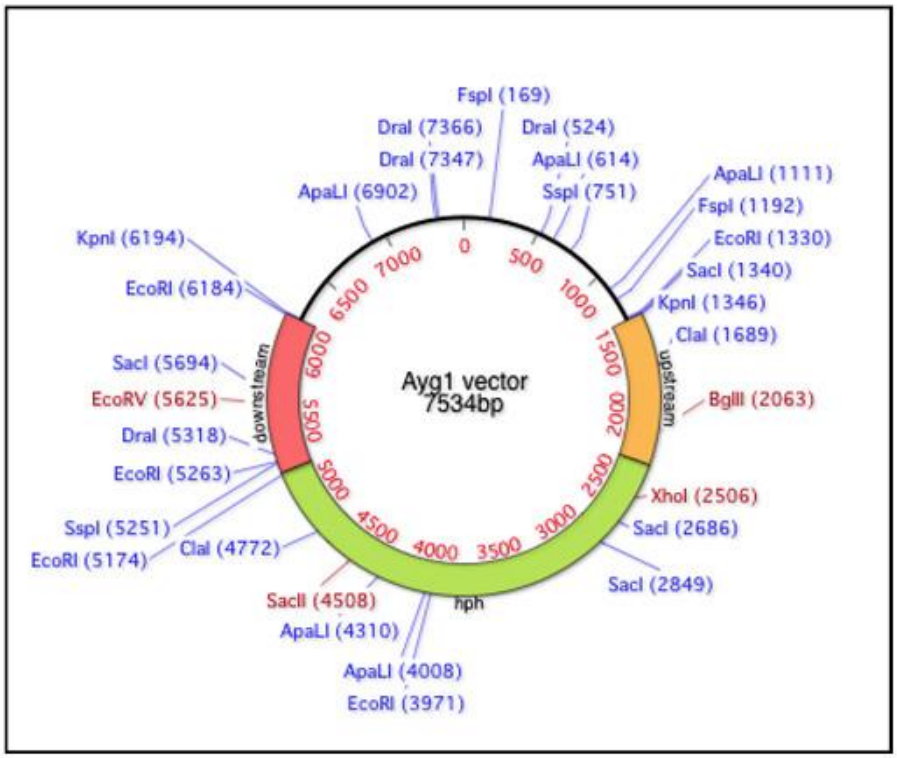


Figure 6.1. Representative gene deletion *ayg1* plasmid vector containing pUC19 backbone, upstream region, hygromycin-B resistance gene, and downstream region. *Kpn*I RE sites located at 1346 bp and 6194 bp.

The pUC19 plasmid was cut with *Kpn*I to linearize the DNA and was then gel purified. The hygromycin sequence along with each upstream and downstream tail for all six genes was amplified via standard PCR cycle (annealing temperature of 63 °C). The PCR clean up was slightly altered by eluting the DNA with 30 µl of elution buffer and placing it in a heat block at 72 °C for 3 minutes. This was then centrifuged at 13,000 rpm/1 min. The elution buffer was removed from the Eppendorf tube, placed back on the membrane, and heated at 72 °C for 3 minutes again. This was spun down at 13,000 rpm/1 min and then the product concentrations determined.

In order to assemble the fragments into a cloning vector 50 ng of pUC19 plasmid, 160 ng of hygromycin DNA, 55 ng of upstream DNA, and 55 ng of downstream DNA were added to a PCR tube. This was mixed with 10 µl of 2X HIFI DNA-assembling cloning solution (New England Biolabs). All six tubes were incubated in the thermo cycler at 50 °C for 30 minutes.

6.2.3 Transformation into *E. coli* via Gibson Cloning Kit

2 µl of the assembling mixture was mixed via pipetting with 50 µl of chemical competent cells of *E. coli* and incubated on ice for 30 minutes (section 2.2.1). The *E. coli* was transferred to 42 °C for 30 seconds to induce heat shock and then incubated on ice for 2 minutes. 950 µl of SOC solution was added and shaken for 60 minutes under incubation at 37 °C. Afterwards, 50-300 µl of mixture was added to LB agar plates containing ampicillin (1 µl/ml) and spread (media according to Strategene instructions, section 2.2.1). Plates were incubated overnight at 37 °C.

6.2.4 Colony PCR

Plates were removed from the incubator and examined for colonies. 8-16 colonies were chosen, plucked with a sterile toothpick, swirled in 15 µl of ddH_2O in a 1.5 Eppendorf tube, and then samples spread onto a segment of an LB agar plate containing ampicillin (1 µl/ml). Remaining liquid suspensions were heated to 95 °C for 15 minutes. 10 µl PCR reactions were made for each sample (6 µl SDW [Sterile Distilled Water], 2 µl 5x Phire buffer, 0.2 µl 10 mM dNTP mix, 0.2 µl 50mM $MgCl_2$, 0.2 µl 100 µM forward primer, 0.2 µl 100 µM reverse primer, 1 µl gDNA template and 0.2 µl 2x Fidelity Phire Polymerase) and run on the Analytikjena Speedcycler2 set to: 105 °C heated lid, initial denaturation at 98 °C for 60 sec., 32 cycles of 2 sec. at 98 °C (ramp 10 °C) for denaturation, 2 sec. at 61 °C (ramp 8 °C) for annealing, and 10 sec. at 72 °C (ramp 10 °C) for elongation, followed by a 72 °C final extension for 60 sec. and a hold of 10 °C. Primers used were nested in the hygromycin resistance gene and designed to produce a band of 833 bp (Appendix 9.5). Positive colonies were further investigated for correct assembly.

6.2.5 Plasmid Extraction Via Alkaline Lysis Solution III

E. coli was wiped up from the plate using a yellow pipette tip and swirled into 100 µl of resuspension buffer solution I (25 mM Tris.HCl pH 8, 10 mM EDTA pH 8, 15% Sucrose/Glucose) in a 1.5 ml Eppendorf tube. 200 µl of lysing solution II (0.5 M NaOH, 1% SDS) was added to each tube and inverted several times to mix (solution was left to stand for no longer than 2 minutes to avoid excessive lysing). 150 µl of potassium acetate neutralizing solution III (3M Potassium Acetate pH 5) was added to each tube and inverted several times to stop the reaction.

Tubes were centrifuged at 4 °C for 10 minutes at 13,000 rpm. 450 µl of supernatant was removed, mixed with 450 µl of propan-2-ol (1:1 mixture), inverted several times, and centrifuged at 13,000 rpm/15 min. The supernatant was removed and 500 µl of 70% ethanol was added. Each tube was inverted several times and spun at 13,000 rpm/5 min. The supernatant was removed again and tubes were dried in heat block at 65 °C for a few minutes. The pellet was finally resuspended in 20 µl of SDW.

6.2.6 Plasmid Extraction Via Promega Miniprep System

Colonies of *E. coli* were plucked with a yellow pipette tip and placed in a universal tube containing 5 ml of LB broth containing ampicillin (1 µl/ml). Tubes were incubated at 37 °C and shaken overnight. 600 µl of sample was placed in a 2 ml Eppendorf tube and spun at 13,00 rpm/30 sec. The supernatant was removed, 600 µl more of sample was added, and the pellet was resuspended by pipetting. 100 µl of cell lysis buffer was added to each tube and inverted several times. Within two minutes 350 µl of cold (4-8 °C) neutralizing solution was added and mixed thoroughly by inverting. Samples were spun at 13,000 rpm/3 min., ~900 µl of supernatant was transferred to a pure yield mini-column, and then centrifuged at 13,000 rpm/15 sec. The flow through was discarded and 200 µl of endotoxin removal wash solution was added to the column and spun at 13,000 rpm/15 sec. The flow through was discarded and 400 µl of column wash solution was added to the column and spun at 13,000 rpm/30 sec. The mini-column was transferred to a new 1.5 Eppendorf tube and 30 µl of elution buffer was added directly to the membrane. This was left to stand for 1 min. and then spun at 13,000 rpm/30 sec. to elute the plasmid DNA.

6.2.7 Plasmid Check Via Restriction Enzyme Digest

All plasmids were checked for correct assembly by a restriction enzyme digest. The fully assembled plasmid DNA was investigated in Mac Vector 12 for restriction enzyme sites resulting in multiple bands >500 bp, none of which were similar in size, and with at least one site located in the deletion cassette. A mixture of restriction enzyme solution (1 µl RE, 2 µl corresponding buffer) was made and mixed with <10 µg of plasmid. Samples were incubated at 37 °C from 1-15 hours and ran on a 1% TAE gel. Samples that produced the desired band sizes were re-inoculated into universal tubes containing 5 ml of LB Broth and ampicillin (1 µl/ml) and shaken overnight at 37 °C. Large quantities of plasmid were extracted via a Promega Miniprep System (according to manufacturer's instructions) with some *E. coli* samples being stored in a 20% glycerol stock at -80 °C for further use.

6.2.8 Gel purification of Deletion Cassette

Each plasmid was digested with its respective restriction enzyme (*Hind*III for *alb1*, *Kpn*I for the other five) at 37 °C for at least 1 hour. Samples were run in a 1% TAE gel and examined under UV light for a band at 4.9 kb (hygromycin resistance gene with 1kb site specific tails). This band was cut out with a scalpel blade, placed in a 2 ml Eppendorf tube, a scale zeroed with an empty tube, and its weight was measured in mg. A Monarch gel purification kit was used to purify the band by adding four times the weight in µl of dissolving solution and heating at 55 °C until uniform. This was spun through a mini-prep column at 13,000 rpm/1 min., flow through discarded, and 200 µl of column wash added. This was spun through at 13,000 rpm/1min., 200 µl of column wash added, and spun through at 13,000 rpm/1 min. The column was transferred to a 1.5 ml Eppendorf tube where 8-20 µl of elution buffer was added and let to sit in the heat block at 50 °C for a few minutes. This was finally spun at 13,000 rpm/1 min.

6.2.9 *Penicillium roqueforti* Transformation Protocol

A protocol for transformation of *P. roqueforti* was devised based on existing protocols used at the University of Nottingham Fungal Genetics and Biology Group, and informed by the recently published protocols of Goarin et al. (2015). The final method is described below, which required preliminary work (data not shown).

At the start, 10 ml PDA slopes in universal tubes were inoculated with *P. roqueforti* and incubated at 28 °C until fully grown (approx. 5-7 days growth). 4-5 ml of Tween 20 was added to the slope and vortexed to remove all the spores. This mix was filtered through a double layer of sterile Miracloth and collected in a fresh universal tube. A 1:100 dilution was made in a new 2 ml Eppendorf tube and spores were counted on a haemocytometer. A spore suspension of 4 x 10^8 spores/ml was made and added to a sterile 2 L flask containing 400 ml of YEPD broth (1x10^8 spores/100 ml). The liquid culture was sealed with a sterile bung and incubated at 28 °C for 17 hours, shaken at 150 rpm. Spores were then checked microscopically for the formation of germ tubes.

The liquid culture was filtered through a double layer of sterile Miracloth and washed with approximately 50 ml of cold (4-8 °C) mycelium wash buffer (0.6M $MgSO_4.7H_2O$). The mycelium was gently blotted with paper towels and 1-2 g transferred into a sterile falcon tube and placed on ice. Next, 0.5 ml of Protoplast F (Megazyme lysing enzyme) was mixed with 9.5 ml of Protoplast F Lysis Buffer (700 mM KCl, 20 mM Potassium Phosphate [25 mM KH_2PO_4 adjusted to pH 5.8 with 25 mM K_2HPO_4]) and filter sterilized through a 0.22 µm filter unit into a sterile 250 ml flask. The mycelium was added to the flask, mixed using a 10 ml pipette, and incubated at 28 °C shaken at 100 rpm. After an hour of incubation, a 10 ml pipette was used to mix the solution up and down. The mixture was checked microscopically every 30 min. until sufficient protoplasting occurred (approximately 2.5 hours).

