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Fungal Sex for Disease Control and Strain Improvement

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I. Abstract

The overarching theme of this thesis was to explore if and how sexual reproduction in fungi could be exploited for disease control and strain improvement. Work focussed on two distantly related members of the *Pezizomycotina*; *Pyrenopeziza brassicae*, an important pathogen of *Brassica napus* (oilseed rape) and other *Brassica* species; and *Penicillium roqueforti*, the famous species used for commercial production of blue cheeses.

First, investigations were undertaken to characterise a putative lipid hormonal compound, "Sex Factor" (SF), involved in controlling the switch between asexual and sexual reproduction in *Pyrenopeziza brassicae*, the causative agent of light leaf spot in *Brassica* plant species. SF shows promise as a novel disease control agent because of its reported ability to repress asexual sporulation, an important factor in the spread of disease. Crude SF was extracted from mated cultures of *P. brassicae* and it was confirmed via bioassays that extracts were able to repress asexual sporulation in this species. By contrast, asexual sporulation of North American "Lineage 2" isolates of *P. brassicae* was unaffected by SF. Biochemical purification work including the use of LC-MS then identified 16-(β-D-Glucopyranosyloxy)-2,15-dihydroxyhexadecanoic acid as a putative candidate for being an active component of crude SF. Finally, progress was made towards identifying key genes which may be involved with regulation of asexual and sexual development. Overall, this work has provided new insights into the asexual and sexual biology of *P. brassicae*, and laid the groundwork for developing new methods of disease control against this important fungal pathogen.

Secondly, investigations were made to characterise sexual progeny of *Penicillium roqueforti*. A sexual cycle has only recently been discovered in this species, and therefore it was of academic and of commercial interest to determine whether sex can lead to the production of progeny with novel

characteristics relevant to blue cheese production. Four pairs of parental isolates and respective progeny were screened for proteolytic activity and lipolytic activity, and production of certain secondary metabolites. It was found that up to 91 % of progeny had altered proteolytic activities and up to 55 % had altered lipolytic activities, compared to parental isolates. No progeny were found to have novel secondary metabolite production capacity, although significant variation was detected, suggesting novelty may be found with further screening. Thus, it was demonstrated that strains of *P. roqueforti* with alterations in desirable characteristics can be generated via the sexual cycle. These results may encourage the use of sexual reproduction for strain improvement of this species and other species used in industrial settings.

Third, work was undertaken to characterise lipase genes involved in blue cheese maturation given that the genetic basis of the lipolytic system of *P. roqueforti*, the most important factor in determining the flavour of blue cheese, was largely unknown. Nine putative lipase genes were identified *in silico*, five of which were found to be more than two-fold upregulated in model blue cheese conditions. Concurrently, a "laboratory strain" of *P. roqueforti* (Δ*ku70*::*pyrG*) was developed which allowed for more efficient genetic manipulation than would be possible using a wild type strain. This laboratory strain was then used to generate lipase knockout strains which led to the identification of two genes, *lipD* and *lipI*, which encode for lipases that likely constitute the main extracellular lipolytic activity of *P. roqueforti*. Finally, the expression of these key genes was determined in parental isolates and sexual progeny of *P. roqueforti.* Novel expression profiles were found for *lipD*, indicating that sexual reproduction can lead to variation in lipolytic activity by alteration of gene expression. The development of a laboratory strain more amenable to genetic manipulation will be of interest to researchers working on this species who can now conduct genetic characterisation studies more easily. In addition, identification of *lipD* and *lipI* will allow more specific screening for strains which show desired expression levels of or mutations

within these genes, and could therefore be of interest to blue cheese producers.

II. Acknowledgements

I would firstly like to thank my supervisor, Professor Paul S. Dyer, who has guided me through the world of fungal sex since my undergraduate research project in 2015, and whose enthusiasm for his field will stay with me long into the future. I would also like to thank Dr. Matthias Brock, who provided advice for the genetic aspects of this thesis; Dr. David Withall, who provided much-needed biochemical expertise; and Dr. Alison Ashby, who was kind enough to share her knowledge of *Pyrenopeziza brassicae* sex. A special mention must go to the technical support staff of the group, without whom the lab couldn't function (particularly evident during a paternity leave and the banning of technical support within the lab because of the COVID pandemic!). I would also like to acknowledge BBSRC and the University of Nottingham for financial support.

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My biggest thanks are to my family, whose love and support has been unwavering and without whom none of this would've been possible.

"***Young hearts run free***"

- Candi Staton

Table of Contents

Chapter 1 – Introduction to Sexual Reproduction in Ascomycete Fungi

1.1 Introduction

Sexual reproduction via outbreeding is a process whereby new living organisms are produced due to recombination of genetic material from two separate parents. This process is conserved throughout the eukaryotic kingdom despite the evolutionary costs it entails; normally only one parent from the pair acts as the maternal partner to produce offspring and there are energetic costs required to find a mating partner (Maynard Smith, 1978). However, there are considerable evolutionary advantages to sex, such as facilitating adaptation to new environments or the removal of deleterious mutations which may have developed through asexual reproduction (Bernstein et al., 2018; Gray and Goddard, 2012; Reyes et al., 2012). In addition, specifically for fungi, sexual reproduction can coincide with the production of more hardy spores or dormant survival structures which may help the fungus survive in adverse environmental conditions (Billiard et al., 2012). Therefore, the persistent of sex is thought to be owed to the fact that the advantages likely outweigh the disadvantages in many environments.

Fungi, in particular ascomycetes, have long been used as model organisms to study the process of sexual reproduction. One reason for this is that most fungi are predominantly haploid, have ubiquitous genetic structures and contain few repetitive sequences, making experimental genetic work relatively easy (Dyer et al., 2017). Despite this genetic simplicity however, fungi maintain most of the features of sex found in other eukaryotic lineages (Lee et al., 2010). This means that processes discovered in fungi are directly related to those which take place in “higher” eukaryotes, especially because fungi are the most closely related clade to the metazoans (Heitman et al., 2013). In addition, sexual reproduction within the fungal kingdom is incredibly varied, with several different sexual lifestyles and developmentally complex sexual

structures having evolved, making them interesting and attractive organisms in which to study this process. Finally, the fungal kingdom contains species which are tremendously important for human life, including species involved in pathogenicity, industrial chemical production and food production. Thus, studying sexual reproduction in this kingdom is vital in working towards a complete understanding of their biology.

A key aim of the present thesis is to exploit knowledge about fungal sexual reproduction for beneficial purposes. Therefore an overview of fungal sexual reproduction will now be provided, focussing particularly on the Ascomycotina, which include the study organisms of the present thesis.

1.1.1 Sexual reproduction in the Ascomycotina

The Ascomycotina is a fungal clade which consists of over 64,000 species which makes it the largest phylum of fungi (Bennett and Turgeon, 2016). This clade can be further subdivided into three major subphyla, namely *Taphrinomycotina*, *Saccharomycotina* and *Pezizomycotina* (filamentous ascomycetes), of which the latter is the primary subject of this thesis and the largest of the three subphyla, consisting of over 32,000 species (Kirk et al., 2008).

Sexual reproduction in ascomycetes consists of several carefully coordinated steps which result in the formation of eponymous asci, sac-like structures which contain four (in the case of yeast) or eight (in the case of most filamentous fungi) sexual spores known as ascospores (Figure 1.1). Sex can occur between two separate individuals of opposing mating (*MAT*) type in the case of heterothallic species or occur in a single individual in the case of a homothallic species. *MAT* types and the genetic features which determine whether a species is heterothallic or homothallic are discussed in Section 1.1.2.

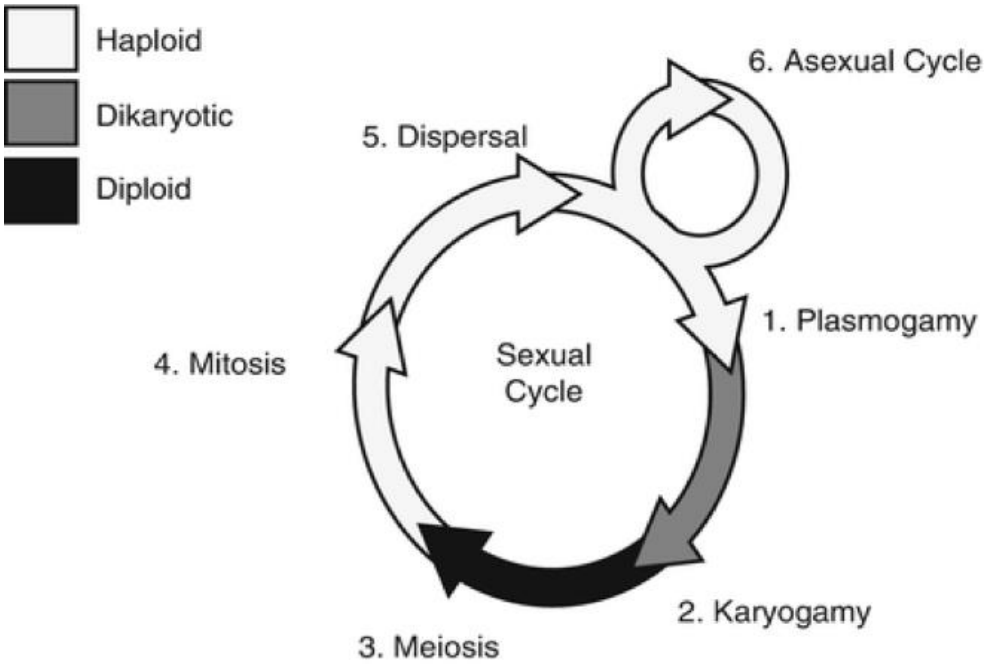

***Figure 1.1**. Generalised ascomycete lifecycle. Sexual reproduction begins with the fusion of two nuclei-containing haploid cells in a process known as plasmogamy (1). Nuclei then fuse in a process known as karyogamy to form diploid zygotes (2) which rapidly undergo meiosis to produce four genetically variable haploid nuclei (3). In filamentous ascomycetes (*Pezizomycotina*) but not yeasts (*Saccharomycotina*), there is a post-meoitic mitotic event which produces eight haploid ascospores (4). These are dispersed into the environment (5) before germinating and entering the asexual cycle if conditions are favourable (6). Taken from Ashton and Dyer (2016).*

The sexual cycle begins with plasmogamy, a process which involves fusion between two cells containing haploid nuclei. In the case of *Saccharomycotina* yeasts such as *S. cerevisiae*, fusion is between two individual cells of opposing mating type (*MAT* type), *MAT*α and *MAT****a***. Plasmogamy is more complex in *Pezizomycotina* such as *Neurospora crassa*, which have specialised multicellular structures involved in this process (Figure 1.2) (Metzenberg and Glass, 1990). Here, fusion is between a specialised sexual hypha known as a trichogyne and either a vegetative hypha or a conidium. Trichogynes extend from a multicellular reproductive structure known as the protoperithecium, which forms under certain conditions such as nitrogen starvation and light. The trichogyne, as a nuclear recipient, is distinguished as the “female” partner and the condium or vegetative hypha, as a nuclear donor, is distinguished as the “male” partner. Male and female partners are not apparent in yeast sexual systems. Plasmogamy in both yeasts and filamentous ascomycetes is initiated by pheromone signalling, whereby a mating cell responds to peptides

produced by the opposite mating partner by growing towards it (Jones and Bennett, 2011). Pheromone signalling is discussed in more detail in Section 2.1.

After successful plasmogamy, two nuclei, which are haploid (N) in most ascomycetes but diploid (2N) in some yeasts, from the two individual mating cells fuse, in a process known as karyogamy. This is a three-staged process which involves sequential fusion of the outer nuclear envelope, inner nuclear envelope and the spindle pole bodies (Melloy et al., 2007). The result of nuclear fusion is the formation of a diploid (or tetraploid) zygote, which for most *Pezizomycotina* is the only stage of their lifecycle in which they contain diploid cells. In *Pezizomycotina*, karyogamy is preceded by the two nuclei undergoing a round of mitosis to produce four nuclei in total. Two of these nuclei, each functionally of opposing *MAT* type (discussed in Section 1.1.2), are compartmentalised into the most apical cell of a specialised hook-like structure called a crozier, before karyogamy occurs (Figure 1.2) (Raju, 1992).

Zygotes exist only shortly, before a meiotic division occurs leading to separation into four genetically variable haploid nuclei. In filamentous ascomycetes, these nuclei usually undergo a subsequent mitotic event to produce eight haploid ascospores. Under favourable conditions, ascospores can germinate and enter the normal vegetative (asexual) lifestyle.

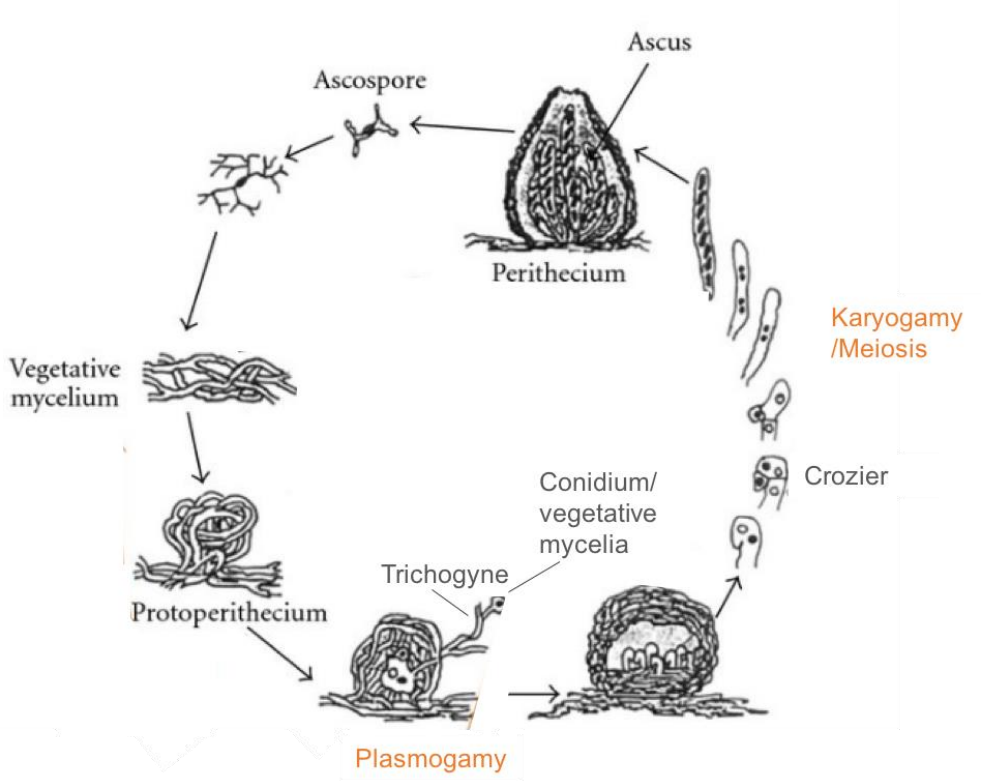

***Figure 1.2**. Sexual cycle of* Neurospora crassa*, a Pezizomycotina.* Pezizomycotina *produce specialised sexual structures including protoperithecia, trichogynes, croziers and a perithecium (fruiting body). Edited from Belozerskaya et al. (2012).*

The sexual cycle in filamentous ascomycetes takes place largely in specialised sexual structures known as fruiting bodies. Fruiting bodies are some of the most developmentally complex structures found in filamentous fungi (Pöggeler et al., 2018). Several forms of fruiting bodies exist, including cleistotheica (enclosed, globular structure); perithecia (open, flask-like structure), apothecia (open, disc like structure); and pseudothecia (similar structure to perithecia but with differing asci organisation) (Figure 1.3) (Pöggeler et al., 2018). Ascospores can be either passively or forcibly ejected from the fruiting bodies (Trail and Seminara, 2014).

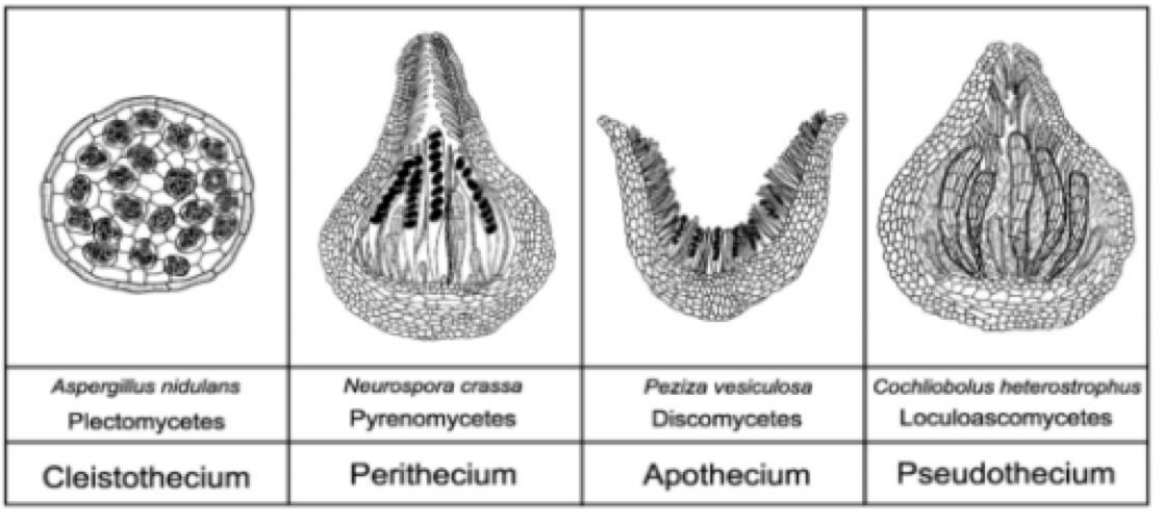

Figure 1.3. *Representation of sexual fruiting bodies produced by various* Pezizomycotina*. Taken from Pöggeler et al. (2018).*

1.1.2 The *MAT* locus and sexual lifestyle

Species within the ascomycetes exhibit a diverse array of different sexual lifestyles. Species may be heterothallic, in which two mating partners are required for sexual reproduction, or homothallic, in which sexual reproduction can occur in single individuals without the need for a mating partner. Homothallism itself is an umbrella term for a variety of sexual lifestyles including primary homothallism, secondary (pseudo-) homothallism and unisexual reproduction (Wilson et al., 2015b). The sexual lifestyle of a species is determined by the specific genetic structure of a region of the genome known as the *MAT* locus (Figure 1.4) (Dyer et al., 2016). This region contains genes that encode transcription factors which help coordinate the sexual cycle (Bennett and Turgeon, 2016).

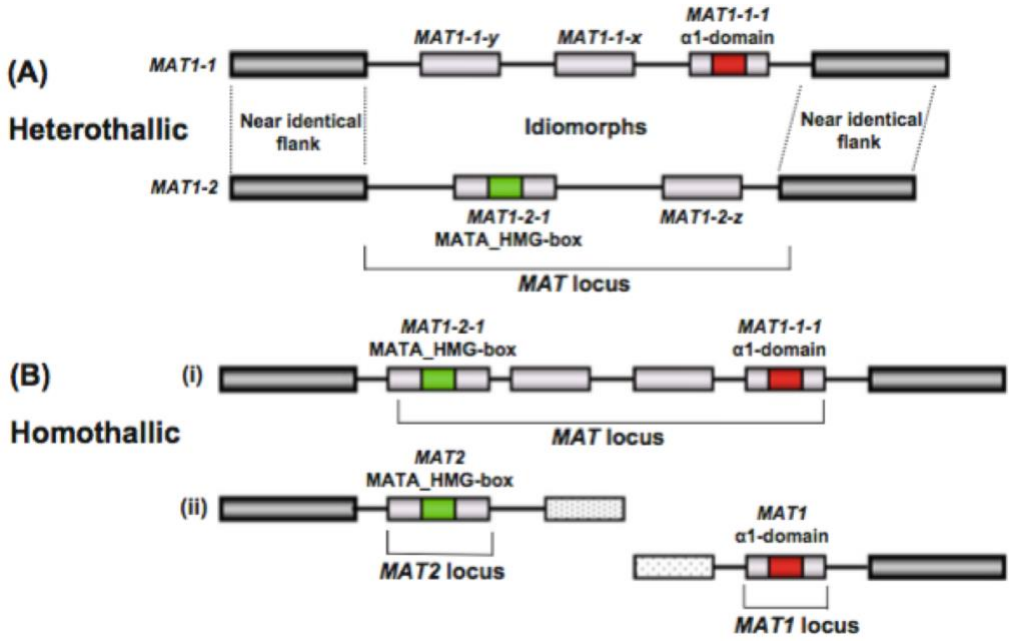

Figure 1.4. *Genomic structures of* MAT *loci in the* Pezizomycotina*. A) Example* MAT *loci structure of heterothallic species. The* MAT1-1 *locus contains at minimum the* MAT1-1-1 *gene but may also contain additional genes, denoted here as* MAT1-1-x *and* MAT1-2-y*. The* MAT1-2 *locus contains at minimum the* MAT1-2-1 *gene but may also contain additional genes, denoted here as* MAT1-2-z*. B) Example* MAT *loci structures of homothallic species. i) Some species harbour* MAT *loci where the two idiomorphs have fused together. ii) Some species harbour the two idiomorphs at disparate locations in the genome. Taken from Dyer et al. (2016).*

In heterothallic ascomycetes, two types of *MAT* loci exist, which are typically called *MAT*α and *MATa* in yeast species and *MAT1-1* and *MAT1-2* in *Pezizomycotina*. In *Neurospora spp.* the nomenclature differs and the two loci are referred to as *mat A* and *mat a* and in *Podospora spp.* they are referred to as *mat+* and *mat-* (Turgeon and Yoder, 2000). There is high sequence dissimilarity between the two types of locus, which has led to the term "idiomorph" rather than allele being used to describe them (Metzenberg and Glass, 1990). At minimum, the *MAT1-1* locus contains a core *MAT1-1-1* α-domain-containing gene encoding the MAT1-1-1 protein and the *MAT1-2* locus contains a core *MAT1-2-1* MATA_HMG-box gene encoding the MAT1-2-1 protein, but other genes may also be present (Table 1.1) (Dyer et al., 2016; Wilken et al., 2017). Individuals from heterothallic species harbour only one idiomorph in their genome, and are referred to as *MAT1-1* if they harbour the *MAT1-1* locus and *MAT1-2* if they harbour the *MAT1-2* locus. Both core *MAT*

genes are required for the full sexual cycle to occur, which explains the inability for individuals from heterothallic species to self-fertilise (Wilson et al., 2019).

By contrast, individuals of species which exhibit primary homothallism harbour both core *MAT* genes in their genome, which enables them to complete the sexual cycle without the need for a mating partner. Some homothallic species, such as *Aspergillus nidulans*, also retain the ability to outcross if certain conditions arise (Hoffmann et al., 2001). The architecture of the *MAT* locus in homothallic species is much more varied than in heterothallic species. Indeed, even homothallic species in the same genus often exhibit drastically different *MAT* locus structure, an example being *Cochliobolus spp.* (Yun et al., 1999). In *Cochliobolus homomorphus*, the two *MAT* idiomorphs are found fused together resulting in the core *MAT* genes existing in a single open reading frame, whereas in *Cochliobolus cymbopogonis* the idiomorphs are present at disparate locations (Yun et al., 1999). The latter is also true in *A. nidulans*, where the two idiomorphs are located on two separate chromosomes (Dyer et al., 2003; Paoletti et al., 2007). In addition, *MAT* loci from some homothallic species contain inverted or truncated versions of *MAT* genes (Bennett and Turgeon, 2016; Inderbitzin et al., 2005). The increased diversity of homothallic *MAT* loci structures compared to heterothallic *MAT* loci structures, paired with phylogenetic studies on *MAT* gene origins within genera containing both homothallic and heterothallic species, has led to the conclusion that homothallism likely evolved from heterothallism (Inderbitzin et al., 2005; Ojeda-López et al., 2018; Rydholm et al., 2007; Yun et al., 1999). Interestingly, although homothallic individuals harbour both core *MAT* genes, it has been theorised that individual nuclei may be functionally heterothallic owing to epigenetic silencing of one of these genes (Metzenberg and Glass, 1990; Scazzocchio, 2006).

Another type of homothallism is secondary homothallism, also known as pseudo-homothallism. Individuals of species which exhibit this sexual lifestyle, such as *Neurospora tetrasperma* and *Podospora anserina*, are heterokaryotic

in that they contain nuclei of opposing *MAT* type within the same cytoplasm (Raju and Perkins, 1994). These species are not truly homothallic, demonstrated by the observation that cultures which have resolved into monokaryons though cytoplasmic shearing or natural procedures are unable to complete the sexual cycle (Dyer et al., 2016). Pseudo-homothallic species produce asci containing only four ascospores as opposed to the usual eight, because each ascospore contains two nuclei (one of each *MAT* type) (Grognet and Silar, 2015). The maintenance of opposing *MAT* type heterokaryons in pseudo-homothallic species is thought to be owed to recombination suppression of the genomic region containing the *MAT* loci, caused by inversions in *N. tetrasperma* and as yet unidentified mechanisms in *P. anserina* (Ellison et al., 2011; Grognet and Silar, 2015).

Homothallism can also be achieved through *MAT* type switching, which is seen in certain yeast species. *MAT* identity in *S. cerevisiae* is determined as mentioned previously by the presence of either the *MAT*α or *MATa* idiomorph being present at the *MAT* locus, however individuals harbour two extra loci called *HML* and *HMR* that contain additional copies of the *MAT*α and *MATa* genes, respectively (Mahoney and Broach, 1989). Genes which reside within the *HML* and *HMR* loci are not usually expressed, as both loci are transcriptionally silenced by heterochromatin (Hickman et al., 2011; Ravindra et al., 1999; Weiss and Simpson, 1998). *MAT* type switching is achieved via homologous recombination between the *MAT* locus and either the *HML* or *HMR* locus, initiated by a double-strand break (DSB) caused by the HO endonuclease (Haber, 2012). The presence of a recombination enhancer, which is active in *MATa* cells and inactivated in *MAT*α cells, helps to ensure that *MATa* cells preferentially recombine with *HML*α and *MAT*α cells preferentially recombine with *HMRa*, because it induces chromosomal restructuring resulting in the DSB in *MATa* cells being brought in close proximity to *HML*α (Li et al., 2012).

Another form of homothallism is unisexuality, which is seen in the ascomycetous yeast *Candida albicans* and the filamentous ascomycetes *N. africana* and *Huntiella moniliformis*, among others (Alby et al., 2009; Glass and Smith, 1994; Wilson et al., 2015a). This form of homothallism is unusual in that individuals of unisexual species only harbour a single *MAT* idiomorph in their genome, making them typically heterothallic (Wilson et al., 2015b). Despite this, individuals of unisexual species are able to sexually reproduce without a mating partner. In *C. albicans*, unisexuality can be caused by altered pheromone signalling (Alby et al., 2009). Altered pheromone signalling is also thought to contribute to unisexuality in *H. moniliformis* (Wilson et al., 2018). This will be discussed in more detail in Section 1.1.5.

***Table 1.1**. Secondary* MAT *genes of filamentous ascomycetes*

Idiomorph	Gene	Example species	Function	Reference
MAT1-1	*MAT1-1-2*	*Fusarium graminearum*	Hyphal fusion, ascosporogenesis	Zheng et al., 2013
		Podospora anserina	Fruiting body development	Debuchy et al., 1993
	MAT1-1-3	*Neurospora crassa*	Normal sexual development	Debuchy et al., 2010
		Sodaria macrospora	Influences sexual reproduction, non-essential	Klix et al., 2010
	MAT1-1-4	*Pyrenopeziza brassicae*	Unknown, essential	Singh et al., 1999
		Tapesia yallundae	Unknown, essential	Singh et al., 1999
	MAT1-1-5	*Botyrtis cinerea*	Fruiting body development	Rodenburg et al., 2018
		Sclerotinia sclerotiorum	Normal sexual development	Doughan and Rollins, 2016

		Coccidiodes spp.	*COX13* gene, not true *MAT* gene. Involved with respiration	Mandel et al., 2007
	MAT1-1-6	*Pseudogymnoascus destructans*	Unknown	Palmer et al., 2014
		Coccidiodes spp.	*APN2* gene, not true *MAT* gene. Involved with DNA repair	Mandel et al., 2007
	MAT1-1-7	*Coccidiodes spp.*	Unknown	Mandel et al., 2007
	MAT1-1-8	*Sphaeropsis sapinea*	Unknown	Bihon et al., 2014
	MAT1-1-9	*Sphaeropsis sapinea*	Unknown	Bihon et al., 2014
MAT1-2	*MAT1-2-2*	*Neurospora crassa*	Unknown	Pöggeler and Kück, 2001
		Magnoporthe oryzae	Unknown	Kanamori et al., 2007
	MAT1-2-3	*Coccidiodes spp.*	Involved with membrane integrity and secretion	Mandel et al., 2007
		Fusarium spp.	Unknown	Martin et al., 2011
	MAT1-2-4	*Aspergillus fumigatus*	Fruiting body development, regulates	Yu et al., 2017

			expression of *preA* and *preB*	
		Coccidiodes spp.	Unknown	Mandel et al., 2007
	MAT1-2-5	*Sphaeropsis sapinea*	Unknown	Bihon et al., 2014
		Coccidiodes spp.	*COX13* gene, not true *MAT* gene. Involved with respiration	Mandel et al., 2007
	MAT1-2-6	*Coccidiodes spp.*	*APN2* gene, not true *MAT* gene. Involved with DNA repair	Mandel et al., 2007
	MAT1-2-7	*Huntiella spp.*	Fruiting body development. Influences pheromone signaling	Wilson et al., 2020
	MAT1-2-8	*Villosiclava virens*	Unknown	Yu et al., 2015

Aside from the core *MAT* genes, *MAT* loci of different ascomycetes may also contain additional, secondary *MAT* genes (Table 1.1) (Dyer et al., 2016; Wilken et al., 2017). These genes have a number of functions and are thought to provide finer control of sexual reproduction than the core genes can alone (Yu et al., 2017). Nomenclature of secondary *MAT* genes is often not straightforward. Two genes in different species may have the same name even though they share low or no sequence homology and have different functions, for example *MAT1-1-5* from both *Botrytis cinerea* and *Coccidioides spp.* (Mandel et al., 2007; Rodenburg et al., 2018). In addition, the so-called *MAT*

genes *MAT1-1-5* and *MAT1-2-5* from *Coccidioides spp.* do not actually have an active role in sexual reproduction, but were named as such because of their proximity to the *MAT* locus (Mandel et al., 2007). Novel *MAT* genes, when discovered, should be named according to Turgeon and Yoder (2000) after homology to known *MAT* genes is assessed, in order to prevent redundancy (Turgeon and Yoder, 2000; Wilken et al., 2017).

1.1.3 Non-*MAT* genes involved in sex

Sexual reproduction in ascomycetes is also influenced by genes found outside of the *MAT* locus (Dyer and O'Gorman, 2012). These non-*MAT* genes are involved in a variety of processes that link to sexual reproduction (Table 1.2). Many of these genes were characterised in *Aspergillus nidulans* and therefore gene nomenclature in Table 1.2 is specific for this species, however homologues can be found throughout the Ascomycotina e.g. *preB* (*A. nidulans*) = *STE2* (*S. cerevisiae*) = *pre2* (*N. crassa*) (Kim et al., 2012; Wang and Dohlman, 2004).

***Table 1.2**. Examples of non-*MAT *genes and processes involved with sexual reproduction in* Aspergillus nidulans.

Process		**Gene(s) involved**	**Role in sexual reproduction**	**Reference(s)**
Environmental sensing	Light	*velA, velB, laeA*	Control activation of sexual reproduction in the dark	Bayram et al., (2008); Kim et al., 2002
	Nutrient	*phoA, cpcA, cpcB, lsdA*	Control activation of sexual reproduction in suitable richness of phosphorus, amino acids and salt	Bussink and Osmani (1998); Hoffmann et al., (2000); Lee et al., (2001)

	Stress	*fhbA, fhbB*	Repress sexual reproduction by detoxifying nitric oxide	Baidya et al., (2011)
Pheromone signaling	Pheromone/ receptor	*ppgA, ppgB, preA, preB*	See Section 2.1	Dyer et al., (2003)
	Signal transduction	*STE20, steC, STE7, mpkB, steA*	Mitogen-Activated Protein (MAP) Kinase cascade which transduces signal from pheromone-receptor coupling into activation of genes required for sexual reproduction	Dyer (2007)
Lipoid hormone signaling		*ppoA, ppoB, ppoC*	See Section 2.1	Tsitsigiannis et al., (2005); Brodhun and Feussner (2011)
Non-*MAT* genetic regulation		*nsdC, nsdD*	Transcription factors that influence initial stages of sexual development	Han et al., (2001); Kim et al., (2009)

The developmental network involved with coordinating sexual reproduction in fungi is very complex, as demonstrated in Figure 1.5. A more detailed exploration of non-*MAT* genes controlling sexual reproduction is beyond the scope of this study, however readers are directed to an excellent review by Dyer and O'Gorman (2012) for further information.

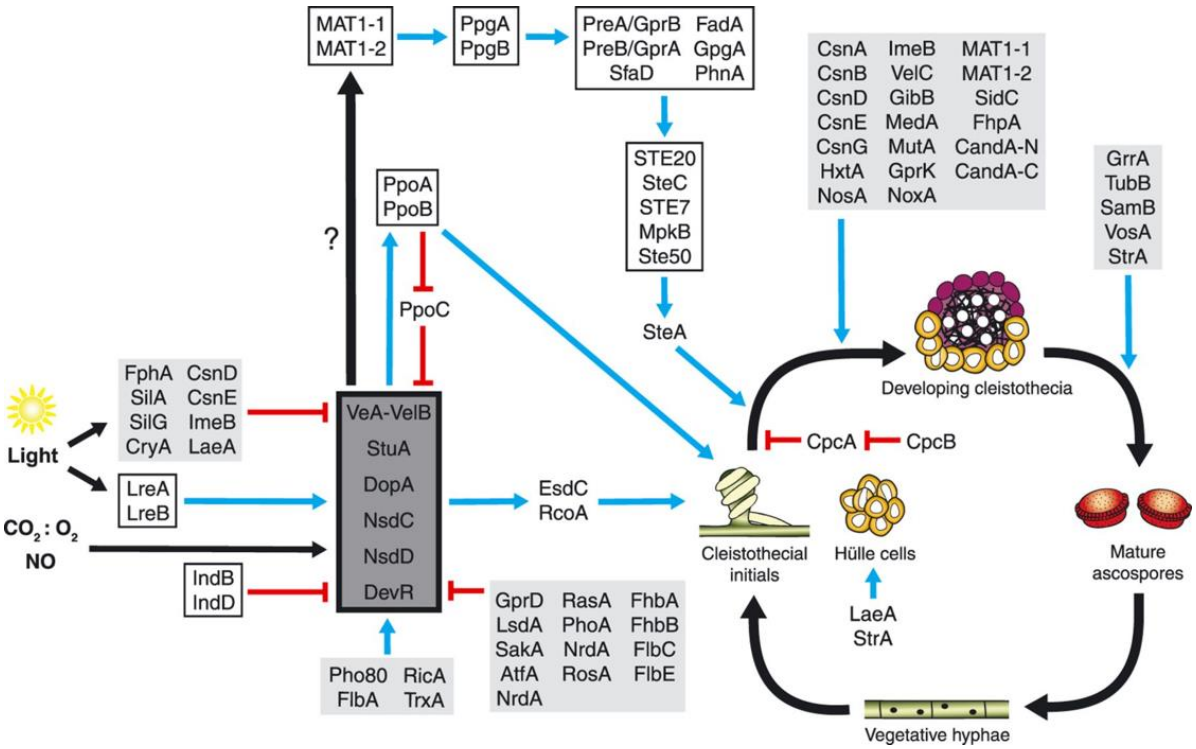

***Figure 1.5**. Developmental network coordinating sexual reproduction in* Aspergillus nidulans*. Proteins are grouped based on the developmental stage they act in, and may not be related. Blue lines represent gene activation and red line represent gene repression. Taken from Dyer and O'Gorman (2012).*

1.1.4 Asexuality, cryptic sexuality and parasexuality

Although most fungi are able to undergo sexual reproduction, about 20 % of fungal species are thought to be asexual (Dyer and O'Gorman, 2011). Asexuality is particularly prevalent in *Aspergillus* species, where approximately two-thirds of taxa are thought to rely solely on asexual reproduction for propagation (Dyer and O'Gorman, 2012). This considerable occurrence of asexuality is perhaps surprising because of the evolutionary benefits that sexual reproduction affords a species (Lee et al., 2010). A number of theories as to how asexuality has evolved have been posited. One theory speculates that in asexual species, mutations have arisen in *MAT* genes rendering them inactive. This appears fairly unlikely, as studies have found that *MAT* genes of asexual species do not differ from sexual species of the same genus and are therefore predicted to be functional (Yun et al., 2000). A more plausible explanation for asexuality is that extant asexual species have evolved from a single *MAT* type of a heterothallic ancestor, and therefore only one of the two *MAT* idiomorphs usually required for a complete sexual cycle is found. While

both *MAT* idiomorphs are found for some apparently asexual species, an explanation for this could be that unrecorded sexual reproduction is occurring. There are numerous examples of asexual species in which only a single *MAT* type has been isolated, including *Rhynchosporium orthosporum* and *Cercospora apii* of which only *MAT1-1* isolates were found and *Cochliobolus victoriae* of which only *MAT1-2* isolates were found, among others (Christiansen et al., 1998; Groenewald et al., 2006; King et al., 2015). Interestingly, as mentioned previously, despite only *MAT1-2* isolates of *H. moniliformis* being isolated from the wild, a sexual cycle has been observed in this species (Discussed in Section 1.1.5) (Wilson et al., 2015a).

It has been speculated that many asexual species may in fact be able to undergo sexual reproduction, and therefore exhibit "cryptic" sexuality (Dyer and O'Gorman, 2012). Several key indicators of cryptic sexuality have been used in the past to provide evidence for an extant sexual cycle. One such indicator is the presence of functional meiosis and *MAT* genes in the genome and active expression of these genes (Gow, 2005; Paoletti et al., 2005; Schurko and Logsdon, 2008). Another indicator of sex is the presence of both *MAT* idiomorphs in environmental populations of a species, which is normally a requirement for sexual reproduction (O'Gorman et al., 2009; Wilson et al., 2019). Further, the observation of proto-sexual structures may also act as an indicator of cryptic sexuality. These indicators have been important in the initial search for sex over recent years, and have led to sexual cycles being discovered for a number of supposedly asexual species, including *A. fumgiatus*, *Pe. chrysogenum* and *Pe. roqueforti* (Böhm et al., 2013; O'Gorman et al., 2009; Swilaiman, 2013). However, it is important to note that even if some or all of these indicators are found, a true sexual cycle may remain elusive, as seen in studies of the asexual species *Fusarium oxysporum*, *A. oryzae* and *Rhynchosporium spp.* (King et al., 2015; Wada et al., 2012; Yun et al., 2000). A sexual cycle may not have been observed in these species because it is rare or requires very specific conditions to be induced (Houbraken and Dyer, 2015). Alternatively, these species may have evolved to be genuinely asexual, as this

lifestyle does have some evolutionary advantages over sexual reproduction including rapid colonisation of new habitats and being less energetically costly (Drenth et al., 2019; Lee et al., 2010).

Asexual reproduction itself is a developmentally complex process that requires the coordinated effort of hundreds of genes, including most notably the central regulators *brlA*, *abaA* and *wetA* of *A. nidulans* and related homologues and orthologues in other filamentous ascomycetes. The asexual genetic machinery is generally conserved throughout the *Pezizomycotina* aside from *brlA*, which is only found in the *Eurotiomycetes* suggesting a more complex control of asexual development in this clade (Ojeda-López et al., 2018). The *Saccharomycotina* however lack many of the genes involved in asexual development in *Pezizomycotina*, which may in part explain why yeast sporulation is less developmentally complex than sporulation in filamentous ascomycetes (Ojeda-López et al., 2018). Further detail on asexual reproduction is beyond the scope of this study, however readers are directed to an excellent review and study by Ojeda-López *et al*. (2018).

Interestingly, some species which are thought to be genuinely asexual may still be able to undergo genetic recombination without the need for conventional sex, via a process known as the parasexual cycle (Figure 1.6). The parasexual cycle shares some common features with sexual reproduction, including initial plasmogamy (cytoplasmic fusion) between unlike hyphae to form a heterodikaryon and subsequent karyogamy (nuclear fusion) between genetically unlike nuclei to form a diploid nucleus, but does not involve conventional meiosis or fruiting body development. Instead, recombination is caused by mitotic crossing-over between chromosomes within the fusion (2N) nucleus and subsequent haploidisation or aneuploidy (Pontecorvo, 1956). Of note, *C. albicans* is thought to use the parasexual cycle as a key means to generate genetic variation because a full sexual cycle including meiosis has never been observed in this species. Although the parasexual cycle is thought to be a rare event in nature, it may have important evolutionary consequences

including accelerating adaptation and improving fitness in the environment (Schoustra et al., 2007). Further, parasexual recombination is implicated in enabling persistence of *A. fumigatus* in cystic fibrosis patients and therefore may also have serious clinical consequences (Engel et al., 2020).

The parasexual cycle has been exploited for strain development and genetic studies in fungal species where sexual reproduction was thought not to occur, such as *P. chrysogenum* and *A. niger* (Arentshorst and Ram, 2018; Ball, 1971). Use of the sexual or parasexual cycle for these purposes is discussed in more detail in Section 1.3.

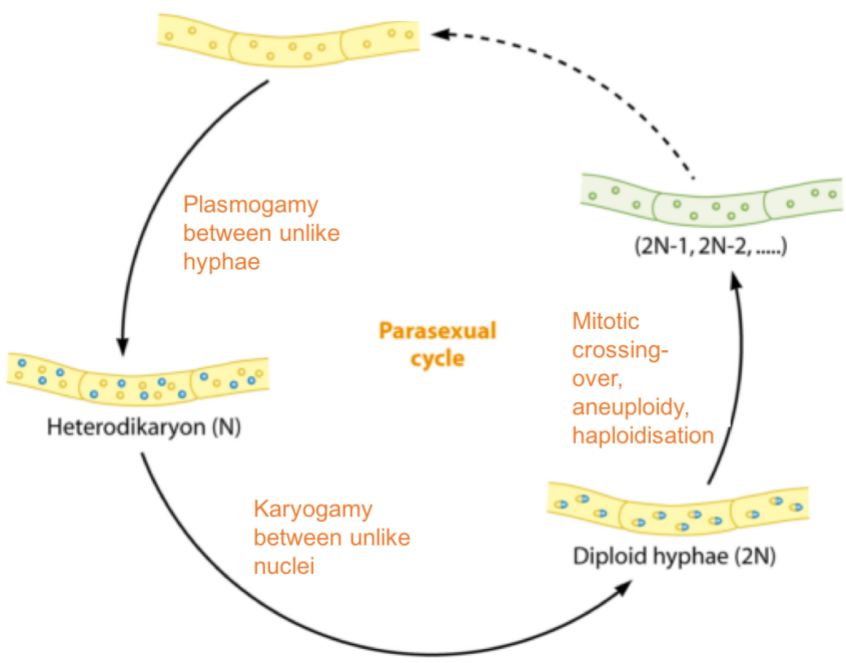

***Figure 1.6**. Parasexual cycle of* Aspergillus nidulans. *The parasexual cycle enables genetic recombination without the need for conventional sexual reproduction, via mitotic crossing-over between genetically distinct chromosomes from two unlike nuclei and subsequent chromosome loss (aneuploidy) or haploidisation. Edited from Lee at al. (2010).*

1.1.5 Hormonal control of sex

An additional level of control of sexual reproduction in ascomycetes is achieved through the production of sexual hormones. Fungal sexual hormones are defined as any diffusible biochemical substance that at low concentrations

can induce a physiological or morphological change forming part of the sexual process (Dyer et al., 1992). These hormones may be peptides, for example **a**-factor and α-factor of *S. cerevisiae*, or lipoid compounds, for example the precocious sexual inducer (*psi*-)factors of *A. nidulans*.

Peptide hormones, commonly referred to as pheromones in the literature, are responsible for the initial attraction of mating partners in heterothallic species (Jones and Bennett, 2011; Wang and Dohlman, 2004). In general, ascomycetes produce two different versions of peptide pheromone, typified by α-factor produced by *MAT*α cells and **a**-factor produced by *MAT*a cells of *S. cerevisiae.* Other yeast and filamentous ascomycetes produce pheromones which have homologous structures and functions to those in *S. cerevisiae* (See Section 2.1). The *S. cerevisiae* **a**-factor initiates the sexual cycle by binding to the Ste3 G-protein-coupled receptor (GPCR) found in the membrane of cells of the opposing mating type, *MAT*α. Conversely, α-factor binds to a different GPCR, Ste2, found in the membrane of *MAT*a cells (Figure 1.7a) (Wang and Dohlman, 2004). Binding of pheromones to their cognate receptor triggers a MAPK signalling cascade which results in the activation of the Ste12 transcription factor, which itself is then responsible for initiating further sexual development (Hoi and Dumas, 2010).

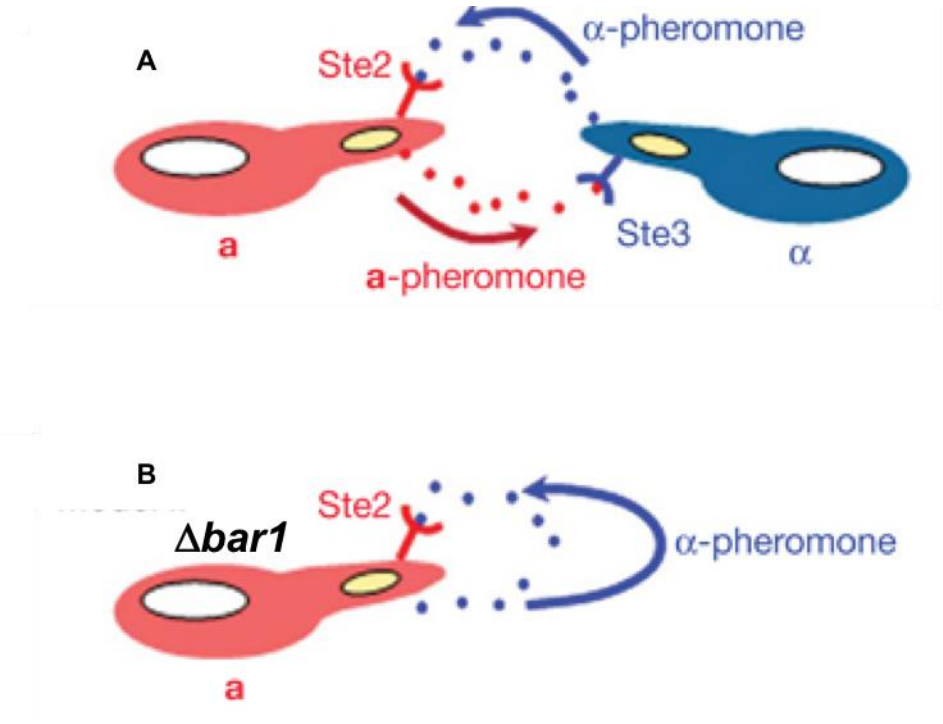

Figure 1.7*. Peptide sexual hormone (pheromone) signaling in ascomycete yeast species. A) a-pheromone (a-factor) is produced by a-cells and binds to the cognate receptor Ste3 within the membrane of α-cells. α-pheromone (α-factor) is produced by α-cells and binds to the cognate receptor Ste2 within the membrane of a-cells. Pheromone-receptor binding triggers a signalling cascade that leads to transcription of genes involved with sexual reproduction. B) In Δbar1 a-cells of* C. albicans*, aberrant pheromone signalling leads to self-stimulation and the initiation of unisexual reproduction. Edited from Alby et al., 2009.*

Altered pheromone signalling is the cause of unisexuality in *C. albicans* (Alby et al., 2009). In normal circumstances, the aspartic protease Bar1 is produced in *MATa* cells of *C. albicans* which acts to cleave α-factor that is constitutively produced in both *MAT* types (Manney, 1983). In Δ*bar1* cells however, α-factor remains active and is free to bind to its cognate receptor Ste2, thus initiating sexual reproduction without the need for a *MAT*α mating partner (Figure 1.7b) (Alby et al., 2009). It is possible that certain conditions within the mammalian host may downregulate Bar1 activity and hence unisexual mating could be clinically relevant (Alby et al., 2009; Tao et al., 2014). Altered pheromone signalling is also thought to contribute to unisexuality in *H. moniliformis*, a member of the Pezizomycotina (Wilson et al., 2015a). This species (of which only *MAT1-2* isolates have been found) was shown to indiscriminately express both α-factor and **a**-factor, in contrast to the closely related heterothallic species *H. omanensis* which expresses the pheromones in a *MAT* type

dependent manner (Wilson et al., 2018). Wilson *et al*. (2018) found that *H. moniliformis* contains a truncated version of a secondary *MAT* gene, *MAT1-2-7*, which is present in its complete form in *H. omanensis*, and thus speculated that this gene influenced pheromone signalling somehow (Wilson et al., 2018). In agreement with this theory, replacement of the native *MAT1-2-7* with the truncated version in *H. omanensis* led to the aberrant expression of both α-factor and **a**-factor as well as decreased expression of **a**-factor, however unisexuality was not achieved, indicating that other factors likely influence unisexuality in this species as well (Wilson et al., 2020).

Other fungal sex hormones are lipoidal in nature, such as the *psi*-factors first characterised in *A. nidulans* or sex factor (SF) in *Pyrenopeziza brassicae* (Ashby, 1998; Champe and el-Zayat, 1989; Siddiq et al., 1990). Less is known about this class of hormones than the peptide pheromones, although it has been shown that they influence sexual morphogenesis and fruiting body development. For example, two oxylipin *psi*-factors, *psi*Bα (8-hydroxyoctadecadienoic acid) and *psi*Cα (5,8-dihydroxyoctadecadienoic acid), act to repress asexual sporulation and induce cleistothecial formation in *A. nidulans* (Champe and el-Zayat, 1989; Mazur et al., 1991). Similarly, SF from *P. brassicae*, an as-yet uncharacterised lipoid compound, has been shown to repress asexual sporulation and induce apothecial formation in this species (Siddiq et al., 1990). Lipoid sexual hormones are thought to elicit their response through GPCRs, although the exact mechanisms of how the compounds bind to the receptors are unknown (Affeldt et al., 2012).

Fungal sexual hormones will be explored in more detail in Chapter 2.

1.1.6 Pleiotropism and repurposing of sexual machinery

Accumulating lines of evidence have demonstrated that core genetic or hormonal machinery which is involved with sexual reproduction also influences a plethora of non-sexual processes. These can include related developmental processes such as asexual sporulation, control of secondary

metabolism or the complete repurposing of sexual pheromone signalling for host-sensing in plant pathogens (Böhm et al., 2015; Turrà et al., 2015).

Deletion strains in which core and secondary *MAT* genes have been knocked out have proved to be important tools in deciphering the function of these genes in sexual reproduction. Often, deletion of these genes also leads to interesting phenotypes unrelated to and alongside aberrant sexual development. Deletion of *MAT1-1-1* in the penicillin-producing ascomycete *Penicillium chrysogenum* led to a 60 % reduction in penicillin production as well as increasing asexual sporulation and hyphal branching (Böhm et al., 2013). Deletion of the other core *MAT* gene, *MAT1-2-1*, in *Pe. chysogenum* was also shown to affect asexual development by increasing asexual sporulation in darkness and changing conidia morphology, however no effect on penicillin production was observed (Böhm et al., 2015). Slight differences in regulatory functions of the two core *MAT* genes was also seen in a study of *A. fumigatus*, where overexpression of *MAT1-1-1* led to a significant increase in production of the secondary metabolite pseurotin and overexpression of *MAT1-2-1* led to a significant increase in production of the secondary metabolite fumagillin (Yidong Yu et al., 2018). Core *MAT* genes have been shown to regulate primary metabolism as well as secondary metabolism, for example *MAT1-2-1* being involved with regulating the production of cellulase in *Trichoderma reesei* (Zheng et al., 2017). Secondary *MAT* genes have also been implicated in controlling non-sexual processes, such as *MAT1-1-2* in *So. macrospora* influencing melanin biosynthesis and *MAT1-2-7* in *Hu. omanensis* influencing colony morphology (Klix et al., 2010; Wilson et al., 2020). The sheer number of cellular processes which appear to be under the control of *MAT* genes has led to them being referred to as “master regulators” (Dyer et al., 2016).

An interesting example of sexual machinery being repurposed involves the use of the pheromone signalling system for host-sensing in the asexual species *F. oxysporum* and the homothallic *F. graminearum* (Sridhar et al., 2020; Turrà et al., 2015; Vitale et al., 2019). *F. oxysporum* is an important plant pathogen

which is able to colonise a broad range of hosts via initial invasion of the plant roots and subsequent travel throughout the plant (Warman and Aitken, 2018). Chemotaxis toward the root of the host plant tomato (*Solanum lycopersicum*) was shown by Turrá *et al.* (2015) to be triggered by a response of the fungus to the catalytic product of various class III peroxidases produced by the plant. Remarkably, chemotaxis was decreased when the *ste2* homologue, encoding the α-factor pheromone receptor, was deleted, indicating that this receptor is required for for the chemotaxis response to the plant derived peroxidases. This phenomenon is not restricted to *F. oxysporum*, as shown by Sridhar *et al.* (2020) in a study using the causative agent of Fusarium Head Blight of wheat, *F. graminearum*. Similar to the former study, deletion of the *F. graminearum ste2* homologue decreased chemotaxis, this time toward the wheat head of *Triticum aestivum*. This indicates that Ste2 mediated host chemotaxis is not specific to soil-borne *Fusarium* species and might be a conserved feature of this fungal group.

The pheromone signalling system of *F. oxysporum* has also been shown to mediate quorum sensing dependent germination (Vitale et al., 2019). Quorum sensing is a process whereby cells modulate their behaviour and gene expression based on population density, and has been well studied in bacteria but less so in fungi (Padder et al., 2018). Vitale *et al.* (2019) found that *F. oxysporum* co-expresses both pheromone and receptor pairs, in contrast to most other ascomycetes which usually differentially express pairs in a *MAT* type dependent manner (Kim et al., 2012). This leads to autocrine pheromone signalling, whereby both pheromones are recognised by the same cell. Intriguingly, the result of this autocrine pheromone signalling is a density dependent germination regulatory system. At low spore densities (hence low pheromone concentrations), Bar1 protease mediated degradation of α factor coupled with a factor-Ste3 competition for MAPK signalling machinery is sufficient in attenuating α factor-Ste2 mediated repression of germination. At high spore densities (hence high pheromone concentration) however, α factor is present at a high enough concentration to counteract degradation and

competition and hence germination is repressed (Vitale et al., 2019). At present, it is unknown whether other species use the pheromone signalling machinery in this way. Discovery of this phenomenon in other species may provide an evolutionary reason as to why the pheromone and pheromone receptor genes are found in many asexual and homothallic species despite their primary function in sexual reproduction being redundant.

As well as the pheromone signalling system, the established lipoid hormonal system has also been shown to influence processes beyond sexual reproduction. *psi*Cα (5,8-dihydroxyoctadecadienoic acid) functions in *A. nidulans* to repress asexual sporulation and induce sexual development (Champe and el-Zayat, 1989; Mazur et al., 1991). Recently, the effects that this hormone has on other species were assessed (Niu et al., 2020). In *A. fumigatus*, treatment with physiologically relevant concentrations of *psi*Cα caused increased lateral branching compared to control. Interestingly, *psi*Cα had a completely different effect in the rice-blast pathogen *Magnoporthe grisea* (*oryzae*), where treatment with this hormone induced cellular differentiation into penetrative structures known as appresoria. However, it should be noted that the effect that *psi*Cα had on sexual reproduction in *A. fumigatus* and *M. grisea* was not tested, and therefore hyphal branching and appresorium induction may represent an additional function of this hormone rather than a complete repurposing. Related oxylipin compounds have been implicated to play a role in many diverse processes, including quorum sensing and host sensing, and it would be of interest to test whether these compounds also influence sexual reproduction (Fischer and Keller, 2016).

Overall, it is clear that the study of fungal sexual machinery may provide insights to more than just sexual reproduction, but also fungal secondary metabolism, non-sexual development and cross kingdom communication.

1.2 Fungal sex and disease

Despite the many positive contributions that fungi make to the environment and the biotechnology industry, some species cause devastating diseases of both animals and plants which cost hundreds of billions of dollars per year in crop loss, disease prevention and disease treatment (Fones et al., 2017). For a number of these species, sexual reproduction is an important part of the life cycle and therefore has an impact on disease progression and evolution of pathogenicity (Drenth et al., 2019).

Many fungal plant pathogens rely on both asexual and sexual sporulation for disease spread and survival (Figure 1.8). Examples of these include; *Zymoseptoria tritici*, causative agent of wheat blotch (Torriani et al., 2015); *Leptosphaeria maculans*, a causative agent of stem canker in oilseed rape (Kaczmarek and Jędryczka, 2012); and *Pyrenopeziza brassicae*, causative agent of light leaf spot in oilseed rape and other *Brassica* species (Dewage et al., 2018). In all three of these species, asexual sporulation is responsible for localised spread of the fungus via rain splash dispersal of conidia, whereas sexual sporulation is responsible for fungal spread over greater distances via forcible ejection and wind dispersal of ascospores (Gilles et al., 2001c; Lô-Pelzer et al., 2009; Zhan et al., 1998). Sexual reproduction primarily occurs on crop debris left over from the harvest in summer, and allows the fungi to overwinter via the production of fruiting bodies (Bousset et al., 2015; Gilles et al., 2001a; Zhan et al., 2001). This sexual development results in the production of ascospores which then act as the primary inoculum for initial infection of new crops in the autumn (Gilles et al., 2000; Lô-Pelzer et al., 2009; Zhan et al., 1998).

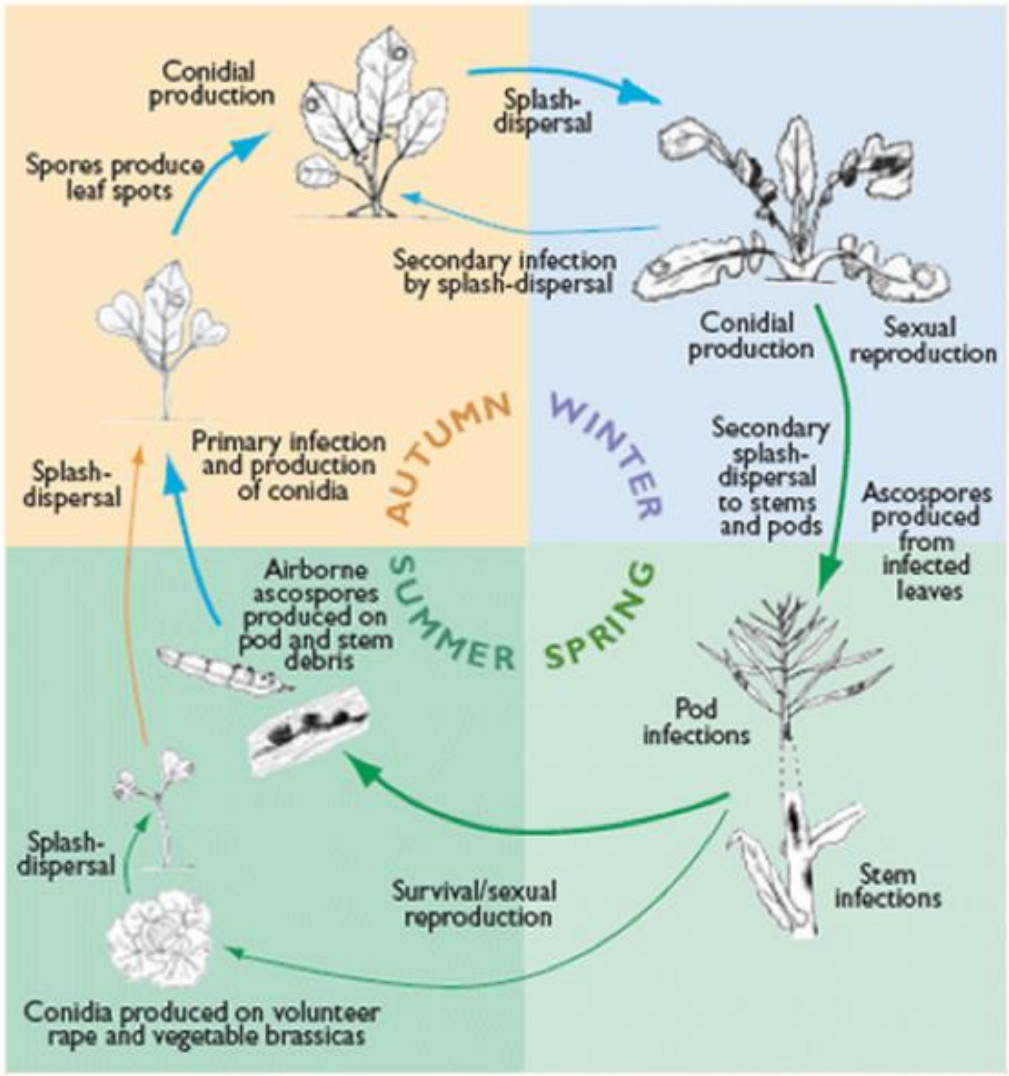

***Figure 1.8**. Life cycle of* Pyrenopeziza brassicae*. Initial infection of host plants is by wind dispersal of ascospores in autumn. Ascospores are ejected from apothecia (fruiting bodies) which have developed on crop debris over the summer. More localised spread of disease is via splash dispersal of conidia throughout winter and spring. Occasionally, sexual reproduction may occur on infected/senescent leaves.* P. brassicae *survives in summer on crop debris and non-primary host species. Taken from resources.rothamsted.ac.uk.*

Knowledge of how, when and where sexual reproduction occurs in plant pathogens is important for developing methods of disease control. As sexual reproduction is a vital part of many species' life cycle, identifying the machinery involved may provide new targets for disease control agents. An example of this would be the pheromone and receptor pairs of *F. oxysporum*, as mentioned in Section 1.1.6, where artificial pheromones used in the field may prevent spore germination (Vitale et al., 2019). Another example would be the identification of a hormone which represses asexual sporulation in *P.*

brassicae, of which an artificial analogue could be used to control spread of disease (Siddiq et al., 1990). This topic will be explored further in Chapter 2. In addition to exploiting the sexual machinery, knowledge of where a species sexually reproduces can also be an important tool. For example, increased removal of wheat stubble post-harvest significantly reduces the rate of sexual reproduction of *Z. tritici* and therefore decreases the amount of biomass available for infection in the next cropping season as well as limiting the emergence of recombinant strains with increased virulence (McDonald and Mundt, 2016).

Sexual reproduction in human fungal pathogens is increasingly being thought of as important in the context of pathogenicity. One of the primary concerns is that new strains may arise through genetic recombination that have additional pathogenicity characteristics such as invasiveness or drug resistance (Heitman et al., 2014). *A. fumigatus*, one of the main causative agent of aspergillosis, serves as a good example of a species in which this may occur. Here there appears to be widespread sexual fertility between worldwide isolates (i.e. little intra-species sterility), indicating that pathogenicity determinants could be readily inherited via meiosis between isolates from different global populations (Swilaiman et al., 2020). In relation to this, it is thought that clinical isolates with resistance to azole fungicides may in some cases have acquired these through sexual reproduction with environmental isolates (Zhang et al., 2017). Sexual (or parasexual) reproduction in *C. albicans*, the main causative agent of thrush, is also a major concern. Evidence of gene flow between global populations has been found and studies have demonstrated that mating lineages show increased fitness compared to clonal lineages (Ropars et al., 2018; Zhang et al., 2015). Further knowledge of sexual reproduction in fungal pathogens of humans will facilitate the development of methods to combat the emergence of more virulent or drug resistance strains.

1.3 Practical applications for the fungal sexual cycle

Aside from the obvious fundamental academic merits discussed so far of studying sexual reproduction in fungi, the sexual cycle can also be exploited for a number of interesting and useful purposes. This includes using the sexual cycle to determine whether a phenotypic trait has mono- or polygenic basis, identifying unknown genes of interest and developing industrial fungal strains with novel desirable characteristics (Ashton and Dyer, 2016).

The genetic basis of a phenotypic trait, in terms of whether it is monogenic (i.e. determined by a single dominant gene) or polygenic (i.e. determined by multiple genes), can be ascertained by mating two parental isolates that differ in the trait of interest and assessing the progeny. If a trait was monogenic, it would be expected that there be a 1:1 segregation pattern, with half of the progeny displaying the phenotype of one parent and the other half displaying the phenotype of the other parent. If a trait was polygenic however, it would be expected that the progeny would show a more complex, continuous distribution of phenotypes (Ashton and Dyer, 2016).

If a trait is found to be monogenic, a technique known as Bulk Segregant Analysis (BSA) can be used to locate and potentially identify the gene of interest (Michelmore et al., 1991). Modern BSA usually involves the use of the sexual cycle paired with next generation sequencing, and has been successfully employed to identify genes involved in processes such as drug resistance and pathogenicity (Ashton, 2018; Camps et al., 2012; Kershaw et al., 2019). The first stage of BSA is to set up a sexual cross between a parental isolate which displays the phenotypic trait of interest and a second parental isolate lacking the trait. Secondly, a fairly large number of progeny (approx. 80-120 individual ascospores) are isolated and separated into pools depending on whether they display the trait of interest or not. DNA is then extracted from all the progeny and pooled according to the trait-pooling done previously. By pooling the DNA in this way, it is expected that the genetic differences between the parents will be “evened out” in the progeny as a result of recombination, aside from the genomic regions linked to and containing the gene of interest. Finally, the two

pools of progeny DNA plus the DNA of the parental isolates are sequenced, and genomic regions which are linked to the trait of interest are identified by searching for differences in the genomes. The amount of genetic variation not including the gene of interest can be lessened by repeated “back-crossing” i.e. sexual crossing of progeny displaying a trait and the parental isolate not displaying a trait. This results in trait-displaying progeny which are more isogenic and may only differ from the non-trait-displaying parent by a few genomic regions (Ashton, 2018). Interestingly, where a full sexual cycle is not available, for example in *A niger*, the parasexual cycle can be successfully used for BSA as an alternative (Arentshorst and Ram, 2018).

Another important use for the fungal sexual cycle is for strain development, which is particularly relevant for fungal species used in the biotechnology or food and drink industries. For this application, sexual crosses are set up between two parental isolates and progeny with novel desirable phenotypes can be screened for and isolated (Ashton and Dyer, 2016). For example, a sexual cross could be set up between a parental isolate that produces a pigment and is slow-growing, and a parental isolate that does not produce the pigment and is fast-growing. The resultant progeny could then be screened and isolates which produce the pigment and are fast growing can be selected for industrial use. In addition, sexual reproduction could be used to generate progeny with novel enzymatic activities or secondary metabolite production capacities. In some cases, sex can lead to the production of progeny which show extreme phenotypes beyond that seen in the parental isolates, a phenomenon known as transgressive segregation (Rieseberg et al., 1999). Sexual reproduction has the added benefit of restoring fitness in progeny by removal of deleterious mutations which may have developed through years of asexual reproduction or mutagenesis programmes (Reyes et al., 2012). Such mutagenesis programmes have traditionally been used to increase enzyme or secondary metabolite production, for example cellulase production in *Trichoderma reeesei* and penicillin production in *P. chrysogenum* (Böhm et al., 2015; Seidl et al., 2009).

Although sexual reproduction has been used for thousands of years to improve agricultural animals and plants, it has almost entirely been overlooked for improvement of fungi (Steensels et al., 2019). This is likely because many of the fungi used in industry were thought to be asexual (Dyer and O'Gorman, 2011). In recent years however, this dogma has been challenged and several industrially important species such as *P. chrysogenum*, *T. reesei* and *P. roqueforti* have been found to have extant sexual cycles, opening up the possibility to use the sexual cycle for strain development (Böhm et al., 2013; Ropars et al., 2014; Seidl et al., 2009; Swilaiman, 2013). This will be explored further in Chapter 3 and Chapter 4.

1.4 Thesis aims

The overall aim of work in this thesis is to explore how sexual reproduction in fungi can be exploited for beneficial purposes, specifically fungal disease control and strain improvement. Work will focus on two members of the Pezizomycotina of economic importance; namely *Pyrenopeziza brassicae*, a plant pathogen, and *Penicillium roqueforti*, a fungus used in production of blue cheese. Specific aims of this study were:

1. To confirm the existence of, and attempt to characterise, a putative 'SF' hormonal compound(s) involved with asexual and sexual reproduction in *Pyrenopeziza brassicae* and assess its suitability for disease control.
2. To identify genes involved with asexual and sexual reproduction in *Pyrenopeziza brassicae* via transcriptomic changes brought about by the putative SF hormonal compound(s).
3. To assess whether sexual reproduction can generate progeny of *Penicillium roqueforti* with novel characteristics relevant to blue cheese production.

4. To characterise the lipolytic system of *Penicillium roqueforti* and determine whether sexual reproduction has led to altered expression of genes in this system.

Chapter 2 - Investigation of Hormones Involved with Asexual and Sexual Reproduction in *Pyrenopeziza brassicae*

2.1 Introduction

Sexual hormones in fungi are defined as diffusible biochemical substances that at low concentrations can induce a physiological or morphological change forming part of the sexual process (Dyer et al., 1992). Fungal sex hormones may work during the initial attraction of mating partners [e.g. **a** factor and α factor in *Saccharomyces cerevisiae* (Wang and Dohlman, 2004)] or act as morphogens to induce fruiting body or sexual structure formation [e.g. Sex Factor in *Pyrenopeziza brassicae* (Siddiq et al., 1990)]. Sexual growth substances, which act as precursor chemicals for the synthesis of sexual signalling compounds or sexual structures, may also be considered together with sex hormones under the umbrella term 'Chemical Sex Factors' (Dyer et al., 1992).

2.1.1 The "first" fungal sex hormones

Most of the early research on fungal sexual hormones was done using non-Ascomycete species. One of the first hormones to be described was sirenin. Sirenin is produced by female gametes of *Allomyces macrogynus* (a Blastocladiomycete) to attract swarming male gametes. This chemotaxis increases the likelihood of fusion (plasmogamy). Male gamete chemotaxis toward sirenin can be observed at concentrations as low as 10^{-10} M (Carlile and Machlis, 1965). A complementary hormone, parisin, is produced by male gametes of *Allomyces* and serves to attract females (Pommerville and Olson, 1987). Sirenin is a bicyclic sesquiterpene (Nutting et al., 1968); the structure of parisin is unknown, but is likely to be similar to sirenin (Pommerville et al., 1990). Although the molecular mechanism of sirenin action is not known in its entirety, it has been shown that the hormone stimulates a Ca^{2+} influx in the

male *Allomyces* gamete (Pommerville et al., 1990). Remarkably, sirenin also stimulates a Ca^{2+} influx in human sperm cells by binding to a cation channel, CatSper (Syeda et al., 2016). Ca^{2+} influx is important for the successful fertilization of the egg cell, therefore non-functional analogues of sirenin have the potential to be used as male contraceptives by blocking CatSper and thus signal transduction (Syeda et al., 2016).

Another sex hormone, trisporic acid, is produced by Zygomycetes and controls early sexual development, including zygophore (sexual structure) formation. Trisporic acid is produced only by mated cultures of Zygomycetes via a number of precursor molecules known as trisporoids. These are β-carotene derivatives formed via cleavage by β-carotene oxygenases (Burmester et al., 2007). The production and cleavage of β-carotene is induced in varying levels by both trisporic acid and the trisporoids in a positive-feedback loop (Sahadevan et al., 2013). Some of the trisporoids are produced in a mating-type specific manner and can only be synthesised into trisporic acid by the opposing mating-type, after diffusion into the cell (Gooday and Carlile, 1997).

Two further sex hormones, antheridiol and oogoniol, initiate and coordinate sexual structure development in *Achlya spp.* (Oomycetes). Antheridiol is produced by female mycelium and induces the development of antheridial initials (male sexual structures), formed from proximal male hyphae (Raper, 1951). These antheridial initials subsequently begin to secrete oogoniol, which induces the formation of oogonial initials (female sexual structures) from female hyphae (McMorris et al., 1975). The antherdial initials grow toward the oogonial initials before the sexual structures fuse and sexual reproduction occurs, producing oospores. Both antheridiol and oogoniol are steroid hormones similar to animal sex hormones such as progesterone and testerone (Arsenault et al., 1968; McMorris et al., 1975).

2.1.2 Peptide sexual hormones of the ascomycetes

Probably the best characterised group of ascomycete sex hormones are the peptide pheromones. These pheromones are encoded as pro-peptides which are post-translationally modified to produce short, mature polypeptides. Peptide pheromones are found throughout the Ascomycota, in both homothallic and heterothallic species. In heterothallic species, these peptides serve both as mate-recognition signals (allowing the attraction of opposing mating types) and as initiators of the sexual process (Jones and Bennett, 2011). The role of peptide pheromones in homothallic species is more ambiguous, as in some species it has been shown that they are not necessary for mating (Kim et al., 2008). Intriguingly, asexual species also possess genes related to the pheromone system. In these species, the pheromone system may have been repurposed for roles outside of sexual reproduction (Bennett and Turgeon, 2016). One such example is evident in the plant pathogen *Fusarium oxysporum*, which uses the sex pheromone system for sensing of plant root signals and as a fungal community quorum sensing mechanism (Turrà et al., 2015; Vitale et al., 2019).

2.1.2.1 Peptide sexual pheromones in *Saccharomyces cerevisiae*

The **a** and α factor pheromones from the ascomycete yeast *Saccharomyces cerevisiae* have been extensively studied since the 1970s. The interaction between the **a** and α factors and their cognate receptors has become a model for fungal pheromone signalling and eukaryotic cellular signalling in general (Wang and Dohlman, 2004). **a** factor is produced by haploid cells of the *MAT***a** mating type and initiates the sexual cycle by binding to the Ste3 G-protein-coupled receptor (GPCR) found in the membrane of cells of the opposing mating type, *MAT*α. Inversely, α factor is produced by *MAT*α cells and binds to a different GPCR, Ste2, found in the membrane of *MAT***a** cells (Wang and Dohlman, 2004). After binding, both pheromones induce a similar MAPK signalling cascade in their cognate cells to activate further stages of sexual development.

Although the two pheromones function in an analogous way, their structures and biogenesis differ substantially. Mature α factor is 13 amino acids in length and is hydrophilic in nature (Stötzler et al., 1976). α factor is encoded by two different genes, *mfα1* and *mfα2*, which code for two different pro-peptides that are 165 and 120 amino acids in length and contain 4 and 2 copies of α factor, respectively (Singh et al., 1983). These pro-peptides are cleaved by the proteases Kex1, Kex2 and Ste13 to release the mature α factor hormone (Figure 2.1a) (Bourbonnais et al., 1991). By contrast, mature **a** factor is 12 amino acids long and hydrophobic in nature due to an incorporation of an additional farnesyl moiety (Anderegg et al., 1988). **a** factor is also encoded by two different genes, *mfa1* and *mfa2* (Michaelis and Herskowitz, 1988). These genes encode pro-peptides much shorter in length than the α factor pro-peptides (36 amino acids compared to 120 or 165). The **a** factor pro-peptides contain C-terminal CAAX motifs which allow for post-translational modification steps including farnesylation by Ram1 and Ram2, proteolysis by Rce1 or Ste14, carboxymethylation by Ste14 and proteolysis by Ste24 and Axl1 which eventually render the mature **a** factor (Figure 2.1b) (Anderegg et al., 1988). The biogenesis of **a** factor has served as a paradigm for studying these posttranslational chemical modifications (Michaelis and Barrowman, 2012).

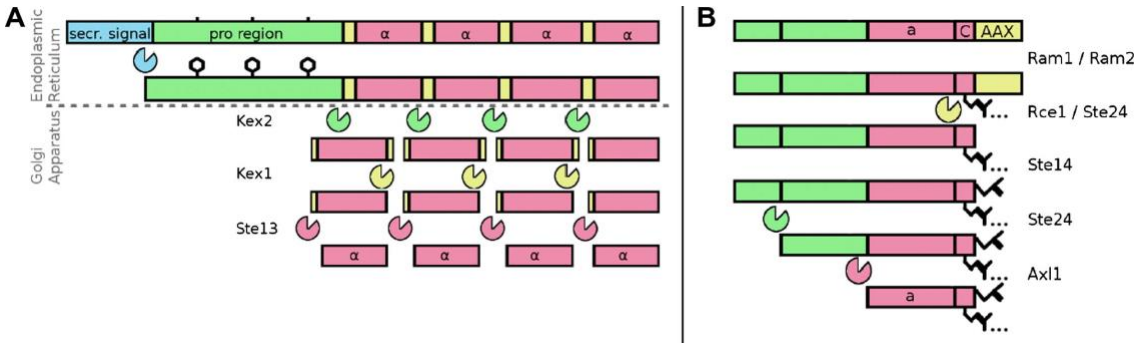

Figure 2.1. *Processing of pheromones of* S. cerevisiae*. A) Mature α factor is produced from a pro-peptide that undergoes proteolytic cleavage. B) Mature* ***a*** *factor is produced from a pro-peptide that undergoes farnesylation, carboxymethylation and proteolysis modifications. Edited from Jones and Bennett (2011).*

2.1.2.2 Peptide sexual pheromones in filamentous fungi

As well as being found in yeast, peptide pheromones are also important in the sexual cycle of filamentous ascomycetes (the Pezizomycotina). Most known filamentous peptide pheromones can be thought of as homologous to either **a** or α factor. **a** factor-like hormones, such as Mfa-1 from *Neurospora crassa*, are around 20 – 25 amino acids in length and are processed from pro-peptides which contain a typical C-terminal CAAX motif allowing for farnesylation and carboxylmethylation (Bobrowicz et al., 2002). Other hormones of this class include Mf1-1 from *Magnaporthe grisea* (Shen et al., 1999) and Mfp from *Podospora anserina* (Coppin et al., 2005). *Cryphonectria parasitica* possesses two separate pheromone precursor genes which encode this class of pheromone, known as *mf2/1* and *mf2/2* (Zhang et al., 1998). The open reading frames of these genes however are identical, and the mature peptides are predicted to be the same. α factor-like hormones e.g. Ccg-4 from *N. crassa* (Bobrowicz et al., 2002) and Mf1/1 from *C. parasitica* (Zhang et al., 1998) are around 11 amino acids in length and are encoded from precursor genes containing several repeats of the mature peptide. These repeats are typically separated by Kex2 protease processing sites, such as with *S. cerevisiae* α factor. It should be noted that in some species at least two different forms of alpha factor pheromone are produced, which differ very slightly in their amino acid composition e.g. the Af1 and Af2 α factor-like pheromones of *Aspergillus fumigatus* and the An1 and An2 α factor-like pheromones of *A. nidulans* (Pöggeler, 2002).