Two purification columns were prepared by loosely packing sterile 20 ml syringe barrels half way with pre-autoclaved polyallomer wool. Using a stand, one column was fixed angling into the other in a hatch-batch formation. The protoplast mixture

was poured into the first column using a sterile funnel allowing separation to occur by gravity. Protoplasts were collected in a 50 ml falcon tube with cold (4-8 °C) NM Buffer (1M NaCl, 20 mM MES) being added to the column until the centrifuge tube was filled to 50 ml. The solution was mixed by gentle inversion and spun at 2,500-4,000 rpm/10 min. at 4 °C. The supernatant was poured off and the pellet was gently resuspended using 1 ml of cold STC. The falcon tube was filled to 50 ml with cold STC, mixed by gentle inversion, and spun at 2,000-4,000 rpm/10 min. at 4 °C. The supernatant was removed and the pellet resuspended in 1 ml cold STC (1.2 M Sorbitol, 10 mM Tris base, 50 mM $CaCl_2.2H_2O$). Protoplasts were counted and ranged from $4x10^6 – 4x10^7$.

For each transformation, 1-4 µg of selection vector (not exceeding 20 µl of sample) and $1.5x10^6 – 8x10^6$ protoplasts (not exceeding 200 µl suspension) was placed in a 15 ml centrifuge tube. A control was made using only protoplast suspension with no selection vector added. This mixture was left to incubate at room temperature for 25 min. 200 µl of PEG Solution (60% [w/v] PEG 4000, 10 mM Tris base, 50 mM $CaCl_2.2H_2O$) was then added gradually, drop by drop with gentle mixing, followed by a second addition of 200 µl of PEG Solution while mixing gently. A final addition of 850 µl of PEG Solution was added, gently mixed by inversion, and incubated at room temperature for 20 minutes. Each tube was filled to 15 ml with cold STC and mixed by gentle inversion until the PEG Solution was completely diluted. This was spun at 2,000-4,000 rpm/10 min. at 4 °C and the supernatant poured off. 5 ml of regeneration broth ([20 g Peptone, 20 g Glucose, 10 g Yeast Extract, 182.12 g Sorbitol]/L SDW) was added to each tube, mixed gently by inversion, and then placed in separate sterile 100 ml flasks. Samples were then incubated overnight at 28 °C, shaken at 80 rpm. 1 ml of sample was spread over PDA plates containing hygromycin (20 µg/ml) until no sample was left. These plates were incubated at 28 °C with their growth observed over the next week. Individuals that formed colonies with irregular pigmentation were selected for further investigation.

6.2.10 Transformation and Confirmation

The chosen method for transformation was the production of protoplasts through the use of lysing enzymes. These protoplasts then uptake the deletion cassette and through a double homologous recombination event, integrate the new piece of DNA into the genome under the stabilization of polyethylene glycol (Figure 6.2). Once the target gene has been replaced for an antifungal resistance marker, the protoplasts are regrown in a regeneration broth containing sorbitol. The cell walls will have begun to reform allowing for better survival under harsher conditions on solid media. Once placed under the selective pressure of hygromycin-B, any colonies that grew on the plates were inspected carefully. It was anticipated that deletion of any of the DHN-melanin biosynthetic pathway genes might result in altered colony colour and therefore any emerging colonies that had a change in pigmentation were further subcultured for evaluation (Figure 6.3).

In order to confirm deletion of the resident gene, PCR using primers designed against the specific target gene (Appendix 9.5) was performed together with a positive 74-88 gDNA control (section 2.2.5). To verify correct integration of the deletion cassette in the *P. roqueforti* genome, a set of primers (Appendix 9.5) were used for positional PCR (Paoletti et al. 2007). Two primers were located outside of the deletion cassette and their compliments were located in the hygromycin-B resistance gene. If the double homologous recombination were correct, a band of the expected size would be given for the new transformant but not for the wild type strain.

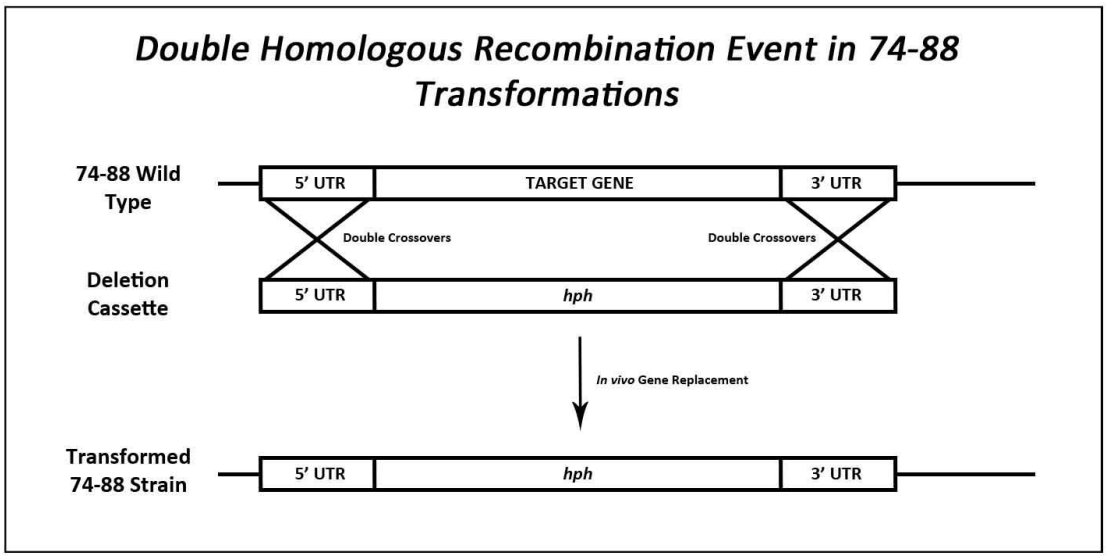


Figure 6.2 Double homologous recombination events during *P. roqueforti* transformations.



Figure 6.3. Representative example of selection of transformant colonies with altered colony colour. Image shows an *arp2* transformation selection plate containing hygromycin-B. Green Arrows indicate putative positive colonies, Red Arrows indicate putative negative colonies.

6.3 Results

6.3.1 Construction of Deletion Cassettes

All six deletion cassettes were successfully constructed and transformed into *E. coli*. Nested primers were used to test for the presence of successful transformants using the assembly mixture as a control. The forward primer was the same one used to amplify the regions upstream of the target genes. The reverse primer was located in the beginning of the hygromycin-B promoter region. The yellow arrow indicates the assembly mixture control, the red arrows indicate negative colonies, and the green arrows indicated strong positive colonies (Figure 6.4). These strong positive colonies were taken for further analysis.

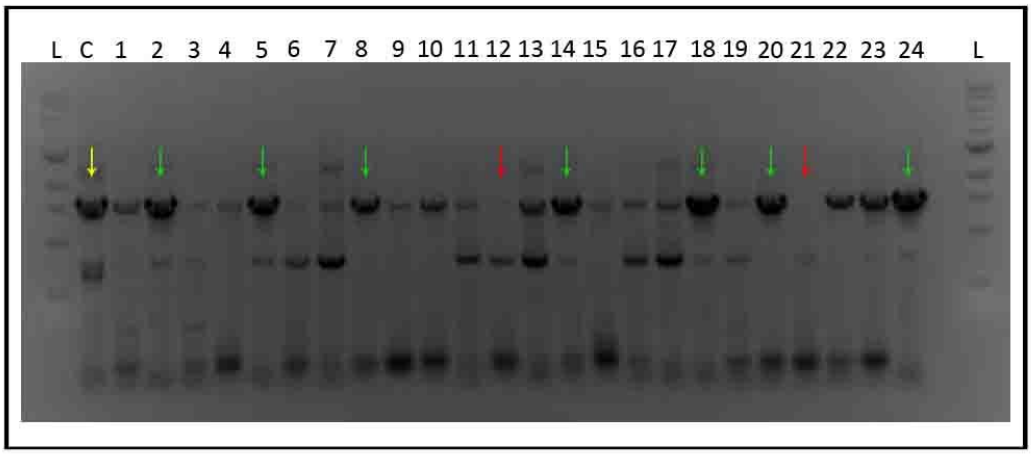


Figure 6.4 Colony PCR for the *arp2* deletion cassette vector; L – 1 kb ladder, C – Assembly mixture control, 1-24 – Individual colonies tested for plasmid.

Plasmids were extracted from the positive colonies using a Promega plasmid extraction kit. Each plasmid was digested with a restriction enzyme to test for correct assembly. MacVector 12 analysis of each plasmid indicated which restriction enzymes would give bands of different lengths (above 500 bp) and cut in at least one piece of the deletion cassette. Both *ayg*1 and *arp*1 were digested with *Msc*I producing bands of 3642 bp, 2119 bp, and 1773 bp for *ayg*1 and 3824 bp, 2593 bp, and 1170 bp for *arp1* (Figure 6.5). *alb*1 was digested with *Eco*RI producing bands of 2794 bp, 1729 bp, 1203 bp, 1083 bp, and 859 bp. *Arp2* was digested with *Sal*I producing bands of 4799 bp and 2669 bp, and digested with *Pvu*II producing bands of 2714 bp, 2364 bp, 1412 bp, and 978 bp. *Abr*1 was digested with *Pst*I producing bands of 3069 bp, 2362 bp, 1527 bp, and 629 bp. *Abr2* was digested with *Sa*ll

producing bands of 4599 bp and 2550 bp and *Eco*RI producing bands of 3405 bp, 1726 bp, 1203 bp, and 815 bp.

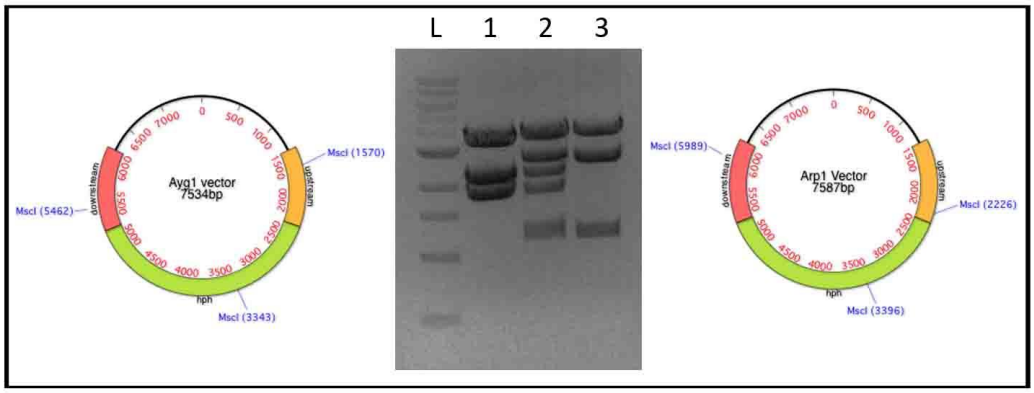


Figure 6.5 Illustrative *ayg1* and *arp2* vector diagrams with the *Msc*I restriction enzyme sites labeled; Gel electrophoresis of digested plasmids L – 1 kb ladder, 1 – Positive *ayg1* digest, 2 – Negative *arp*1 digest, 3 – Positive *arp*1 digest.

All six deletion cassettes for each gene were assembled correctly. These were then digested with a restriction enzyme to remove the pUC19 vector backbone from the cassette. *Hind*III was used to remove the cassette from the *alb1* plasmid. *Kpn*I was used to remove the cassettes from the other correctly assembled plasmids. These digests were run on a gel with the cassette being gel purified.

6.3.2 Transformation Experiments

It was aimed to generate at least two verified gene deletion mutants of each of the six DHN-melanin pathway genes of *P. roqueforti,* to ensure consistency of gene deletion phenotype. To achieve this it was necessary to perform a series of different transformations.

In the first transformation, 4.0 x 10^6 protoplasts were produced in 1.0 ml of STC. 8.0 x 10^5 protoplasts were used per transformation targeting *alb1, ayg1, arp1*, and *abr1*. 1-1.5 µg of selection vector was used per deletion with a blank being used for the control. *Ayg1* produced 28 deletion colonies in 2 successful transformations, based on altered colour of the transformants. *Abr1* produced 87 deletion colonies in 2 successful transformations. *Alb1* produced 24 deletion colonies in 1 successful transformation. *Arp1* produced 3 deletion colonies in 1 successful transformation.

This set of transformations suggested an overall success rate of 11.5% regarding correct gene targeting, according to colony colour.

In the second transformation, 3.6 x 10^7 protoplasts were produced total with 5.0 x10^6 protoplasts used per transformation. All 6 DHN melanin pathway genes were targeted using 4 µg of selection vector for *ayg1*, 1.1 µg for *alb1*, 2.17 µg for *arp1*, 525 ng for *arp2*, 1.6 µg for *abr1*, and 1.9 µg for *abr2*. *Ayg1* produced 8 deletion colonies, *Arp1* produced 6 deletion colonies, and *Alb1* produced 2 deletion colonies. *Abr1, abr2,* and *arp2* transformations were unsuccessful. The success rate of the second round of transformations was approximately 5%.

In the third transformation, 1.25 x 10^7 protoplasts were produced total with 3.125 x10^6 protoplasts used per transformation. *Arp2* was targeted using 3.0 µg and 2.1 µg of selection vector. 8 positive deletions were recorded out of 63 total colonies yielding a transformation success rate of 12.7%.

In the fourth transformation, 1.1 x 10^7 protoplasts were produced for the first set and 1.26 x 10^7 protoplasts for the second set. The entire fourth transformation was performed solely to target the remaining *abr2* gene using 2.75 x 10^6 protoplasts for the first set of three and 3.15 x 10^6 protoplasts for the second set of three transformations. The first set of transformations used 5.3 µg, 3.8 µg, and 3.4 µg of selection vector while the second set used 4.0 µg, 3.2 µg, and 3.8 µg of selection vector. Three positive deletions were recorded out 63 total colonies for a success rate of 4.8 %. The overall success rate of *P. roqueforti* transformations targeting all six genes was 8.5%.

6.3.2 Characterisation of Transformants

The *alb1* deletion produced an all white colony. When *ayg1* was removed a yellowish-green colony was formed. The deletion of *arp1* produced a light reddish-pink colony. The *arp2* deletion produced darker reddish-pink colonies. When *abr1* was deleted the transformed strain produced a dark brown colony. The *abr2*

deletion produced colonies with brown centres that grew outwards in an off green fashion (Figure 6.6).

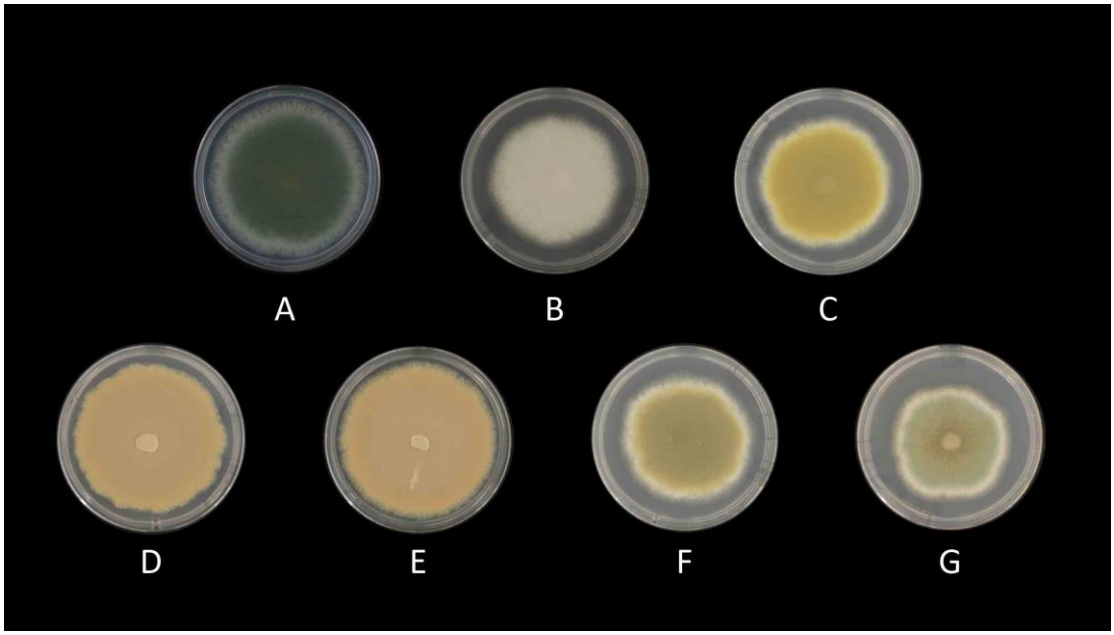


Figure 6.6 *Penicillium roqueforti* wild type strain with pigment gene targeted transformations. (A) 74-88, (B) *alb1* deletion, (C) *ayg1* deletion, (D) a*rp1* deletion, (E) *arp2* deletion, (F) *abr1* deletion, (G) *abr2* deletion.

Positional PCR primers were used to determine the correct point of insertion along the genome. This was performed for both the upstream and downstream region of the target gene. These sets of primers produced bands between 1650 bp and 2056 bp for the upstream position and between 2500 bp and 2768 bp for the downstream position. For example, for *arp1* specifically, a correct position produced a band of 1883 bp for the upstream and 2679 bp for the downstream. For *ayg1* a correct position produced a band of 1833 bp for the upstream and 2599 bp for the downstream (Figure 6.7; Appendix A9.4.2; A9.4.3).

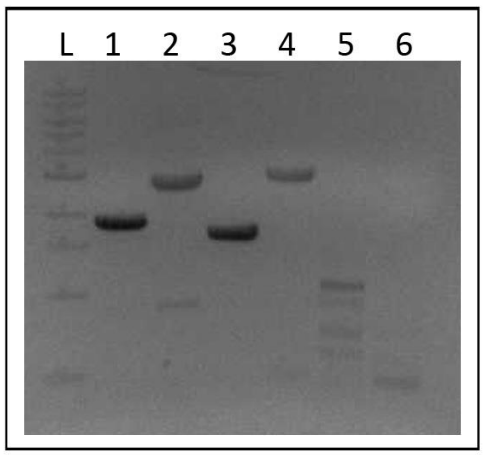


Figure 6.7 *Arp1* and *ayg1* positional PCR gel; L – 1 kb ladder, 1 – *Arp1* upstream, 2 – *Arp1* downstream, 3 – *Ayg1* upstream, 4 – *Ayg1* downstream, 5 – Upstream control, 6 – Downstream control.

6.4 Discussion

With all six genes being successfully knocked out of the *P. roqueforti* genome, strong conclusions can be made about their existence and function. Each deletion cassette was carefully constructed to target a specific region in the genome and remove what was shown through bioinformatic analysis and RNA expression work to be a potentially functional gene. When each putative gene of the DHN-melanin pathway was knocked out, a distinct change was observed in the production of conidial pigments in the resulting gene deletant strains. Indeed, the entire colony had changed dramatically in the expression of colour (Figure 6.6). In order to prove that the deletion occurred in the correct site of the genome, rather than at an ectopic site, positional PCR primers were used with the forward primer upstream of the cassette, the reverse primer in the hygromycin resistance gene, and vice-versa. All six deletions gave positive results that they were located in the correct position and had removed the targeted gene. To further assess the accuracy of the deletion, sequencing work was performed in the genes adjacent to the targeted site to ensure no mutations had occurred in the double homologous recombination event (data not shown). This was important because four of the six genes are clustered so close that the upstream and downstream regions of those cassettes included regions in the adjacent genes. Sequencing results show that no mutations or alterations to the adjacent genes occurred in the deletion of targeted sites. There is therefore considerable evidence to support the fact that each deletion was successful, in the correct position, and without any unintended alterations to neighboring genes.

6.4.1 Evidence for a DHN-Melanin Pathway

Each deletion brings with it a new and unique colour far from that seen in any wild-type strain. Only when all the genes are functioning does *P. roqueforti* produce conidia that are recognisably wild-type in colour expression. The *alb1* gene when deleted produces a pure white fluff of colonies that lack any sort of pigmentation, indicating that this gene encodes a heptaketide naphtopyrone as described in other *Aspergillus* and *Penicilium* species (Tsai et al. 1999; Woo et al. 2010). This gene is also known as the polyketide synthase and is the first step in the DHN-Melanin

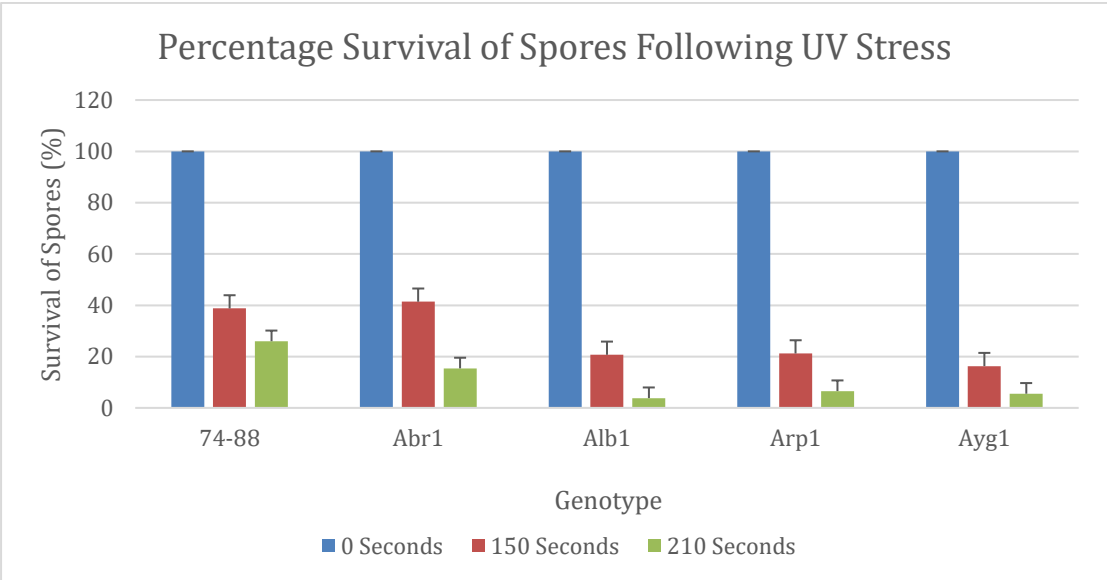
pathway. When the next gene in the pathway, *ayg1*, is deleted, a yellowish-green colony is formed because the *alb1* gene is still active and likely to be producing the heptaketide naphtopyrone, based on homology to other DHN-melanin pathways (Tsai et al. 1999; Langfelder *et al.* 2003; Woo et al. 2010). The removal of the next gene in the pathway, *arp2*, leaves a darker reddish-pink colony with the previous two genes in the pathway likely to actively produce 1,3,6,8,-Tetrahydroxinapthalene. When the subsequent *arp1* gene is knocked out, a lighter reddish-pink colony is formed because *arp2* is likely able to produce scytalone. Without the *abr1* gene the pigment pathway is only able to produce up to vermelone leaving a lighter brown colony. The final gene in the pathway is the *abr2* coding for the laccase in the DHN-Melanin pathway. Without it a colony of brownish-green conidia is formed but when it remains active the whole pathway works together to form the bluish-green colonies observed in the wild type. However, it is cautioned that confirmation of the chemical intermediates (Tsai et al. 1999; Langfelder *et al.* 2003; Woo et al. 2010) will require further biochemical work.

Bioinformatic analysis (Chapter 3) had shown the putative DHN-melanin pathway genes of *P. roqueforti* to share high homology (based on low E value matches) to those genes known to exist in the DHN-Melanin pathway of *Aspergillus fumigatus*. These six genes, when removed, produce similar pigment colours to those implied through the *Aspergillus* nomenclature. Ayg1 stands for *Aspergillus* yellowish-green 1 because when removed the species produces yellowish green colonies. Arp1 and Arp2 when deleted produce reddish-pink colonies and the same goes for Abr1 and 2 for brownish colonies (Tsia et al. 1999). Similar sequencing and deletion results show that *P. roqueforti* has the genes encoding for the polyketide synthase and the laccase. All this evidence indicates that *Penicillium roqueforti* uses a similar DHN-melanin pathway to produce the bluish-green conidia observed in the wild type.