A third class of peptide pheromones produced by filamentous ascomycetes, discovered in *Hypocrea jecorina* (telomorph of *Trichoderma reesei*), are known as hybrid-type peptide pheromones, of which Hpp1 was the first characterised (Schmoll et al., 2010). Hpp1 contains the typical CAAX motif found in **a**-factor like hormones but is encoded by a precursor gene which contains 3 repeats of the mature peptide surrounded by Kex2 protease recognition sites typical of α factor-like hormones. Hpp1, therefore, is structurally a mix of both classes of peptide pheromone, but assumes the **a** factor-like hormone role in this species (Figure 2.2) (Seibel et al., 2012).

Most known pheromone precursor genes from heterothallic filamentous species are expressed in a strictly mating-type dependent manner (Bobrowicz et al., 2002; Shen et al., 1999; Wilson et al., 2018; Zhang et al., 1998). For example, *mfa-1* is expressed only by mat a cells of *N. crassa* and *ccg-4* is expressed only by mat A cells (Bobrowicz et al., 2002). In some species such as *H. jecorina* however, precursor genes are expressed independently of mating-type. Instead, specific mating-types of this species express one precursor gene more highly than the other. Here, *ppg1* (α factor-like pheromone) is more highly expressed in *MAT1-1* cells and *hpp1* is more highly expressed in *MAT1-2* cells, but RNA transcripts of both pheromone precursor genes can be found in either mating type (Figure 2.2) (Seibel et al., 2012). Pheromone receptor genes are expressed in the same manner, with one mating-type generally expressing one particular receptor more highly then the other (Kim et al., 2012). Another species which produces transcripts from both of its pheromone precursor genes regardless of mating-type is *Huntiella monoliformis*, a unisexual species which is genetically heterothallic (i.e. individuals possess only one *MAT* locus idiomorph) (Wilson et al., 2018). *MAT1-2* isolates of this species harbour a truncated version of a *MAT1-2-7* gene which, in its non-truncated form, is thought to be involved with pheromone expression. This truncation may render the encoded protein unable to properly control pheromone expression profile and lead to both being expressed (Wilson et al., 2018, 2020). A difference between the pheromone systems of yeast and filamentous fungi is that in the latter, cells assume male and female roles in sexual development. To this end, pheromones have been shown to be essential for male fertility (Coppin et al., 2005; Kim and Borkovich, 2006) whereas pheromone receptors are essential for female fertility (Kim and Borkovich, 2004; Seibel et al., 2012).

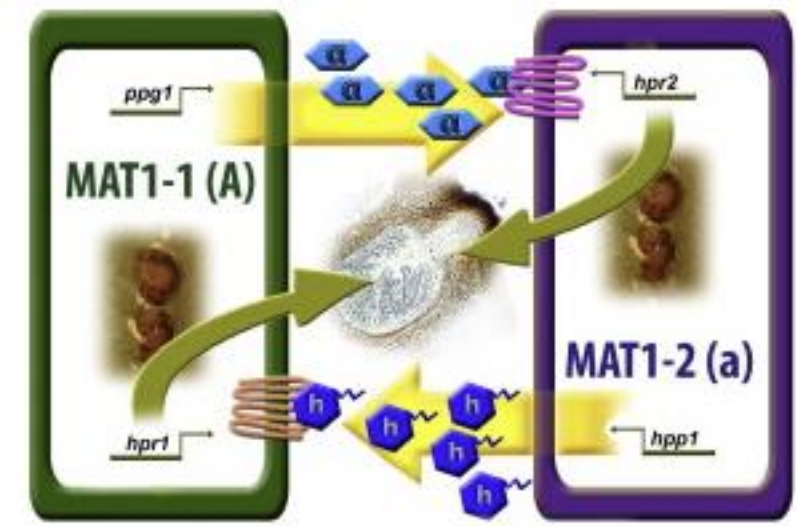

Figure 2.2. *Production of pheromones (ppg1 [MAT1-1] and hpp1 [MAT1-2]) and pheromone receptors (hpr1 [MAT1-1] and hpr2 [MAT1-2]) in* H. jecorina *leads to sexual reproduction. System applies to other ascomycetes. Edited from Seibel et al. (2012).*

2.1.3 Lipid sexual hormones of the Pezizomycotina

Lipids can act as important signalling molecules in organisms from all kingdoms of life. In fungi, lipid signalling is important for processes as diverse as quorum sensing (Polke and Jacobsen, 2017), pathogenicity (Singh and Del Poeta, 2011) and development (Fischer and Keller, 2016). During sexual development, lipids may act as chemical sex factors, either as morphogens to regulate the switch between asexual and sexual development or as sexual growth substances required as precursors to produce hormones (Dyer et al., 1992).

2.1.3.1 Fatty acids, oxylipins and the *psi* factors

Most of the known fungal lipid chemical sex factors are polyunsaturated fatty acids (PUFAs) and oxygenated derivatives of these fatty acids, known as oxylipins. Linoleic acid for example has been shown to act as a chemical sex factor in a range of species (Calvo et al., 1999). In *N. crassa*, addition of micromolar levels of linoleic acid was shown to increase perithecial production by up to 20-fold (Nukina et al., 1981). Similarly, addition of micromolar levels of linoleic acid increased perithecial production in *Nectria haematococca* mating population IV by up to 4-fold (Dyer et al., 1993). The best characterised ascomycete lipoid sex hormones are the precocious sexual inducer (*psi*-) factors of *Aspergillus nidulans*. In *A. nidulans*, asexual and sexual sporulation

are temporally separated with asexual sporulation usually preceding sexual sporulation (Adams et al., 1998). *Psi*-factor however was shown to prematurely induce sexual sporulation when applied via filter paper discs to *A. nidulans* cultures (Champe et al., 1987; Champe and el-Zayat, 1989). *Psi*-factor consists of several oxylipins termed *psi*α and *psi*β, which are derivatives of linoleic and oleic acid, respectively. Both *psi*α and *psi*β can be further classified as either *psi*A, *psi*B or *psi*C depending on the position of the hydroxyl group on the fatty acid backbone (Mazur et al., 1991, 1990). The best characterised of the *psi*-factor compounds are the linoleic acid derivatives *psi*Bα (8-hydroxyoctadecadienoic acid) and *psi*Cα (5,8-dihydroxyoctadecadienoic acid), which were shown to induce sexual sporulation and inhibit asexual sporulation in *A. nidulans*, and *psi*Aα (lactone ring at 5' position of *psi*Cα) which was shown to do the reverse (Champe and el-Zayat, 1989) (Figure 2.3).

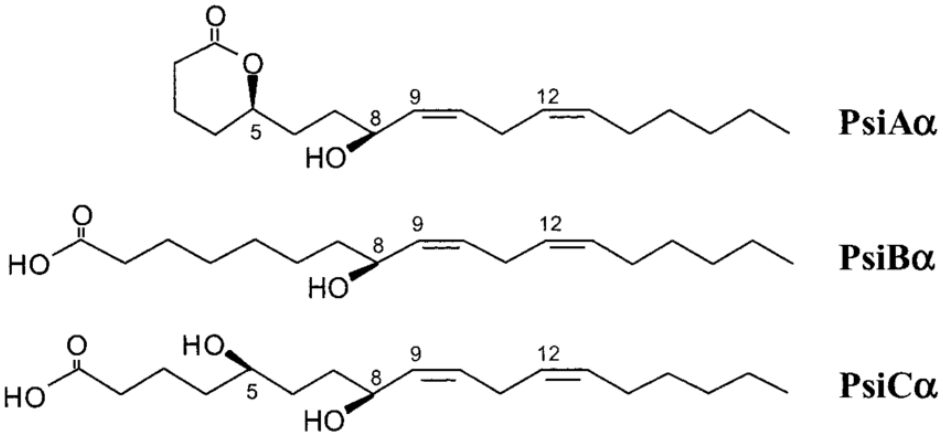

***Figure 2.3**. psi-factor compounds involved in regulating asexual and sexual reproduction in* A. nidulans

The production of *psi*-factor compounds is controlled by the *psi* factor producing (*ppo*) genes *ppoA, ppoB* and *ppoC* which code for fatty acid oxygenases (Tsitsigiannis et al., 2005c). PpoA is responsible for the production *psi*Bα and *psi*Cα, via an N-terminal haem peroxidase domain and a C-terminal P450 haem thiolate domain, respectively (Brodhun et al., 2009). In one study, deletion of *ppo*A resulted in an approximately 10-fold decrease in the level of *psi*Bα, and a 4-fold increase in ratio of asexual sporulation compared to sexual

sporulation (Tsitsigiannis et al., 2004b). PpoB and PpoC are both involved in the production of *psi*Bβ, although their exact role in this production is unknown (Brodhun and Feussner, 2011). Interestingly, although deletion of both *ppo*B and *ppo*C results in decreased levels of *psi*Bβ, the effects of the deletions on sporulation are different. *ppoB* deletion results in increased asexual sporulation and decrease sexual sporulation (Tsitsigiannis et al., 2005c) whereas *ppoC* deletion has the opposite effect (Tsitsigiannis et al., 2004a). A triple mutant (Δ*ppoA*Δ*ppoB*Δ*ppoC*) perhaps surprisingly presented a phenotype with up to a 22-fold decrease in the ratio of asexual sporulation to sexual sporulation in comparison with the wild-type (Tsitsigiannis et al., 2005c). This indicates the importance of the oxylipin products of the *ppo* genes for coordination of sexual development in *A. nidulans*.

Ppo genes and *psi*-factors are found throughout the ascomycetes (Tsitsigiannis et al., 2005c), although research beyond *A. nidulans* has been limited. In related studies manipulation of *ppo* and *lox* gene activity was found to influence sclerotial development and hence morphogenesis in *A. flavus* (Brown et al., 2009, 2008). A more recent study characterised the function of *psi*Cα (5,8-diHODE) and other related dihydroxy oxylipins in *A. fumigatus* and *Magnaporthe grisea* (*oryzae*) where they were shown to induce lateral branching or appressorium formation, respectively (Niu et al., 2020). *Psi*-factors have also been found in other medically important fungi such as *Pencillium chrysogenum* (Shin et al., 2016) as well as the industrially important fungus *A. niger* (Wadman et al., 2009) but their function in these species is not well characterised. Further study of *psi*-factors in diverse fungal species is needed to fully understand their role in fungal development.

2.1.3.2 Sex factor in *Pyrenopeziza brassicae*

Another potential example of a lipoid sex hormone is so-called sex factor (SF), referring to a lipid extract obtained from mated cultures of the plant pathogen *Pyrenopeziza brassicae* (Ilott et al., 1986). When applied to culture media prior to inoculation with single mating-type isolates of *P. brassicae*, SF was found to

both repress asexual sporulation and induce the formation of sterile apothecia in these cultures, with the timing of application important (Ilott et al., 1986; Siddiq et al., 1990). SF also stimulated the formation of an increased number of apothecia in mated cultures of *P. brassicae*, compared to untreated cultures. This is thought to be owed in part to the induction of a protein, Sex Factor Induced 1 (SFI1), which may be a structural component of apothecia (Ashby, 1998). SF could be extracted from mated cultures using organic solvents such as ethanol and ethyl acetate, and although it represents a relatively crude (i.e. non-purified) fraction, its presence mostly in the organic phase and almost complete absence from the aqueous phase of Folch extractions would suggest that any active SF component is lipoid in nature (Siddiq, 1989). Additionally, unpublished work using Thin Layer Chromatography (TLC) analysis of partially purified SF indicates the possible presence of a sugar moiety (Alison Ashby, unpubl. data). It is possible that SF is a mixture of several compounds which may be acting together to elicit the observed effect on sporulation, similar to *psi*-factor. Preliminary evidence indicates however that SF is distinct from *psi*-factor because the latter was not found to have any effect on sporulation in *P. brassicae* (Siddiq, 1989). In follow up work Chamberlain et al. (1995) reported that addition of the crude SF extract could stimulate sexual development in other test ascomycete species, although control extracts from unmated cultures of *P. brassicae* also showed some bioactivity.

Thus, at the onset of the present studies, the exact chemical structure and encoding gene(s) of SF were unknown. However, the observed bioactivity of SF in repressing asexual sporulation in *P. brassicae* offers the possibility that SF might provide an exciting new means of disease management of *P. brassicae* by preventing spread of disease by formation and dispersal of asexual spores.

2.1.4 The plant pathogen *Pyrenopeziza brassicae*

Pyrenopeziza brassicae (anamorph *Cylindrosporium concentricum*) is a heterothallic, hemibiotrophic pathogen of *Brassica* plant species which include the crops winter oilseed rape (*Brassica napus*) and cabbage (*Brassica oleracea*). This pathogen is the causal agent of the disease "light leaf spot", a disease which causes up to £160 million worth of crop yield loss annually in the UK alone (Dewage et al., 2018; Thomas et al., 2019). The economic importance of *P. brassicae* has resulted in the recent genome sequencing of the species (C. S. K. Dewage, Y. Huang, B. Fitt, University of Hertfordshire, unpubl. results). *P. brassicae* is part of the *Leotiomycete* class of fungi, which includes other important plant pathogens such as *Rhynchosporium commune*, *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Johnston et al., 2019). This group was traditionally known as the inoperculate discomycetes and species within it were characterised by the production of apothecia and unitunicate asci, although more recent molecular phylogeny has revealed that the group contains species which do not fit these criteria (Johnston et al., 2019). Light leaf spot is most problematic in temperate regions of the world where temperatures and rainfall suit the growing conditions of the fungus (Gilles et al., 2001b). This includes the UK, Northern European countries, Canada and New Zealand. Light leaf spot has also recently been found in the Pacific Northwest of USA (Carmody et al., 2020; Ocamb et al., 2015).

Pyrenopeziza brassicae has both an asexual and sexual stage, the spores of which both contribute to disease (Gilles et al., 2001c). Initial crop infection occurs in autumn by ascospores which are forcibly ejected by apothecia that have developed on crop debris over the summer (Figure 2.4a) (Gilles et al., 2001a, 2001c). Such apothecia are formed as a result of sexual reproduction between compatible *MAT1-1* and *MAT1-2* isolates of the species i.e. a heterothallic sexual breeding system (Ilott et al., 1984). Infecting ascospores germinate on crops and directly penetrate the plant cuticle using a cutinase enzyme (Davies et al., 2000; Li et al., 2003). The fungus then continues to grow asymptomatically in the subcuticular space until around winter/early spring time, when asexual sporulation can be observed on leaf surfaces (Figure 2.4b)

(Boys et al., 2007; Gilles et al., 2001c). The asexual spores, often visible in masses known as acervuli, can be splash dispersed by rain and cause secondary infections throughout the remainder of the year until harvest in the summer (Gilles et al., 2001c, 2000). It is also possible that secondary ascospore infections can occur if apothecia develop on senescent plant matter that has fallen to the canopy (Gilles et al., 2001c, 2000).

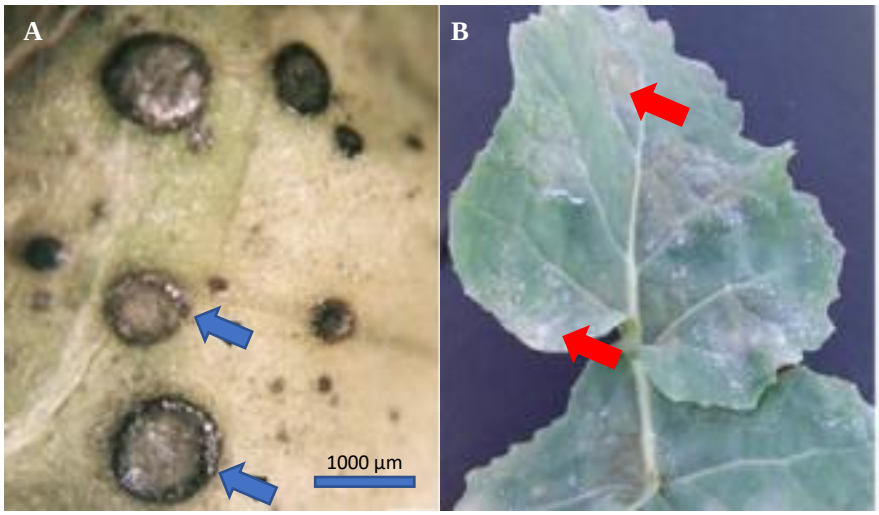

Figure 2.4. P. brassicae *infection on host plants. A) Apothecia (blue arrows) on* Brassica olaracea *leaf. B) Masses of asexual spores (acervuli) (red arrows) on* B. napus *leaf. Edited from Dewage et al. (2018).*

Recently, a very closely related species *Pyrenopeziza cascadia* (also termed *P. brassicae* Lineage 2) has been described from infected *Brassica* plant material in the Pacific North-West of the USA (Carmody et al., 2020). This is the only worldwide geographic location from which *P. cascadia* has so far been identified. The species is almost indistinguishable from *P. brassicae* in terms of morphology and disease symptoms, but does not yet have a described sexual state.

2.1.4.1 Methods of detection and disease control

P. brassicae has a long asymptomatic phase of infection and can normally only be visually detected *in situ* in January/February by the presence of light green necrotic legions on infected plant tissue (Fitt et al., 1998a). At this stage, the disease burden is already high and crop yield losses are difficult to circumvent.

Therefore, several studies have been undertaken to develop methods to detect the fungus in plants which are not yet showing symptoms of infection.

A common means to detect *P. brassicae* in infected tissue is by PCR diagnostic methods. This involves the designing of PCR primers which are specific to a *P. brassicae* target locus such as the *MAT* locus (Foster et al., 1999, 2002), internal transcribed spacer (ITS) (Karolewski et al., 2006) or cutinase gene (Thomas et al., 2019). Total DNA can be extracted from plant tissue from the field and amplified by PCR, and if *P. brassicae* is present then a PCR product should be produced. This method has been able to detect *P. brassicae* DNA from as little as 10 pg of total DNA from symptomatic plant tissue and 10 ng of total DNA from plants with low severity of infection (Thomas et al., 2019). However, PCR diagnostics can be time-consuming and require specialist equipment such as thermal cyclers which limit the use of this procedure in the field (King et al., 2017). An alternative DNA-detection based method called loop-mediated isothermal diagnostic (LAMP) assay has been developed for detection of *P. brassicae*. This diagnostic also makes use of DNA primers which are specific to *P. brassicae* loci, but amplification of the PCR product is much faster (less than 30 mins) and is done at a single constant temperature, making it more suitable for use in the field (King et al., 2017). Another recently developed diagnostic tool is multispectral imaging (MSI) which collects light reflected from the surface of a leaf and uses this spectrum to predict whether a plant is infected (Veys et al., 2019). This diagnostic was shown to accurately detect infection before visible features (e.g. necrotic legions) were evident *in vitro*, but further work is required to test its efficacy in the field (Veys et al., 2019). Finally, studies are ongoing to determine whether a diagnostic can be developed based on the detection of infection specific volatile organic compounds (VOCs) produced by plants infected with *P. brassicae* (D. Withall, Rothamsted Research, *pers. comm*.).

Disease control methods employed against *P. brassicae* are two-fold; using resistant cultivars of host plants (Boys et al., 2012) and also chemical control

using fungicides (Carter et al., 2014). At present, the mechanisms that grant a particular cultivar resistance to *P. brassicae* are largely unknown. Potential resistance mechanisms include inhibition of fungal pathogenicity determinants (e.g. cutinases and proteases), inhibition of fungal growth in the subcuticular space, and inhibition of asexual and sexual sporulation (Boys et al., 2007). One resistance phenotype, "no sporulation" (asexual), has been partially characterised and is found in *Brassica napus* cv. Imola (Boys et al., 2012; Bradburne et al., 1999). This phenotype is thought to be conferred by a single *R* gene and limits asexual sporulation but not sexual sporulation (Boys et al., 2012). Further mechanistic characterisation of how this gene limits asexual sporulation has not been undertaken.

The second method used to control disease in host plants is via chemical control using fungicides. These include the use of chemicals such as methyl benzimidazole carbamate (MBC) fungicides and azole fungicides. However, as seen in many other plant pathogens, some isolates of *P. brassicae* have evolved resistance to these fungicides. Resistance to methyl benzimidazole carbamate (MBC) fungicides has been reported and is caused by non-synonymous mutations in the coding region of the MBC target, β-tubulin (Carter et al., 2013). Two mutations were detected in the isolates sequenced by Carter *et al*., causing β-tubulin amino acid substitutions E198A and L240F, of which E198A grants a high level of resistance and L240F a moderate level of resistance (Carter et al., 2013). Resistance to another class of fungicide commonly deployed to control *P. brassicae*, the azoles, has also evolved in some isolates (Carter et al., 2014). Sequencing of the promoter region and coding region of the azole target sterol 14α-demethylase (*cyp51*) revealed the presence of several mutations which granted varying levels of azole resistance. In the coding region of *cyp51*, two non-synonymous mutations causing the amino acid substitutions G460S and S508T were detected which were associated with increased azole resistance of between 7 and 12-fold for G460S and 7 and 17-fold for S508T (Carter et al., 2014). In the promotor region of *cyp51*, 232 bp, 151 bp or 46 bp sequence insertions were detected which were

associated with azole resistance factors of between 8 and 27-fold in isolates containing these insertions (Carter et al., 2014). An isolate containing both the S508T substitution and 151 bp insertion has been detected which was found to be more resistant than either mutation in isolation (Carter et al., 2014). Interestingly, resistance to commonly deployed fungicides appears not to have evolved in North American isolates of *P. brassicae*, although more comprehensive studies are required to confirm this (Carmody et al., 2020). Neverthless, the widespread occurrence of fungicide resistance in European and other non-North American isolates stresses the need for novel methods of disease control, with complementary modes of action to existing fungicides, to be developed.

2.1.5 Aims

The first aim of work in the present chapter was to confirm the findings of Ilott et al. 1986 and Siddiq et al. (1990) i.e. that a hormonal factor(s) (SF) is produced by sexual cultures of *P. brassicae* that has the ability to repress asexual sporulation and induce the formation of sterile apothecia in single isolates of this species. This was previously only shown using a crude lipid extract from a single mating pair of isolates (NH10 x JH26), and thus further validation is needed to prove that production of SF is species-wide. Screening of multiple mating pairs was therefore undertaken to find pairs that were hyper-sexual (i.e. produced many fertile apothecia) and would in turn be hypothesised to produce the most SF. Solvent extractions and bioassays were then used to determine whether SF was a genuine sex hormone and, if so, assays assessing its effect on asexual/sexual sporulation in other closely related species were to be undertaken.

The second aim of this chapter was to purify and characterise the active component(s) of SF. At present it is unknown whether SF, as characterised by Siddiq et al. (1990), is a single compound or a multi-compound factor as seen elsewhere with the *psi*-factor. High pressure liquid chromatography (HPLC)

and liquid chromatography-mass spectrometry (LC-MS) were to be used to purify active fractions and identify candidate active compounds, respectively.

A final aim was to perform transcriptomic studies in order to assess the effects that SF has on gene transcription. This would make use of the recent unpublished *P. brassicae* genome (C. S. K. Dewage, Y. Huang, B. Fitt, University of Hertfordshire, unpubl. data). An underlying goal was to potentially identify genes involved in the asexual and sexual cycle of *P. brassicae* (e.g. genes controlling sporulation). This would hopefully serve to improve our understanding of the *P. brassicae* lifecycle and identify new targets for use in controlling light leafspot crop disease and hopefully fungal diseases more generally.

2.2 Methods

2.2.1 Media and chemical supplier

Media compositions are as specified in text. All media were autoclaved at 121 °c for 20 mins. Chemicals for use in media were routinely purchased from Sigma, UK unless otherwise specified.

2.2.2 Isolates and strain maintenance

Isolates were routinely cultured on slopes of 3 % Malt Extract Agar (MEA) (3 % Malt Extract, 1.5% agar) at 18 °C in the dark. For long term storage cultures were stored under liquid nitrogen in the Nottingham culture collection. Conidia for use in crosses and bioassays were harvested from cultures on 3 % MEA plates using 0.1 % Tween 80 in sterile distilled water (v/v) by scraping the agar surface with a sterile inoculating loop after 14 days growth. The resulting spore suspensions were filtered through sterile miracloth and the concentrations estimated using an improved Neubauer haemocytometer, before diluting to a working concentration of 1 x 10^6 spores mL^{-1}. Any

remaining excess spore suspensions (of various concentrations) were mixed with glycerol (final concentration 15 %) and stored at -80 °c for future use.

Isolates used in this study can be found in Table 2.1. *Pyrenopeziza brassicae* (112-*x*) and "Lineage 2" (*Pyrenopeziza cascadia*) (117-*x*) isolates were kindly donated by Kevin King at Rothamsted Research and Bruce Fitt at the University of Hertfordshire. All isolates were obtained from diseased plant tissues from either UK or USA sites as listed in Table 2.1.

Table 2.1. Pyrenopeziza brassicae *Isolates used in this study.*

Isolate	**Origin**
112-1	East Lothian, UK
112-2	Hertfordshire, UK
112-3	Hertfordshire, UK
112-4	Hertfordshire, UK
112-5	Hertfordshire, UK
112-6	Hertfordshire, UK
112-7	Hertfordshire, UK
112-8	Hertfordshire, UK
112-9	Hertfordshire, UK
112-10	Hertfordshire, UK
112-11	Harpenden, UK
112-12	Harpenden, UK
112-13	Harpenden, UK
112-14	Harpenden, UK
112-15	Northumberland, UK
112-16	Northumberland, UK
112--17	Northumberland, UK
112-18	Northumberland, UK
117-1	Pacific North West, USA
117-3	Pacific North West, USA
117-4	Pacific North West, USA
117-5	Pacific North West, USA

2.2.3 Mating-type (*MAT*) determination

As *P. brassicae* is a heterothallic species, the mating type (*MAT*) of each isolate was established before setting up sexual crosses. This was achieved using a previously published diagnostic multiplex PCR (Foster et al., 2002), which utilises a common reverse primer (Mt3) and two forward primers (PbM-1-3, PbM-2) (Table 2.2). PbM-1-3 binds specifically in *MAT1-1* isolates and produces a 687 bp product whereas PbM-2 binds specifically in *MAT1-2* isolates to produce an 858 bp product. Resolving the products on an agarose gel allows the mating (*MAT*) type of isolates to be determined.

Table 2.2. *Primers used to determine* MAT *type of* Pyrenopeziza brassicae *isolates*

Primer name	Primer sequence
PbM1-3	GATCAAGAGACGCAAGACCAAG
PbM-2	CCCGAAATCATTGAGCATTACAAG
Mt3	CCAAATCAGGCCCCAAAATATG

Genomic DNA was extracted using the Wizard® Genomic DNA purification kit (Promega, UK) to manufacturer's instructions. Reactions were performed in 50 µL reactions containing: 1X Phusion HF reaction buffer (New England Biolabs, UK), 200 µM dNTP, 500 nM each primer, 1 unit Phusion polymerase, 50 ng genomic DNA and distilled water up to 50 µL. The reactions were performed using a Techne TC-5000 thermal cycler (Applied Biosystems, USA) with the following program: 98 °c for 30 s, 32X [98 °c for 10 s, 66 °c for 20 s, 72 °c for 30 s], followed by a final extension of 72 °c for 5 mins. PCR products were resolved on 1.0 % agarose gels and visualised using UV light.

2.2.4 Induction of sex *in vitro*

Attempts were made to induce sexual reproduction *in vitro* using methods and media based on Houbraken and Dyer (2015) and previous experience with crossing of *P. brassicae* (Ashby, 1998). Spore suspensions (1 x 10^6 spores mL^{-1}) from opposing *MAT* types were mixed in equal proportions and a 50 µL aliquot spotted onto the centre of a 5 cm diameter agar Petri plates (i.e. a mixed spore

crossing method); alternatively, 2.5 µL of single *MAT* type spore suspensions were spotted perpendicular to opposing *MAT* types on agar plates (i.e. a barrage crossing method) (Houbraken and Dyer, 2015). Several media were used in crossing attempts (Table 2.3). Agar plates inoculated with conidial suspension from single *MAT* types (1 x 10^6 spores mL^{-1}) were set up as controls. All cultures were incubated at 18 °c in the dark for 5 weeks and examined periodically for the presence of apothecia. After 5 weeks, the numbers of apothecia per 100 mm^2 were estimated using a dissecting microscope. It was considered that if a sexual hormonal compound existed, crosses which produced a greater number of apothecia would produce a greater amount of this compound.

Table 2.3. *Media used to induce sexual reproduction. Mushroom compost was a kind gift from G's Mushrooms, Cambridgeshire and was dried for 3 days before use.*

Media	**Composition (per L distilled water)**	**Method**
3 % Malt Extract Agar (MEA)	30 g malt extract, 15 g agar	Dissolve components in distilled water
V8 Medium	175 mL V8 Juice, 3 g $CaCO_3$, 0.01 g $ZnSO_4 \cdot 7\ H_20$, 0.005 g $CuSO_4$, 20 g agar	Dissolve components in distilled water, add V8 juice
Compost Malt Medium (CMM)	50 g mushroom compost, 7.5 g malt extract, 15 g agar	Boil compost in distilled water for 3 min. Filter compost mixture through muslin cloth. Dissolve malt extract and agar.

2.2.5 Hormone extraction

Crosses and single isolate cultures of *P. brassicae* were set up using the mixed spore crossing method in combination with CMM (owing to ongoing results, see Section 2.3.2). Approximately 75 of the 5 cm plates of CMM were inoculated for each extraction. Fungal material and surrounding agar was harvested after 35 days with a sterile spoon and placed in a 500 mL Duran

bottle. Approximately 200 mL of ethyl acetate (Fisher Scientific, UK) (or enough to submerge all fungal material) was then added to the Duran and the mixture homogenised using an UltraTurrex at 14,000 rpm for approximately 3 mins. The resulting homogenate was filtered through Whatman no. 1 filter paper and this filtrate mixed with a small amount (ca. 5 g) of sodium sulphate to absorb residual water. This mixture was then filtered as before into a 250 mL round-bottomed flask and the ethyl acetate removed under vacuum using a Rotovapor rotary evaporator (Buchi, UK). The resultant dry residue was weighed and resuspended at 50 mg mL^{-1} in ethyl acetate and stored in glass vials at -20 °c.

2.2.6 Bioassay for repression of asexual sporulation *in vitro*

Assays were undertaken to determine the effects that crude sexual hormone extracts (SF) from mated cultures of *Pyrenopeziza brassicae* had on the development of non-mated, single isolate cultures of *P. brassicae* and *P. cascadia* (*P. brassicae* Lineage 2). This included observation both of the effect on levels of asexual sporulation and any impact on sexual development.

1 mL of 3 % MEA was pipetted into individual wells of a 24-well plate and allowed to solidify. 20 µl of SF (at various concentrations in ethyl acetate) was then spotted onto the agar surface and the ethyl acetate solvent allowed to evaporate passively. 20 µL of a conidial suspension (1 x 10^6 spore mL^{-1}) of *P. brassicae* or *P. cascadia* was then spotted onto the medium and the plates were incubated at 18 °c in the dark for 15 days. Control conditions consisted of the addition of (a) crude ethyl acetate lipid extracts from single *MAT* isolate cultures; (b) 20 µL ethyl acetate to the medium; and (c) untreated medium. Cultures were checked periodically after 7 days for any physiological changes. After 15 days, conidia were harvested from each multiwell using 400 µL of water and gentle agitation with a sterile brush. The number of conidia were then estimated using an improved Neubauer haemocytometer. All assays were performed in triplicate. Statistical tests were performed using GraphPad Prism 8.0.

Crude hormone extracts used were from mated cultures of *P. brassicae* isolates 112-15 x 112-17, as well as single isolate cultures of these isolates (owing to ongoing results, see Section 2.3.2). Conidial suspensions used for bioassays were obtained from *P. brassicae* isolates 112-16 and 112-18 and *P. cascadia* isolates 117-1, 117-3, 117-4, and 117-5. Isolates 112-16 and 112-18 were chosen for bioassays because they presented abundant asexual sporulation *in vitro* (results not shown), and therefore any changes in sporulation as a result of treatment with the various extracts would be more obvious than using isolates 112-15 and 112-17 which did not asexually sporulate as well.

2.2.7 High Performance-Liquid Chromatography fractionation

Crude lipid extracts from sexually reproducing cultures of *P. brassicae* were separated using a Shimadzu Prominence UFLC HPLC system. Before fractionation, all extracts were filtered through cotton wool to remove undissolved material. 500 µL samples (12.5 mg mL^{-1}) were injected in the system and chromatographic separations were carried out as described in Table 2.4 using a ACE 5AQ reverse-phase column (10 × 250 mm, 5 µm). The flow rate was set to 4.0 mL min^{-1} using as mobile phase: solvent A (HPLC grade water) and solvent B (acetonitrile). A PDA detector was used to monitor the UV trace at 254 nm and 270 nm.

Table 2.4. HPLC conditions used for fractionation of crude SF

Time (min)	% Solvent A (Water)	% Solvent B (Acetonitrile)
0.00	95	5
5.00	95	5
45.00	45	55
50.00	0	100
55.00	0	100
58.00	95	5

Fractions eluting over various timespans were collected in round-bottomed flasks. The solvent was removed using a Rotovapor rotary evaporator (Buchi, UK) and the resultant dry residue was resuspended in 1 mL acetonitrile. These fractions were assayed as described previously (Section 2.2.6) to determine the effects they had on the development of non-mated, single isolate cultures of *P. brassicae*.

2.2.8 Liquid Chromatography-Mass Spectrometry

Selected fractions were analysed using UPLC-Q-ToF mass spectrometry. UPLC-Q-ToF mass spectrometry was preformed using a Waters Acquity ultra pressure liquid chromatography system (UPLC) coupled to a Waters Synapt G2 Si Q-ToF mass spectrometer with an electrospray ionisation source. The system was controlled via Masslynx 4.1 software (Waters). Chromatographic separations were carried out as described in Table 2.5 using an Acquity UPLC BEH C18 Waters column attached (2.1 x 150 mm, 1.7 µm) coupled to a C18 VanGuard pre-column (2.1 x 5 mm, 1.7 µm). The column was maintained at 45 °C and the injection volume was 4 µl of analyte. The flow rate was set to 0.21 mL min^{-1} using as mobile phase: solvent A [LCMS grade water (0.01 % formic acid)] and solvent B [methanol (0.01 % formic acid)). Samples were

infused in both negative and positive ionization modes (ESI^- and ESI^+) with two blank methanol samples run between to allow for stabilization of ionization modes. The Acquity PDA detector was used to monitor the UV trace range 200 nm – 400 nm. MS detection was operated in sensitivity mode using a full MS scan from 50 – 1200 Da. The following instrument conditions were applied; capillary voltage 2.5 kV, source temperature 100 °C, desolvation temperature 350 °C, desolvation gas flow 800 L h^{-1}, cone gas flow 60 L h^{-1}. The Synapt was calibrated by infusing a sodium formate solution and accurate mass detection was ensured by infusing the peptide leucine enkephalin during each run.

HRMS was used to calculate potential molecular formulae from peaks of interest. Molecular formulae were discarded if they had an error larger than 5 ppm of the detected value. These were then used to probe the online chemical databases Reaxys (www.reaxys.com) and ChemSpider (www.chemspider.com).

Table 2.5. *Elution profile for LCMS used for analysis of fractions*

Time (min)	% Solvent A [Water (0.01 % formic acid)	% Solvent B [Methanol (0.01 % formic acid)
0.00	95	5
2.40	95	5
29.40	0	100
32.40	0	100
35.40	95	5
38.00	95	5

2.2.9 RNA extraction and quantitative reverse transcriptase PCR (qRT-PCR) analysis of sex-associated genes

The expression of *MAT1-1-1* (*pad1*) in cultures of 112-18 grown on media treated with SF or on untreated media was analysed using qRT-PCR. 200 µL of SF was pipetted on 10 mL of 3 % MEA in a 5 cm agar Petri plate (yielding a final concentration of 25 µg mL^{-1}). Sterile 11 µm nylon discs (Merck Millipore, Ireland) were placed on the agar surface after the evaporation of the solvent. Control plates were set up the same way except using 200 µL of ethyl acetate. 200 µL of 112-18 conidial suspension (1 x 10^6 spore mL^{-1}) was then spotted onto the nylon covered agar plates and the cultures were incubated at 18 °c in the dark for 15 days. Fungal material was harvested by scraping nylon discs with a sterile metal spatula before being frozen in liquid nitrogen and ground to a fine powder under liquid nitrogen using a sterile pestle and mortar.

Total RNA was then extracted from the ground fungal material as follows. The ground material from one 5 cm plate was added to 1 mL of Tri-reagent (Sigma, UK) in a 2 mL microcentrifuge tube, mixed by inversion and incubated for 10 minutes at room temperature. 200 µL of chloroform (Fisher Scientific, UK) was added and the suspension was vortexed and incubated at room temperature for 3 mins. The suspension was then microcentrifuged at 13,000 rpm for 10 mins, after which approximately 750 µL of the upper aqueous phase was removed and added to 750 µL of isopropanol. This suspension was inverted several times and incubated at -20 °c for 20 mins. The suspension was then centrifuged at 13,000 rpm for 10 mins to collect precipitated nucleic acid at the bottom and side of the microcentrifuge tube. The supernatant was subsequently removed and the precipitated pellet washed with 700 µL of 70 % ethanol in sterile distilled water (v/v). The suspension was then centrifuged again at 13,000 rpm for 10 mins, before removing the supernatant and drying the nucleic acid in sterile air. The nucleic acid was then resuspended in 100 µL of DEPC treated water, before cleaning up and digesting contaminating DNA using an NucleoSpin RNA clean up kit (Macherey Nagel, Germany) according to manufacturer's instructions. RNA samples were quantified using a P33 nanophotometer (Implen, Germany), and RNA Integrity Number (RIN)

calculated using RNA ScreenTape (Agilent, USA). Samples were stored at -80 °c.

cDNA was synthesised using GoScript Reverse Transcription Mix (Promega, USA) in 20 µL reactions containing: 4 µL GoScript reaction buffer, 2 µL GoScript enzyme mix, 2 µg total RNA and nuclease-free water up to 20 µL. The reaction was performed using a Techne TC-5000 thermal cycler (Applied Biosystems, USA) with the following program: 25 °c for 5 mins, 42 °c for 60 mins and 70 °c for 15 mins.

Primers for use in qRT-PCR were designed with the aid of Primer3 (Untergasser et al., 2012) (Table 2.6). qRT-PCR experiments were performed using Fast SYBR Green Master Mix (Applied Biosystems, USA) in 10 µL reactions containing: 1X Fast SYBR Green Master Mix, 500 nM each primer, 0.5 µL cDNA and water to 10 µL. Amplifications were performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, USA) with the following program: 95 °c for 20 s, followed by 40 cycles of 95 °c for 3 s and 60 °c for 30 s, followed by a melt-curve consisting of an increase of 1 °c min^{-1} for 35 mins.