EPILOGUE - WHY BE BLUE?

A brief extra epilogue is now included. Full details and statistical analysis are not included as this work has yet to be fully written up with the co-investigator.

Jack Whittaker was a third year summer student who was doing a 10-week project in the laboratory in the summer of 2016 and was assigned under the author's supervision. He was taught standard laboratory sterile techniques, laboratory safety, and then brought up to date on the current project. He was shown how perform kill curves and interpret data directly related to the pigmentation of *Penicillium roqueforti*. He performed a set of experiments that tested the resistance of 74-88 and the 74-88 transformants against UV radiation. This was to assess if the production on fungal melanin and the quality of "being blue" had a direct evolutionary advantage for spore survival in *P. roqueforti* as has been suggested elsewhere for DHN-melanins (Wheeler and Bell, 1988; Casadevall et al. 2000; Langfelder et al. 2003). Jack Whittaker performed the following experiment and data evaluation under direct supervision (Epilogue Figure).



Epilogue Figure - Percentage survival of spores following UV stress (data provided by Jack Whittaker).

The results of this experiment show that the 74-88 wild type had a 26% survival rate after 210 seconds of exposure to UV radiation. By contrast, “naked” spores of the *alb1* deletion strain, which had no production at all of intermediates in the DNH-melanin pathway, survive at significantly lower rates than the wild type (statistical data not shown). After 150 seconds of exposure survival rate had decreased to 20% and by 210 seconds of exposure survival rate dropped to around 4%. A similar affect can be seen in the *ayg1* and *arp1* deletions strains, most likely because they also occur early in the pigment biosynthetic pathway. In contrast, the *abr1* deletion strain shows much higher levels of survival closer to that of the wild type. At 150 seconds of exposure there is a survival rate of 41% and by 210 seconds of exposure over 15% of the spores are still alive. This could be due to the fact that the *abr1* gene deletion being second to last in the pigment pathway, that by then the pigment produced was able to more effectively absorb UV radiation and therefore a much more robust conidial shell has formed. Also melanins are in themselves thought to be important for the structural integrity of the spore wall (Langfelder et al. 2003). Thus, there may be ecological survival reasons why it is of evolutionary advantage to “be blue”.

7. GENERAL DISCUSSION

7.1 Existence of a DHN-Melanin Pathway in *P. roqueforti*

Given the industrial importance of the fungal species *Penicillium roqueforti* in the production of blue veined cheeses, the current research was conducted to determine the genetic basis of conidial pigmentation. Research findings could have very valuable applications should it be possible to manipulate spore colour in *P. roqueforti* as this could have an impact on how cheese producers make and market their product. Understanding the mechanisms behind the production of fungal melanin in *P. roqueforti* is also of academic interest for furthering the knowledge of this common fungus. Details were known about pigment biosynthesis in related *Aspergillus* and *Pencillium* species, which had implications for *P. roqueforti* but definitive conclusions could not be drawn until now. An overall hypothesis of this study was that the production of fungal melanin in *P. roqueforti* is achieved through a DHN-Melanin pathway utilizing a gene cluster containing the six genes *alb1, ayg1, arp1, arp2, abr1,* and *abr2* found in the genus *Aspergillus* (Tsai et al. 1999). A set of aims were laid out in order to test this hypothesis and produce conclusive evidence as to the existence and function of this pathway.

The use of bioinformatics has been a key factor in understanding and interpreting the proposed pathway in *P. roqueforti*. The recent publication of the *P. roqueforti* genome (Cheeseman et al. 2014) along with identification of the *A. fumigatus* pigment pathway (Tsai et al. 1999) allowed direct genetic comparisons to be made. The BLAST searching of the *P. roqueforti* genome using the *A. fumigatus* pathway exposed the existence of all six genes with sequences of high homology. However, variations in the ordering of some genes and the direction in which they read were detected. Where *A. fumigatus* has a much more compact gene cluster in relative order, *P. roqueforti* has only four genes closely clustered and in no particular order. The *alb1* and *abr2* genes are much farther away with *abr2* being located on another Super contig. All six genes found in the French genome were then sequenced for the strain primarily used in this study (74-88) to ensure that the genomes were

consistent and guard against errors. The same set of six putative DHN-melanin pathway genes were again detected. Thus, two confirmed sets of sequences for *P. roqueforti* containing the six genes found in the *A. fumigatus* pathway now exist.

7.2 Functionality of the DHN-Melanin Pathway in *P. roqueforti*

The use of enzyme inhibitors has been documented as a tool for either surveying the existence of a gene or to imply its function (Wheeler and Klich; 1995; Pihet *et al.* 2009). A set of UV colour mutants of *P. roqueforti* were available from previous work at the University of Nottingham, produced by mutagenesis of the blue cheese production strain 74-88. Therefore the parental strain and these mutants were tested using known enzyme inhibitors of the DHN-melanin pathway. The results of these tests (section 3.2) began to provide clear evidence for some of the genes believed to be involved in the pigment biosynthesis pathway. The ability of tricyclazole and pyroquilon to target and inhibit the *arp2* gene in the production of scytalone resulted in the growth of reddish-pink colonies of 74-88 when exposed to these chemicals. These colonies when compared to the 74-88-1 mutant show a close similarity in pigmentation implying a mutation in that specific gene. On the other hand, when specific UV mutants like 74-88-12 that produce yellowish-green colonies were exposed to the enzyme inhibitors, no change was seen. Ayg1 stands for *Aspergillus* yellowish-green 1 because when this gene is rendered inactive a yellowish-green colony is formed. The *ayg1* gene also occurs before the *arp2* gene in the DHN-Melanin pathway. It can be implied that tricyclazole and pyroquilon have no effect on the 74-88-12 mutant because a mutation in the *ayg1* gene prevents the 1,3,6,8- Tetrahydroxinapthalene intermediate from being turned into scytalone by the *arp2* gene. This evidence supports the functionality of at least 2 of the DHN-melanin pathway genes detected in the initial bioinformatic analysis.

In order to tie together insights from the colours of the UV mutants, the enzyme inhibitor work, and the initial bioinformatic analysis, all of the DHN-melanin pathways genes from the UV mutants had to be sequenced and compared to those in the wild-type parent 74-88. This sequencing work confirmed the mutations

proposed during the enzyme inhibitor work. 74-88-1 was found to have a mutation in the *arp2* gene and 74-88-12 had a mutation in the *ayg1* gene. The B20-1 strain of *P. roqueforti* had a mutation in the *alb1* gene resulting in pure white colonies of growth. The 74-88-2 had a mutation in the *arp2* gene producing a reddish-pink colony congruent with the *Aspergillus* reddish-pink 2 name given to the gene.

To gain further evidence of gene function, the production of cDNA from varying substrates was tested to see if gene expression could be demonstrated. It was thought that the genes producing pigments might be suppressed under non-sporulating conditions and be turned on once sporulation begins, as was demonstrated from pigment expression in *A. terreus* (Geib et al. 2016). Although it was not possible to reduce the non-sporulating conditions to no gene expression at all, it was shown for three of the six genes that a greater gene expression was evident during sporulation. Non-sporulating conditions did show a small level of expression but that activity was greatly increased during the formation of conidia. This evidence suggests that the genes examined were in some way involved with conidiation because of their increased expression during sporulation.

The final and most important method used to test the given hypothesis, of the presence of a functional DHN-melanin pathway, involved targeted gene deletions of the entire proposed pathway. Specific deletion cassettes containing a hygromycin-B resistance marker were assembled for each gene. Through a double homologous recombination event mediated by fungal protoplast transformation, these cassettes were integrated into the genome removing the targeted gene sequence. All six of these gene deletions were successful, resulting in a drastic change in conidial pigmentation. Three pieces of evidence stand out to support the proposed existence and function of this pigment pathway. First, each gene deletion resulted in a specific change in conidial pigmentation different from the wild type. For five of the six gene deletions, multiple replicates of the same transformation produced the same result in pigmentation. Further evidence was garnered to support the proper placement of the deletion cassettes without affecting any near by genes or sequences. Therefore, It can be concluded that the function of these six genes are likely to be directly

involved in the production of fungal melanin during conidial pigmentation. Secondly, these genes are nearly identical to the ones found in the *A. fumigatus* pigment pathway. The names of these genes were given to reflect the colouration of a colony when that gene was specifically rendered inactive. All six of the gene deletions in *P. roqueforti* produced the same colouration of colonies implied by their *A. fumigatus* equivalents. For example, when the Albino gene was targeted, white colonies were formed. When *ayg1* was removed, yellowish-green colonies were formed. The same can be said for the reddish-pink and the brown genes. Finally, four of the six genes believed to be in the pathway were directly affected among the original UV colour mutants. The mutations in the genes have stopped them from functioning properly resulting in altered colony pigmentation. Sequencing work and bioinformatics have proven these mutations exist and affect only one of the candidate genes per strain. These are the *alb1, ayg1, arp2*, and *abr1* genes. When the four gene deletion strains that targeted these regions are compared to the UV mutants, a striking similarity in conidial pigmentation can be seen (Figure 8.1). Both the *Alb1* deletion and the B20-1 strain containing an *alb1* mutation produced all white colonies. The *ayg1* deletion has produced yellowish-green colonies the same as the 74-88-12 strain, which contained an *ayg1* mutation. The 74-88-1 UV mutant containing an *arp2* mutation had similar pigmentation to that of the targeted *arp2* deletion strain. The *abr1* transformant when compared to the 74-88-2 strain containing an *abr1* mutation produces nearly identical colonies (Figure 7.1).

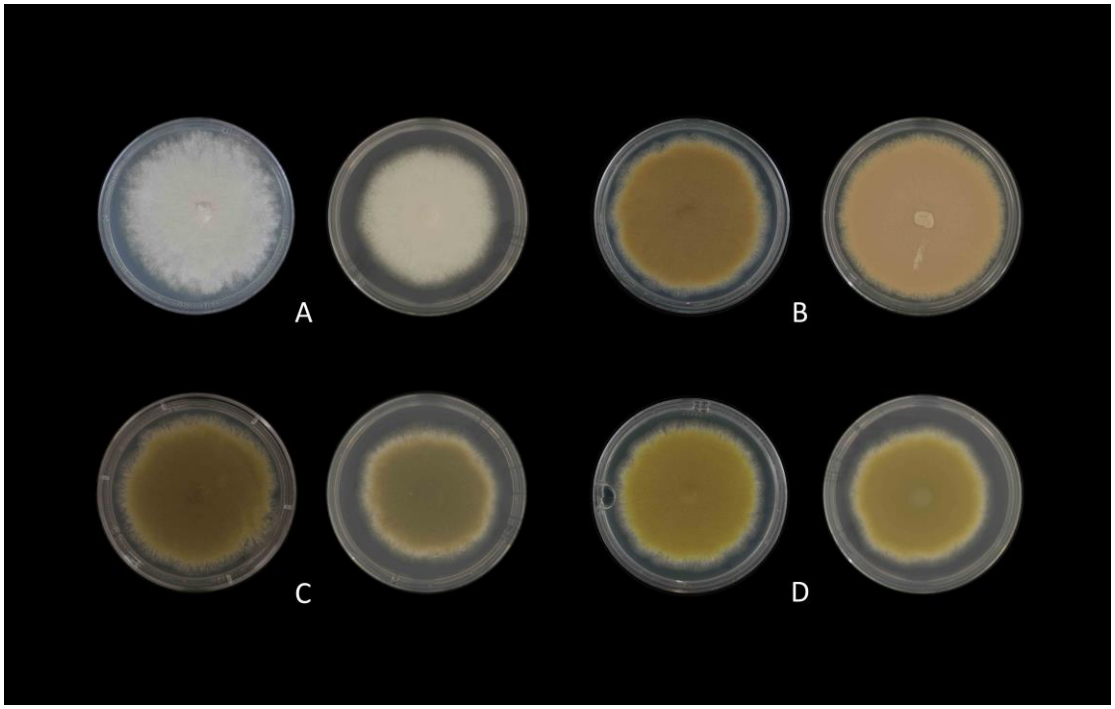


Figure 7.1 Transformation strains compared to UV mutants with the same effected gene. (A) B20-1 [left] and *alb1* deletion [Right], (B) 74-88-1 [left] and *arp2* deletion [Right], (C) 74-88-2 [left] and *abr1* deletion [Right], (D) 74-88-12 [left] and *ayg1* deletion [Right].