All experiments were performed using three biological replicates and three technical replicates. *β-tubulin* was used as a reference gene in this study. Expression levels were calculated as fold change between cultures grown on untreated media and SF treated media using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Student t-tests were calculated to compare the means of the ΔCt levels for *MAT1-1-1* between cultures grown on untreated media and SF treated media using GraphPad Prism 8.0.

***Table 2.6**. Primers used in qRT-PCR experiment*

Gene	Primer (sequence)
MAT1-1-1 (*pad1*)	Pb_pad1_F (ttcaggtatcggtctagcgg)
	Pb_pad1_R (gctcagacaccatacaacgc)
β-tubulin	Pb_bt_F (ctctttccgtgccgttactg)
	Pb_bt_R (aggacatcttgagaccacgg)

2.2.10 Transcriptomic analysis of crude hormone extract-treated cultures

RNA sequencing of *P. brassicae* cultures was due to be performed to assess the global transcriptional changes that occur from treatment with SF. Sequencing was due to be performed on the RNA extracted using the methodology in Section 2.2.9 by Nottingham DeepSeq.

2.3 Results

2.3.1 Mating-type (*MAT*) determination of *Pyrenopeziza brassicae* isolates

The mating type (*MAT*) of individual *P. brassicae* isolates was determined using a multiplex PCR diagnostic test followed by resolving PCR products on an agarose gel (Figure 2.5). Of the 18 isolates tested, 11 were found to be *MAT1-1* and 7 were *MAT1-2* (Table 2.7).

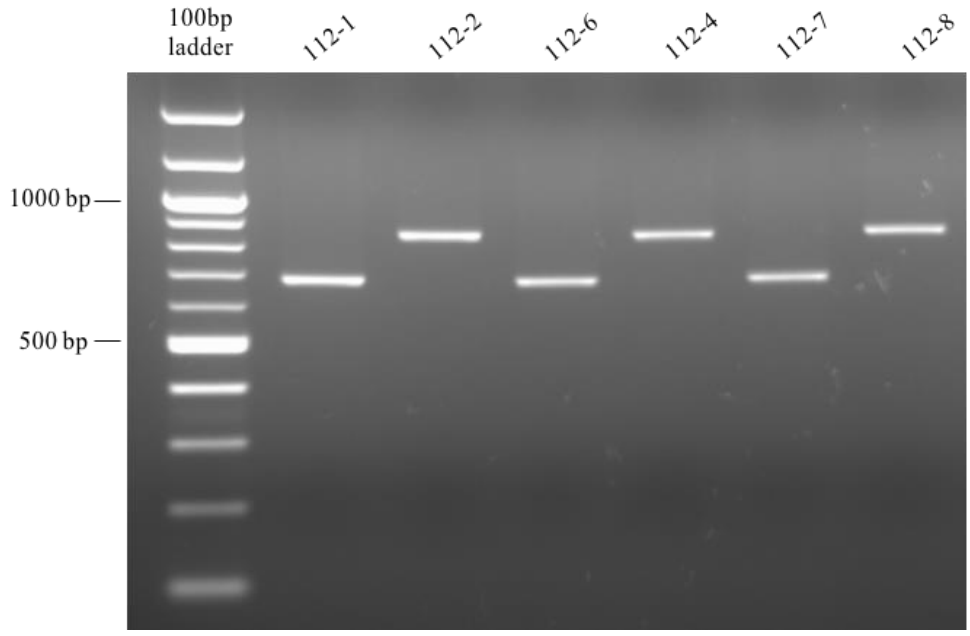

Figure 2.5. *Representative MAT typing diagnostic PCR. Mating types can be distinguished via gel electrophoresis by the size of the fragment produced from PCR: MAT1-1 = 687 bp; MAT1-2 = 858 bp. Using this diagnostic PCR, isolates 112-1, 112-6 and 112-7 were identified as being MAT1-1 and isolates 112-2, 112-4 and 112-8 were identified as being MAT1-2.*

Table 2.7. *Mating type (*MAT*) of* P. brassicae *isolates used in this study, determined using PCR diagnostic test*

Isolate	***MAT* type**
112-1	*MAT1-1*
112-2	*MAT1-2*
112-3	*MAT1-2*
112-4	*MAT1-2*
112-5	*MAT1-2*
112-6	*MAT1-1*
112-7	*MAT1-1*
112-8	*MAT1-2*
112-9	*MAT1-1*
112-10	*MAT1-1*
112-11	*MAT1-1*
112-12	*MAT1-1*
112-13	*MAT1-1*
112-14	*MAT1-1*
112-15	*MAT1-1*
112-16	*MAT1-2*
112--17	*MAT1-2*
112-18	*MAT1-1*

2.3.2 Sexual reproduction in *Pyrenopeziza brassicae* is induced by compost malt media

3 % malt extract agar (MEA) (Siddiq et al., 1990), V8 medium (Houbraken and Dyer, 2015) and compost malt medium (CMM) (Ashby, 1998) were all used to attempt to induce sexual reproduction in *P. brassicae*. Two crossing methods were also used: mixed spore and barrage (Houbraken and Dyer, 2015). Sexual reproduction was not induced using 3 % MEA or V8 media in combination with either of the crossing methods, or by using the barrage crossing method in combination with CMM (results not shown). By contrast, use of the mixed spore crossing method in combination with CMM resulted in induction of sexual reproduction (Figure 2.6), with the development of mature apothecia after 35 days of incubation (Figure 2.6c). The presence of asci containing ascospores confirmed the fertility of the crosses (Figure 2.6d). Although sexual reproduction was induced in all crossing combinations tested, there was considerable variability in the numbers of apothecia produced between crosses (Table 2.8). The cross 112-15 (*MAT1-1*) X 112-17 (*MAT1-2*) produced the most apothecia [100.6 ± 7.3 (SEM) per 100 mm^{-2}] and so was selected for further experimentation.

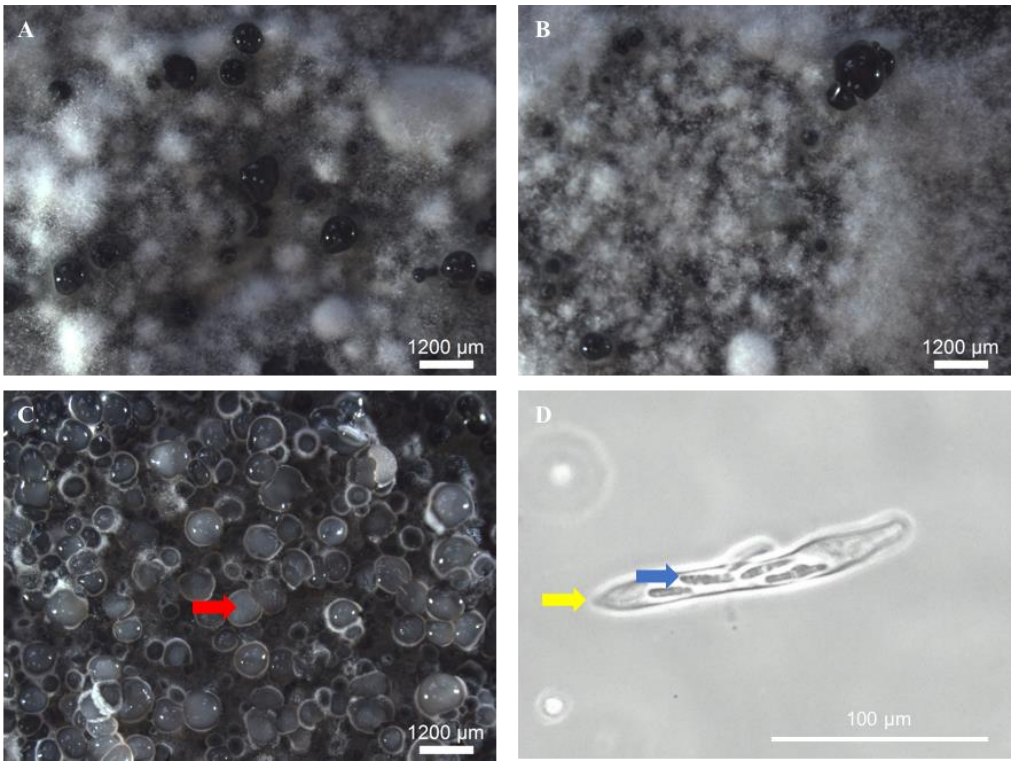

Figure 2.6. *Individual MAT cultures and sexual cultures after 35 days' growth on compost malt medium. Sexual cultures were established by using a mixed spore crossing methodology whereby a 1:1 (MAT1-1:MAT1-2) mix of conidia was spotted onto the centre of the CMM agar plate. A) 112-15 (MAT1-1). B)*

112-17 (MAT1-2). C) Sexual culture (112-15 X 112-17). Abundant apothecia (sexual fruiting bodies) (red arrow) apparent. D) Ascus (yellow arrow) bearing ascospores (sexual spores) (blue arrow), confirming fertility.

Table 2.8. *Number of apothecia (per 100 mm^2) produced by each cross using the mixed spore crossing method on compost malt medium (± SEM). n = 5.*

X		*MAT1-1*					
		112-6	**112-7**	**112-9**	**112-10**	**112-15**	**112-18**
MAT1-2	**112-2**	37.2 ± 5.5	10.0 ± 0.6	38.0 ± 7.3	10.4 ± 2.1	64.2 ± 3.7	38.8 ± 4.7
	112-3	59.2 ± 5.2	23.2 ± 5.3	74.6 ± 8.5	68.6 ± 4.6	92.6 ± 4.2	40.8 ± 2.8
	112-4	62.2 ± 4.0	3.8 ± 1.9	10.6 ± 2.7	3.2 ± 0.7	75.8 ± 7.6	8.6 ± 1.5
	112-8	83.8 ± 8.8	20.6 ± 2.3	71.8 ± 3.8	46.4 ± 7.4	85.6 ± 5.3	30.0 ± 5.5
	112-16	21.4 ± 3.1	4.6 ± 0.9	15.6 ± 1.5	32.4 ± 7.8	20.6 ± 1.8	10.2 ± 2.6
	112-17	85.6 ± 9.1	38.6 ± 4.1	86.8 ± 6.7	70.0 ± 4.2	100.6 ± 7.4	42.6 ± 3.3

2.3.3 A hormonal compound(s) (SF) that represses asexual sporulation is confirmed to be produced by sexual cultures of *Pyrenopeziza brassicae*

Solvent extractions of sexual cultures were undertaken and the resulting lipid extracts (putative SF) assayed for activity in terms of any observed morphological changes and impact on asexual sporulation after 15 day's growth on media supplemented with various extracts.

Addition of increasing amounts of the crude lipid SF extract caused a visually detectable darkening of cultures of both 112-16 (*MAT1-2*) and 112-18 (*MAT1-1*) relative to the control, although no gross sexual morphogenesis was apparent at this point (Figure 2.7b). A Welch's ANOVA revealed that there were significant differences in the number of conidia produced per plate by cultures of 112-16 (*MAT1-2*) [Welch's ANOVA, W (DFn, DFd) = 46.59 (5.000, 5.294), $p < 0.001$] and 112-18 (*MAT1*-1) [Welch's ANOVA, W (DFn, DFd) = 30.78 (5.000, 5.218), $p < 0.001$] when treated with different concentrations of extract from sexual cultures (SF) (extracted from cross 112-15 X 112-17) (Figure 2.7a). The number of conidia produced per plate by cultures of 112-16 treated with SF at all tested concentrations was significantly reduced compared to control cultures. At the lowest SF concentration [25 µg mL^{-1} (SF final concentration in media)], conidial production was 2.6 ± 0.3 % (SEM) that of the control (0 µg

mL^{-1}) (Dunnett's T3 multiple comparisons test, t =16.07, DF = 2.013, $p < 0.05$). Conidial production was further repressed at higher SF concentrations to a minimum of 0.07 ± 0.03 % (SEM) that of the control in cultures treated with 100 µg mL^{-1} SF (Dunnett's T3 multiple comparisons test, t = 16.51, DF = 2.000, $p < 0.05$). The number of conidia produced per plate by cultures of 112-18 treated with SF followed a similar trend. At the lowest SF concentration (25 µg mL^{-1}), conidial production was 1.9 ± 0.5 % (SEM) that of the control (0 µg mL^{-1}) (Dunnett's T3 multiple comparisons test, t = 13.99, DF = 2.016, $p < 0.05$). Conidial production was lowest in cultures treated with 125 µg mL^{-1} SF, at 0.02 ± 0.01 % (SEM) that of the control (Dunnett's T3 multiple comparisons test, t = 14.29, DF = 2.000, $p < 0.05$).

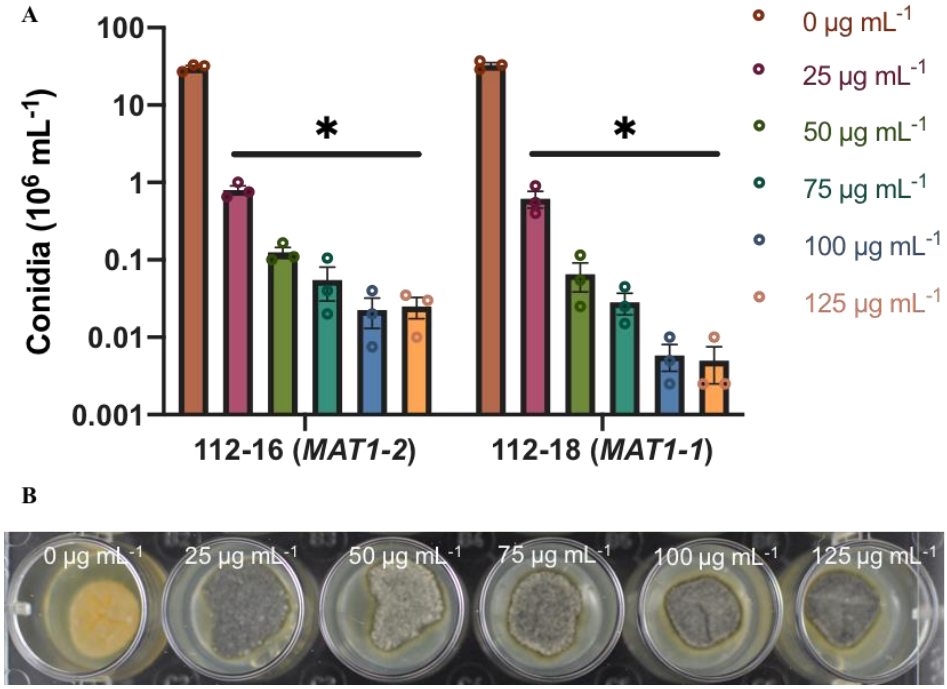

***Figure 2.7**. Effects of crude SF extracts on single isolate cultures of* Pyrenopeziza brassicae. *A) Conidial production was significantly repressed in cultures of both MAT types amended with SF compared to control conditions (Dunnett's T3 multiple comparisons test, p < 0.05). B) Cultures treated with SF appeared darker than control cultures. Figure shows cultures of 112-16 as example.* * = p < 0.05. Error bars represent SEM. n = 3*

2.3.4 SF is not produced by single *MAT* cultures of *Pyrenopeziza brassicae*

Solvent extractions of single *MAT* cultures were undertaken and the resulting extracts assayed for activity. Numbers of conidia per plate were estimated after 15 day's growth on media supplemented with various extracts.

A Welch's ANOVA revealed that there were significant differences in the number of conidia produced per plate by cultures of 112-16 (*MAT1-2*) [Welch's ANOVA, W (DFn, DFd) = 714.4 (4.000, 4.031), $p < 0.0001$] and 112-18 (*MAT1-1*) [Welch's ANOVA, W (DFn, DFd) = 749.1 (4.000, 4.034), $p < 0.0001$] treated with ethyl acetate (control), extracts from sexual cultures (SF), extracts from single *MAT* cultures or a 1:1 mix of extracts from single *MAT* cultures (25 µg mL^{-1}) (Figure 2.8a). The number of conidia produced per plate by cultures of 112-16 treated with SF was 2.4 ± 0.4 % (SEM) (Dunnett's T3 multiple comparisons test, t = 17.42, DF = 3.883, $p < 0.01$) that of the control (Figure 2.8a). By contrast, there were significant increases in the numbers of conidia produced per plate by cultures of 112-16 treated with extracts from *MAT1-1* cultures [276.3 ± 4.7 % (SEM); Dunnett's T3 multiple comparisons test, t = 24.18, DF = 3.883, $p < 0.0001$]; *MAT1-2* cultures [236.6 ± 16.9 % (SEM); Dunnett's T3 multiple comparisons test, t = 7.672, DF = 2.432, $p < 0.05$] and a 1:1 mix of single *MAT* extracts [277.4 ± 13.4 % (SEM); Dunnett's T3 multiple comparisons test, t = 12.20, DF = 2.672, $p < 0.01$] (Figure 2.8a). Meanwhile, the number of conidia produced per plate by cultures of 112-18 treated with SF was 4.8 ± 0.4 % (SEM) (Dunnett's T3 multiple comparisons test, t = 23.14, DF = 2.037, $p < 0.01$) that of the control (Figure 2.8a). There were again significant increases in the numbers of conidia produced by cultures of 112-18 treated with extracts from *MAT1-1* cultures [265.9 ± 9.0 % (SEM); Dunnett's T3 multiple comparisons test, t = 16.75, DF = 2.792, $p < 0.01$]; *MAT1-2* cultures [292.1 ± 6.0 % (SEM); Dunnett's T3 multiple comparisons test, t = 26.39, DF = 3.528, $p < 0.0001$] and a 1:1 mix of single *MAT* extracts [273.7 ± 9.0 % (SEM); Dunnett's T3 multiple comparisons test, t = 17.44, DF = 2.792, $p < 0.01$] (Figure 2.8a). It is possible that the increase in number of conidia produced per plate is a result of increased biomass of cultures, as can be seen in Figure 2.8. Consistent with previous results, cultures of both 112-16 (Figure 2.8b) and 112-

18 treated with SF were darker than control conditions. However, no darkening was seen in cultures treated with extracts from single *MAT* cultures or a 1:1 mix of single *MAT* extracts (Figure 2.8b).

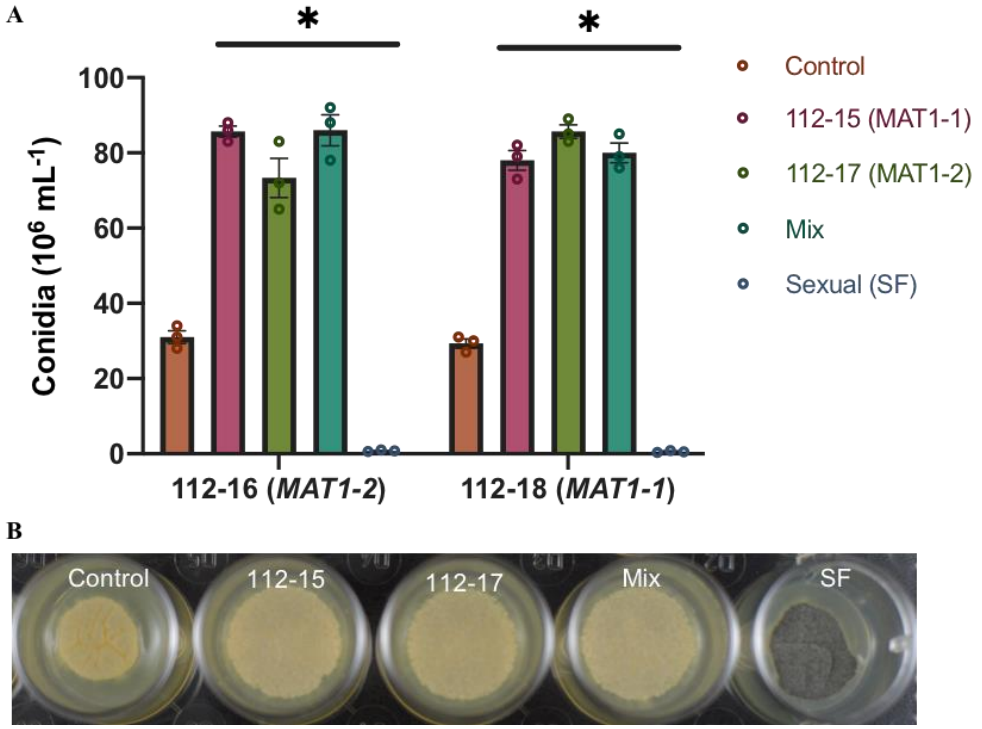

Figure 2.8. *Effects of crude extracts (25 µg mL^{-1}) on single isolate cultures of* Pyrenopeziza brassicae. *A) Conidial production was significantly repressed in cultures of both MAT types amended with SF compared to control conditions, whereas conidial production was significantly increased in cultures of both MAT types treated with single MAT extracts or a mix of single MAT extracts (Dunnett's T3 multiple comparisons test, $p < 0.05$). B) Cultures treated with SF appeared darker than control cultures whereas cultures treated with single MAT extracts were the same colour as control conditions. Figure shows cultures of 112-16 as example. * = $p < 0.05$. Error bars represent SEM. n = 3*

2.3.5 SF has no effect on cultures of Lineage 2 (*Pyrenopeziza cascadia*) isolates

An assay was undertaken to determine whether the effects that SF had on single *MAT* cultures of *Pyrenopeziza brassicae* (i.e. repression of asexual sporulation) were also apparent when single *MAT* cultures of Lineage 2 (*Pyrenopeziza cascadia*) isolates were treated with SF (25 µg mL^{-1}). There was no observable difference between cultures of Lineage 2 isolates treated with crude SF or control conditions (ethyl acetate) (Figure 2.9).

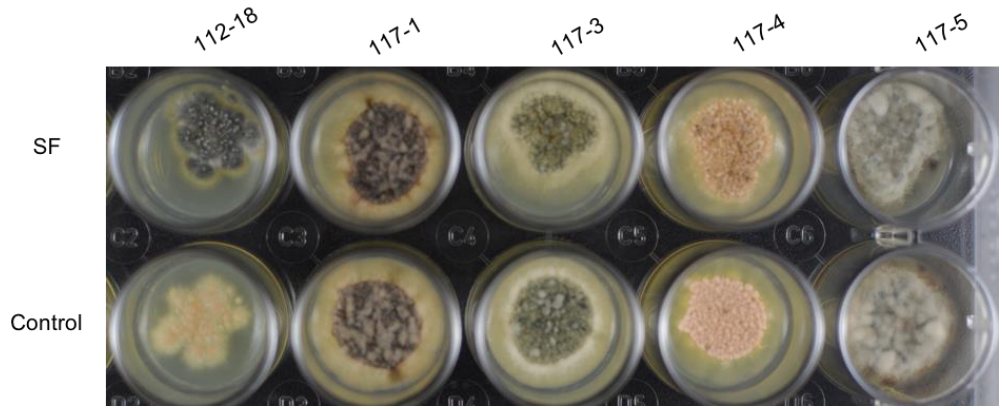

Figure 2.9*. Effects of crude SF extracts on asexual sporulation and culture appearance in single isolate cultures of* P. brassicae *and Lineage 2 (*P. cascadia*) after 15 days' growth. Asexual sporulation was repressed in cultures of 112-18 (*P. brassicae; MAT1-1*) treated with SF compared to control conditions. There was no observable difference between cultures of 117-1 (*P. cascadia; MAT1-2*), 117-3 (*P. cascadia; MAT1-1*), 117-4 (*P. cascadia; MAT1-1*) or 117-5 (*P. cascadia; MAT1-2*) treated with SF compared to control conditions.*

2.3.6 Several candidate active compounds, including 16-(β-D-Glucopyranosyloxy)-2,15-dihydroxyhexadecanoic acid, are revealed by HPLC fractionation and LCMS

Attempts were made to purify the bioactive compound(s) within the crude sexual extract that was responsible for the repression of asexual sporulation. Culture darkening was always associated with repression of asexual sporulation and so was used as an indicator of the activity of HPLC fractions. Sequential rounds of fractionations were undertaken with each resulting fraction assayed for activity. In the first round of fractionation, the active compound(s) was found to elute between 25.0 – 55.0 % acetonitrile. The second round of fractionation revealed that the active compound(s) eluted between 41.0 – 49.0 % acetonitrile. In a final round of fractionation, two different fractions eluting between 33 – 35 mins (40.0 – 42.5% acetonitrile) ("Fraction 1"; Figure 2.10a) and 35 – 37 mins (42.5 – 45.0 % acetonitrile) ("Fraction 2"; Figure 2.10a) had the ability to cause culture darkening and repression of asexual sporulation. However, the phenotypes of cultures treated with either Fraction 1 or Fraction 2 were noticeably different, after 19 days' growth. There were no obvious complex structures observed in cultures treated with Fraction 1, although a degree of hyphal aggregation was apparent (Figure 2.10b). By contrast, cultures treated with Fraction 2 had developed

sterile (i.e. lacking asci and ascospores when checked by microscopy), yet structurally mature, apothecia (Figure 2.10c). Cultures amended with a 1:1 mix of Fraction 1 and Fraction 2 had developed early apothecial initials (densely clustered mycelial structures) but no structurally mature apothecia (Figure 2.10d).

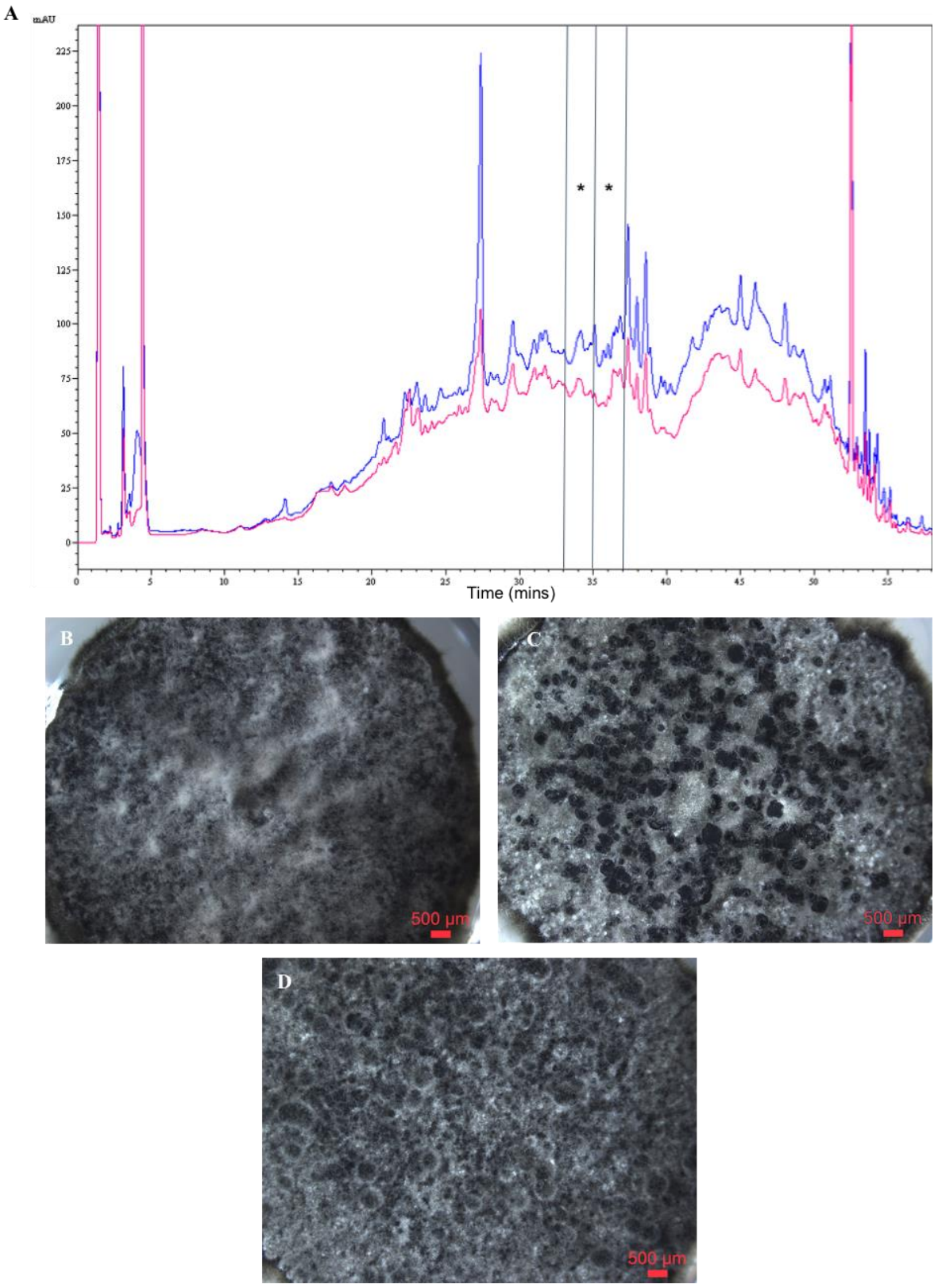

Figure 2.10. *HPLC chromatogram of crude SF and effects of selected SF fractions on colony appearance in single isolate cultures of* Pyrenopeziza brassicae *after 19 days' growth. A) HPLC chromatogram of crude SF. Blue plot = absorption at 254 nm, Pink plot = 270 nm, x axis represents time of elution (mins). Asterisk denotes active fraction. B) "Fraction 1"-induced culture morphology. C) "Fraction 2"-induced culture morphology. D) Fraction 1 + Fraction 2-induced culture morphology.*

Fraction 1 and Fraction 2 were further analysed using LCMS. Molecular formulae were calculated for peaks which either appeared solely in Fraction 1 (Figure 2.11; Figure 2.12; red arrow), appeared solely in Fraction 2 (Figure 2.11; Figure 2.12; green arrow) or appeared in both Fractions 1 and 2 but did not appear in other fractions (Figure 2.11; Figure 2.12; blue arrow). The most likely molecular formulae of peaks of interest are presented in Table 2.9.

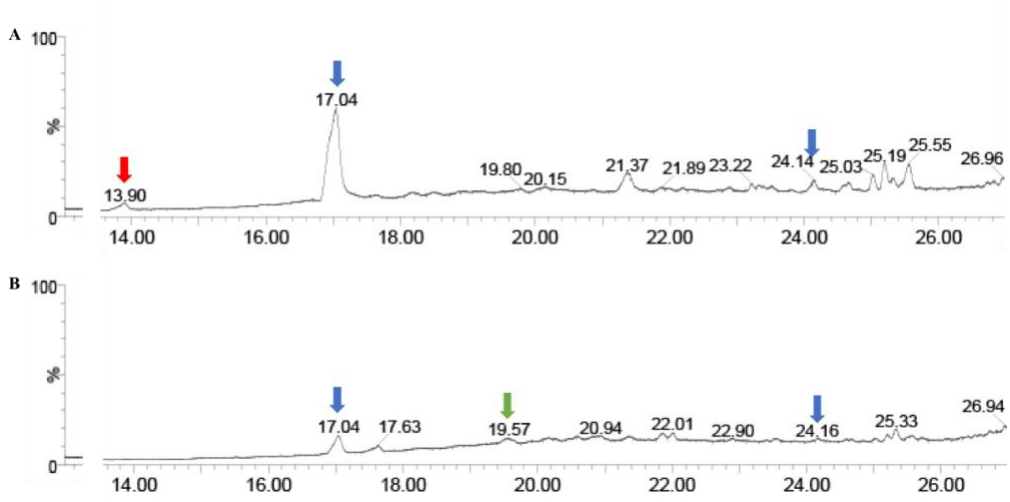

***Figure 2.11**. Positive ion mode LCMS chromatograms. A) chromatogram from fraction 1. B) chromatogram from fraction 2.*

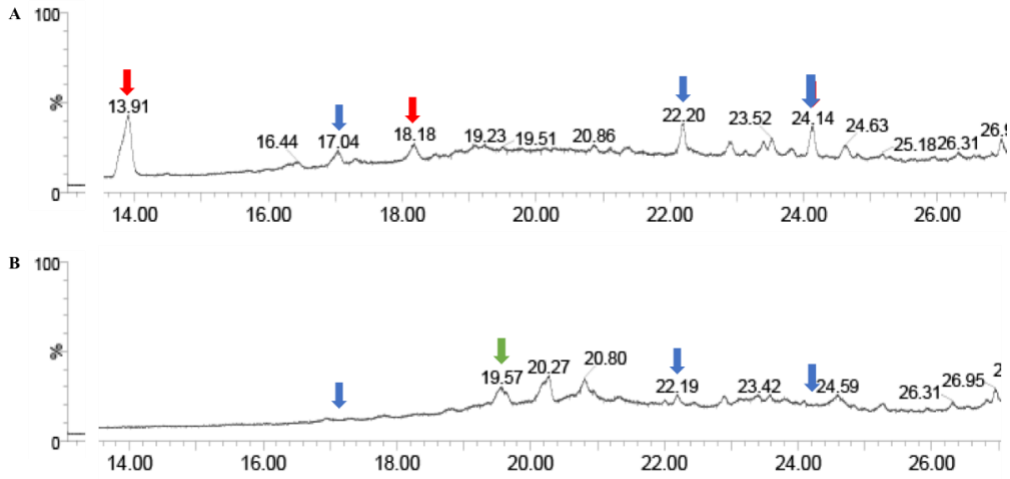

***Figure 2.12**. Negative ion mode LCMS chromatogram. A) chromatogram from fraction 1. B) chromatogram from fraction 2.*

***Table 2.9**. Most likely molecular formulae predicted for peaks of interest.*

Peak	Fraction found (ionisation mode)	Most likely molecular formula
13.90	1 (Pos, Neg)	$C_4H_{10}NO_6$
17.04	1 (Pos, Neg), 2 (Pos)	$C_5H_5NO_2S_2$
18.18	1 (Neg)	$C_{28}H_{48}N_3O_{13}$
19.57	2 (Pos, Neg)	$C_{22}H_{42}O_{10}$
22.20	1 (Neg), 2 (Neg)	$C_{10}H_{25}N_6O_4$
24.14	1 (Pos, Neg), 2 (Pos)	$C_{20}H_{38}NO_{11}$

Molecular formulae of peaks of interest (Table 2.9) were queried using the online databases Reaxys and ChemSpider. There were 0 hits for $C_4H_{10}NO_6$, $C_{28}H_{48}N_3O_{13}$, $C_{10}H_{25}N_6O_4$ and $C_{20}H_{38}NO_{11}$ on either database. However, there were 35 hits for $C_5H_5NO_2S_2$ and 15 hits for $C_{22}H_{42}O_{10}$ on the Reaxys database, and 45 hits for $C_5H_5NO_2S_2$ and 10 hits for $C_{22}H_{42}O_{10}$ on the ChemSpider database. One compound showing as a hit for $C_{22}H_{42}O_{10}$, 16-(β-D-Glucopyranosyloxy)-2,15-dihydroxyhexadecanoic acid (Figure 2.12), stood out as being a strong candidate for being one of the potential active components of SF in Fraction 2, owing to its similarity to previously identified sexual hormones (e.g. *psi* factors; Champe and el-Zayat, 1989 and Mazur et al., 1991)) as a dihydroxy- fatty acid and the presence of a sugar moiety (Alison Ashby, unpubl. data).

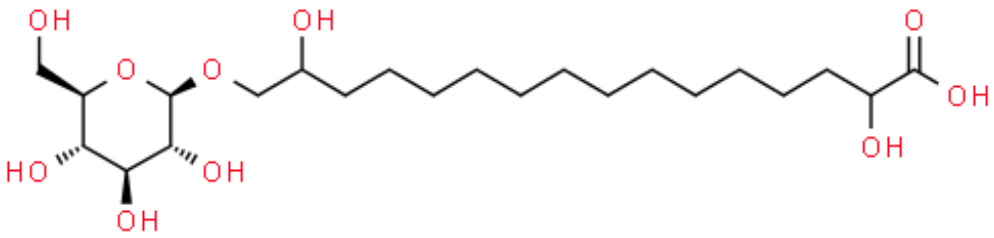

***Figure 2.12**. Chemical structure of 16-(β-D-Glucopyranosyloxy)-2,15-dihydroxyhexadecanoic acid ($C_{22}H_{42}O_{10}$), possible compound responsible for peak at ~19.57 on LCMS chromatogram of Fraction 2 and a potential active component of SF.*

2.3.7 SF does not influence expression of the *MAT1-1* master regulator of sex, *MAT1-1-1* (*pad1*)

qRT-PCR was used to determine whether SF influences the expression of the *MAT1-1* master regulator of sexual reproduction, *MAT1-1-1* (*pad1*). Expression in cultures treated with SF (25 µg mL^{-1}) was slightly increased compared to control conditions, but not significantly (Figure 2.13) (1.2 fold, Student's t-test with Welch's correction, t = 2.053, df = 3.96, p = 0.1).

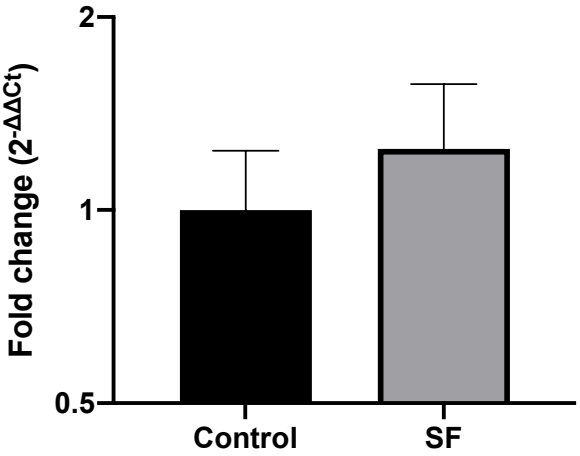

***Figure 2.13**. Expression of* MAT1-1-1 *(*pad1*) in cultures treated with SF. Expression was statistically unchanged compared to control conditions (Student's t-test with Welch's correction, p > 0.05). Error bars represent SEM. n = 3*

2.3.8 Transcriptomic changes in *Pyrenopeziza brassicae* resulting from exposure to SF

Unfortunately, owing to the understandable prioritisation of COVID-19 sequencing by Nottingham DeepSeq, the results of this transcriptomics experiment have not been received by the thesis submission date.

2.4 **Discussion**

This study supports the findings of Ilott et al. (1986) and Siddiq et al. (1990) that a hormonal factor(s) (known as 'SF') is produced by sexually reproducing cultures of *Pyrenopeziza brassicae* that has the ability to repress asexual sporulation and induce the formation of sterile apothecia in single isolates of

this species. In addition, several candidate compounds which may be active constituents of SF were identified, the most promising of which is 16-(β-D-Glucopyranosyloxy)-2,15-dihydroxyhexadecanoic acid. Finally, transcriptomics was due to be performed on cultures of *P. brassicae* exposed to SF with the aim of revealing genes involved in the asexual and sexual cycle of this species (study ongoing).

2.4.1 Bioassay of SF activity and partial purification

Asexual sporulation was repressed to less than 3 % of that observed in control conditions in cultures of both mating type when treated with crude SF at concentrations as low as 25 µg mL^{-1} (Figure 2.7). Previously, a comparable level of repression was not achieved at lower than a crude SF concentration of 1,000 µg mL^{-1} (Siddiq et al., 1990). This is likely because of the differences in extraction methodology between this work and that employed by Siddiq et al. (1990), in which ethyl acetate was used in this study and methanol used in the latter. Interestingly, SF extraction using ethyl acetate was used in the later publication by Ashby (1998), which suggests that this solvent was found to be more efficient in extracting SF by the latter author. As ethyl acetate is less polar than methanol, it favours the solving of less polar compounds (e.g. lipids) over more polar compounds which would solve better in methanol. It is possible that the active compound(s) within crude SF are similar to the *psi* factors of *Aspergillus nidulans* which are oxylipins (Champe and el-Zayat, 1989; Mazur et al., 1991), and therefore ethyl acetate probably extracted more of these compound(s) than in the previous work using methanol.

A fractionation approach using HPLC was used to attempt to purify the active component of crude SF. This approach has previously been successful in identifying active components of crude solvent extractions from fungi (Patkar et al., 2015). After several rounds of fractionation coupled with confirmation of fraction activity using bioassays, two fractions were identified that both repressed asexual sporulation (Figure 2.10). interestingly, Fraction 2 but not Fraction 1 also induced the formation of sterile apothecial structures (Figure

2.10c). One possible explanation for this is that both fractions contain a single active compound, but Fraction 2 contains more than Fraction 1. It may be that a threshold level of active compound must be reached before triggering the induction of sexual structures, but low amounts (as may be present in Fraction 1) are still able to repress asexual sporulation. In work studying *psi*-factor in *A. nidulans*, the number of cleistothecia induced by *psi*-factor was consistent in fungal tissue up to 20 mm away from a *psi*-factor-soaked disc, but in distances beyond this, the number of cleistothecia reduced in a non-linear fashion (Champe et al., 1987). This suggests that a threshold level of hormone is required to trigger sexual morphogenesis, which is not reached at distances beyond 20 mm from the *psi*-soaked disc. Sensitivity assays with the purified component of SF may act to determine whether this is the case for *P. brassicae*. Another possibility is that SF consists of two or more active components, each with slightly different biological activity as seen with the various *psi* factors of *A. nidulans* ((Mazur et al., 1991, 1990). Thus, it is possible that there is a separate compound in Fraction 1 which represses formation of sexual structures, similar to *psi*Aα (Champe and el-Zayat, 1989). The inability of the mixed Fraction 1:Fraction 2 extract to induce sterile apothecia (Figure 2.10d) may provide evidence for this theory, with different components affecting the bioactivity of each other, although it is also possible that a single active compound is simply diluted by mixing the fractions and hence the threshold for induction of sterile cleistothecia is no longer achieved.

2.4.2 Putative identification of 16-(β-D-Glucopyranosyloxy)-2,15-dihydroxyhexadecanoic acid as an active component of SF

Fraction 1 and Fraction 2 were analysed using LCMS and molecular formulae were calculated for compounds responsible for causing peaks of interest (See Section 2.3.6). This led to the identification of $C_{22}H_{42}O_{10}$ which was responsible for a prominent peak found in Fraction 2 (Figure 2.11; Figure 2.12). Querying this molecular formula on online databases brought up matches with several compounds, of which 16-(β-D-Glucopyranosyloxy)-2,15-dihydroxyhexadecanoic acid showed promise for being an active compound.

This compound contains a dihdroxylated fatty acid (2,15-dihydroxyhexadecanoic acid) as a backbone, similar to *psi*Aα (5,8-dihydroxyoctadecadienoic acid) but differing in the backbone length (C16 not C18) and the positions of the hydroxyl groups (Mazur et al., 1991). In addition, 16-(β-D-Glucopyranosyloxy)-2,15-dihydroxyhexadecanoic acid contains a sugar moiety, which was implicated as forming part of an active compound in previous attempted SF purification work (Alison Ashby, unpubl. data). In the work by Ashby, partially purified SF was analysed using Thin Layer Chromatography (TLC) and a band which had bioactivity was visualised when stained with anthrone, indicative of a sugar moiety.

Glycolipids are produced by many different fungal species and have been shown to have antifungal activity (Abdel-Mawgoud and Stephanopoulos, 2018). For example, ustilagic acid, a glycolipid produced by *Ustilago maydis* and other basidiomycete yeasts, has antifungal activity against a number of yeast and filamentous fungal species (Kulakovskaya et al., 2005; Teichmann et al., 2010). However, there is to the best of our knowledge no evidence in the literature for glycolipids having a role in fungal asexual or sexual development. 16-(β-D-Glucopyranosyloxy)-2,15-dihydroxyhexadecanoic acid, then, would be the first example of a glycolipid involved in fungal sexual development. Although this compound is an exciting prospect, it is important to note a couple of things. Firstly, it is currently unknown whether the compound which is responsible for the peak at ~19.57 in Fraction 2 is in fact 16-(β-D-Glucopyranosyloxy)-2,15-dihydroxyhexadecanoic acid, as mass spectral data does not give any structural information. Secondly, a completely pure fraction containing $C_{22}H_{42}O_{10}$ was not obtained in this study, and it is also possible that one or more of the compounds in Table 2.9, or even compounds which could not be detected in this analysis, play some role in initiating the developmental responses brought about by the fractions. Therefore, total purification and Nuclear Magnetic Resonance to give structural information, coupled with bioassays to check for activity will be required to definitively determine that

16-(β-D-Glucopyranosyloxy)-2,15-dihydroxyhexadecanoic acid is a principal active component of SF.

2.4.3 Effect on Lineage 2 *Pyrenopeziza brassicae* (*Pyrenopeziza cascadia*)

SF was tested for bioactivity on isolates from the closely related North American "Lineage 2" of *P. brassicae* to determine whether the extract had any effect on these isolates. After 15 days, there was no darkening, repression of asexual sporulation or induction of sterile apothecia observed in any of the cultures treated with SF (Figure 2.9). This is perhaps unsurprising as SF appears to act by inducing the sexual cycle in *P. brassicae*, whereas no sexual state (teleomorph) has yet been identified in Lineage 2 (Carmody et al., 2020). Although an expected 1:1 ratio of *MAT1-1*:*MAT1-2* was found in the isolates screened by the authors, it was not possible to induce a sexual cycle *in vitro* under various conditions tested (Carmody et al., 2020). This may be because integral machinery required for sexual reproduction has been mutated or lost in this lineage (Dyer and O'Gorman, 2011; Dyer and Kück, 2017). The inability of the Lineage 2 isolates tested in this study to respond to SF could be due to a mutation in an SF receptor. Consistent with this hypothesis is that certain *P. brassicae* mutants were previously generated that were unable to respond to SF (Siddiq et al., 1990). Therefore it would be interesting to determine the specific mutations in these *P. brassicae* isolates to ascertain whether the same mutations are found in the Lineage 2 isolates.

Another possible reason for Lineage 2 isolates being unresponsive to SF is that Lineage 2 actually represents a separate distinct species, putatively named *Pyrenopeziza cascadia* (K. King, Rothamsted Research, *pers. comm.*). However, even if this is the case it is possible that SF may influence the development of Lineage 2, or indeed other species, in ways beyond asexual and sexual reproduction. For example, *psi*Cα (5,8-DiHODE), which acts similarly to SF in that it represses asexual sporulation and induces the formation of cleistothecia *in Aspergillus nidulans*, induces hyphal branching in *A. fumigatus* and appressorium formation in *Magnaporthe grisea* (Niu et al., 2020). Testing

purified SF on diverse fungi may serve to improve our understanding of cross-species and cross-genera developmental signalling molecules. Indeed, preliminary studies by Chamberlain et al. (1995) demonstrated the bioactivity of lipid extracts from both mated and control cultures of *P. brassicae* on sexual development in a series of 30 ascomycete species with different reproductive modes.

2.4.4 qRT-PCR and RNAseq studies of SF-induced gene expression

Despite *P. brassicae* being the major fungal disease threat to *Brassica napus*, causing over £160 million in annual yield losses in the UK by infection of this crop and other *Brassica* plant species (Dewage et al., 2018), a genome sequence has until now been unavailable. This study was due to make use of the recent unpublished *P. brassicae* genome (C. S. K. Dewage, Y. Huang, B. Fitt, University of Hertfordshire, unpubl. data) to interrogate the molecular pathways and genes involved with asexual and sexual reproduction in this species. Unfortunately, owing to the understandable prioritisation of COVID-19 sequencing by Nottingham DeepSeq, the results of this transcriptomics experiment have not been received by the thesis submission date. Therefore, this work is still in progress but should hopefully serve to contribute to the knowledge of asexual and sexual reproduction in *P. brassicae* once data becomes available.

Perhaps surprisingly, results from pilot qRT-PCR work suggest that SF has no impact on the expression of one the major regulators of sex, *MAT1-1-1*. However, it may be that SF acts on genes downstream of *MAT1-1-1* to illicit its effects, and the *MAT* locus is not involved in SF signal transduction. It will be intriguing to determine whether there are expression changes in any of the other *MAT* genes found in *P. brassicae* once transcriptomic data is made available.

2.4.5 Use in disease control and management

The confirmation that SF can repress asexual sporulation makes the active compound(s) promising disease control agents, because *P. brassicae* spreads predominantly in Spring and Summer via splash dispersal of conidia (Gilles et al., 2001c, 2000). In addition, the disease load (fungal biomass) at the end of a harvest is related to the severity of disease in autumn because higher densities of fungi make it more likely that sexual reproduction will occur on crop debris post-harvest, thus increasing the amount of ascospores that can be released to cause initial infections in Autumn (Gilles et al., 2001c). Limiting asexual sporulation could therefore not only prevent disease spread and crop damage during the Spring and Summer of one cropping season, but also dampen the initial severity of infection in the next. Repression of asexual sporulation is an already established method of disease control, seen in the Imola cultivar of *B. napus* (Boys et al., 2012; Bradburne et al., 1999), and so it is likely that if SF has a viable effect *in planta* it would be useful in controlling disease by suppressing asexual sporulation and therefore spread of disease, although further testing is required beyond the *in vitro* assays used in this study.

Interestingly, the mechanisms involved with the “no sporulation” phenotype of *B. napus* cv. Imola are unknown. It is possible that this is orchestrated by cross-kingdom lipid signalling (Fischer and Keller, 2016). The same or similar compounds have been shown to be produced by plants and fungi which affect immune responses or development in each other (Battilani et al., 2018; Patkar et al., 2015; Scarpari et al., 2014). *B. napus* cv. Imola may therefore produce SF or an analogous compound which acts to repress asexual sporulation of *P. brassicae* residing in the subcuticular space. Metabolomic profiling of this cultivar compared to cultivars which do not show the “no sporulation” phenotype could be used to test this theory.

2.4.6 Concluding remarks

In summary, this work has expanded on the work of Ilott et al. (1986) and Siddiq et al. (1990) and given new insights. First, the existence of a lipid fraction from sexually reproducing cultures with bioactivity in repressing asexual

reproduction and inducing sexual development was substantiated, despite this previous work being overlooked for several decades. Second, modern separation methods were applied, not previously available to the previous researchers. This led to the potential identification of a novel sexual hormone from *P. brassicae*, 16-(β-D-Glucopyranosyloxy)-2,15-dihydroxyhexadecanoic acid, of one possible candidate having the ability to repress asexual sporulation and induce the formation of sexual structures. Lastly, work is currently ongoing to assess transcriptional changes induced by SF exposure, which will hopefully reveal genes involved with asexual and sexual development in *P. brassicae.*

Further studies are now required to fully isolate and characterise the active compound(s) within SF. Once fully characterised, attempts could be made to synthesise the active compound(s) and to test the efficacy for repression of asexual sporulation *in planta*, which may lead to a novel method to control fungal disease in important *Brassica* crop species. The SF component(s) might also have activity against a broader range of fungal pathogens, a very enticing prospect.

Chapter 3 - Investigation of Enzyme Activity and Secondary Metabolite Production in Sexual Progeny of *Penicillium roqueforti*

3.1 Introduction

Sexual reproduction has been used for the improvement of crops and livestock for thousands of years. Organisms with extreme or desirable characteristics can be selectively bred with the aim of producing offspring with the desired phenotypes. There is also the possibility of 'transgressive segregation' whereby genome-wide recombination during the sexual cycle can lead to progeny with unexpected improvement(s) in traits of interest (Rieseberg et al., 1999; Ashton and Dyer, 2016). Examples of the outcomes of this process, known as artificial selection or selective breeding, include improved meat yields in cattle (Wu et al., 2018) and chicken (Rubin et al., 2010), and improved flavours in fruits such as peach (Yang Yu et al., 2018) and cacao (Cornejo et al., 2018). Despite the obvious merits of selective breeding, using this process for improvement of industrially important microbes such as yeast and filamentous fungi has often been overlooked (Steensels et al., 2019).

3.1.1 Sexual reproduction for strain improvement of industrially relevant fungi

Many species of filamentous fungi are used in industry for the production of food stuffs, enzymes and secondary metabolites. Historically, many of these species were considered to be asexual and hence there has been a reliance on the use of single clonal strains (Dyer and O'Gorman, 2011). However, over the past decade or so several industrially relevant fungal species have had sexual cycles discovered, opening up the possibility of strain improvement via sexual reproduction (Böhm et al., 2013; Horn et al., 2013; Seidl et al., 2009). Sexual reproduction in these fungal species can be used to generate progeny which produce higher yields of desired products (Ashton and Dyer, 2016). Sexual

reproduction could also be used to cleanse strains of deleterious mutations which may have accumulated as a result of strain improvement by classical mutagenesis (Ashton and Dyer, 2016).

Penicillium chrysogenum (*P. rubens*), the famous producer of the antibiotic penicillin, is an example of a species that was long thought to be asexual. Evidence for "cryptic sexuality" was presented by Hoff *et al*. (2008) who found that the *MAT* genes of this species were transcriptionally expressed and homologues of pheromone and pheromone receptor genes (See Section 2.1) were present in the genome. A sexual cycle was subsequently induced by growing isolates of complementary *MAT* type at opposite sides on a plate of oatmeal agar supplemented with biotin, followed by incubation for 5 weeks at 20 °c in the dark (Böhm et al., 2013). Owing to this discovery, it is now possible for sexual reproduction to be used to produce isolates with improved characteristics such as increased penicillin production (Dahlmann et al., 2015).

Another example of an industrially important fungus in which sexual reproduction was relatively recently discovered is *Trichoderma reesei*, a species well known for the production of cellulase enzymes, which has been exploited by the biotechnology industry (Seidl et al., 2009). A sexual cycle was first reported by Seidl *et al*. (2009) who induced the formation of perithecia by growing isolates of opposing *MAT* type on opposite sides on a plate of 3 % malt extract agar in 12-hour light-dark cycles at 20 – 22 °c. Subsequent studies of sexual reproduction in this species has uncovered a number of interesting aspects. This includes the discovery that sexual machinery is important for enzyme production (Tisch et al., 2017; Zheng et al., 2017) and the presence of a novel pheromone peptide deviating from the canonical **a** and α forms (Schmoll et al., 2010). Sexual reproduction has already been used to produce progeny with higher xylanase producing capacity (Chuang et al., 2015)

Other examples of species in which sexual reproduction has been recently discovered and could be used for strain improvement are the citric acid

producer *Aspergillus tubingensis* (Horn et al., 2013; Olarte et al., 2015) and the fungus used in blue cheese production, *Penicillium roqueforti* (Swilaiman, 2013; Ropars et al., 2014)

3.1.2 Sexual reproduction in *Penicillium roqueforti*

Penicillium roqueforti is a saprotrophic fungus commonly found in soil, silage and as a food spoilage organism (Pitt and Hocking, 2009). This species is best known as the mould used to produce blue cheeses such as Stilton, Roquefort and Gorgonzola, some of which have been produced for over a thousand years (Cantor et al., 2017). Phylogenetic analysis has revealed that *P. roqueforti* strains which are used for cheese production form two distinct populations, separate from non-cheese (e.g. silage) strains (Dumas et al., 2020). As well as providing evidence for two domestication events, this also indicates that all the strains used in blue cheese production today likely originate from a few clonal lineages (Dumas et al., 2020). This lack of genetic diversity amongst cheese production strains was seemingly fixed owing to the reliance on asexual propagation as a result of there being no identified sexual cycle.

Indirect evidence that sexual reproduction was possible was first presented from PhD studies of Eagle (2009) at the University of Nottingham who found that *MAT1-1* and *MAT1-2* amplicons could be obtained from different isolates of *P. roqueforti*, consistent with the presence of a heterothallic breeding system. In subsequent studies at Nottingham, Swilaiman (2013) was then able to amplify and clone entire *MAT1-1* and *MAT1-2* idiomorph regions from different isolates of *P. roqueforti,* and show a near 1:1 ratio of complementary *MAT1-1* and *MAT1-2* isolates in a collection of over 70 food production and environmental isolates. In parallel, evidence that sexual reproduction may be occurring or have occurred recently in the *P. roqueforti* population was presented by the French group of Ropars *et al*. (2012). Firstly, It was shown that both *MAT* types were present in all subsets of strain collections tested and that in 2/3 subsets there was an approximate 1:1 segregation of *MAT1-1*:*MAT1-2* idiomorphs. Secondly, all genes which are considered important for

meiosis in the sexually reproducing *Neurospora crassa* were identified in the *P. roqueforti* genome. These genes were shown to be subject to strong purifying selection which suggests they are functional and in active use. Finally, footprints of repeat-induced point mutation (RIP), a process which only occurs during meiosis, were also found in the *P. roqueforti* genome. Together, these lines of evidence lead to further studies which attempted to induce sexual reproduction under laboratory conditions (Swilaiman, 2013; Ropars et al., 2014). A sexual cycle was successfully induced by both the Nottingham and French groups when isolates of opposing *MAT* type were inoculated at opposite sides on a plate of oatmeal agar and incubated under very specific growth conditions (Swilaiman, 2013; Ropars et al., 2014). Sexual structures including cleistothecia (the characteristic fruiting body of sexual *Penicillium spp*. and *Aspergillus spp*.), asci and ascospores were observed (Figure 3.1) and genotyping analysis revealed that progeny growing from the ascospores showed evidence of recombination, thus confirming sexual reproduction had occurred.

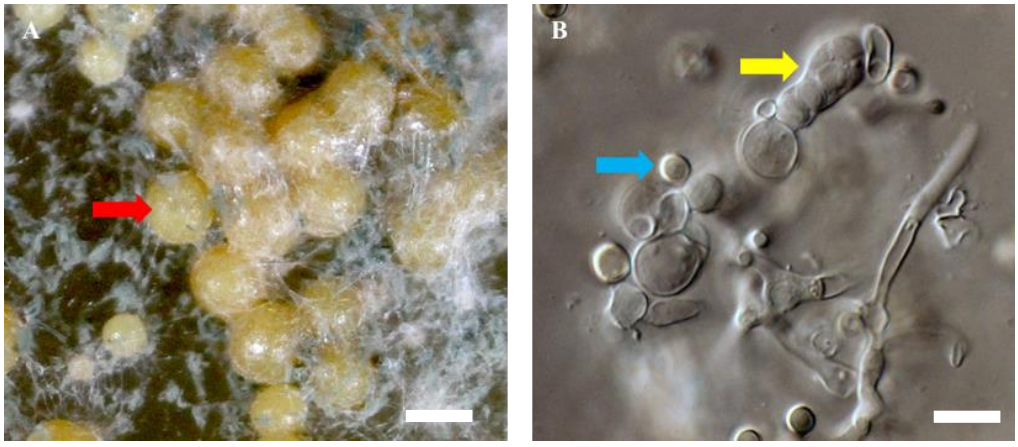

Figure 3.1. *Sexual structures from P. roqueforti sexual cross. A) Cleistothecia (red arrow). Scale bar = 300 µm. B) Asci (yellow arrow) containing ascospores (blue arrow). Scale bar = 50 µm.*

3.1.3 Sexual reproduction for strain improvement of *Penicillium roqueforti*

Sexual reproduction could be used to cleanse clonal cheese production strains of any deleterious mutations that may have arisen as a result of continuous asexual reproduction (Muller, 1964). However, perhaps the main practical application for inducing sexual reproduction in *P. roqueforti* would be to

generate progeny with novel desirable characteristics such as altered enzymatic activity (with subsequent impact on production of flavour volatiles), secondary metabolite production or growth rate. This could be of major interest to blue cheese manufacturers.

3.1.3.1 Enzymatic activity

Aside from the eponymous blue-green veins found in blue cheese, formed as a result of fungal asexual sporulation, the main contribution of *P. roqueforti* to blue cheese production is the unique texture and flavour generated via its enzymatic activities, in particular proteolytic and lipolytic activities (Cantor et al., 2017). The overall levels of proteolysis and lipolysis by *P. roqueforti* are vital for the characteristics of the final blue cheese product.

Proteolysis by *P. roqueforti* is responsible for the hydrolysis of casein micelles found within milk. These contain four major types of related casein phosphoproteins, with the main constituents being α_{s1}-casein and β-casein, but with α_{s2}-casein and κ-casein also present (Ardö et al., 2017). Proteolysis is important for the gradual breakdown in structure of the cheese during ripening, yielding characteristic creamy to crumbly blue cheese textures (Ardö et al., 2017). Casein hydrolysis produces peptides and amino acids which, although only having a minor influence on background flavour themselves, are used as substrate for other microorganisms that convert them into more important flavour compounds (Yvon and Rijnen, 2001). The overall proteolysis of casein can have a negative effect on flavour due to certain peptides having a bitter taste as a result of the presence of a high proportion of hydrophobic amino acids such as leucine, proline and phenylalanine. However, these can be hydrolysed to non-bitter peptides and amino acids by the action of peptidases, so the overall accumulation of bitter peptides depends on the relative rates of production and degradation during ripening (Ardö et al., 2017).

Lipolysis by *P. roqueforti* is responsible for the production of the majority of the flavour compounds found within blue cheese (Coghill, 1979). Triglycerides

within the milk are hydrolysed by lipases into free fatty acids (FFA) which, as well as contributing to flavour themselves, are further metabolised into other flavour compounds such as methyl ketones, secondary alcohols, esters and lactones (Figure 3.2). Of these flavour compounds, methyl ketones have been shown to be the most abundant in blue cheese, accounting for up to 75% of the total volatile flavour compounds found, and are therefore the most important to the overall flavour (Gallois and Langlois, 1990; Gkatzionis et al., 2009). Methyl ketones can be generated by both spores and mycelia of *P. roqueforti* (Chalier and Crouzet, 1998; Fan et al., 1976).

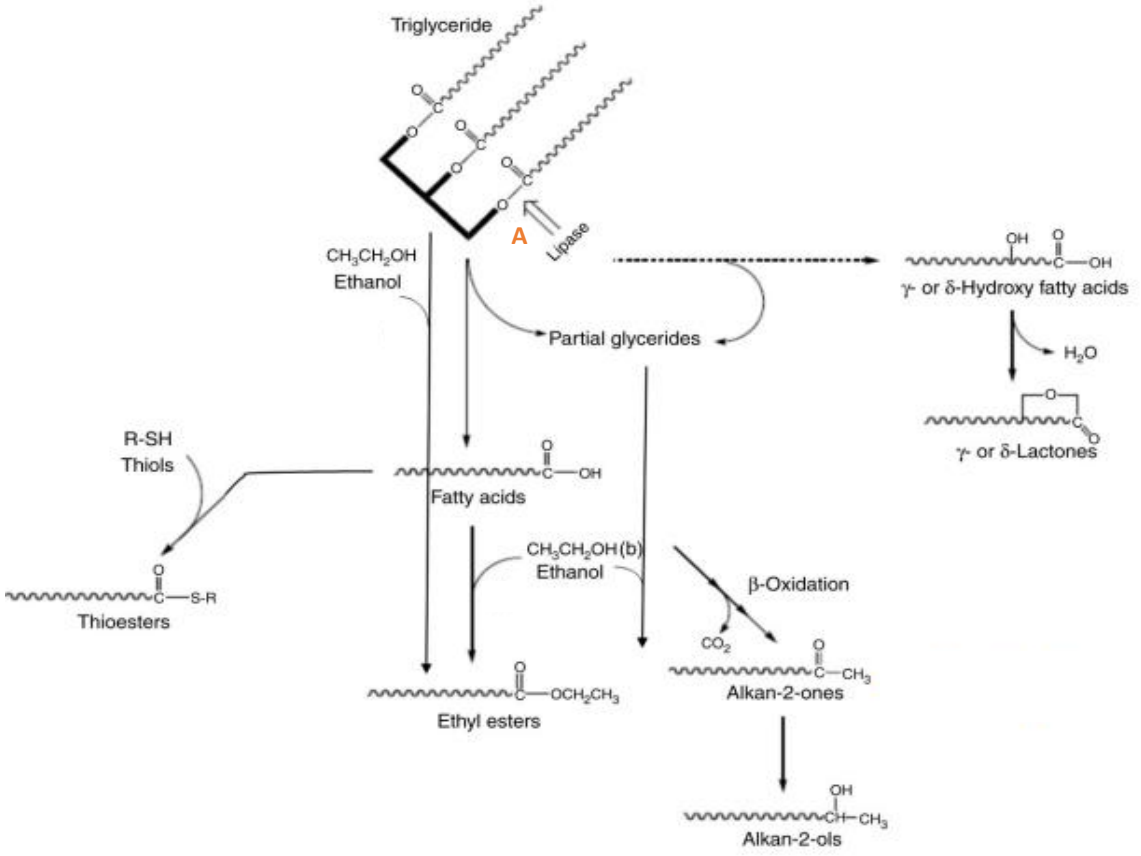

***Figure 3.2**. Edited from Thierry et al. 2017. Various flavour compounds found within cheese. Initial hydrolysis of triglycerides by lipases (A) releases fatty acids which can be converted into lactones, thioesters, ethyl esters, methyl ketones (alkan-2-ones) and secondary alcohols (alkan-2-ols) by enzymatic activity.*

Additional detail of the specific enzymes involved in the *P. roqueforti* proteolytic and lipolytic systems is given in Section 4.1.

Proteolytic and lipolytic activities been shown to vary considerably between strains (Dumas et al., 2020; Fernández-Bodega et al., 2009; Gillot et al., 2017a; Larsen et al., 1998; Larsen and Jensen, 1999). The selection of a *P. roqueforti* strain, the enzymatic activity of which will be the main driver of proteolysis and lipolysis in the blue cheese, is therefore a vital decision when considering the desired characteristics of the final product. It would be of considerable interest to ascertain whether progeny with novel levels of these enzymatic activities could be generated by sexual reproduction. If possible, sexual progeny could be used to produce blue cheeses which may in turn have novel characteristics including flavour and texture.

3.1.3.2 Secondary metabolites

P. roqueforti is able to produce several secondary metabolites, some of which are potentially harmful and some of which are potentially beneficial for human health (Martín and Coton, 2017). The best characterised of these are PR toxin, roquefortine C, mycophenolic acid and andrastin A (Figure 3.3). The level of secondary metabolite production is highly dependent upon strain (Fontaine et al., 2015b; Gillot et al., 2017a) and physiological conditions (Casquete et al., 2018; Hammerl et al., 2019).

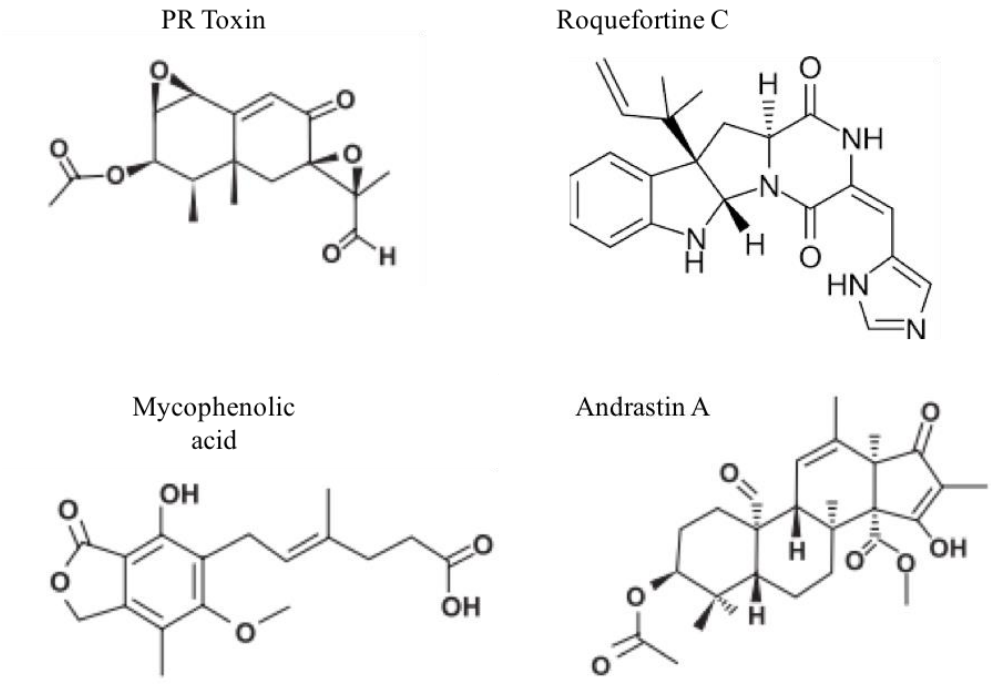

***Figure 3.3**. Secondary metabolites known to be produced by* P. roqueforti

PR toxin is a bicyclic sesquiterpene that has potent toxigenic activity against rats, mice, cats and human cells (Chang et al., 1993; Hymery et al., 2017). Despite its toxigenicity, PR toxin does not usually pose a health risk to blue cheese consumers because the physiological conditions found within the cheese cause PR toxin degradation to the less toxic PR-amide, PR-imine or PR-acid (Chang et al., 2003). The gene cluster responsible for PR toxin biosynthesis was elucidated recently and was found to be 22.4 kb in length and consists of 10 putative proteins (Hidalgo et al., 2017).

Roquefortine C is an indole alkaloid compound that has also been shown to be somewhat toxic in mice, but considerably less so than PR toxin (Arnold et al., 1978). Quantifiable levels of this mycotoxin were found in 97.7 % of blue cheeses tested in one study but the levels detected were not high enough to pose a health risk to consumers (Fontaine et al., 2015a). Roquefortine C, as well as having some toxigenic activity against mice, is a fairly potent antibacterial which may aid the fungus in interspecies competition with bacterial organisms found within blue cheese or other habited environments

(Hammerl et al., 2019). The gene cluster for roquefortine C biosynthesis is thought to have evolved via gene loss from the related meleagrin gene cluster found in other *Penicillium spp.* and is approximately 16.6 kb and consists of 4 genes (Kosalková et al., 2015).

A secondary metabolite that has the potential to be beneficial for human health is mycophenolic acid. Mycophenolic acid is a meroterpenoid compound that has a number of interesting biological activities including antibacterial, antifungal and immunosuppressive properties (Bentley, 2000). Indeed, mycophenolic acid is a commonly used drug to prevent organ rejection following kidney, heart and liver transplantations. It works by inhibiting inosine monophosphate dehydrogenase which is responsible for *de novo* synthesis of purines, the process that lymphocytes rely on for proliferation (Fulton and Markham, 1996). Mycophenolic acid has been found at detectable levels in only ~40 % of blue cheeses, and there was considerable variation in concentrations between cheeses where it was present (Fontaine et al., 2015a; Lafont et al., 1979). This attests to the variability in mycophenolic acid production levels between strains (Engel et al., 1982; Gillot et al., 2017a). This variation can be partially explained by a 174 bp deletion in one of the key genes in the mycophenolic acid gene cluster in some strains, which led to a concomitant major decrease or lack of production (Gillot et al., 2017b). However, variation in production levels is nevertheless seen between strains without this deletion, indicating that other factors must also be influential. The mycophenolic acid gene cluster is approximately 24.0 kb and contains 7 genes (Del-Cid et al., 2016; Gillot et al., 2017b).

Andrastin A is also a meroterpenoid compound that has the potential to be beneficial for human health. This secondary metabolite is a potent inhibitor of RAS farnesyltransferase, an enzyme responsible for the maturation of RAS proteins which are overactive in tumours, and thus has the potential to be used as an anti-cancer drug (Omura et al., 1996). In addition, andrastin A may also inhibit the efflux of drugs from multi-drug resistant cancer cells (Rho et

al., 1998). Almost all blue cheeses contain detectable levels of andrastin A, but the amounts are highly variable (Fernández-Bodega et al., 2009; Nielsen et al., 2005). As is the case with mycophenolic acid, this can largely be explained by the considerable variability in andrastin A production levels between strains (Fernández-Bodega et al., 2009; Gillot et al., 2017a). The gene cluster responsible for andrastin A biosynthesis is approximately 29.4 kb and consists of 10 genes (Rojas-Aedo et al., 2017).

Owing to the beneficial uses of mycophenolic acid and andrastin A, it would be of interest to determine whether sexual reproduction could be used to generate progeny with increased production levels of these secondary metabolites. Conversely, although the levels of PR toxin and roquefortine C found within blue cheese are not thought to pose a health concern to consumers, it may also be commercially beneficial to produce strains with lower production levels of these metabolites.

3.1.3.3 Growth rate and adaption to cheese environment

Phylogenetic analysis of *P. roqueforti* populations has found that some strains used for blue cheese manufacture harbour horizontally transferred genomic islands which are associated with adaption to the cheese environment (Cheeseman et al., 2014; Ropars et al., 2015). One, *Wallaby*, contains genes which may aid in competition with other microorganisms found in cheese; a second, *CheesyTer*, contains genes which are associated with assimilation of compounds found within the cheese substrate such as lactose (Cheeseman et al., 2014; Ropars et al., 2015). Strains that harbour *Wallaby* and *CheesyTer* appeared to have a greater growth rate on cheese (Dumas et al., 2020; Ropars et al., 2015). Interestingly, almost all strains used to produce the French Roquefort blue cheese variety do not harbour these genomic islands (Dumas et al., 2020; Ropars et al., 2017). Therefore, sexual reproduction could be used to introduce the genomic islands into Roquefort lineage or other *Wallaby*$^{-}$ *CheesyTer*$^{-}$ strains to improve their growth rate in cheese if this is desired.

Indeed, sexual progeny with novel combinations of enzymatic activities, secondary metabolite production levels and growth rate may be envisioned, which could lead to more variety in the type of blue cheeses able to be produced.

3.1.4 Aims

The first aim of this chapter was to investigate whether isolates of *P. roqueforti* with novel enzymatic activities could be produced via sexual reproduction. This was to be tested using simple and cheap enzymatic assays which would allow a number of parental crosses and the sexual progeny resulting from those crosses to be screened. As the enzymatic activities of an isolate are related to the qualities of a cheese, isolates with novel activities could be used to produce novel cheeses, which could be of great commercial interest.

The second aim of this chapter was to investigate whether sexual reproduction could generate progeny with altered secondary metabolite production levels. This was to be tested using solvent extractions and quantifying secondary metabolites using High Pressure Liquid Chromatography. Work would focus on the potentially harmful roquefortine C, of which it could be of commercial interest if isolates with reduced levels of production could be generated, and the beneficial mycophenolic acid, of which it could be of commercial interest if isolates with increased levels of production could be generated.

Taken together, the results of this work were anticipated to be of academic interest in terms of investigating the effects that sexual reproduction can have on phenotype, and of industrial interest in terms of ascertaining whether novel strains of *P. roqueforti* with desirable characteristics could be produced.

3.2 Methods

3.2.1 Media and chemical supplier

Media compositions are as specified in text. 1000X trace elements (per L: 40 mg $Na_2B_40_7$, 800 mg $CuSO_4$, 800 mg $FePO_4$, 800 mg $MnSO_4$, 800 mg $NaMoO_4$, 8 g $ZnSO_4$) was prepared for use in some media. All media were autoclaved at 121 °c for 20 mins. Standard chemicals for use in media were purchased from Sigma, UK unless otherwise specified.

3.2.2 Isolates and strain maintenance

Isolates were routinely cultured on slopes of Potato dextrose agar (PDA) at 28 °C. Conidia were harvested from slopes using 0.1 % Tween 80 in sterile distilled water (v/v) after 5 – 7 days growth. The resulting spore suspensions were filtered through sterile Miracloth, and spore concentration determined using an improved Neubauer haemocytometer, before diluting to a working concentration of 1 x 10^8 spores mL^{-1}. Long-term stocks of *P. roqueforti* were prepared by harvesting mixed hyphal and conidia material, which was then stored in a 10 % glycerol solution both at -80 °C and under liquid nitrogen.

Isolates used in this study can be found in Table 3.1. Parental isolates were obtained as a gift from Danisco (Denmark) or isolated from various commercial blue cheeses. Sexual progeny were isolated from crosses between parental isolates following the protocols of Swilaiman (2013). These particular parental strains were chosen because preliminary trials indicated that they presented a good range of enzymatic activities.