This thesis therefore proposes that the production of fungal melanin in *Penicillium roqueforti* is achieved through a DHN-Melanin pathway utilizing a gene cluster containing the six genes *alb1, ayg1, arp1, arp2, abr1*, and *abr2* as found in members of the genus *Aspergillus*. Bioinformatics revealed the existence of all six genes of the *A. fumigatus* gene cluster in *P. roqueforti*. The production of UV mutant strains of *P. roqueforti* and their subsequent examination through enzyme inhibitors has indicated the functionality of at least two of the genes in the pathway. Sequence analysis of each UV mutant strain showed mutations in four of the six genes of the proposed pathway. These mutations have rendered the specific gene inoperable causing a change in conidial pigmentation. Bioinformatics also shows that these specific genes are the ones found in DHN-Melanin pigment pathways versus the DOPA-melanin pathway found in some filamentous fungi (Langfelder *et al.* 2003). An expression study to ascertain the function of these genes was performed comparing sporulating and non-sporulating conditions. It was shown that three of the six genes showed increased expression during sporulation. Finally, targeted gene deletions were performed knocking out each of the six genes believed to be in the pathway.

Each deletion produced a specific change in conidial pigmentation congruent with all the previous work thus far. These transformations have shown that each gene plays a necessary role in the production of fungal melanin. Only when all six genes are working correctly does the wild type conidial pigmentation form. Given the evidence provided by this study, it can be concluded that proposed hypothesis be accepted. It is concluded that *P. roqueforti* uses a DHN-Melanin pigment pathway containing the six genes *alb1, ayg1, arp1, arp2, abr1,* and *abr2* to produce fungal melanin.

7.3 Outliers and Errors

While enough evidence has been gathered to support the hypothesis, there are still some confounding outliers of these experiments that are worth discussion. While the enzyme inhibitors tricyclazole and pyroquilon successfully targeted pigment formation, phthalide was unsuccessful in doing so. This could be due to a different mechanism phthalide uses to deactivate enzymes that it was unable to perform on *P. roqueforti* unlike other fungi tested where it did inhibit *arp2* gene-product activity (Wheeler and Klich, 1995). The same goes for fenoxanil, which in previous studies was shown to inhibit the *arp1* gene product (Pihet *et al.* 2009). However, the only affect seen on *P. roqueforti* was a reduced radial growth in colony size.

When the gene cluster for *P. roqueforti* was assembled after BLAST searching the genome, it was spread more widely in the genome than the tightly clustered one found in *A. fumigatus* and some *Pencillium* species (Woo et al. 2010). The genome sequence was checked for gaps and while none were found between the smaller gene cluster it is possible the Super contigs were not assembled correctly. The entire *P. roqueforti* genome as a whole does contain some incomplete sequencing. This could all contribute to a partially incorrect assembly where the genes are not exactly as far apart as it would seem. Given there is only one published genome for this species, it is hard to compare or have multiple sources to prove whether this is the correct assembly.

The original *alb1* UV mutant was actually produced from a different strain than 74-88. While B20 was still sequenced for the B20-1 *alb1* gene comparison, the entire pathway was not sequenced and checked for further mutations.

During the gene expression testing, it was not possible to reduce the non-sporulating conditions to no gene expression at all. This could be from a small carry over of undetected sporulating RNA during the culturing process.

Despite the fact that the transformations were integrated successfully, time constraints meant that it was not possible to test to see how many times an integration event may have occurred. The target gene may have been specifically delayed, but there is the possibility that another recombination event could have taken place somewhere else in the genome. This would affect the conclusion that only the target gene was responsible for the observed results. It could be that this multiple integration at different sites is necessary in combination to produce the observed results. Given that multiple replicate transformants were seen for 5 of the 6 genes, all with the same phenotype, it is however unlikely that the same multiple integration occurred in all the colonies. Although it is worth noting that only one positive colony was produced during the *abr1* transformation.

The most interesting possibility is that while all the research thus far is correct, other genes involved in pigment production may exist that have yet to be discovered. This could involve either known genes that serve multiple purposes or unidentified genes in the genome. Indeed, it was highly significant that three of the UV colour mutants (78-88-4, 74-88-6, and 74-88-10) had no mutation in the DHN-melanin pathway genes and yet exhibited different colony colouration. If such discoveries are made in the future, a subset of the DHN-Melanin pathway could be proposed. It is possible they have yet to be identified through UV mutation or could even be highly resistant to externally induced alterations. It is noted that a non-canonical pigment biosynthesis pathway has recently been reported from *A. terreus* (Geib et al. 2016).

7.4 Effects on the Cheese Industry

The applications of this research in the production and marketing of novel varieties of cheese will have industrial relevance. Interest in the life cycle of *P. roqueforti* has led to the discovery and use of the sexual cycle for strain improvement (Swilaiman, 2013; Ropars et al. 2014). The investigation of new avenues of product design will be furthered by the discoveries made during this research. Now that the mechanism by which blue cheese fungal strains make their colour has been shown, further exploration into harnessing its potential can be explored. Businesses strive to exploit new niches in the marketplace in order to increase revenue and generate new products. Although genetically modified foods and application of methods such as CRISPR are still hotly debated and banned in many places, UV induced mutations is a much greyer area. If the cheese producers can create new flavours through sexual crossing while altering the pigmentation through either UV induced or genetic modification, they can create a novel product that will stand out from the rest. Such a potential edge over the competition has created a vested interest in this research. Now that the pigment pathway has been identified, producers can know with greater certainty the effect they are making on pigmentation or how to influence colouration with predictable accuracy.

7.5 Future Prospects

The work produced in this study has revealed the principal conidial pigment pathway *P. roqueforti* uses to produce fungal melanin. While this is an accomplishment, it has left many questions unanswered and allows for further research to be done. The primary follow up to this study should be the retransformation of the gene knockouts back to their wild type expression. This would be through complementation cassettes containing the original gene deleted with a separate marker other than hygromycin such as phleomycin. This verification would establish conclusively each gene function as it has been proposed in this research. Follow up studies using the enzyme inhibitors on each transformant would add additional information on the effect they have on “cleaner mutants” versus the UV mutant strains which might contain unknown extra confounding gene deletions.

One of the most interesting question left unanswered in this study concerns the levels of expression each gene has under varying conditions of growth. A proper course of action would be to run a set of Real-Time PCRs to accurately determine the levels of expression. Further work could also be done to see if the genes are constitutively expressed at low levels at all times. If so, the implications this could have for other gene functions and how they operate could be of interest.

Finally, a unique incident occurred with the 74-88-5 and 74-88-12 strains which contained mutations in the *ayg1* gene but which resulted in different pigmentations. The possibility of not just removing a gene but altering its structure while remaining active at some level could open up a whole realm of possibilities in influencing colouration. Transforming this altered gene into a wild type to see if the point mutation alone is responsible for this unique occurrence of colour could test this. That information could provide new academic knowledge on the potential of influencing pigment gene structure to create never before seen phenotypes.

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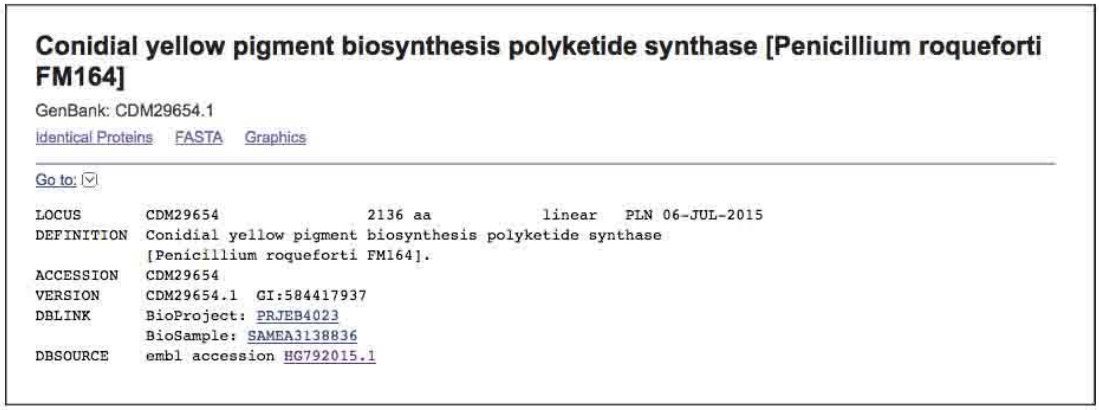
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9. APPENDICES

9.1 Chapter 3 Supplementary Data



Conidial yellow pigment biosynthesis polyketide synthase [Penicillium roqueforti FM164]

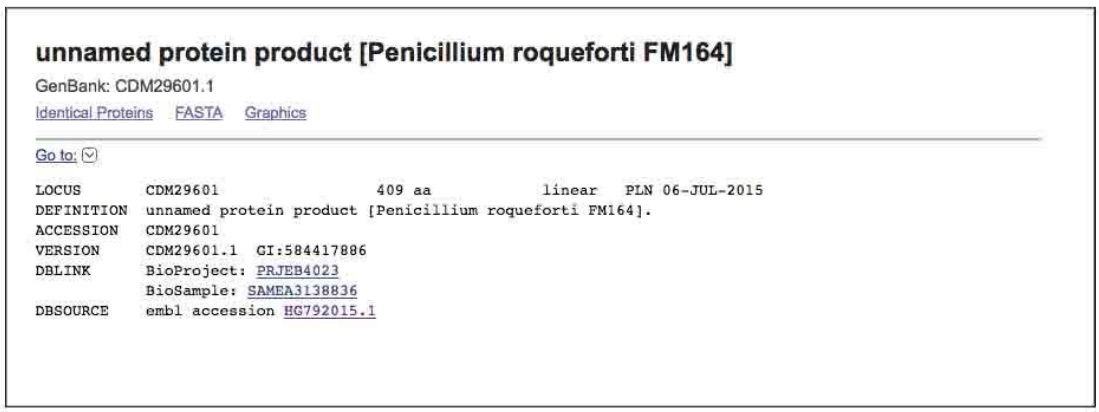
GenBank: CDM29654.1

Identical Proteins FASTA Graphics

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            [Penicillium roqueforti FM164].
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DBLINK      BioProject: PRJEB4023
            BioSample: SAMEA3138836
DBSOURCE    embl accession HG792015.1
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Figure A9.1.1 *A. fumigatus alb1* PKS gene identified in *P. roqueforti* through BLAST search analysis.



unnamed protein product [Penicillium roqueforti FM164]

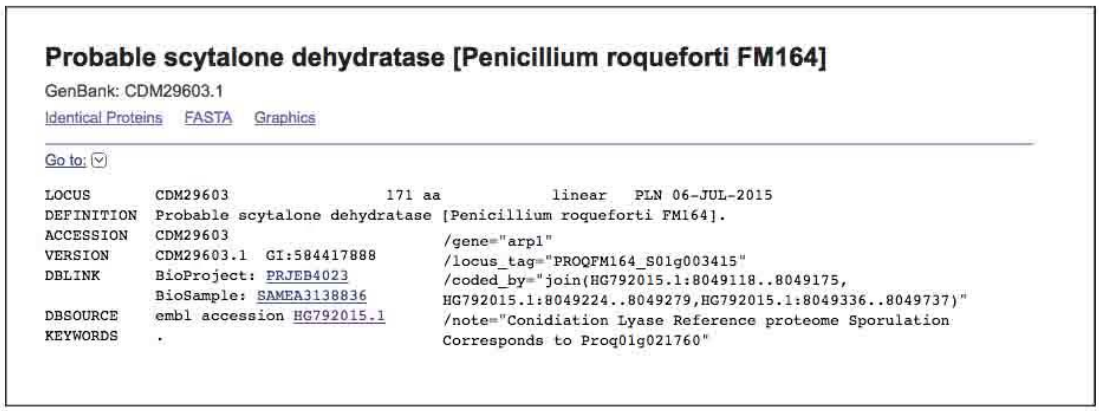
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VERSION     CDM29601.1  GI:584417886
DBLINK      BioProject: PRJEB4023
            BioSample: SAMEA3138836
DBSOURCE    embl accession HG792015.1
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Figure A9.1.2 *A. fumigatus ayg1* gene identified in *P. roqueforti* through BLAST search analysis.