Table 3.1*. Isolates used in this study*

Isolate	Origin
74-88	Danisco type strain
74-92	Danisco type strain
74-118	74-88 X 74-92
74-119	74-88 X 74-92
74-120	74-88 X 74-92
74-121	74-88 X 74-92
74-122	74-88 X 74-92
74-123	74-88 X 74-92
74-124	74-88 X 74-92

74-125	74-88 X 74-92
74-126	74-88 X 74-92
74-127	74-88 X 74-92
74-128	74-88 X 74-92
74-130	Stilton cheese strain
74-133	Gorgonzola cheese strain
74-136	Blue cheese strain
74-137	Blue cheese strain
74-144	Roquefort cheese strain
74-146	Roquefort cheese strain
A3	74-130 X 74-144
A7	74-130 X 74-144
A10	74-130 X 74-144
A11	74-130 X 74-144
A12	74-130 X 74-144
A14	74-130 X 74-144
A15	74-130 X 74-144
A18	74-130 X 74-144
A19	74-130 X 74-144
A22	74-130 X 74-144
B2	74-133 X 74-146
B4	74-133 X 74-146
B6	74-133 X 74-146
B8	74-133 X 74-146
B9	74-133 X 74-146
B10	74-133 X 74-146
B11	74-133 X 74-146
B12	74-133 X 74-146
B13	74-133 X 74-146
B15	74-133 X 74-146
B19	74-133 X 74-146
B20	74-133 X 74-146
B22	74-133 X 74-146
B25	74-133 X 74-146
B29	74-133 X 74-146
C1	74-136 X 74-137
C2	74-136 X 74-137
C5	74-136 X 74-137
C7	74-136 X 74-137
C8	74-136 X 74-137

C10	74-136 X 74-137
C11	74-136 X 74-137
C14	74-136 X 74-137
C15	74-136 X 74-137
C16	74-136 X 74-137
C17	74-136 X 74-137
C20	74-136 X 74-137
C21	74-136 X 74-137
C23	74-136 X 74-137
C24	74-136 X 74-137

3.2.3 Total proteolytic activity analysis

3.2.3.1 Casein agar diffusion assay

Total proteolytic activity was determined using an agar diffusion assay adapted from published methods (Larsen et al., 1998). Conidial suspensions of *P. roqueforti* were prepared as described in Section 3.2.2. 10 µL of conidial suspension (1 x 10^8 spores mL^{-1}) was used to spot-inoculate 25 mL casein agar (0.1 % casein, 0.02 % $CaCl_2$, 0.03 % CaOH, 1.5 % agar, pH 5.8) which had been aliquoted into 9 cm Petri dishes. Cultures were incubated for 10 days at 28 °c in the light, at which point an image was taken of each individual plate from an exact height (30 cm). The area of clearance was measured by image analysis using ImageJ computer software using the macro found below (courtesy of David Foster, unpubl. data). All experiments were performed using three biological replicates. Statistical tests were performed using GraphPad Prism 8.0.

```
run("Clear Results");
roiManager("Reset");
run("Duplicate...", "title=Copy");
title=getTitle();
run("Set Scale...", "distance=1 known=0.02448 unit=mm global");
run("Set Measurements...", "area fit redirect=Copy decimal=1");


setTool("oval");
run("Select None");
 if (selectionType()!=1){
```

```
 waitForUser("Circle colony, then press OK.");
 }
run("Crop");

delimiters =".";
stringResult = split(title, delimiters);
title2=stringResult[0];
        selectWindow(title);
        s=nSlices();
        run("Duplicate...", "title="+title2+"_mask duplicate range=1-"+s+"");
        run("8-bit");
        run("Despeckle");

        //If subtract is messing with thresholding, try diff thres or subtraction
        //Answer=getBoolean("Apply Background Subtraction?");
//run("Subtract Background...", "rolling=1000000000 sliding disable stack");
                // if (Answer==true) run("Subtract Background...",
"rolling=10000000 sliding disable stack");
        //run("Remove Outliers...", "radius=50 threshold=50 which=Dark");
        //setAutoThreshold("IJ_IsoData dark");
        //run("Threshold...");                  // to open the threshold window if
not opened yet
//waitForUser("Set the threshold and press OK.");

run("Auto Threshold...", "method=Minimum");
        run("Convert to Mask");
        run("Fill Holes");
        run("Options...", "iterations=4 count=1 edm=Overwrite do=Open");

//overlay ROI on original image
setTool("wand");
//waitForUser("Click on clearance zone, then OK");
doWand(678, 630);
roiManager("add");
roiManager("Measure");
close();
selectWindow(title);
roiManager("Show All with labels");

                //run("Invert", "stack");

                //run("Analyze Particles...", "size=1000-250000000000
circularity=0.2-1.00 show=Outlines display exclude add stack");

                //selectWindow(title);
//roiManager("Show All with labels");
//run("Flatten");
```

3.2.3.2 Azocasein spectrophotometry assay

Total proteolytic activity was also determined for the parental strains 74-88 and 74-92 plus sexual progeny (See Table 3.1) using a spectrophotometry assay adapted from published methods (Larsen et al., 1998). Conidial

suspensions of *P. roqueforti* were prepared as described in Section 3.2.2. 100 µL of conidial suspension (1 x 10^8 spores mL^{-1}) was used to inoculate flasks containing 100 mL Czapek-Dox broth (3.5 % Czapex-Dox powder, 0.5 % bactopeptone, 1X trace elements, pH 4.0). Cultures were incubated for 3 days at 28 °c in the light at 150 rpm. After 3 days, cultures were filtered through sterile Miracloth to remove mycelium and 50 mL of filtrate collected in sterile falcon tubes. Falcon tubes were centrifuged at 4000 rpm for 20 min at 4 °c, after which the top 15 mL of culture filtrate was decanted and filtered through 0.45 µm filters (Sartorius, Germany). 2 mL of sterile filtrate was concentrated six times using a Savant SPD121P SpeedVac Concentrator (Thermo Scientific, USA) to give concentrated culture filtrate (CCF)

In sterile Eppendorf tubes, 100 µL of CFF was added to 500 µL of 1 % azocasein in phosphate buffered saline (pH adjusted to 5.8) and incubated for 1 hour at 35 °c, after which the reaction was stopped by the addition of 600 µL 10 % trichloroacetic acid. Reaction mixtures were then centrifuged for 10 min at 13000 rpm and the absorbance of the supernatant measured at 336 nm using a spectrophotometer (Eppendorf, UK). Absorbance was measured against a control reaction mixture containing 100 µL PBS instead of CCF. Proteolytic activity was expressed as PAu/mL CCF, with one unit of proteolytic activity defined as an increase in absorbance of 0.01 per mL of CCF. All experiments were performed using three biological replicates. Statistical tests were performed using GraphPad Prism 8.0.

3.2.4 Total lipolytic activity analysis

Total lipolytic activity was determined using a deep agar diffusion assay adapted from published methods (Larsen and Jensen, 1999). Conidial suspensions of *P. roqueforti* were prepared as described in Section 3.2.2. 10 µL of conidial suspension (1 x 10^8 spores mL^{-1}) was used to spot-inoculate 10 mL tributyrin agar (0.5 % peptone, 0.3 % yeast extract, 0.1 % tributyrin, 1.0 % agar) which had been aliquoted into sterile 15 mL falcon tubes. Cultures were incubated for 4 days at 28 °c in the light, before the depth of tributyrin

clearance was measured using engineering callipers. All experiments were performed using four biological replicates. Statistical tests were performed using GraphPad Prism 8.0.

3.2.5 Secondary metabolite extraction and quantitation by High Performance-Liquid Chromatography

1 µl of spore suspension (1 x 10^6 spores mL^{-1}) was inoculated onto 25 ml yeast extract sucrose (YES) agar (15 % sucrose, 2 % yeast extract, 15 % agar) in 9 cm Petri dishes and grown for 7 days at 28 °C in darkness. Secondary metabolite extraction and detection was performed according to Houbraken et al. (2012) with some modifications. Six agar plugs along the colony diameter were taken and submerged in 800 µl of ethyl acetate/dichloromethane/methanol (3:2:1 v/v/v with 1 % formic acid) and sonicated for 45 min. The extracts were dried in a Savant SPD121P SpeedVac Concentrator (Thermo Scientific, USA), re-dissolved in 400 µl methanol, sonicated for 10 min and filtered through 0.2 µm filters (Sartorius, Germany) into amber vials. Samples were stored at -20 °C. Extracts were obtained from the parents and selected progeny of cross 2 (74-130 x 74-144), chosen because isolates A7 and A22 are of commercial interest.

Analysis was performed by UHPLC Dionex Ultimate 3000 (Thermo Scientific) equipped with diode array detector (DAD). 5 µL samples were injected into the system and chromatographic separations were carried out as described in Table 3.2 using an Accucore C18 column (150x2.1 mm, particle size 2.6 µm, Thermo Scientific). The column temperature was held at 60 °C. The flow rate was set to 0.8 mL min^{-1} using as mobile phase: solvent A (HPLC grade water) and solvent B (acetonitrile). Secondary metabolites were identified by their retention times and UV-VIS spectra with standards. Secondary metabolites were quantified from a standard curve generated from known concentrations of pure standards. Statistical tests were performed using GraphPad Prism 8.0.

Table 3.2. HPLC conditions used for separation of secondary metabolites

Time (min)	% Solvent A [Water (0.01 % formic acid)]	% Solvent B (Acetonitrile)
0.00	85	15
5.00	35	65
6.00	0	100
7.00	0	100
8.00	85	15

3.3 Results

3.3.1 Sexual reproduction can generate progeny with altered proteolytic activity

Total proteolytic activity was determined for parental isolates of *P. roqueforti* and a selection of progeny resulting from sexual crosses between parental isolates, using a casein agar diffusion assay (Figure 3.4). Overall, the isolates tested in this study showed a considerable range of activities. The area of clearance (mm^2 ± SEM) ranged from 0 ± 0 (i.e. no clearing zone detected) through to 1048.7 ± 32.5 in Cross 1 (74-88 x 74-92) (Table 3.3); 612.7 ± 12.6 to 1156.6 ± 48.0 in Cross 2 (74-130 x 74-144) (Table 3.4); 0 ± 0 to 1071.5 ± 20.6 in Cross 3 (74-133 x 74-146) (Table 3.5) and 0 ± 0 to 1087.1 ± 31.3 in Cross 4 (74-136 x 74-137) (Table 3.6). There were significant differences between the proteolytic activities of sexual progeny from Cross 1 (One-way ANOVA, F (DFn, DFd) = 220.1 (10, 22), $p < 0.0001$), Cross 2 (One-way ANOVA, F (DFn, DFd) = 31.56 (9, 20), $p < 0.0001$), Cross 3 (One-way ANOVA, F (DFn, DFd) = 494.0 (7, 16), $p < 0.0001$) and Cross 4 (One-way ANOVA, F (DFn, DFd) = 323.9 (14, 30), $p < 0.0001$). Post-hoc analysis was used to determine whether the proteolytic activities of any sexual progeny differed significantly to that of the corresponding parental isolates. Progeny with significantly different proteolytic activities to both parental isolates (Dunnett's multiple comparisons test, $p < 0.05$) (denoted by "a,b" above bar in Figure 3.5) were considered to have altered activities. In total, 90.9 % ($n = 11$) of progeny from Cross 1 (Table 3.3; Figure 3.5a); 50.0 % ($n = 10$) of progeny from Cross 2 (Table 3.4; Figure

3.5b); 62.5 % (*n* = 8) of progeny from Cross 3 (Table 3.5; Figure 3.5c) and 46.6 % (*n* = 15) of progeny from Cross 4 (Table 3.6; Figure 3.5d) had altered proteolytic activity, showing either an increase or decrease relative to the parental isolates.

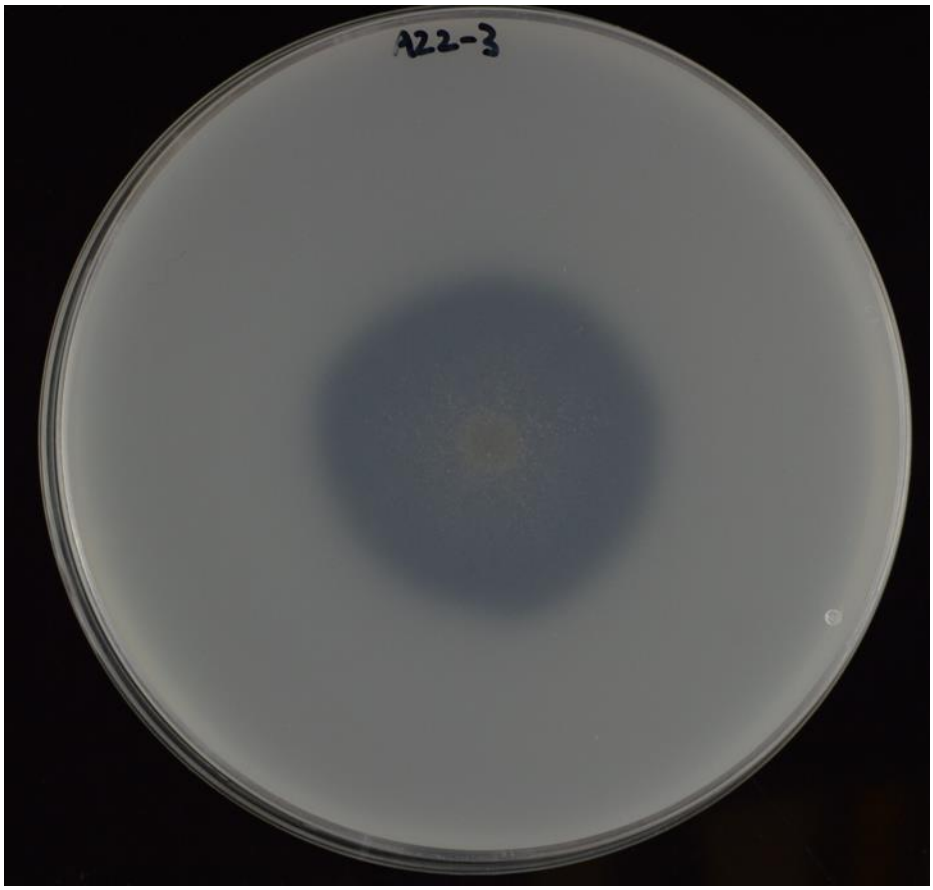

Figure 3.4. *Representative casein agar diffusion assay.*

***Table 3.3**. Total proteolytic activities of parental isolates 74-88 and 74-92 (Cross 1) and sexual progeny based on a casein agar diffusion assay. ns = not significant. ** = p < 0.01. **** = p < 0.0001 (according to Dunnett's multiple comparisons test). n = 3*

Isolate	Area of clearance (mm^2 ± SEM)	*P* value vs P1	*P* value vs P2	Altered activity?
P1 (74-88)	927.8 ± 23.7	n/a	****	n/a
P2 (74-92)	663.3 ± 7.6	****	n/a	n/a
74-118	0 ± 0	****	****	Yes
74-119	0 ± 0	****	****	Yes
74-120	0 ± 0	****	****	Yes
74-121	631.9 ± 71.8	****	ns	No
74-122	0 ± 0	****	****	Yes
74-123	0 ± 0	****	****	Yes
74-124	0 ± 0	****	****	Yes
74-125	1048.7 ± 32.5	**	****	Yes
74-126	0 ± 0	****	****	Yes
74-127	0 ± 0	****	****	Yes
74-128	0 ± 0	****	****	Yes

***Table 3.4**. Total proteolytic activities of parental isolates 74-130 and 74-144 (Cross 2) and sexual progeny based on a casein agar diffusion assay. ns = not significant. * = p < 0.05. ** = p < 0.01. *** = p < 0.001 . **** = p < 0.0001 (according to Dunnett's multiple comparisons test). n = 3*

Isolate	Area of clearance (mm^2 ± SEM)	*P* value vs P1	*P* value vs P2	Altered activity?
P1 (74-130)	906.6 ± 18.3	n/a	ns	n/a
P2 (74-144)	851.2 ± 29.2	ns	n/a	n/a
A3	1023.8 ± 14.5	ns	*	No
A7	612.7 ± 12.6	****	***	Yes
A10	838.2 ± 59.1	ns	ns	No
A11	789.7 ± 22.6	ns	ns	No
A12	781.5 ± 29.3	ns	ns	No
A14	1156.6 ± 48.0	***	****	Yes
A15	1105.1 ± 31.4	**	***	Yes
A18	639.1 ± 58.6	***	**	Yes
A19	791.6 ± 15.4	ns	ns	No
A22	1134.3 ± 27.8	***	****	Yes

***Table 3.5**. Total proteolytic activities of parental isolates 74-133 and 74-146 (Cross 3) and sexual progeny based on a casein agar diffusion assay. ns = not significant. * = p < 0.05. **** = p < 0.0001 (according to Dunnett's multiple comparisons test). n = 3*

Isolate	Area of clearance (mm^2 ± SEM)	*P* value vs P1	*P* value vs P2	Altered activity?
P1 (74-133)	719.4 ± 16.3	n/a	****	n/a
P2 (74-146)	0 ± 0	****	n/a	n/a
B6	1071.5 ± 20.6	****	****	Yes
B8	886.4 ± 2.5	****	****	Yes
B9	641.3 ± 21.9	*	****	Yes
B12	0 ± 0	****	ns	No
B14	0 ± 0	****	ns	No
B15	303.9 ± 26.5	****	****	Yes
B20	745.6 ± 3.4	ns	****	No
B22	481.9 ± 30.0	****	****	Yes

***Table 3.6**. Total proteolytic activities of parental isolates 74-136 and 74-137 (Cross 4) and sexual progeny based on a casein agar diffusion assay. ns = not significant. * = p < 0.05. ** = p < 0.01. **** = p < 0.0001 (according to Dunnett's multiple comparisons test). n = 3*

Isolate	Area of clearance (mm^2 ± SEM)	*P* value vs P1	*P* value vs P2	Altered activity?
P1 (74-136)	0 ± 0	n/a	****	n/a
P2 (74-137)	864.5 ± 14.2	****	n/a	n/a
C1	0 ± 0	ns	****	No
C2	0 ± 0	ns	****	No
C5	939.4 ± 22.6	****	ns	No
C6	756.1 ± 20.8	****	**	Yes
C7	1085.6 ± 14.8	****	****	Yes
C8	869.2 ± 24.4	****	ns	No
C12	0 ± 0	ns	****	No
C14	1045.0 ± 14.5	****	****	Yes
C15	960.3 ± 26.7	****	*	Yes
C16	1087.1 ± 31.3	****	****	Yes
C17	847.1 ± 7.6	****	ns	No
C20	897.6 ± 20.6	****	ns	No
C21	831.2 ± 44.3	****	ns	No
C23	631.4 ± 12.6	****	****	Yes
C24	627.5 ± 30.0	****	****	Yes

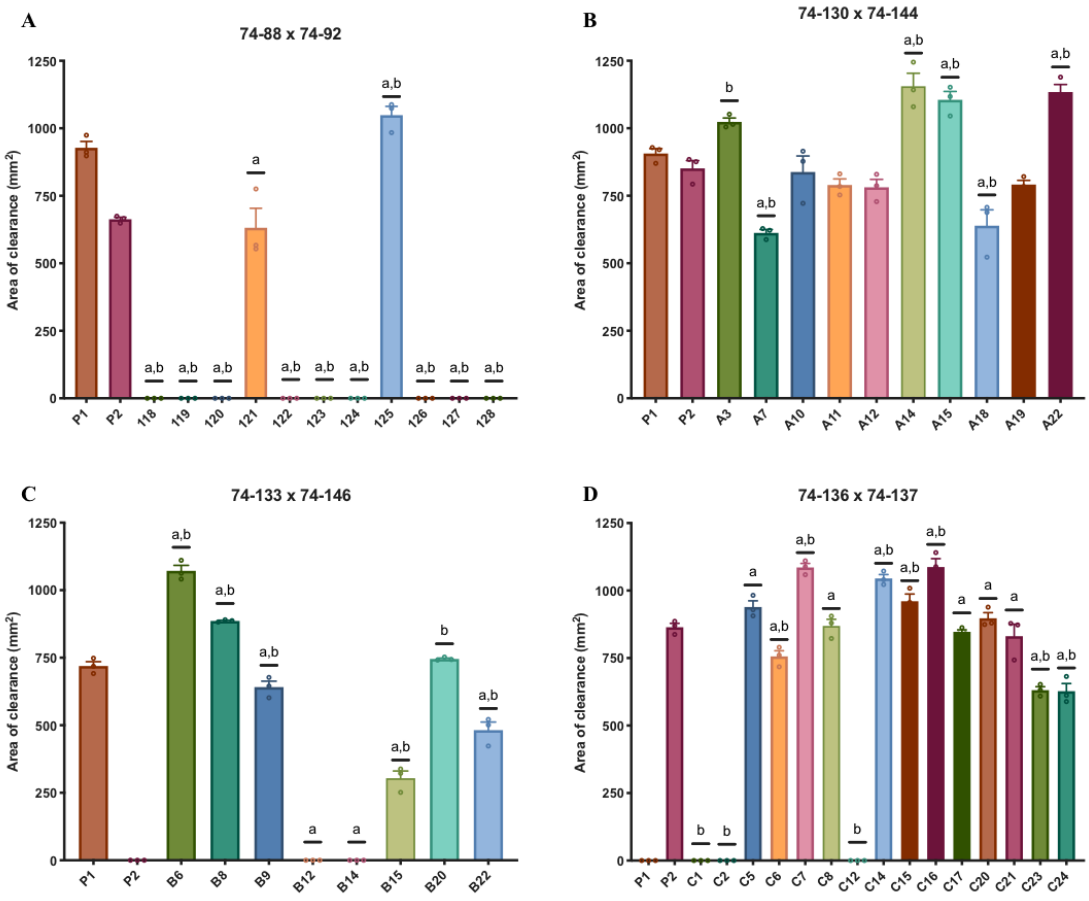

***Figure 3.5**. Total proteolytic activities of: (A) Cross 1 isolates; (B) Cross 2 isolates; (C) Cross 3 isolates; (D) Cross 4 isolates. based on a casein agar diffusion assay (see label above each figure for the respective crossing partners). **a** = significantly different to P1 (Dunnett's multiple comparisons test, $p < 0.05$). **b** = significantly different to P2 (Dunnett's multiple comparisons test, $p < 0.05$). **a,b** = significantly different to P1 and P2 (Dunnett's multiple comparisons test, $p < 0.05$). Error bars represent SEM. n = 3*

In addition, proteolytic activities of Cross 1 isolates (parents 74-88 x 74-92) were further assessed using an azocasein spectrophotometry assay. There were significant differences in proteolytic activities between sexual progeny (Welch's ANOVA, W (DFn, DFd) = 7.795 (10.00, 21.84), $p < 0.0001$). However, no sexual progeny had novel proteolytic activity (Dunnett's T3 multiple comparisons test, $p > 0.05$) (Table 3.7; Figure 3.6). Notably, all offspring showed proteolytic ability, unlike the results from the casein agar diffusion assay (Table 3.3; Figure 3.5a).

***Table 3.7**. Total proteolytic activities of parental isolates 74-88 and 74-92 (Cross 1) and sexual progeny, determined via azocasein spectrophotometry assay. ns = not significant. * = p < 0.01. ** = p < 0.01. *** = p < 0.001. **** = p < 0.0001 (according to Dunnett's T3 multiple comparisons test). n = 6*

Isolate	Proteolytic activity (PAU mL^{-1} CCF ± SEM)	P value vs P1	P value vs P2	Novel activity?
P1 (74-88)	350.3 ± 8.0	n/a	*	n/a
P2 (74-92)	216.7 ± 24.3	*	n/a	n/a
74-118	195 ± 11.4	****	ns	No
74-119	204.5 ± 17.0	**	ns	No
74-120	238.0 ± 15.0	**	ns	No
74-121	221.7 ± 14.2	***	ns	No
74-122	192.8 ± 10.3	****	ns	No
74-123	221.0 ± 17.2	**	ns	No
74-124	174.3 ± 29.7	*	ns	No
74-125	314.8 ± 12.6	ns	ns	No
74-126	233.2 ± 6.6	****	ns	No
74-127	293.2 ± 12.6	*	ns	No
74-128	237.0 ± 20.6	*	ns	No

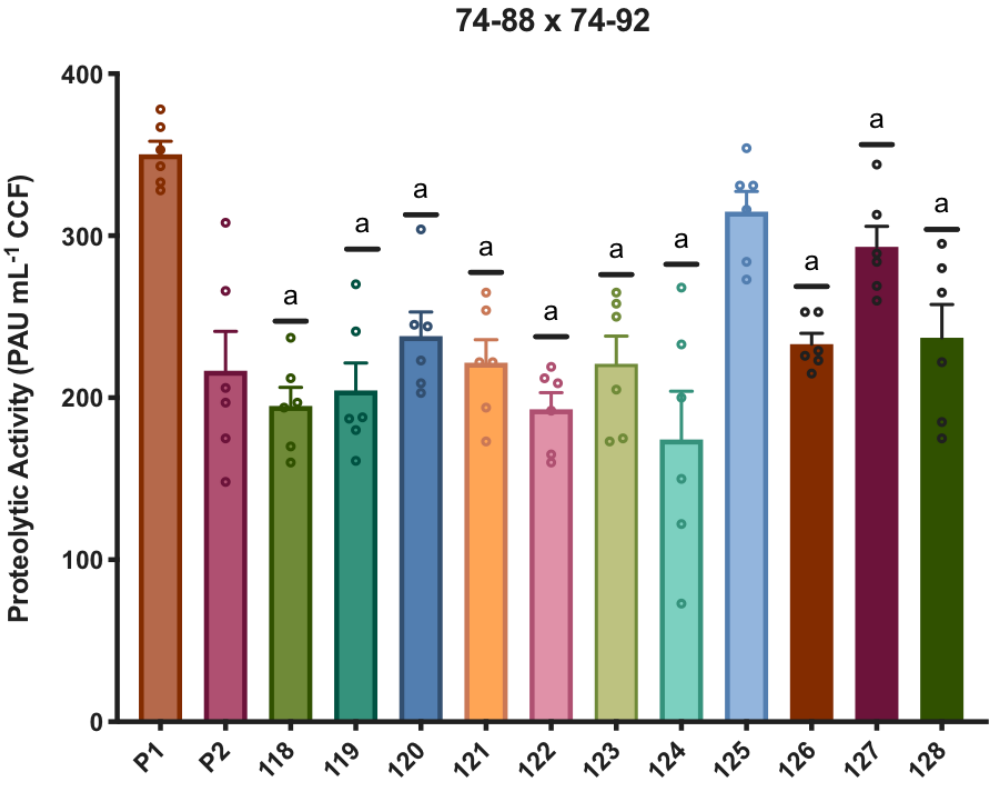

***Figure 3.6**. Proteolytic activity of 74-88 and 74-92 (Cross 1) and sexual progeny, determined via azocasein spectrophotometry assay. **a** = significantly different to P1 (Dunnett's T3 multiple comparisons test, p < 0.05). Error bars represent SEM. n = 6*

3.3.2 Sexual reproduction can generate progeny with altered lipolytic activity

Total lipolytic activity was determined for parental isolates and a selection of progeny resulting from sexual crosses between parental isolates using a tributyrin deep agar diffusion assay (Figure 3.7). Overall, the isolates tested in this study showed a considerable range of activities. Depth of clearance (mm ± SEM) ranged from 7.0 ± 0.10 to 14.3 ± 0.12 in Cross 1 (74-88 x 74-92) (Table 3.8); 6.7 ± 0.12 to 14.7 ± 0.15 in Cross 2 (74-130 x 74-144) (Table 3.9); 6.3 ± 0.10 to 13.8 ± 0.12 in Cross 3 (74-133 x 74-146) (Table 3.10) and 10.1 ± 0.53 to 16.1 ± 0.29 in Cross 4 (74-136 x 74-137) (Table 3.11). There were significant differences between the lipolytic activities of sexual progeny from Cross 1 (Welch's ANOVA, W (DFn, DFd) = 97.70 (10.00, 13.09), $p < 0.0001$), Cross 2 (Welch's ANOVA, W (DFn, DFd) = 186.3 (9.00, 12.13), $p < 0.0001$), Cross 3 (Welch's ANOVA, W (DFn, DFd) = 103.6 (14.00, 17.05), $p < 0.0001$) and Cross 4 (Welch's ANOVA, W (DFn, DFd) = 49.26 (14.00, 16.84), $p < 0.0001$) and several progeny had altered activities (Dunnett's T3 multiple comparisons test, $p < 0.05$) (denoted by "a,b" above bar in Figure 3.8). In total, 54.5 % (n = 11) of progeny from Cross 1 (Table 3.8; Figure 3.8a); 10 % (n = 10) of progeny from Cross 2 (Table 3.9; Figure 3.8b); 53.3 % (n = 15) of progeny from Cross 3 (Table 3.10; Figure 3.8c) and 33.3 % (n = 15) of progeny from Cross 4 (Table 3.11; Figure 3.8d) had novel lipolytic activity.

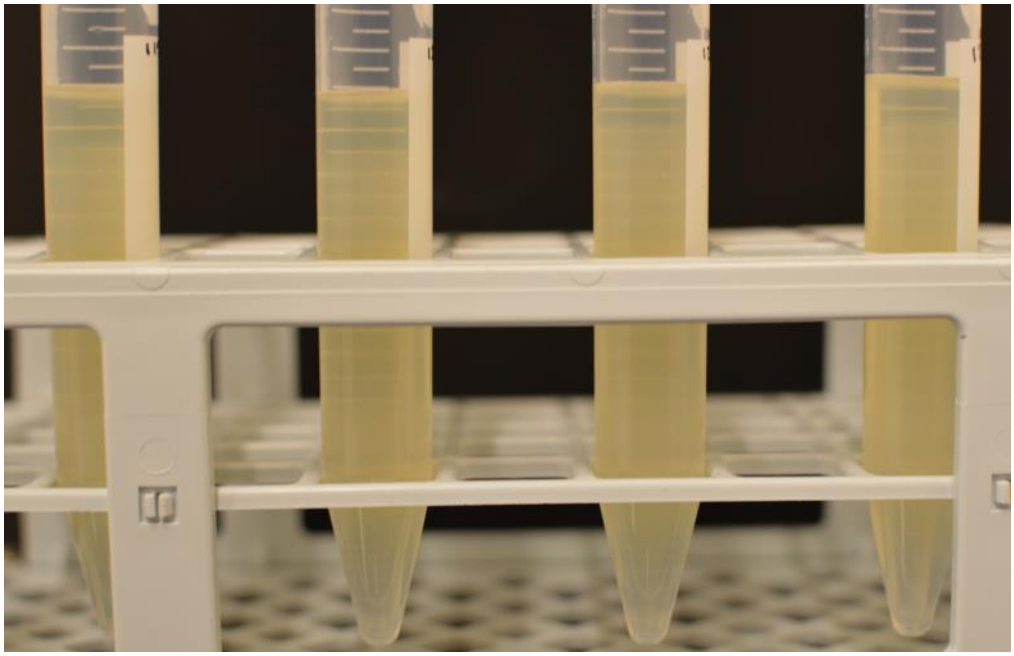

***Figure 3.7**. Representative tributyrin deep agar diffusion assay.*

***Table 3.8**. Total lipolytic activities of parental isolates 74-88 and 74-92 (Cross 1) and sexual progeny according to a tributyrin deep agar diffusion assay. ns = not significant. * = p < 0.05. ** = p < 0.01. *** = p < 0.001. **** = p < 0.0001 (according to Dunnett's T3 multiple comparisons test). n = 4*

Isolate	Depth of clearance (mm ± SEM)	*P* value vs P1	*P* value vs P2	Altered activity?
P1 (74-88)	10.8 ± 0.04	n/a	****	n/a
P2 (74-92)	7.0 ± 0.09	****	n/a	n/a
74-118	12.9 ± 0.36	ns	**	No
74-119	12.5 ± 0.30	ns	***	No
74-120	13.5 ± 0.28	*	***	Yes
74-121	8.5 ± 0.15	**	**	Yes
74-122	9.0 ± 0.33	ns	ns	No
74-123	14.3 ± 0.12	****	****	Yes
74-124	9.1 ± 0.18	*	**	Yes
74-125	8.8 ± 0.36	ns	ns	No
74-126	12.0 ± 0.12	**	****	Yes
74-127	9.1 ± 0.33	ns	*	No
74-128	8.7 ± 0.31	*	*	Yes

***Table 3.9**. Total lipolytic activities of parental isolates 74-130 and 74-144 (Cross 2) and sexual progeny according to a tributyrin deep agar diffusion assay.. ns = not significant. * = p < 0.05. ** = p < 0.01. *** = p < 0.001. **** = p < 0.0001 (according to Dunnett's T3 multiple comparisons test). n = 4*

Isolate	Depth of clearance (mm ± SEM)	*P* value vs P1	*P* value vs P2	Altered activity?
P1 (74-130)	14.7 ± 0.12	n/a	***	n/a
P2 (74-144)	12.7 ± 0.16	***	n/a	n/a
A3	14.5 ± 0.33	ns	*	No
A7	6.7 ± 0.12	****	****	Yes
A10	12.2 ± 0.20	***	ns	No
A11	12.1 ± 0.26	**	ns	No
A12	12.6 ± 0.20	**	ns	No
A14	14.4 ± 0.20	ns	**	No
A15	14.7 ± 0.15	ns	***	No
A18	11.3 ± 0.55	*	ns	No
A19	10.4 ± 0.47	*	ns	No
A22	14.2 ± 0.25	ns	*	No

***Table 3.10**. Total lipolytic activities of parental isolates 74-133 and 74-146 (Cross 3) and sexual progeny according to a tributyrin deep agar diffusion assay.. ns = not significant. * = p < 0.05. ** = p < 0.01. *** = p < 0.001. **** = p < 0.0001 (according to Dunnett's T3 multiple comparisons test). n = 4*

Isolate	Depth of clearance (mm ± SEM)	*P* value vs P1	*P* value vs P2	Altered activity?
P1 (74-133)	6.3 ± 0.10	n/a	***	n/a
P2 (74-146)	12.0 ± 0.27	***	n/a	n/a
B2	7.8 ± 0.13	**	**	Yes
B4	13.8 ± 0.12	****	*	Yes
B6	8.6 ± 0.28	*	**	Yes
B8	10.5 ± 0.48	*	ns	No
B9	12.0 ± 0.17	****	ns	No
B10	13.4 ± 0.36	**	ns	No
B11	8.7 ± 0.10	****	**	Yes
B12	8.7 ± 0.09	****	**	Yes
B13	9.0 ± 0.33	*	**	Yes
B15	10.8 ± 0.39	*	ns	No
B19	7.3 ± 0.27	ns	***	No
B20	9.7 ± 0.43	*	ns	No
B22	12.6 ± 0.20	****	ns	No
B25	8.2 ± 0.24	*	***	Yes
B29	10.4 ± 0.15	****	*	Yes

***Table 3.11**. Total lipolytic activities of parental isolates 74-136 and 74-137 (Cross 4) and sexual progeny according to a tributyrin deep agar diffusion assay.. ns = not significant. * = p < 0.05. ** = p < 0.01. *** = p < 0.001. **** = p < 0.0001 (according to Dunnett's T3 multiple comparisons test). n = 4*

Isolate	Depth of clearance (mm ± SEM)	*P* value vs P1	*P* value vs P2	Altered activity?
P1 (74-136)	16.1 ± 0.13	n/a	****	n/a
P2 (74-137)	12.2 ± 0.12	****	n/a	n/a
C1	13.0 ± 0.07	***	*	Yes
C2	10.3 ± 0.43	**	ns	No
C5	15.7 ± 0.11	ns	****	No
C7	12.6 ± 0.04	***	ns	No
C8	12.0 ± 0.22	***	ns	No
C10	13.6 ± 0.16	***	**	Yes
C11	10.0 ± 0.53	**	ns	No
C14	12.0 ± 0.16	****	ns	No
C15	13.2 ± 0.17	***	*	Yes
C16	16.1 ± 0.29	ns	**	No
C17	11.8 ± 0.25	***	ns	No
C20	13.5 ± 0.26	**	ns	No
C21	14.3 ± 0.18	**	**	Yes
C23	12.6 ± 0.39	**	ns	No
C24	14.4 ± 0.18	**	**	Yes

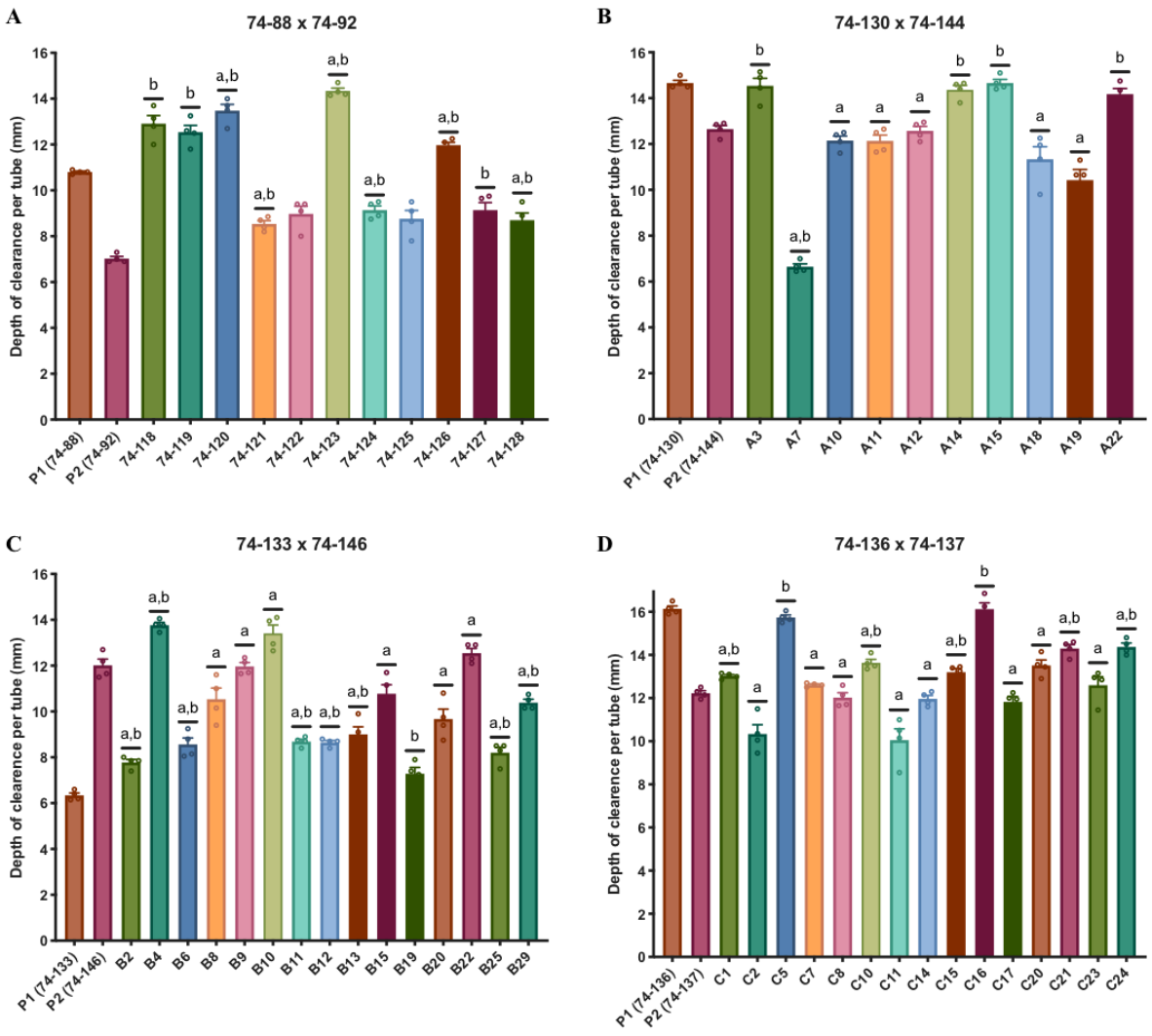

***Figure 3.8**. Total lipolytic activities of: (A) Cross 1; (B) Cross 2; (C) Cross 3; (D) Cross 4, according to a tributyrin deep agar diffusion assay (see label above each figure for the respective crossing partners). **a** = significantly different to P1 (Dunnett's T3 multiple comparisons test, $p < 0.05$). **b** = significantly different to P2 (Dunnett's T3 multiple comparisons test, $p < 0.05$). **a,b** = significantly different to P1 and P2 (Dunnett's T3 multiple comparisons test, $p < 0.05$). Error bars represent SEM. n = 4*

3.3.3 Roquefortine C production is not altered in sexual progeny

Selected parental isolates and sexual progeny were screened for secondary metabolite production. Roquefortine C was produced in quantifiable amounts by all isolates tested and production amounts varied between isolates (Table 3.12; Figure 3.9. There were significant differences between the amounts of roquefortine C produced by sexual progeny (Welch's ANOVA, W (DFn, DFd) = 8.225 (4.00, 6.99), $p < 0.01$). However, no sexual progeny produced a significantly different amount of roquefortine C to both parental isolates (Dunnett's T3 multiple comparisons test, $p > 0.05$) (Table 3.12; Figure 3.9).

Isolates were also screened for mycophenolic acid production, but no isolates were found to produce this secondary metabolite in detectable amounts under the growth conditions used (data not shown).