Probable scytalone dehydratase [Penicillium roqueforti FM164]

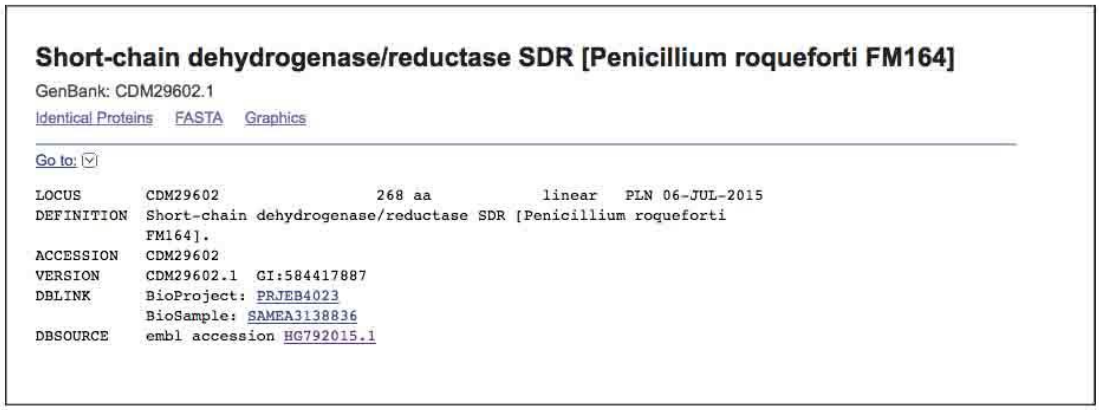
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            BioSample: SAMEA3138836         HG792015.1:8049224..8049279,HG792015.1:8049336..8049737)"
DBSOURCE    embl accession HG792015.1       /note="Conidiation Lyase Reference proteome Sporulation
KEYWORDS    .                               Corresponds to Proq01g021760"
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Figure A9.1.3 *A. fumigatus arp1* scytalone dehydratase gene identified in *P. roqueforti* through BLAST search analysis.



Short-chain dehydrogenase/reductase SDR [Penicillium roqueforti FM164]

GenBank: CDM29602.1

Identical Proteins FASTA Graphics

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VERSION     CDM29602.1  GI:584417887
DBLINK      BioProject: PRJEB4023
            BioSample: SAMEA3138836
DBSOURCE    embl accession HG792015.1
```

Figure A9.1.4 *A. fumigatus arp2* HN-reductase gene identified in *P. roqueforti* through BLAST search analysis.



Cupredoxin [Penicillium roqueforti FM164]

GenBank: CDM29606.1

Identical Proteins FASTA Graphics

Go to:

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VERSION     CDM29606.1  GI:584417891
DBLINK      BioProject: PRJEB4023
            BioSample: SAMEA3138836
DBSOURCE    embl accession HG792015.1
```

Figure A9.1.5 *A. fumigatus abr1* Cupredoxin gene identified in *P. roqueforti* through BLAST search analysis.



Laccase-1 [Penicillium roqueforti FM164]

GenBank: CDM30362.1

Identical Proteins FASTA Graphics

Go to:

```
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DBLINK      BioProject: PRJEB4023
            BioSample: SAMEA3138836
DBSOURCE    embl accession HG792016.1
  AUTHORS   Cheeseman,K., Ropars,J., Renault,P., Dupont,J., Gouzy,J.,
            Branca,A., Abraham,A.L., Ceppi,M., Conseiller,E., Debuchy,R.,
            Malagnac,F., Goarin,A., Silar,P., Lacoste,S., Sallet,E.,
            Bensimon,A., Giraud,T. and Brygoo,Y.
  TITLE     Multiple recent horizontal transfers of a large genomic region in
            cheese making fungi
  JOURNAL   Nat Commun 5, 2876 (2014)
   PUBMED   24407037
REFERENCE   2  (residues 1 to 2136)
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Figure A9.1.6 *A. fumigatus abr2* Laccase gene identified in *P. roqueforti* through BLAST search analysis.

9.2 Chapter 4 Supplementary Data

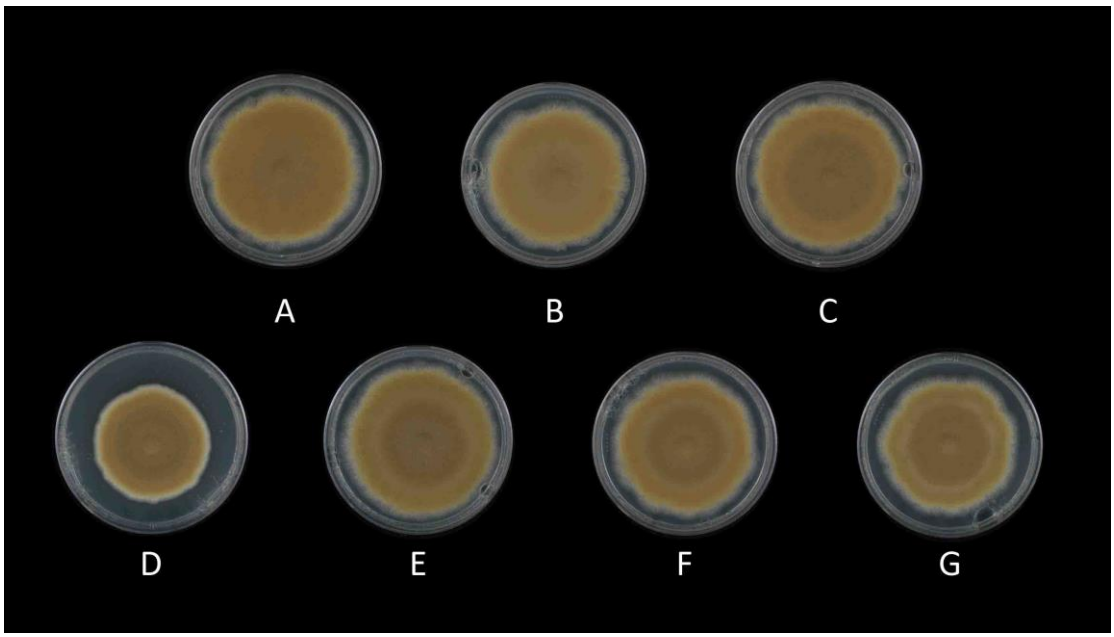


Figure A9.2.1 Enzyme inhibitions of 74-88-1. (A) Control, (B) DMSO, (C) Ethanol Control, (D) Fenoxanil, (E) Phthalide, (F) Pyroquilon, (G) Tricyclazole.

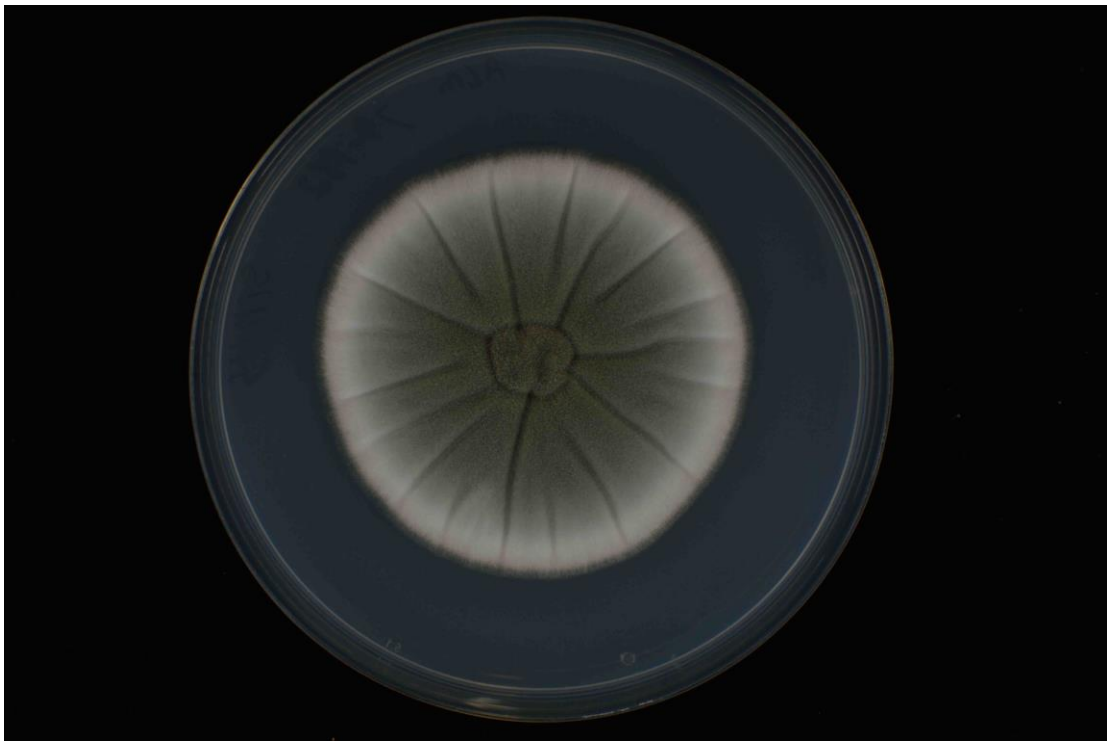


Figure A9.2.2 74-182 Isolated from a soft Beauvale blue cheese.

9.3 Chapter 5 Supplementary Data

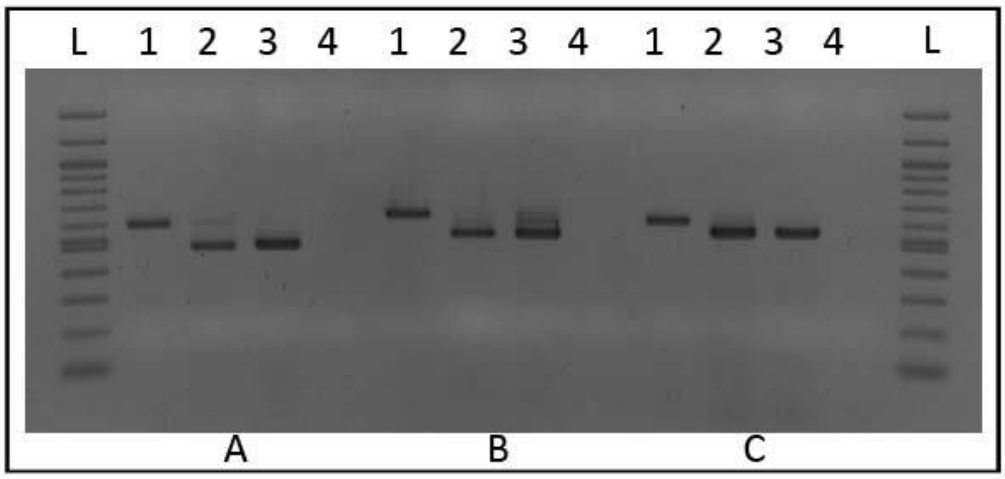


Figure A9.3.1 RNA Expression Gels: 30 Cycles (A) Ayg1 (B) Arp1 (C) Arp2; L – 100 bp ladder, 1 – genomic DNA, 2 – cDNA from solid media RNA extraction, 3 – cDNA from liquid media RNA extraction, 4 – water control.

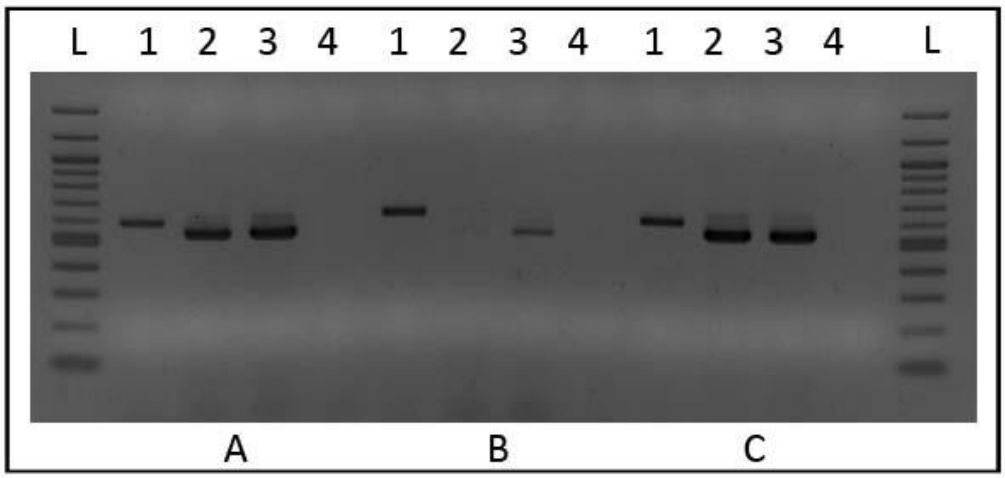


Figure A9.3.2 RNA Expression Gels: 30 Cycles (A) Abr1 (B) Abr2 (C) Alb1; L – 100 bp ladder, 1 – genomic DNA, 2 – cDNA from solid media RNA extraction, 3 – cDNA from liquid media RNA extraction, 4 – water control.

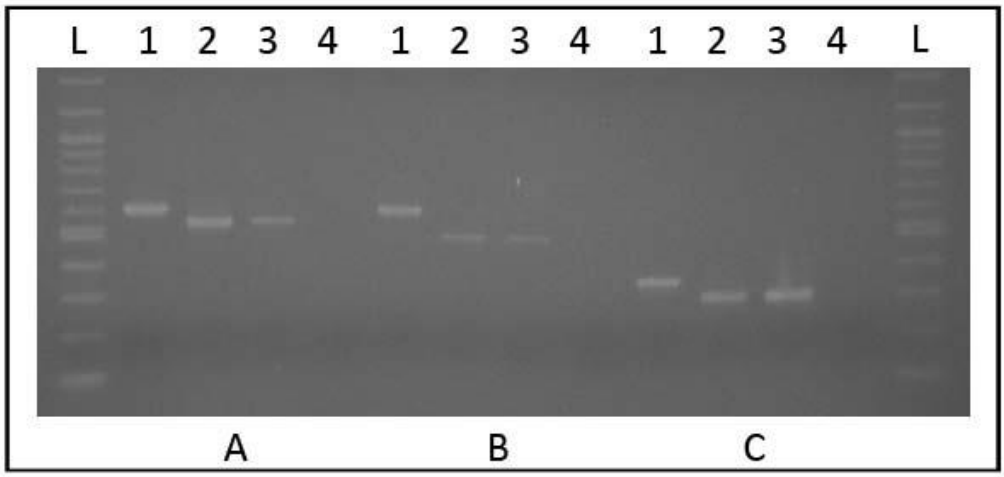


Figure A9.3.3 RNA Expression Gels: 25 Cycles (A) Alb1 (B) Ayg1 (C) Tubulin HKG; L – 100 bp ladder, 1 – genomic DNA, 2 – cDNA from solid media RNA extraction, 3 – cDNA from liquid media RNA extraction, 4 – water control.

9.4 Chapter 6 Supplementary Data

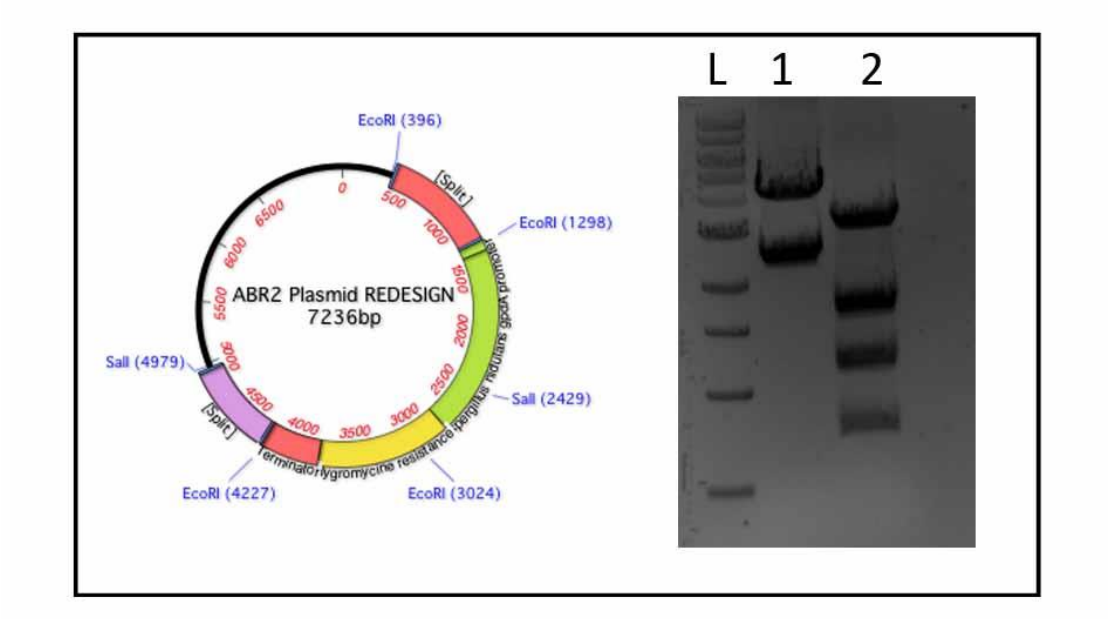


Figure A9.4.1 Abr2 vector diagram with the SalI and EcoRI restriction enzyme sites labeled; Gel electrophoresis of digested plasmids; L – 1 kb ladder, 1 – SalI digest, 2 – EcoRI digest.

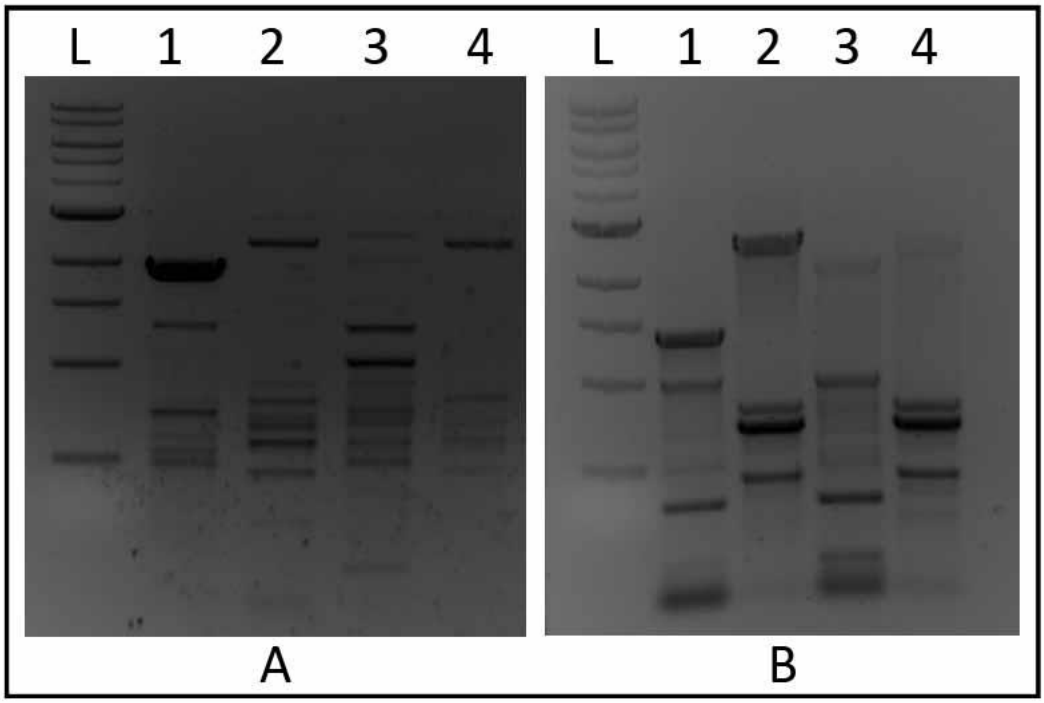


Figure A9.4.2 Positional PCR (A) Arp2 (B) Abr2; L – 1 kb ladder, 1 – transformation upstream, 2 – transformation downstream, 3 – 74-88 upstream, 4 – 74-88 downstream.

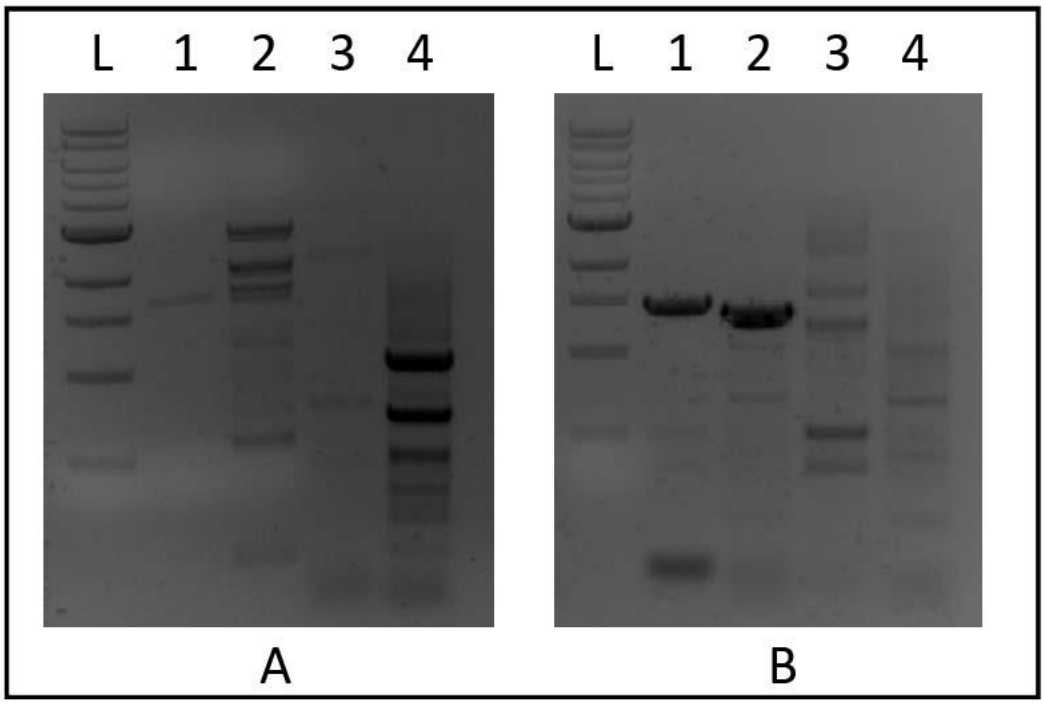


Figure A9.4.3 Positional PCR (A) Alb1 (B) Abr1; L – 1 kb ladder, 1 – transformation upstream, 2 – transformation downstream, 3 – 74-88 upstream, 4 – 74-88 downstream.

9.5 Primers Master List

Sequencing Primers				
Primer Set	**Oligo Name**	**Sequence 5' to 3' (include modification codes if applicable)**	**Scale (µmole)**	**Purification**
1	MCayg1aF	CCTTACCCTCGTCATCTTGG	0.025	DST
	MCayg1aR	TTGATGATCTCGTGGGAGCG	0.025	DST
2	MCayg1bF	AAGCAGGTGTACCTGAAAGC	0.025	DST
	MCayg1bR	GGCTGCGACTCTTTAAAGCT	0.025	DST
3	arp2F	TCAACACGATTCCACACCCA	0.025	DST
	arp2R	TCAGATGGTAATTGGCCCTGA	0.025	DST
4	MCarp1F	GAAATATGCTGGCCAGATCC	0.025	DST
	MCarp1R	GTTGTACCAGTTTCCCTTCG	0.025	DST
5	MCarb1aF	AGCATGGCAGTGGAGTTATG	0.025	DST
	MCarb1aR	CTGCAGAACCTGGAATTGGT	0.025	DST
6	MCarb1bF	ATCGCTCTACACTGCCAACA	0.025	DST
	MCarb1bR	AGACTTGCGGGTATTATCGG	0.025	DST
7	MCalb1aF	CCTTAAGTTTTCGCCTGACC	0.025	DST
	MCalb1aR	CTTGCCTGTCTCACTTGACA	0.025	DST
8	alb1bF	GTTCCAATCCACGGCCCTTA	0.025	DST
	alb1bR	AACCGACTGCATCTCCACTG	0.025	DST
8/9 Gap	MCalb1b/cF	TAGTGTGGATACCGCTTGCT	0.025	DST
	MCalb1b/cR	CACATTCTCGTCATTGAGCG	0.025	DST
9	alb1cF	TCCAACAGCGACCCAAAAGA	0.025	DST
	alb1cR	GCTGGGCGCATTGATACAAG	0.025	DST
10	alb1dF	ACACAATCTACCTTGCCGGG	0.025	DST
	alb1dR	GATGAGGTAAGAGCTGCGCT	0.025	DST
10/11 Gap	MCalb1d/eF	GCTGCGCCAGTTTCTGTTTT	0.025	DST
	MCalb1d/eR	ACGCTCGGATCGAACTTCTT	0.025	DST
11	MCalb1eF	GAAGAAGATCCTTGACCACG	0.025	DST
	MCalb1eR	TGCTGTCATTTTGCTCCTGC	0.025	DST
11/12 Gap	MCalb1e/fF	TTCCAAGCTCTGGCACGAAA	0.025	DST
	MCalb1e/fR	ACGCCGAATCTCAGCAATGT	0.025	DST
12	alb1fF	TCGAGTCCAAACAGCAGTGG	0.025	DST
	alb1fR	ATACGTTTACGCCGAAGCCA	0.025	DST
13	arb2aF	AGGAAGGCGGTAAGAAAGGC	0.025	DST
	arb2aR	GGGCAAATTGTTGGTCACGT	0.025	DST
14	MCarb2bF	GTCGAGAGGGAGATGATCTT	0.025	DST
	MCarb2bR	GCAGGTAGGATAGCAGGATA	0.025	DST
15	arb2cF	GCGCTACTCTGCCATGATCA	0.025	DST
	arb2cR	CGATGATGATGCCAGTCCCA	0.025	DST
Mix	Alb1mixF	AAGGTGCTCAGTACACAGGC	0.025	DST
	Alb1mixR	CGCCCTTTCCAATTTCAGCC	0.025	DST
RNA Expression Primers				
Primer Set	**Oligo Name**	**Sequence 5' to 3' (include modification codes if applicable)**	**Scale (µmole)**	**Purification**
Ayg1	Ayg1intronF	AGCACACTCTCCAACCACTT	0.025	DST
	Ayg1intronR	TGGATTACATGGATCGGCGG	0.025	DST