***Table 3.12**. Roquefortine C produced by parental isolates 74-130 and 74-144 (Cross 2) and sexual progeny. ns = not significant. * = p < 0.05 (according to Dunnett's T3 multiple comparison test). n = 4*

Isolate	RoqC produced (ng/extraction ± SEM)	*P* value vs P1	*P* value vs P2	Novel activity?
P1 (74-130)	900.9 ± 115.0	n/a	ns	n/a
P2 (74-144)	1309.2 ± 101.5	ns	n/a	n/a
A3	656.8 ± 92.6	ns	*	No
A7	1223.2 ± 24.8	ns	ns	No
A18	1186.8 ± 57.6	ns	ns	No
A19	1065.3 ± 38.3	ns	ns	No
A22	1153.4 ± 300.0	ns	ns	No

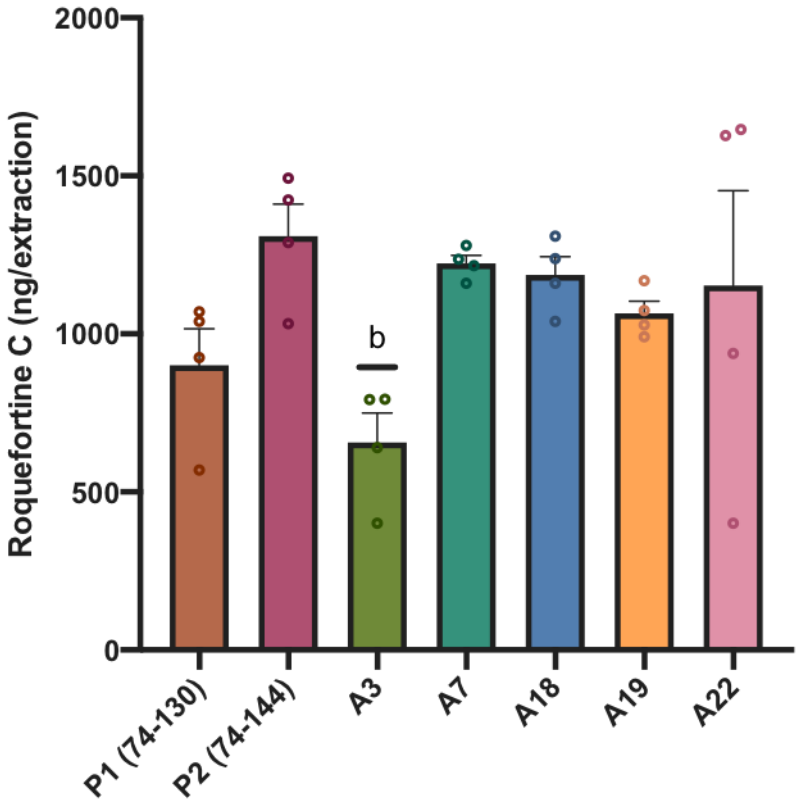

***Figure 3.9**. Roquefortine C produced by parental isolates 74-130 and 74-144 (Cross 2) and sexual progeny. **b** = significantly different to P2 (Dunnett's T3 multiple comparisons test, p < 0.05). Error bars represent SEM. n = 4*

3.4 Discussion

The discovery of a sexual cycle in *Penicillium roqueforti* by Swilaiman (2013) and Ropars *et al.* (2014) paved the way for targeted sexual crossing to be performed in this species for the first time, therefore making it possible to generate progeny with novel desirable characteristics. In this present study, it was found that isolates with altered proteolytic and lipolytic activities, the key enzymatic processes involved with blue cheese production, could be generated by sexual reproduction. Secondary metabolite production of sexual progeny was also investigated, and although no altered production levels were found in the isolates tested, the significant variation between isolates suggests that sexual reproduction could also be used to generate progeny with novel secondary metabolite-producing capacity.

3.4.1 Proteolytic activity

Proteolytic activity was tested using a casein agar diffusion assay modified from Larsen *et al.* (1998). One modification to the assay was to use agar plates rather than tubes so that clearance could be measured by ImageJ computer software rather than manually. This modification may increase the sensitivity of the assay as more subtle changes in clearance could be detected using area (mm^2) rather than depth (mm). Secondly, conidia rather than established agar plugs were used to inoculate casein agar because spores have been shown to secrete metabolic enzymes in their own right (Chalier and Crouzet, 1998). Overall, this assay proved suitable to measure differences between isolates.

All parental crosses tested in this study produced some progeny with novel proteolytic activity (i.e. activity that was significantly different to both parental isolates), but the percentage of novel progeny generated varied from 46.6 – 90.9 % depending on the cross. This variation could be due to greater phenotypic divergence of parental isolates (Hung et al., 2012). Looking solely at proteolytic activity as a phenotype, this theory is challenged as roughly the same percentage of progeny had novel activities in Cross 2 (50 %) and Cross 4 (46.6 %), even though the parental isolates of Cross 2 (74-130 and 74-146)

have much closer activities than the parental isolates of Cross 4 (74-136 and 74-137). However, it is possible that untested genetic or phenotypic divergence, such as growth rate, has some influence on the variation seen in proteolytic activity (Hung et al., 2012). In order to fully investigate whether there is link between parental divergence in proteolytic activity and progeny diversity, more crosses will need to be tested.

A surprising result was that several progeny of 74-88 X 74-92 (Cross 1) were unable to clear casein in the assay, even though both parental isolates were able to (Figure 3.5a). This phenotype has been seen in previous studies (Fernández-Bodega et al., 2009; Larsen et al., 1998). Non-clearance is most likely the result of low production of the protein AspA in these progeny. AspA is a protease which is thought to constitute the main extracellular proteolytic activity in *P. roqueforti* (see Section 4.1 for additional detail), and a lack of detectable AspA has been shown via Western blotting to correspond to no casein clearance in the *P. roqueforti* isolate CECT 2905 (Fernández-Bodega et al., 2009). Congruent with this theory, *aspA* expression of Cross 1 progeny was screened using qRT-PCR. It was found that low expression of *aspA* in both non-inducing (minimal media) and inducing (minimal media + casein) conditions was correlated with an isolates lack of ability to cause clearance in the casein agar diffusion assay (Matthew Kokolski, unpubl. data). Since a threshold level of constitutive expression is required to produce enough AspA to begin breaking down casein and release inducing peptides (Gente et al., 2001), it is reasonable to postulate that the progeny of Cross 1 tested in this study do not cause casein clearance because of reduced *aspA* expression compared to both parental isolates. Overall, this observation suggests that changes in gene expression resulting from sexual reproduction are a major factor in determining proteolytic activity in progeny. Analysis of *aspA* expression in other parental isolates and progeny is required to test this theory further.

The proteolytic activities of Cross 1 isolates were also tested using an azocasein spectrophotometry assay. This assay has been suggested to be more sensitive

than the casein diffusion assay, although reasonable correlations between the results of both assays has been seen in previous studies with *P. roqueforti* (Larsen et al., 1998). In agreement with the latter statement, the two Cross 1 isolates with highest proteolytic activity as shown by the casein agar diffusion assay (74-88 and 74-125) were also ranked highest using the azocasein assay (Table 2.3.3;Table 2.3.7). In agreement with the former statement, progeny which showed no clearance in the casein diffusion assay did show proteolytic activity using the azocasein assay. However, owing to the significant ease and less time-intensive nature of the casein diffusion assay compared to the azocasein assay, coupled with the fact that differences in proteolytic activity were successfully shown using either, the remaining crosses were assessed using only the diffusion assay.

Although the casein diffusion assay is useful in determining total proteolytic activity, it does not provide information about the contributions of individual components of the proteolytic system [e.g. asparatic protease (AspA), metalloprotease, peptidases etc. (Cantor et al., 2017)]. Results from this study and others (e.g. Fernández-Bodega et al., 2009) suggest that AspA is the main driving force of *P. roqueforti* proteolytic activity, but it would nonetheless be interesting to determine the extent to which other enzymes affect proteolysis. Indeed, rather than a clear 1:1 segregation of phenotypic activity relative to the parental isolates, as might be predicted for different variants (alleles) of *aspA* alone, a more polygenic spread of activity was seen in the offspring of most crosses consistent with the contribution of multiple genes to the proteolytic phenotype (Ashton and Dyer, 2016). Thus, further work is needed, such as determination of the expression levels of other genes which encode casein proteolytic enzymes, although to date only *aspA* has been genetically characterised (See Section 4.1). Sequencing of *aspA* from different isolates might also reveal possible allelic variation in populations of *P. roqueforti.*

3.4.2 Lipolytic activity

Lipolytic activity was tested via a deep agar diffusion assay using tributyrin as a lipase substrate (Larsen and Jensen, 1999). As with the casein agar diffusion assay, conidia (rather than the previous standard inoculum of agar plugs) were used as inoculum because of their metabolic activity (Chalier and Crouzet, 1998). Initially, attempts were made to also use the ImageJ computer software and agar plate methodology to measure area of clearance, but the software was not sensitive enough to determine the boundaries of the clearance. This is most likely because there is only a slight change in agar turbidity when tributyrin is metabolised. The original deep agar diffusion assay using tubes was therefore used and proved suitable for this study.

Progeny with novel lipolytic activities were generated from all parental crosses tested in this study, but there were differences between the percentages of novel progeny generated depending on cross (Figure 3.8). As mentioned previously, this could be because of greater divergence in lipolytic activity between parental isolates (Hung et al., 2012). In agreement with this, Cross 2 parental isolates (74-130 and 74-146), with the lowest difference in lipolytic activity of the parental isolates pairs, produced the fewest progeny with novel lipolytic activity (10.0 %) (Table 3.9). However, the difference in percentages of progeny with novel lipolytic activity generated between Cross 1 (54.5 %) and Cross 4 (33.3 %) confounds this explanation given that the parents of these crosses exhibited almost identical differences in lipolytic activity. More crosses will need to be tested to assess whether parental divergence in lipolytic activity is linked to progeny diversity.

The parental isolates tested in this study and all isolates tested by Larsen *et al.* (1999) are used for blue cheese production. Despite different isolates being tested, the ranges of lipolytic activity recorded in this study (6.3 ± 0.1 - 16.1 ± 0.3 mm) and that of Larsen *et al.* (1999) (5.0 ± 0 – 13.8 ± 0.2 mm) were fairly similar. This similarity may reflect the clonal ancestry of isolates used for blue cheese production (Dumas et al., 2020; Ropars et al., 2017). Clonal ancestry of all blue cheese production isolates except those used for French Roquefort has

been suggested based on genotypic and phenotypic evidence, including lipolytic activity which has been reported to be lower in Roquefort isolates (Dumas et al., 2020). The results of this present study appear to contradict this, as the Roquefort isolates used (74-144 and 74-146) had greater lipolytic activity than both Danisco type strains (74-88 and 74-92) and the Gorgonzola isolate (74-133) (Figure 3.8). However, it is possible that use of a lower growth temperature (20 °C) by Dumas *et al.* (2020) in the deep agar diffusion assay compared to this present study may have affected activity rankings, and therefore direct comparisons cannot be made. Further studies testing lipolytic activity at different temperatures, including commercially relevant temperatures, are required to fully establish whether isolates used in Roquefort production have significantly different activities to non-Roquefort isolates.

Similarly to the assay used in this study to determine proteolytic activity, the tributyrin agar diffusion assay measures *total* lipolytic activity and does not give any information about the contribution of individual lipases to lipolysis. As seen with the proteolysis results, rather than a clear 1:1 segregation of phenotypic activity relative to the parental isolates, as might be predicted for different variants (alleles) of a single gene, a characteristic polygenic spread of activity was seen in the offspring of crosses consistent with the contribution of multiple genes to the lipolytic phenotype (Ashton and Dyer, 2016). It has been reported that *P. roqueforti* produces two extracellular lipases (Lamberet and Menassa, 1983b; Mase et al., 1995; Menassa and Lamberet, 1982), but the genes which encode these lipases or other components of the lipolytic system are unknown (See Section 4.1). If these were to be identified, expression analysis could be undertaken to determine whether the differences in lipolytic activity found in this study result from altered expression in one or more of the genes involved in the *P. roqueforti* lipolytic system. This is investigated in Chapter 4 of the present study.

3.4.3 Secondary metabolite production

Parental isolates and selected progeny from cross 2 (74-130 x 74-144) were screened for roquefortine C production on artificial media. Unlike the bioassays of proteolytic and lipolytic activity, no progeny were found to produce significantly different amounts of roquefortine C as compared to both parental isolates (Figure 3.9). This may be a result of the small sample size (5 progeny) used, and if more progeny were tested one or more isolates with novel activity might be found. In agreement with this, there were significant differences in roquefortine C production levels between progeny, which suggests sexual reproduction does have the potential to produce progeny with significantly altered production levels. Another explanation could be that there is low phenotypic and/or genotypic divergence between the parental isolates (Hung et al., 2012). Congruent with this theory, this cross produced the lowest percentage of progeny with altered lipolytic activity (10.0 %) and second lowest percentage of progeny with altered proteolytic activity (50.0 %) (Figures 3.8 and 3.5). Roquefortine C production levels should be tested for additional parental crosses and progeny in the future to conclusively determine whether sexual reproduction can lead to novel variation in this trait.

The production levels of an additional secondary metabolite, mycophenolic acid (MPA), was to be determined for parental isolates and sexual progeny. However, none of these isolates produced any MPA at detectable amounts under the growth conditions assayed. This was not entirely unexpected, as previous studies have shown that a fair percentage of isolates do not produce detectable quantities of MPA (Engel et al., 1982; Gillot et al., 2017a). Gillot *et al*. (2017a) found that 16 % of the *P. roqueforti* isolates they tested did not produce any MPA, whereas Engel *et al*. (1982) found that the same was true for 75 % of their sample isolates. Low or no MPA production has been linked to a 174 bp deletion in one of the key genes in the mycophenolic acid gene cluster (Gillot et al., 2017b). It would be interesting to determine whether this deletion is present in the isolates tested in this present study which may explain why no MPA could be detected. It is also possible that the isolates produce MPA in very low amounts and the methods used in this study were

not sensitive enough to detect this. Although 84 % of isolates tested by Gillot *et al*. (2017a) produced detectable amounts of MPA, only 42 % produced quantifiable amounts which suggests that many isolates have very low production levels. Future studies should focus on crosses of which both or one of the parental isolates are known to produce MPA.

It would also be of interest to determine whether the production of other important *P. roqueforti* secondary metabolites such as Andrastin A and PR toxin can be significantly altered by sexual reproduction. Intriguingly, parallel work on a different set of sexual progeny has even shown the appearance of novel secondary metabolites in progeny of *P. roqueforti* not seen in either parent of the cross, indicating the major role of the sexual cycle in generating phenotypic diversity (H Darbyshir, J Frisvad and PS Dyer, unpub. data).

3.4.4 Concluding remarks

Although the assays used in this study to determine enzymatic activity and secondary metabolite production capacity have the benefit of being low cost and relatively easy to use, it is important to note that these are only models and may not reflect what might happen during the blue cheese maturation process. For example, several isolates were found via the casein diffusion assay to be non-proteolytic (unable to degrade casein). However, this does not mean that these isolates would lack proteolytic activity during the blue cheese manufacturing process, because non-fungal proteases such as those native to milk may release the peptides required for *aspA* induction (Gente et al., 2001). These peptides are probably not found in the media used for the casein diffusion assay and so isolates with low constitutive expression, which may be unable to release the threshold level of inducing peptides, would have presented (incorrectly) as non-proteolytic. Similarly, the media used in this study to determine lipolytic activity and secondary metabolite production may also not give a true indication of activity in milk, because the inducing agents required for production of *P. roqueforti* lipase(s) or secondary metabolites may not be present or present at the wrong concentrations. It would be useful to

conduct studies to ascertain how closely the phenotypic traits determined using the model assays correlate with actual proteolysis, lipolysis and secondary metabolite production in blue cheese.

The parental isolates tested in this study were all derived from blue cheese, and as such have likely been subjected to domestication events (Dumas et al., 2020; Ropars et al., 2017). This may mean that the characteristics which have been tested in this study (enzymatic activity, secondary metabolite production) do not vary as much between these isolates as they would between wild isolates because they are likely to have derived from a common ancestor. Wild yeast isolates have been shown to have a more diverse aroma profile than domesticated strains, a discovery which has received interest from wine producers (Dzialo et al., 2017). It would be interesting therefore to test the phenotypic traits of progeny from crosses between wild isolates or from crosses between domesticated and wild isolates to determine whether the percentage of progeny with novel characteristics increases compared to that seen in this study.

In conclusion, this study has provided evidence that isolates with altered enzymatic activities and secondary metabolite production capacities can be generated by sexual reproduction. Future studies should aim to assess whether these novel phenotypes translate into blue cheeses with novel characteristics such as improved taste and texture. The results of this study may be of interest to blue cheese producers because of availability of an increased variety of *P. roqueforti* isolates of which to choose from to produce cheese.

Chapter 4 – Genetic Characterisation of the *Penicillium roqueforti* Lipolytic System

4.1 Introduction

Penicillium roqueforti is a saprotrophic ascomycete mould found in various environments including soil, silage and in food as a spoilage agent (Pitt and Hocking, 2009). However, this species is most famous as the mould that is used in blue cheese production, a procedure that has been going on for over a thousand years (Cantor et al., 2017). The availability of full genome sequences of *P. roqueforti* strain FM164 (Cheeseman et al., 2014) and later CECT 2095 (Rojas-Aedo et al., 2017) has allowed for functional characterisation of genes of this fungus to be performed with much greater ease than previously possible. Indeed, several recent studies have made use of these genomes to characterise genes involved in secondary metabolite biosynthesis (Gillot et al., 2017a; Hidalgo et al., 2017; Kosalková et al., 2015; Rojas-Aedo et al., 2017) and pigment production (Cleere, 2017). Nevertheless, the vast majority of genes involved in the biology of *P. roqueforti* remain to be elucidated.

4.1.1 *Penicillium roqueforti* enzymes involved with blue cheese production

As explained in Section 3.1, the main contribution of *P. roqueforti* to blue cheese production is the unique texture and flavour generated via its enzymatic activities (Cantor et al., 2017). Although enzymes originating from milk, rennet, starter lactic acid bacteria and yeast are also likely to contribute to blue cheese maturation, those produced by *P. roqueforti* are thought to be the most important (Coghill, 1979). The best studied of these enzymes include proteases, peptidases and lipases.

Proteolysis by *P. roqueforti* affects both the flavour and texture of blue cheese (See Section 3.1.3.1). *P. roqueforti* produces several proteases and peptidases which hydrolyse casein micelles (made up of α_{s1}-casein, β-casein, α_{s2}-casein and κ-casein) found within milk. Two extracellular proteases have been

identified; an aspartic protease which has peak activity at pH 3.5 and 5.5 (Le Bars and Gripon, 1981), and a metalloprotease which has peak activity at pH 5.5 but is active up to pH 8.5 (Trieu-Cuot et al., 1982b). The aspartic protease is able to hydrolyse α_{s1}-casein and β-casein, whereas the metalloprotease can hydrolyse a broader range of substrates (Cantor et al., 2017). Of the two proteases, the aspartic protease is thought to be the most important for overall proteolytic activity. This was demonstrated by Fernández-Bodega *et al*. (2009) who showed that the intensity of an immuno-stained band of aspartic protease correlated with overall casein degradation capability in a diffusion assay (Fernández-Bodega et al., 2009). The gene encoding the *P. roqueforti* aspartic protease (*aspA*) has been characterised and shown to produce a 397 amino acid polypeptide (Gente et al., 1997). The gene encoding for the metalloprotease has not yet been identified. Two *P. roqueforti* extracellular peptidases have also been identified; a serine carboxypeptidase which has a peak activity at pH 3.5, and a metalloaminopeptidase which has a peak activity at pH 8.0. The carboxypeptidase liberates acidic, basic and hydrophobic amino acids, whereas the metalloaminopeptidase liberates apolar amino acids (Cantor et al., 2017; Gripon, 1993). Neither of the genes encoding for these peptidases have been elucidated.

The majority of the flavour compounds found within blue cheese can be attributed to the lipolytic activity of *P. roqueforti* (see Section 3.1.3.1). *P. roqueforti* produces at least two extracellular lipases; an acidic lipase which has two peak activities at pH 2.8 and 6.0, and an alkaline lipase which has peak activity between pH 8.8 and 10.0 but is active down to pH 4.5 (Lamberet and Menassa, 1983b; Mase et al., 1995; Menassa and Lamberet, 1982). *P. roqueforti* also produces at least one intracellular lipase, as intracellular lipolytic activity has been detected (Stepaniak et al., 1980). The activity of *P. roqueforti* lipases is the first stage of a 3-stage enzymatic process which results in the formation of the most important blue cheese-associated flavour compounds, methyl ketones [e.g. 2-heptanone and 2-nonanone (Figure 4.1)] (Gallois and Langlois, 1990; Gkatzionis et al., 2009). Firstly, free fatty acids are

released by lipases; secondly, the fatty acids are converted into β-keto acids via oxidation by a thiohydrolase; Thirdly, the β-keto acids are converted into methyl ketones via decarboxylation by a β-ketoacyldecarboxylase. A subsequent reduction reaction by a reductase may occur which converts the methyl ketone into the corresponding secondary alcohol (e.g. 2-heptanone → 2-heptanol) (Figure 4.2) (Thierry et al., 2017). The genes encoding for the enzymes involved with methyl ketone formation have not yet been identified.

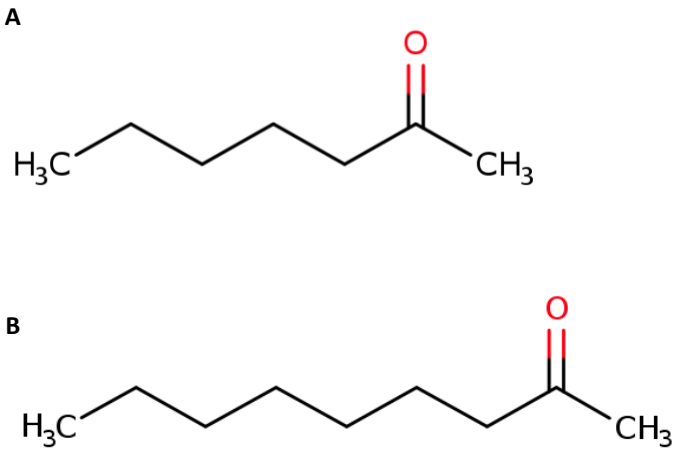

Figure 4.1. *Chemical structure of the methyl ketones 2-heptanone (A) and 2-nonanone (B).*

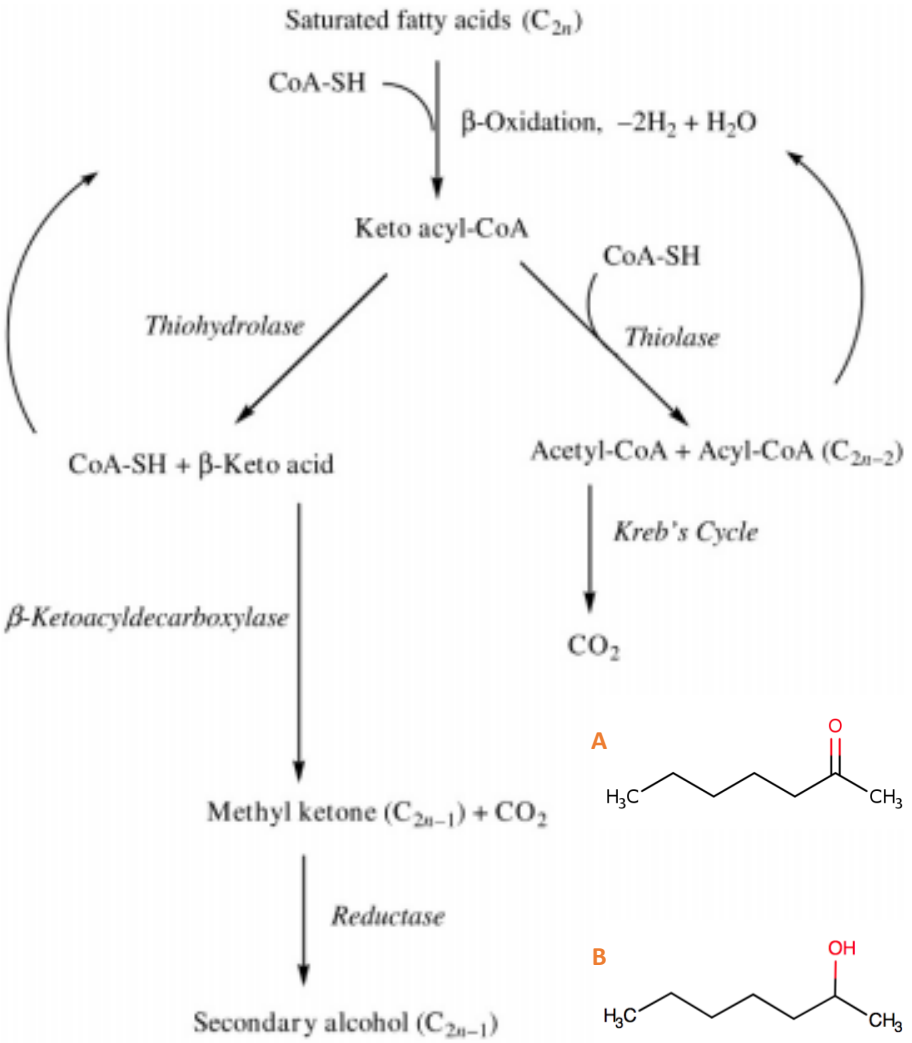

***Figure 4.2**. Enzymatic production of methyl ketones [e.g. 2-heptanone (A)] and secondary alcohols [e.g. 2-heptanol (B)] by* Penicillium roqueforti. *Edited from Thierry et al. (2017).*

The relative importance of the individual *P. roqueforti* lipases for the production of blue cheese-associated flavour compounds is currently unknown, and no functional characterisation has been undertaken to identify the genes which encode for these lipases. Further genetic characterisation of the *P. roqueforti* lipolytic system may prove useful for identifying strains with altered expression of, or mutations within, specific lipase genes, which could impact on flavour production and therefore have implications for isolate selection for blue cheese manufacture.

4.1.2 Methods for genetic manipulation and functional gene characterisation in filamentous fungi and their application in *P. roqueforti*

Several diverse methods have been developed to genetically manipulate and characterise gene function in filamentous ascomycetes (Kück and Hoff, 2010; Mei et al., 2019).

Traditionally, random mutagenesis was used to characterise gene function in fungi. This process involves exposing a fungus to either radiation or mutagenic chemicals and looking for changes in phenotype in the mutants (Casselton and Zolan, 2002). A famous example of this is the use of random X-ray mutagenesis in formulating the “one gene-one enzyme” hypothesis in a filamentous fungus, *Neurospora crassa* (Beadle and Tatum, 1941). A major disadvantage of this method is that it can be difficult and time-consuming to identify the gene or genes responsible for the phenotype (Wang et al., 2017).

One of the most commonly applied methods for gene characterisation in fungi involves gene replacement by homologous recombination, facilitated by polyethylene glycol (PEG)-mediated transformation (Liu and Friesen, 2012). This process relies on the initial formation of fungal protoplasts, which are fungal cells in which the cell wall has been degraded by cell wall digesting enzymes (Peberdy, 1995). Protoplast are then incubated with PEG, $CaCl_2$ and exogenous DNA, of which the latter is hopefully taken up by the protoplasts. The exogenous DNA is usually designed to contain a selectable genetic marker flanked on either side by regions of homology to a gene of interest, so that the DNA fragment can be targeted specifically to this gene. The gene of interest is then replaced by the selectable genetic marker through homologous recombination (Kück and Hoff, 2010). Phenotypes of these mutants are assessed and can then be attributed to the gene which has been replaced.

Aside from disruption of genes via manipulation of the genome itself, as described in the examples above, gene characterisation in fungi has also been undertaken using RNA interference (RNAi). RNAi is an RNA-based post-transcriptional gene silencing mechanism that is conserved throughout the eukaryote domain (Davis et al., 2010; Fire et al., 1998; Romano and Macino,

1992; van der Krol et al., 1990). RNAi begins with the production of double-stranded RNA (dsRNA) molecules from mRNA by an RNA-depended RNA polymerase. These dsRNA molecules are recognised by a Dicer enzyme, which cleaves the dsRNA into small interfering RNA (siRNA) of length 21 – 25 bp. siRNAs guide the degradation of the cognate mRNA by associating with the RNA-induced silencing complex (RISC). The now degraded mRNA is no longer functional and the corresponding gene is silenced or "quelled" (Chang et al., 2012). RNAi can be harnessed for gene characterisation by designing DNA fragments that when transcribed will produce both a sense and an antisense strand of RNA corresponding to a gene of interest (Kück and Hoff, 2010). This is often achieved by cloning a section of the gene of interest between two opposite-acting promotors (Ullán et al., 2008). The fungal RNAi machinery will then proceed as detailed to silence the gene of interest, and transformants can be screened for phenotypes which can be attributed to the silencing of that particular gene.

Several other methods of genetic manipulation to study gene function in fungi exist. These include *Agrobacterium*-mediated transformation (De Groot et al., 1998), electroporation (Chakraborty and Kapoor, 1990), biolistic transformation (Barcellos et al., 1998), shock-wave-mediated transformation (Magaña-Ortíz et al., 2013) and the more recently described CRISPR system, which has begun to be applied in filamentous fungi (Wang and Coleman, 2019).

The only published methods described for the genetic manipulation of *P. roqueforti* thus far are gene replacement by homologous recombination facilitated by PEG-mediated transformation and RNAi. The latter strategy has been employed in several recent studies (Gil-Durán et al., 2015; Gillot et al., 2017a; Kosalková et al., 2015; Rojas-Aedo et al., 2017) whereas the former has only been used in two studies for targeted gene replacement (Cleere, 2017; Goarin et al., 2015) although an earlier study used PEG-mediated transformation for random integration of exogenous DNA (Durand et al., 1991). A disadvantage of RNAi is that the silencing cassette integration is non-

directed, so there is the possibility of unintended off-target effects (Gillot et al., 2017a). The predominant use of RNAi even with this disadvantage might indicate that gene replacement in *P. roqueforti* is difficult. This fact, alongside the inherent lack of selectable genetic markers available for fungi (Mei et al., 2019) and low rates of homologous recombination often observed (Qiao et al., 2019), makes expansion of the genetic toolbox for *P. roqueforti* an important pursuit.

4.1.2.1 Auxotrophic mutants and *pyrG*-blaster marker recycling system

Auxotrophic mutants are organisms that are unable to produce an essential nutrient *de novo*, and therefore require the supplementation of this nutrient in growth media. Auxotrophic mutants are useful for gene characterisation studies because genes of interest can be replaced by a copy of a gene which functionally complements the auxotrophy. Positive transformants can then be selected based on their restored prototrophy. This has advantages over selectable genetic markers which confer resistance to fungicides (e.g. hygromycin B) because these fungicides are often expensive and may have undesired effects on cell biology even in resistant strains (Pronk, 2002). Examples of auxotrophies commonly used in fungal genetic studies include inability to produce arginine, lysine or pyrimidines (Boeke et al., 1984; Xue et al., 2004).

There are a relatively limited number of selectable genetic markers available for use in fungi, including ones which are used to functionally complement auxotrophies (Mei et al., 2019). This hampers the ability to make multiple gene deletions in a single strain because such markers may be 'used up' in each successive round of transformation and selection. One way in which to circumvent this limitation is by using a marker recycling system, whereby the same selectable genetic marker can be removed from the genome and used again in subsequent gene deletions. The most commonly used system in fungi is the so called *pyrG*-blaster system (also known as *URA*-blaster) (Figure 4.3). This uses a selectable genetic marker that generally consists of a functional

pyrG/*URA3* gene (which complements uridine and uracil auxotrophy) flanked on either side by a direct repeat sequence of bacterial origin (Alani et al., 1987; Brock et al., 2007). At a rate of around 10^{-5}, a random homologous recombination event can occur between the direct repeats, thus excising the *pyrG*/*URA3* gene (Brock et al., 2007). This can be directly selected for by culturing a transformant strain which possesses the *pyrG*-blaster on media contain 5-fluroorotic acid (5-FOA). 5-FOA is converted into the cytotoxic compound flurodeoxyuridine in *pyrG*/*URA3*$^{+}$ prototrophic strains, therefore inclusion of 5-FOA in the growth media ensures that only cells where the functional *pyrG*/*URA3* has been excised can form colonies. These colonies can then be used in subsequent genetic deletion experiments using the *pyrG*-blaster system again. The *pyrG*-blaster system was first established in yeast (Alani et al., 1987; Wilson et al., 2000), but has now been applied successfully to numerous fungi including *Aspergillus fumigatus*, *A. niger*, *A. oryzae*, *Mucor circirelloides* and *Colletotrichum orbiculae* (D'Enfert, 1996; Delmas et al., 2014; Garcia et al., 2017; Geib et al., 2019; Kumakura et al., 2019).

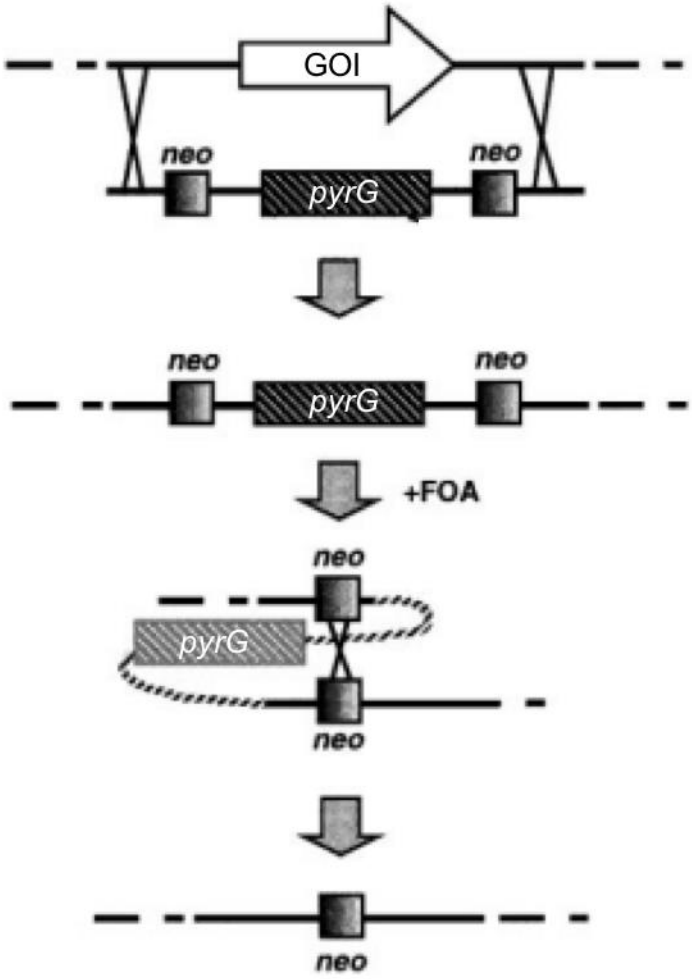

***Figure 4.3**. pyrG-blaster marker recycling system. Allows iterative rounds of gene deletions using same selection marker (pyrG). Edited from Brock et al. 2007.*

4.1.2.2 Non-homologous end joining deficient mutants and improving gene targeting efficiency

Non-homologous end joining (NHEJ) is a DNA repair mechanism by which double-strand breaks in DNA are repaired by direct ligation of the DNA ends either side of the break (Daley et al., 2005). This process differs to the other canonical double-strand break repair mechanism, homology direct repair (HDR), by not being reliant on a homologous sequence to guide repair. In eukaryotes, the NHEJ system is composed of several proteins including the Ku heterodimer (consisting of Ku70 and Ku80), DNA-dependent protein kinase (DNA-PKcs), DNA ligase IV (Lig4) and the exonuclease Artemis (Kück and Hoff, 2010). The Ku heterodimer functions as a molecular bridge between the two

DNA ends and acts to recruit the DNA-PKcs to the break site, which itself induces conformational changes to allow the DNA to be accessed by end-processing enzymes such as Artemis (Davis et al., 2014). The DNA ends are then ligated together by Lig4 (Costantini et al., 2007).

NHEJ is the preferred method to repair DNA double-strand breaks in filamentous fungi and, as indicated by the name, it allows for non-homologous DNA fragments to be joined together. This accounts for the low frequency of successful gene targeting commonly seen during genetic transformation experiments in these organisms, because ectopic (i.e. non target site) DNA integration can occur, whereas targeted gene deletion requires homologous recombination (Qiao et al., 2019). A common method of increasing gene targeting efficiency in filamentous fungi is to disrupt the NHEJ system (Kück and Hoff, 2010). This is usually done by deleting the homologues of either the *ku70*, *ku80* or *lig4* genes. NHEJ disruption has been undertaken in a plethora of filamentous fungi, including model organisms (El-Khoury et al., 2008; Nayak et al., 2006), pathogens (Choquer et al., 2008; Krappmann et al., 2006) and fungi used in the biotechnology industry (Meyer et al., 2007; Snoek et al., 2009). In all cases, the rate of successful gene targeting was increased in the NHEJ-impaired strain compared to the wild type. In some studies, the efficiency increased to over 90 % (El-Khoury et al., 2008; Nayak et al., 2006). Disruption of the NHEJ system should therefore be considered as one strategy in any filamentous fungi in which genetic manipulation experiments are to be undertaken, as significantly less transformants would need to be screened for successful gene targeting compared to wild-type, thus saving time and resources.

4.1.3 Aims

The first aim of this present chapter was to develop a “laboratory strain” of *Penicillium roqueforti* which would be more amenable to genetic manipulation studies than a wild-type strain. This was planned to be achieved in two steps, both of which have been employed in other filamentous fungi and have been

successful in improving the efficiency of genetic manipulation. In the first step, a *pyrG*-blaster marker recycling system would be established which could be used in multiple rounds of gene deletions (D'Enfert, 1996). In the second step, gene targeting efficiency would be increased by disrupting the NHEJ system in this fungus (Qiao et al., 2019). The creation of this "lab-strain" could prove to be a valuable asset for research groups wanting to undertake genetic functional characterisation studies in *P. roqueforti* in the future.

The second aim of this chapter was to attempt to genetically characterise the lipolytic system of *P. roqueforti*, specifically in the context of blue cheese production. Lipolysis of triglycerides in milk by *P. roqueforti* is the main driver for the production of blue cheese-associated flavour compounds, but to date the genes and enzymes involved in this process remains largely uncharacterised (Cantor et al., 2017). Therefore, the present study set out to elucidate which lipases were important for this process by deleting several genes encoding putative lipases and assessing the resulting phenotypes of the deletion mutants. Knowledge gained from this study was then to be used to gain insight into the genetic causes for the differences in total lipolytic activities of sexual progeny as shown in Chapter 3. Further knowledge of the *P. roqueforti* lipolytic system could be of use to blue cheese producers, because it may allow for more tailored selection for strains with altered expression of, or mutations within, specific lipase genes which have been identified as being important for blue cheese-associated flavour compound production.

4.2 Methods

4.2.1 Media and chemical supplier

Media compositions are specified in text. 1000X trace elements (per L: 40 mg $Na_2B_40_7$, 800 mg $CuSO_4$, 800 mg $FePO_4$, 800 mg $MnSO_4$, 800 mg $NaMoO_4$, 8 g $ZnSO_4$) and 20X salt solution (per L: 26 KCl, 26 g $MgSO_4.7H_2O$, 76 g KH_2PO_4, 10

mL 1000X trace elements) were prepared for use in several media. All media were autoclaved at 121 °c for 20 mins. Standard chemicals for use in media were purchased from Sigma, UK unless otherwise specified.

4.2.2 Isolates and strain maintenance

Isolates were routinely cultured on slopes of potato dextrose agar (PDA; Sigma Aldrich, UK), GG10 (50 mM glucose, 10 mM glutamine, 1X salt solution, 1X trace elements, 1.5 % agar, pH 6.5) or GG10 + 10 mM uridine + 10 mM uracil at 28 °C in light. Conidia were harvested from slopes using 0.1 % Tween 80 in sterile distilled water (v/v) after 5 – 7 days growth. The resulting spore suspensions were filtered through sterile Miracloth, and spore concentration determined using an improved Neubauer haemocytometer, before diluting to a working concentration of 1 x 10^8 spores mL^{-1}. For long-term storage, spores and mycelium of isolates were suspended in a 10 % glycerol solution and stored at -80 °C and/or under liquid nitrogen.