Arp2	Arp2intronF	TGGTAATTGGCCCTGATCCT	0.025	DST
	Arp2intronR	TGTTCGACCTCAACACTCGG	0.025	DST
Arp1	Arp1intronF	TTGCCGAACAACCATGTCTC	0.025	DST
	Arp1intronR	AAGGCCCTTGAAAACCTCGT	0.025	DST
Abr1	Abr1intronF	CCGACCAAATGGCTGGCATT	0.025	DST
	Abr1intronR	GCAGAGCCATTACGACACCT	0.025	DST
Alb1	Alb1intronF	TCAGCGAGAGAAGGGAGTCA	0.025	DST
	Alb1intronR	CTCCTCCGGGCAACTTTCAT	0.025	DST
Abr2	Abr2intronF	GAATGCGAGGATTGTGTGGC	0.025	DST
	Abr2intronR	CGTGGGCATGGTACCAGTAA	0.025	DST

Transformation Primers				
Arp2				
Primer Set	**Oligo Name**	**Sequence 5' to 3' (include modification codes if applicable)**	**Scale (µmole)**	**Purification**
Upstream	Arp2-up/FWD	GTGAATTCGAGCTCGGTACCGCATGAGAGTGACCACGAAG	0.025	DST
	Arp2-up/RV	TCCATCACACTGGCGGCCGCGGATTGTAGATGACTGGGTG	0.025	DST
Downstream	Arp2-dwn/FWD	TCCATCACACTGGCGGCCGCGGCCATGTATAGGATCAGGG	0.025	DST
	Arp2-dwn/RV	CTAGAGGATCCCCGGGTACCCCTGTTGTTGTTAGTCGTGG	0.025	DST
Abr2				
Primer Set	**Oligo Name**	**Sequence 5' to 3' (include modification codes if applicable)**	**Scale (µmole)**	**Purification**
Upstream	Abr2-up/FWD	GTGAATTCGAGCTCGGTACCCGTTACCCTAAATTAGGCGG	0.025	DST
	Abr2-up/RV	TCCATCACACTGGCGGCCGCCGTGTCCAGAAAATGCCTCC	0.025	DST
Downstream	Abr2-dwn/FWD	TCCATCACACTGGCGGCCGCGGCTCTCGACCTACATATCG	0.025	DST
	Abr2-dwn/RV	TCTAGAGGATCCCCGGGTACCCCTGATCAACGGGTATGCG	0.025	DST
Alb1				
Primer Set	**Oligo Name**	**Sequence 5' to 3' (include modification codes if applicable)**	**Scale (µmole)**	**Purification**
Upstream	Upstream_Alb1 Fwd	acctgcaggcatgcaagcttCAGATGATGCCTGCAGATG	0.025	DST
	Upstream_Alb1 Rev	gcagagctAATCTTCCTGGTAGCAGATTAATG	0.025	DST
hph	hph_Alb1 Fwd	aggaagattAGCTCTGCAAAGGGCGTT	0.025	DST
	hph_Alb1 Rev	gcaatacgtCAGTGTGATGGAATTCGC	0.025	DST
Downstream	Downstream_Alb1 Fwd	tcacactgACGTATTGCATTCGGGGAC	0.025	DST
	Downstream_Alb1 Rev	gaccatgattacgccaagcttACCGAAAACCGCCATTTG	0.025	DST
Ayg1				
Primer Set	**Oligo Name**	**Sequence 5' to 3' (include modification codes if applicable)**	**Scale (µmole)**	**Purification**
Upstream	Upstream_Ayg1 Fwd	aattcgagctcggtaccACTCGCTGCTTTGCTGCT	0.025	DST
	Upstream_Ayg1 Rev	gcagagctTTTGAGCGGGATATAGACG	0.025	DST
hph	hph_Ayg1 Fwd	cgctcaaaAGCTCTGCAAAGGGCGTT	0.025	DST
	hph_Ayg1 Rev	gcacccacCAGTGTGATGGAATTCGC	0.025	DST
Downstream	Downstream_Ayg1 Fwd	tcacactgGTGGGTGCTGTGTCAATATAG	0.025	DST
	Downstream_Ayg1 Rev	aggatccccgggtaccGAATTCAGGAAGCTGAAGATG	0.025	DST
Abr1				
Primer Set	**Oligo Name**	**Sequence 5' to 3' (include modification codes if applicable)**	**Scale (µmole)**	**Purification**
Upstream	Upstream_Abr1 Fwd	aattcgagctcggtacCGACTATTCTTCTTCATCATG	0.025	DST
	Upstream_Abr1 Rev	gcagagctATGTTCATCTTTTCCACCG	0.025	DST
hph	hph_Abr1 Fwd	gatgaacatAGCTCTGCAAAGGGCGTT	0.025	DST
	hph_Abr1 Rev	tgctggcaCAGTGTGATGGAATTCGC	0.025	DST
Downstream	Downstream_Abr1 Fwd	tcacactgTGCCAGCACCTGTGATATG	0.025	DST
	Downstream_Abr1 Rev	aggatccccgggtaccATCCCCACTCAGAGTCGTATC	0.025	DST
Arp1				
Primer Set	**Oligo Name**	**Sequence 5' to 3' (include modification codes if applicable)**	**Scale (µmole)**	**Purification**
Upstream	Upstream_Arp1 Fwd	aattcgagctcggtacCTCTTGCTGCCCGCATAC	0.025	DST
	Upstream_Arp1 Rev	gcagagctGGTTGTTCGGCAATCTTTG	0.025	DST
hph	hph_Arp1 Fwd	gaacaaccAGCTCTGCAAAGGGCGTT	0.025	DST
	hph_Arp1 Rev	cattgacatCAGTGTGATGGAATTCGC	0.025	DST
Downstream	Downstream_Arp1 Fwd	tcacactgATGTCAATGGGCTTTTGC	0.025	DST
	Downstream_Arp1 Rev	aggatccccgggtaccTTAAATTATCTGAAAGAAGTGTCG	0.025	DST

Universal Hygromycin Primers				
Primer Set	**Oligo Name**	**Sequence 5' to 3' (include modification codes if applicable)**	**Scale (µmole)**	**Purification**
hph	Forward	AGCTCTGCAAAGGGCGTT	0.025	DST
	Reverse	CAGTGTGATGGAATTCGCCCTT	0.025	DST
Hygromycin Colony PCR Primers				
Primer Set	**Oligo Name**	**Sequence 5' to 3' (include modification codes if applicable)**	**Scale (µmole)**	**Purification**
hph	hph_for	CGATGTAGGAGGGCGTGGATA	0.025	DST
	hph_rev	GCTTCTGCGGGCGATTTGTGT	0.025	DST
Positional PCR Primers				
Primer Set	**Oligo Name**	**Sequence 5' to 3' (include modification codes if applicable)**	**Scale (µmole)**	**Purification**
Abr1	Abr1-FF	TTCAGTCCCATCATCAGGAGCC	0.025	DST
	Abr1-FR	ATCTCCAGTCAATCTCCCGC	0.025	DST
Abr2	Abr2-Ff	ACCTCTACCATTGACCGCTC	0.025	DST
	Abr2-FR	ACTCGGTGCCTGGTTCATTG	0.025	DST
Alb1	Alb1-FF	CGGCAGATCTTATTCCGTCG	0.025	DST
	Alb1-FR	CCATGTAACTCGCCAAGAGC	0.025	DST
Ayg1	Ayg1-FF	CCATCAACAGCATTTATCGTCC	0.025	DST
	Ayg1-FR	TTGTCCGAGACTACACTGGAGG	0.025	DST
Arp1	Arp1-FF	TTGGCTCCACCGTTACAAGG	0.025	DST
	Arp1-FR	TGTCCCGTTTTGCGTCCAAG	0.025	DST
Arp2	Arp2-FF	AGCCGCCTCACTATGGAAAC	0.025	DST
	Arp2-FR	GGCAGAGAAGAAGGTGTATCTATC	0.025	DST
Hph1	Hph1-FF	ACAATGCCTGAACTCACCGC	0.025	DST
	Hph1-FR	TCTACAAATGGTGGAGGCGG	0.025	DST
Pos/hph	Hph2-FF	GCATTAATGCATTGGACCTCG	0.025	DST
	Hph2-FR	GCTGAAGTCGATTTGAGTCC	0.025	DST