Isolate 74-88 (an industrial production strain from Danisco Foods) was used for initial gene expression studies, routine PCR generation of flanking regions for replacement cassettes and initial replacement of the putative *P. roqueforti pyrG* (orotidine 5-phosphate decarboxylase) gene. Where a flanking region could not be amplified from genomic DNA (gDNA) from isolate 74-88, gDNA from isolate A22 was used. Isolate 74-88Δ*pyrG:hph* (from this thesis) was used as the parental strain for attempts to replace the putative *P. roqueforti ku70* (ATP-dependent DNA helicase II subunit 1) gene. All other gene replacements were performed in 74-88Δ*pyrG*Δ*ku70*. Parental isolates 74-130 and 74-144 and progeny isolates A3, A7, A18, A19 and A22 (see Section 3.2.2) were used for *lipD* and *lipI* expression studies.

4.2.3 *In silico* studies

All genes of interest were identified *in silico* using the *Penicillium roqueforti* FM164 annotated genome on the NCBI online database (www.ncbi.nlm.nih.gov) (Cheeseman et al., 2014). The putative *P. roqueforti*

pyrG and *ku70* nucleotide sequences were searched for using the search terms "penicillium roqueforti AND orotidine" and "penicillium roqueforti AND ku70", respectively. Putative lipase nucleotide sequences were searched for using the search term "penicillium roqueforti AND lipase". ORF and flanking region nucleotides sequences of all genes of interest were identified from protein sequences using NCBI GenBank function and searching genome scaffolds. Subcellular locations of proteins of interest were predicted using DeepLoc-1.0 (Almagro Armenteros et al., 2017). The NCBI BLASTP function (https://blast.ncbi.nlm.nih.gov/Blast) was used to screen some proteins for homology to other known proteins and AcalPred software (Lin et al., 2013) was used to predict optimum pH conditions for the function of these proteins.

4.2.4 Quantitative reverse transcriptase PCR analysis of lipase gene expression

The expression of lipase genes identified *in silico* was analysed using qRT-PCR. Conidial suspensions of *P. roqueforti* 74-88 were prepared as described above (Section 4.2.2). 1 mL of conidial suspension (1 x 10^8 spores mL^{-1}) was used to inoculate 100 mL of minimal media (50 mM glucose, 10 mM glutamine, 1X salt solution, 1X trace elements, pH 5.0 using DL-lactic acid) or 100 mL of milk medium [whole UHT milk (Co-op, UK), 2 % NaCl, pH 5.0 using DL-lactic acid]. Cultures were incubated for 7 days at 24.0 °c in darkness, at 80 rpm. 7 days incubation allowed time for sufficient fungal biomass to develop and was shown in *P. camemberti* to be amenable for lipase expression (Lessard et al., 2014). Cultures were set up in triplicate.

After 7 days, fungal material was harvested using sterile Miracloth, frozen in liquid nitrogen and ground to a fine powder under liquid nitrogen using a sterile pestle and mortar. Total RNA extraction, cDNA synthesis and qRT-PCR experiments were performed as described in Section 2.2.9.

Primers for use in qRT-PCR were designed with the aid of Primer3 (Untergasser et al., 2012) and can be found in Table 4.1.

***Table 4.1**. Primers used for qRT-PCR of putative lipase genes of* P. roqueforti

Gene	**Primers used (sequence 5' – 3')**
lipA	LipA_F (atgccgatgactacacagct)
	LipA_R (gaatatggcatcgctgaccc)
lipB	LipB_F (cacgttgggaggtattgtgc)
	LipB_R (ccgcatttccagacttcgag)
lipC	LipC_F (ccgtcgcttctcctgactat)
	LipC_R (agcccttgatgttcagctct)
lipD	LipD_F (tcggttatcatgaggggctc)
	LipD_R (cgtgggcaatgtatgtgagg)
lipE	LipE_F (agctacgactttgaccagct)
	LipE_R (agaagtgcctgcgagagatt)
lipF	LipF_F (agcccaaaacaagacgaacc)
	LipF_R (gttattgagtaggtgccgcg)
lipG	LipG_F (gcgtgacatccattgcatct)
	LipG_R (ctaaccgaggtgagagcgat)
lipH	LipH_F (atctcgaacatttggtccgc)
	LipH_R (agcgttgttaaatgaggcgg)
lipI	LipI_F (cgaagtgctcaataccgtcg)
	LipI_R (tgggcaagtatcctggatcc)
sac7	RhoGTP-F (aatgggaaaacctcccaatc)
	RhoGTP-R (gtcgtaatcgcggaagaact)

All experiments were performed using three biological replicates and three technical replicates. *sac*7, a gene encoding a Rho GTPase activator involved in signal transduction, was used as a reference gene in this study. This gene was taken from a previous study which found it to be stably expressed under different experimental conditions and therefore be suitable for use in qRT-PCR experiments (Gillot et al., 2017a). Expression levels were calculated as fold change between minimal media and milk media using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Statistical tests to compare the means of the ΔCt levels for each lipase gene between minimal media and milk media were performed using GraphPad Prism 8.0.

4.2.5 Construction of gene replacement cassettes

Gene replacement cassettes were constructed for each individual gene of interest. Each cassette consisted of: 5' flanking region of the gene of interest ORF (approximately 1 kb), selectable marker, 3' flanking region of the gene of interest ORF (approximately 1 kb). All replacement cassettes were cloned into the pUC19 plasmid. The online tool NEBuilder was used to guide *in silico* construction of gene replacement cassettes and primer design. Primers used to generate flanking regions are detailed in Table 4.2. Specific restriction sites, which did not appear within the flanking regions themselves, were included on the forward primer used for the amplification of the 5' flanking region and the reverse primer used for the amplification of the 3' flanking region. Reactions were performed in 50 µL reactions containing: 1X Phusion HF reaction buffer (New England Biolabs, UK), 200 µM dNTP, 500 nM each primer, 1 unit Phusion polymerase, 50 ng genomic DNA or 1.0 ng plasmid DNA, and distilled water up to 50 µL. The reactions were performed using a Techne TC-5000 thermal cycler (Applied Biosystems, USA) with the following program: 98 °c for 30 s, 32X [98 °c for 10 s, 62 °c for 20 s, 72 °c for 30 s], followed by a final extension of 72 °c for 5 mins. Up to four 50 µL reactions were set up for each fragment to increase the total amount of DNA produced.

Table 4.2. *Primers used to generate 5' (upstream) and 3' (downstream) flanking regions of genes of interest. * = A22 gDNA used to generate flanking region.*

Gene	Flanking region (bp)	Primers used (sequence 5' – 3')
pyrG	5' (upstream) (980)	PyrG_up_F (taggcccttcgtgagttgac)
		PyrG_up_R.1 (tacgccgtctgacttttgtggtgagagtccacgttccactgtatg)
	3' (downstream) (925)	PyrG_dw_F (ggcggggtgggttgatatg)
		PyrG_dw_R (aacggagagccaccatatcg)
ku70	5' (upstream) (1143)	Ku70_up_F.1 (gccagtgaattcgagctcggtacccgtatctatgctgaggagggag)
		Ku70_up_R.1 (cgttgatggtgccaacaatctgcgcgcttaggatcatcttggtgg)
	3' (downstream) (1052)	Ku70_dw_F.1 (acgaagagaagctcgacaacctggcctctctgacgcggacaacac)
		Ku70_dw_R.1 (tgcaggtcgactctagaggatccccgcctccacaagaccagagtg)
lipA	5' (upstream) (1132)	LipA_up_F (cctctagagtcgacctgcaggcatgcggttccccatctacttcccc)
		LipA_up_R (cgttgatggtgccaacaatctgcgctcaatcgcggtgcctaattg)

	3’ (downstream) (1079)	LipA_dw_F (acgaagagaagctcgacaacctggcgagttatgcaaggccactgg)
		LipA_dw_R (accatgattacgccaagcttgcatgcgtctggcttggagtatgtgc)
lipB	5’ (upstream) (1171)	LipB_up_F (gccagtgaattcgagctcggtacccggggtcagtatgcgagacgaaag)
		LipB_up_R (cgttgatggtgccaacaatctgcgctgtaggtagtatgtcctggg)
	3’ (downstream) (1082)	LipB_dw_F (acgaagagaagctcgacaacctggcagtagggctttctcaacggg)
		LipB_dw_R (tgcaggtcgactctagaggatccccgggagaagatggacaggacaccg)
lipC	5’ (upstream) (1010)	LipC_up_F.1 (atactcatactcttccttttttcaatattagctggaaaatgttgaccgaag)
		LipC_up_R.1 (cgttgatggtgccaacaatctgcgcaacagtgcgacgatcatcac)
	3’ (downstream) (1010)	LipC_dw_F.1 (acgaagagaagctcgacaacctggcgagccgcaactgtttctgatatg)
		LipC_dw_R.1 (taaccctgataaatgcttcaataatattaccgccggaatctgttatcag)
lipD	5’ (upstream) (1114)	LipD_up_F (gccagtgaattcgagctcggtacccgggctgtccaggtgatgctctg)
		LipD_up_R (cgttgatggtgccaacaatctgcgccttgagcgctgttttggatg)
	3’ (downstream) (1154)	LipD_dw_F (acgaagagaagctcgacaacctggcagaagtggacacaactgg)
		LipD_dw_R (tgcaggtcgactctagaggatccccggggtctcactcgtcaggcaaac)
lipE	5’ (upstream) (1079)	LipE_up_F (cctctagagtcgacctgcaggcatgccattattgtagccccggtcg)
		LipE_up_R (cgttgatggtgccaacaatctgcgcacatgtgatcgacgtccaag)
	3’ (downstream) (1001)	LipE_dw_F (acgaagagaagctcgacaacctggcccttggaattgtgaggctcag)
		LipE_dw_R (accatgattacgccaagcttgcatgccaacggctcaatccacatcc)
lipF	5’ (upstream) (1091)*	LipF_up_F (gccagtgaattcgagctcggtacccgggaggagaacgactggtggatc)
		LipF_up_R (cgttgatggtgccaacaatctgcgcgagaggtgaccagaatccgg)
	3’ (downstream) (1058)*	LipF_dw_F (acgaagagaagctcgacaacctggctattctgttggatgctggcg)
		LipF_dw_R (tgcaggtcgactctagaggatccccgggcacgagtcgaaggtttgtcc)
lipG	5’ (upstream) (1077)	LipG_up_F (gccagtgaattcgagctcggtacccggggcagccctacctacatcctc)
		LipG_up_R (cgttgatggtgccaacaatctgcgccagcatgcatagtccaacgg)
	3’ (downstream) (1026)	LipG_dw_F.1 (acgaagagaagctcgacaacctggctttcccaccgtcaacaacag)
		LipG_dw_R.1 (tgcaggtcgactctagaggatccccgggaggttgtactgttccctggttg)
lipH	5’ (upstream) (1075)	LipH_up_F (cctctagagtcgacctgcaggcatgcgctaccctacactgcctacc)
		LipH_up_R (cgttgatggtgccaacaatctgcgccggcagatttgtttactcgc)
	3’ (downstream) (1143)	LipH_dw_F (acgaagagaagctcgacaacctggctgtgtgaaatgcgtggagtg)
		LipH_dw_R (accatgattacgccaagcttgcatgcctccaatgtgccttgctgtc)
lipI	5’ (upstream) (1074)	LipI_up_F (gccagtgaattcgagctcggtacccggggcgcacaagagatggctatc)
		LipI_up_R (cgttgatggtgccaacaatctgcgcgggattctacaggtacgcag)
	3’ (downstream) (1085)	LipI_dw_F (acgaagagaagctcgacaacctggctcccatcattgttcggcttc)
		LipI_dw_R (tgcaggtcgactctagaggatccccggggatgaaaagatcgggcggac)

For replacement of *pyrG*, the selectable marker used was a hygromycin B resistance cassette consisting of *A. nidulans gpdA* promoter, *E. coli hph* and *A. nidulans trpC* terminator which was amplified from the hph_mut_pJet1.2 plasmid via PCR using primers hph_F (ctcaccacaaaagtcagacg) and hph_R.1 (caaatacatatcaacccaccccgcccagtgtgatggaattcgcc). The reaction was performed in a 50 µL reaction containing: 1X Phusion HF reaction buffer, 200 µM dNTP, 500 nM each primer, 1 unit Phusion polymerase, 1.0 ng plasmid and distilled water up to 50 µL. The reaction was performed using a Techne TC-5000 thermal cycler (Applied Biosystems, USA) with the following program: 98 °c for 30 s, 32X [98 °c for 10 s, 65 °c for 20 s, 72 °c for 90 s], followed by a final extension of 72 °c for 5 mins. For all other gene replacements, the selectable marker was a *pyrG*-blaster consisting of a functional *A. nidulans pyrG* gene flanked on either side by direct repeats from the *E. coli neo* gene (Geib et al., 2019). This was generated via restriction digest of the URAblasterAsp(short)_pUC19 plasmid with *NotI* which excises the *pyrG*-blaster.

The *pyrG* replacement cassette was constructed using NEB HiFi assembly master mix (New England Biolabs, UK) in a 20 µL reaction containing: 1X NEB HiFi assembly master mix, 0.1 pmol 5' flanking region, 0.1 pmol hygromycin B resistance cassette, 0.1 pmol 3' flanking region and distilled water up to 20 µL. The reaction was performed using a Techne TC-5000 thermal cycler (Applied Biosystems, USA) with the following program: 50 °c for 60 mins. The *pyrG* replacement cassette was subsequently amplified directly from the NEB HiFi reaction using primers pyrG_up_F_KpnI (taagcaggtacctaggcccttcgtgagttgac) and pyrG_dw_R_HindIII (taagcaaagcttaacggagagccaccatatcg). The reaction was performed in a 50 µL reaction containing: 1X Phusion HF reaction buffer, 200 µM dNTP, 500 nM each primer, 1 unit Phusion polymerase, 1 µL HiFi reaction (10^{-1}) and distilled water up to 50 µL. The reaction was performed using a Techne TC-5000 thermal cycler (Applied Biosystems, USA) with the following program: 98 °c for 30 s, 32X [98 °c for 10 s, 67 °c for 20 s, 72 °c for 75 s], followed by a final extension of 72 °c for 5 mins. The PCR products were

separated via gel electrophoresis and the replacement cassette (~4.7 kb) was excised using a Gel Clean Up Kit (Macherey Nagel, Germany). The purified DNA fragment was digested using *Kpn*I and *Hin*dIII restriction enzymes and cloned into a pUC19 plasmid using T4 DNA ligase (New England Biolabs, UK) in a 20 µL reaction containing: 1X T4 DNA ligase buffer, 400 units T4 DNA ligase, 50 ng pUC19, 88.9 ng *pyrG* replacement cassette and distilled water to 20 µL. The reaction was performed at room temperature for 2 hrs and heat inactivated at 65 °c for 10 mins. This generated the pUC19_pyrG_hph plasmid.

All other gene replacement cassettes were also constructed using NEB HiFi assembly master mix in 20 µL reactions containing: 1X NEB HiFi assembly master mix, 0.1 pmol 5' flanking region, 0.1 pmol *pyrG*-blaster, 0.1 pmol 3' flanking region, 0.1 pmol pUC19 (pre-digested with various restriction enzymes) and distilled water up to 20 µL. pUC19 was digested with: *Sma*I to generate the ku70, LipB, LipD, LipF, LipG and LipI plasmids; *Sph*I to generate the LipA, LipE and LipH plasmids; and *Ssp*I to generate the LipC plasmid. The reactions were performed using a Techne TC-5000 thermal cycler (Applied Biosystems, USA) with the following program: 50 °c for 60 mins.

4.2.6 Bacterial transformation

HiFi reactions (containing the gene replacement plasmids) were transformed into XL1 Blue *E. coli* competent cells (Agilent, Canada) by electroporation. Each HiFi reaction was diluted 1:4 in sterile distilled water and 2 µL was mixed with 20 µL of XL1 Blue *E. coli* competent cells on ice before electroporation with a MicroPulser electroporation machine (Bio-Rad, USA). Bacteria were incubated at 37°c for up to 1 hr in SOC (2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) before spreading on Luria Bertani (LB) agar (1 % tryptone, 0.5 % yeast extract, 171.1 mM NaCl, 1.5 % agar and pH 7.0) + 100 µg mL^{-1} ampicillin and incubation at 37 °c overnight.

4.2.7 Gene replacement cassette preparation

Gene replacement plasmids were extracted using a NucleoSpin plasmid extraction kit (Macherey Nagel, Germany) according to manufacturer's instructions. Plasmids were digested with restriction enzymes (Table 4.3) and separated via gel electrophoresis. The replacement cassettes were then excised using a Gel Clean Up Kit (Macherey Nagel, Germany) according to manufacturer's instructions.

***Table 4.3**. Restriction enzyme(s) used to excise gene replacement cassette from plasmid.*

Gene	**Restriction enzyme(s) used**
pyrG	*Kpn*I, *Hin*dIII
ku70	*Xba*I, *Ehe*I
lipA	*Sph*I
lipB	*Sma*I
lipC	*SspI*
lipD	*Sma*I
lipE	*Sph*I
lipF	*Sma*I
lipG	*Sma*I
lipH	*Sph*I
lipI	*Sma*I

4.2.8 Fungal genetic transformation

A protocol for genetic transformation of *P. roqueforti* was developed based on existing protocols from the Nottingham Fungal Biology and Genetics group (Matthias Brock, *pers. comms*.) and published protocols (Gillot et al., 2017a; Goarin et al., 2015; Hidalgo et al., 2017).

Conidial suspensions of *P. roqueforti* were prepared as described in Section 3.2.2. 6 mL of conidial suspension (1 x 10^8 spores mL^{-1}) was used to inoculate 600 mL of YEPD broth or YEPD broth + 10 mM uridine/uracil and the culture incubated for between 20 – 24 hrs at 28 °C in light at 150 rpm or until the germ tube increased to approximately 10X length of the conidium (Hidalgo et al.,

2017). Fungal material was collected using sterile Miracloth and washed with 0.9 % KCl in sterile distilled water to remove un-germinated conidia. Protoplast formation was performed using Protoplast F (Megazyme, Ireland) according to manufacturer's instructions with some adaptions. Washed fungal material was added to 9.5 mL lysing buffer (20 mM potassium phosphate, 700 mM KCl, pH 5.8) with 0.5 mL Protoplast F enzyme mixture and incubated for between 2 – 3 hrs at 28 °c in light, at 80 rpm. After protoplast release (monitored microscopically every 30 minutes), the protoplasting solution was filtered through sterile Miracloth and the protoplasts were collected in a sterile 50 mL falcon tube. The protoplast solution was made up to 50 mL using KCM solution [700 mM KCl, 50 mM $CaCl_2$, 10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.8] and centrifuged at 4,000 rpm for 8 minutes at 4 °c. The resultant protoplast pellet was washed twice in KCM by centrifugation at 4,000 rpm for 8 minutes at 4 °c, before being suspended in KCM to a final concentration of between 2 x 10^7 protoplasts mL^{-1} – 2 x 10^8 protoplasts mL^{-1}.

Up to 10 µL of replacement cassette (2 – 10 µg DNA), or 10 µL sterile distilled water for negative controls, was mixed with 100 µL protoplast solution and 15 µL PCM solution (50 % PEG6000, 50 mM $CaCl_2$, 10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.8) and incubated for 20 minutes on ice. 500 µL PCM was then added and the protoplast solution was mixed via gentle pipetting and incubated for a further 20 minutes at room temperature. The protoplast solution was then diluted in 600 µL KCM, mixed via inversion and 500 µl spread onto GG10 + 1.2 M sorbitol agar selection plates. For replacement of *pyrG*, selection plates contained hygromycin B (100 µg mL^{-1}) (Invitrogen, UK) and 10 mM uridine + 10 mM uracil. Positive control plates, to check protoplast viability, contained no hygromycin B. For all other gene replacements, selection plates contained no uridine or uracil source. Positive control plates contained 10 mM uridine + 10 mM uracil. Plates were incubated for between 5 – 7 days at 28 °c in light, and checked periodically for the formation of colonies.

4.2.9 Gene replacement cassette integration confirmation

Transformants were screened for correct target or entopic integration of replacement cassettes using two PCR diagnostic tests. Internal PCR diagnostics used primers which bind within the gene of interest and should therefore not produce a product in a successful gene knockout. Positional PCR diagnostics used a forward primer which binds 5' of the replacement cassette and a reverse primer which binds within the replacement cassette and so will only produce a product in the case of an entopic integration. Primers used in these diagnostics are found in Table 4.4. For rapid confirmation screening of transformants, colony PCRs were undertaken. Mycelia from transformants were harvested using a sterile toothpick and incubated in 100 µL of colony PCR mix [98 µL solution 1 (10 mM NaCl, 10 mM tri-HCl pH 7.5, 1 mM ethylenediaminetetraacetic acid) + 2 µL 1 M NaOH] at 99 °c for 10 min. This colony PCR reaction was used as the template for PCR. PCR reactions were performed in 50 µL reactions containing: 1X Phire reaction buffer (Thermo Scientific, UK), 200 µM dNTP, 500 nM each primer, 1 µL Phire Hot Start II polymerase, 2 µL colony PCR reaction and distilled water up to 50 µL. The reaction for the internal PCR diagnostic was performed using a Techne TC-5000 thermal cycler (Applied Biosystems, USA) with the following program: 98 °c for 30 s, 32X [98 °c for 10 s, 62 °c for 10 s, 72 °c for 10 s], followed by a final extension of 72 °c for 5 mins. The reaction for the positional PCR diagnostic was performed using a Techne TC-5000 thermal cycler (Applied Biosystems, USA) with the following program: 98 °c for 30 s, 32X [98 °c for 10 s, 62 °c for 10 s, 72 °c for 30 s], followed by a final extension of 72 °c for 5 mins.

Table 4.4. *Primers used for internal and positional PCR diagnostic tests.*

Gene	PCR diagnostic	Primers used (sequence 5' – 3')
pyrG	Internal	PyrG_in_F (agacaaagccgagcacaaag)
		PyrG_in_R (caatgttaccgtctccgctg)
	Positional	PyrG_ex_F (gccatggtgtcggttctatg)
		hph_pos_R (catccactgcacctcagagc)
ku70	Internal	Ku70_in_F (ctcaccgccgaacttgtatg)

		Ku70_in_R (caacgataatccccacgcag)
	Positional	Ku70_ex_F (gaggacttctagtgtgcgtg)
		URA_pos_R (gctttacattgctcaatgctgg)
lipA	Internal	LipA_in_F (aggcagtcgttctctccttc)
		LipA_in_R (ccagttccagtgtttccagc)
	Positional	LipA_ex_F (caagcatgatcagctggttc)
		URA_pos_R (gctttacattgctcaatgctgg)
lipB	Internal	LipB_in_F (ccgcatttccagacttcgag)
		LipB_in_R (actggatcgcgaacatcaac)
	Positional	LipB_ex_F (gctcgagtcagtatgcgaga)
		URA_pos_R (gctttacattgctcaatgctgg)
lipC	Internal	LipC_in_F (acgactgcaatcaatggctc)
		LipC_in_R (acattgtgccagtgacgttc)
	Positional	LipC_ex_F (ctgcagcagtttagaagagc)
		URA_pos_R (gctttacattgctcaatgctgg)
lipD	Internal	LipD_in_F (cttttggagtcgagggcaac)
		LipD_in_R (cgtgggcaatgtatgtgagg)
	Positional	LipD_ex_F (ctaaagttccaaggctgagc)
		URA_pos_R (gctttacattgctcaatgctgg)
lipE	Internal	LipE_in_F (cctgtactccctagcgactg)
		LipE_in_R (aagcctgtctctgtctcacc)
	Positional	LipE_ex_F (cgtctcggtcgagctcttc)
		URA_pos_R (gctttacattgctcaatgctgg)
lipF	Internal	LipF_in_F (agcccaaaacaagacgaacc)
		LipF_in_R (gttattgagtaggtgccgcg)
	Positional	LipF_ex_F (gctcgagtgattgcagtctg)
		URA_pos_R (gctttacattgctcaatgctgg)
lipG	Internal	LipG_in_F (tcggccagcttcaaatttcc)
		LipG_in_R (atcgctctcacctcggttag)
	Positional	LipG_ex_F (ccaatctcgccgacgatgc)
		URA_pos_R (gctttacattgctcaatgctgg)
lipH	Internal	LipH_in_F (tctacgatcccacaggcatc)
		LipH_in_R (cgtcgccagcttgctatatc)
	Positional	LipH_ex_F (cagatcgacttcctcgatgg)
		URA_pos_R (gctttacattgctcaatgctgg)

lipI	Internal	LipI_in_F (tcgtcgcaacaggaatcaac)
		LipI_in_R (cgacggtattgagcacttcg)
	Positional	LipI_ex_F (gggcttactggcagtatcag)
		URA_pos_R (gctttacattgctcaatgctgg)

Southern blots were also undertaken to check for single integration of *pyrG* and *ku70* gene replacement cassettes into the genome. Southern DNA probes of size 980 bp (for *pyrG*) and 850 bp (for *ku70*) were generated via PCR using primers and plasmids specific to each gene of interest (Table 4.5). PCR reactions were performed in 50 µL reactions containing: 1X ThermoPol reaction buffer (New England Biolabs, USA), 10 µM digoxigenin-11-dUTP (Roche, USA), 200 µM dNTP, 200 nM each primer, 2.5 units Taq DNA polymerase, 30 ng plasmid DNA and distilled water up to 50 µL. The reactions were performed using a Techne TC-5000 thermal cycler (Applied Biosystems, USA) with the following program: 95 °c for 30 s, 32X [95 °c for 30 s, 50 °c for 30 s, 72 °c for 60 s], followed by a final extension of 72 °c for 5 mins. Probes were purified via gel electrophoresis and excised using a Gel Clean Up Kit (Macherey Nagel, Germany) to manufacturer's instructions. Before use, the Southern DNA probe was incubated for 10 mins at 95 °c followed by 5 mins on ice.

10 µg of transformant and wild-type gDNA was digested overnight with suitable restriction enzymes (Table 4.5). Digested gDNA was then separated via gel electrophoresis using an ethidium bromide stained 0.8 % (w/v) agarose gel in TAE buffer at 100 V for approximately 3 hrs. The gel was then washed in depurination solution (0.25 M HCl) for 2X 7.5 mins, denaturation solution (0.5M NaOH, 1.5 M NaCl) for 2X 20 mins and neutralisation solution (1.5M NaCl, 0.5M tris-HCl, pH 7.5) for 2X 15 mins. DNA was then transferred to a Nylon membrane (Hybond, UK) under vacuum for 1 hr under 10X saline-sodium citrate (SSC) buffer (0.15 M $Na_3C_6H_5O_7$, 1.5 M NaCl). DNA was crosslinked using a GS Gene Linker UV Chamber (Bio-Rad, USA).

Subsequent incubation and washing steps were performed with constant rotation in a hybridisation oven (Stuart, UK) and were performed at room temperature unless otherwise specified. The nylon membrane was rolled and placed into a hybridisation tube and incubated in 20 mL hybridisation solution at 65 °c for 1 hr. 10 µL of Southern probe was then added to the hybridisation solution and left to hybridise at 65 °c overnight. After, the hybridisation solution was discarded and the membrane was washed in washing solution I [2X SSC, 0.1 % sodium dodecyl sulphate (SDS)] for 2X 5 mins at 65 °c, wash solution II (0.1X SSC, 0.1 % SDS) for 2X 10 mins at 65 °c, solution A (0.1 M maleic acid, 0.15 M NaCl, 0.3 % Tween 20, pH 7.5) for 5 mins and maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 2 mins. The membrane was then incubated in 20 mL blocking solution [1X Western Blocking Reagent (Roche, USA) in maleic acid buffer] for 30 mins, after which the blocking solution was discarded and there was a secondary incubation in 20 mL of fresh blocking solution with the addition of 0.8 µL anti-digoxigenin-AP Fab fragments (Roche, USA) for 30 mins. The membrane was then washed in solution A for 2X 10 mins, maleic acid buffer for 5 mins and detection buffer (0.1 M tris-HCl, 0.1 M NaCl, pH 9.5) for 1 minute. The nylon membrane was then stained using CDP-Star reagent (Roche, USA) and visualised using a Universal Hood II chemiluminescence detector (Bio-Rad, USA). A secondary staining was performed by washing the membrane briefly in maleic acid buffer and then staining overnight with NBT/BCIP (Roche, USA).

***Table 4.5**. Primers used to generate Southern blot probe and restriction enzyme used on gDNA*

Gene	Primers used (sequence 5' – 3')	Restriction enzyme(s) used
pyrG	PyrG_Up_F (taggcccttcgtgagttgac)	*BglII*
	PyrG_Up_R.1 (tacgccgtctgacttttgtggtgagagtccacgttccactgtatg)	
ku70	Ku70_probe_F (gggagaggaggagtgttagc)	*NdeI*
	Ku70_probe_R (atgtggggtacgtactgtgg)	

4.2.10 *PyrG*-blaster marker recycling

For generation of multiple gene replacements in a single isolate, a marker recycling approach was used (D'Enfert, 1996). Conidial suspensions of *P. roqueforti* transformants were prepared as described previously (Section 4.2.2). 500 µL of conidial suspension (1 x 10^8 spores mL^{-1}) was used to inoculate 9 cm Petri dishes with GG10 + 10 mM uridine + 10 mM uracil medium containing 1.0 mg mL^{-1} 5-fluroorotic acid (5-FOA). Cultures were incubated for between 5 – 7 days at 28 °c in light and checked periodically for the formation of colonies. Selected colonies were further streaked onto GG10 + 10 mM uridine + 10 mM uracil containing 1.0 mg mL^{-1} 5-FOA, and then single isolates arising were maintained on GG10 + 10 mM uridine + 10 mM uracil.

4.2.11 Growth rate of transformants

Growth rates of transformants were determined by measuring the diameter of fungal colonies. Conidial suspensions of *P. roqueforti* were prepared as described previously (Section 4.2.2). 10 µL of conidial suspension (1 x 10^8 spores mL^{-1}) was used to spot-inoculate potato dextrose agar or potato dextrose agar + 10 mM uridine + 10 mM uracil in the case of 74-88Δ*pyrG:hph*. Cultures were incubated for 7 days at 28 °c in the light, before the diameters of the fungal colonies were measured in two perpendicular cross sections using engineering callipers. All experiments were performed using three biological replicates.

4.2.12 Total lipolytic activity determination

Total lipolytic activity of lipase knockout strains was determined using a deep agar diffusion assay as described in Section 3.2.4.

4.2.13 SPME-GCMS profiling of lipase knockouts

Conidial suspensions of *P. roqueforti* were prepared as described previously (Section 4.2.2). 1 mL of conidial suspension (1 x 10^8 spores mL^{-1}) was used to inoculate 100 mL of whole UHT milk (Co-op, UK) in a 250 ml sterile conical flask. This acted as a laboratory model for induction of volatile flavour production in cheese, and was developed from the earlier artificial cheese model of

Professor Christine Dodd et al. (pers. comms.) who also used UHT milk to assess volatile production by *P. roqueforti.* Cultures were incubated for 7 days at 24.0 °c in darkness, at 80 rpm. 3 biological replicates were performed for each strain. The content of each flask was then homogenized using an Ultraturrax at 14,000 rpm for 15 s, before 10 ml of homogenate was transferred into 20 ml headspace vials. Vials were sealed with magnetic caps and stored at -20 °C until SPME-GCMS analysis. GCMS was performed according to Gkatzionis et al. (2009) with some modifications, on a TRACE 1310 Gas Chromatograph. Extraction occurred at 50 °C for 20 min at continuous shaking using a Stableflex 50/30mm DVB/CAR/PDMS fibre and desorption at 230 °C for 0.1min in splitless mode. Volatiles were separated on a TG-5MS column (length 30m, I.D. 0.25mm, film thickness 0.1µm) using helium as the carrier gas at constant pressure of 18psi. Initial oven temperature 40 °C was held for 2 min, increasing 6 °C/min to final temperature 220 °C. The chromatograph was coupled to an ISQ mass spectrometer with transfer line held at 250 °C. Mass spectrometry was performed in EI+ mode at 70eV, the ion source temperature was 200 °C and the detector was scanning between 45-250 m/z at a scan rate 2 scan/s. Volatile compounds were identified by their retention times and comparing mass spectra with those of the National Institute of Standards and Technology (NIST) library.

All data were processed using Chromeleon 7.2 software (Thermo Scientific, UK). Volatile compound levels were calculated as peak area (counts/min) of each volatile and are presented as a percentage of peak areas obtained for parental homogenates (counts/min). Statistical tests were performed using GraphPad Prism 8.0.

4.2.14 Complementation of *lipD* and *lipI*

As a result of ongoing studies, it was also necessary to construct complementation cassettes. Complementation cassettes were constructed as per **Section 4.2.5** using primers detailed in Table 4.6. These cassettes contained in addition to the replacement cassette, the gene of interest ORF, 5’

regulatory sequences and ~200 bp 3' of the stop codon. The selectable marker was the *pyrG*-blaster. Complementation was achieved via transformation as detailed in **Section 4.2.8** and transformants were screened via positional PCR as detailed in **Section 4.2.9** using primers LipD_ex_F and LipD_in_R for *lipD* complementation and LipI_ex_F and LipI_in_R for *lipI* complementation. Confirmed complemented transformants were screened using SPME-GCMS as in Section 4.2.13.

Table 4.6. *Primers used to generate 5' (upstream + GOI) and 3' (downstream) flanking region of lipD and lipI for complementation cassettes.*

Gene complementation cassette	Fragments (length bp)	Primers used (sequence)
lipD complementation	Upstream + GOI (2423)	LipD_comp_up_F (cctctagagtcgacctgcaggcatgccgcccgacgatctttcttg)
		LipD_comp_gene_R (cgttgatggtgccaacaatctgcgccaagccatagtagagtgtgattg)
	Downstream (974)	LipD_comp_dw_F (acgaagagaagctcgacaacctggcggcgctcttaacaaagatac)
		LipD_comp_dw_R (accatgattacgccaagcttgcatgccaatcgaataggcaggattg)
lipI complementation	Upstream + GOI (2054)	LipI_comp_up_F (gccagtgaattcgagctcggtacccgggggcgcacaagagatggctatc)
		LipI_comp_gene_R (cgttgatggtgccaacaatctgcgcgccgaacaatgatgggataac)
	Downstream (1038)	LipI_comp_dw_F (acgaagagaagctcgacaacctggccagtggcccatagtacgg)
		LipI_comp_dw_R (tgcaggtcgactctagaggatccccggggatgaaaagatcgggcgg)

4.2.15 Expression of *lipD* and *lipI* in sexual progeny

Expression levels of *lipD* and *lipI* in milk media were determined via qRT-PCR as described in **Section 4.2.4.** Data are presented as ΔCt values to allow for direct comparison between isolates. Expression fold changes between parental isolates and progeny were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

4.3 Results

4.3.1 *In silico* identification of putative *pyrG*, *ku70* and lipase genes

The *Penicillium roqueforti* putative *pyrG* gene (encoding orotidine 5-phosphate decarboxylase) was identified using the search term "penicillium roqueforti AND orotidine". This search returned a single protein of 259 amino acids, annotated as "orotidine 5-phosphate decarboxylase pyrG-Penicillium chrysogenum", accession number CDM27009. The GenBank function was then

used to identify the nucleotide sequence of the ORF and flanking regions (See Appendix 1.1).

The *P. roqueforti* putative *ku70* (ATP-dependent DNA helicase II subunit 1) gene was identified using the search term "penicillium roqueforti AND ku70". This search returned a single protein of 653 amino acids, annotated as "ATP-dependent DNA helicase II subunit 1", accession number CDM35978. The GenBank function was then used to identify the nucleotide sequence of the ORF and flanking regions (See Appendix 1.2).

Putative *P. roqueforti* lipase genes were identified using the search term "penicillium roqueforti AND lipase". This search returned 14 hits in the identical protein groups resource (contains single entries for each protein translation found over several online resources). Lipases selected for further study are presented in Table 4.7. Five lipase proteins were excluded from selection based on annotations which predicted functions or activities that were not relevant such as glycoprotein modification, autophagy and fatty-acyltransferase activity. Selected lipases were arbitrarily named Lipase A - I based on the order they were found. The GenBank function was then used to identify the nucleotide sequence of the ORFs and flanking regions (See Appendix 1.3).

Table 4.7. *Lipases identified in silico using NCBI online database**

Lipase (accession number)	Annotation	Size (aas)
Lipase A (CDM30754)	Lipase, class 3	305
Lipase B (CDM29794)	Lipase	294
Lipase C (CDM34589)	Lipase, secreted	469
Lipase D (CDM28427)	Lipase, class 3	284
Lipase E (CDM27260)	Lipase, class 3	1140
Lipase F (CDM34977)	Lipase, class 3	399
Lipase G (CDM29966)	Lipase, class 3	305
Lipase H (CDM34335)	Lipase, class 3	549
Lipase I (CDM26301)	Lipase, class 2	274

4.3.2 Predicted subcellular localisation of identified proteins

DeepLoc-1.0 (Almagro Armenteros et al., 2017) was used to predict the subcellular localisations of the putative PyrG, Ku70 and lipase proteins. PyrG was predicted to localise to the cytoplasm (probability = 0.747). Ku70 was predicted to localise to the nucleus (probability = 1.0). LipA, LipB, LipC, LipD, LipF, LipG and LipI were predicted to localise extracellularly (probability = 0.988, 0.912, 0.983, 0.843, 0.624, 0.999 and 0.992, respectively). LipE and LipH were predicted to localise to the cytoplasm (probability = 0.448 and 0.53, respectively).

4.3.3 Growth in milk medium causes expression changes in some lipase genes

To gain insights into whether a particular lipase(s) may actively contribute to the breakdown of fats during the blue cheese manufacturing process, an expression study was undertaken. Expression levels of putative lipase genes were calculated as fold change between minimal medium and milk medium using the $2^{-\Delta\Delta Ct}$ method and *sac*7 as a reference gene (Gillot et al., 2017a). Genes which were upregulated in milk medium compared to minimal medium were considered likely to be important in the breakdown of fats present in milk. All lipase genes were actively expressed in both media conditions, with Ct < 32 for all experiments. ΔCt values (different in expression between lipase and *sac7*) varied. *lipB* (average ΔCt = -0.007) and *lipI* (average ΔCt = -4.5) had the highest relative expression (lowest ΔCt) in milk. Average ΔCt values varied from 4.2 – 7.6 for the other lipase genes, representing relatively low expression. Of the 9 genes tested, 5 were more than 2-fold upregulated in milk medium compared to minimal medium (Figure 4.4). These were *lipB* (6.3-fold, Student's t-test, T = 2.592, DF = 4, p = 0.06), *lipC* (4.7-fold, Student's t-test, T = 6.858, DF = 4, p = 0.002), *lipD* (7.9-fold, Student's t-test, T = 1.453, DF = 4, p = 0.22), *lipH* (2.7-fold, Student's t-test, T = 3.037, DF = 4, p = 0.039) and *lipI* (12.8-fold, Student's t-test, T = 4.861, DF = 4, p = 0.008). Due to considerable variability in expression levels between biological replicates, the only

differences in expression level that were statistically significant were those between minimal medium and milk medium of *lipC* ($p < 0.01$), *lipH* ($p < 0.05$) and *lipI* ($p < 0.01$).

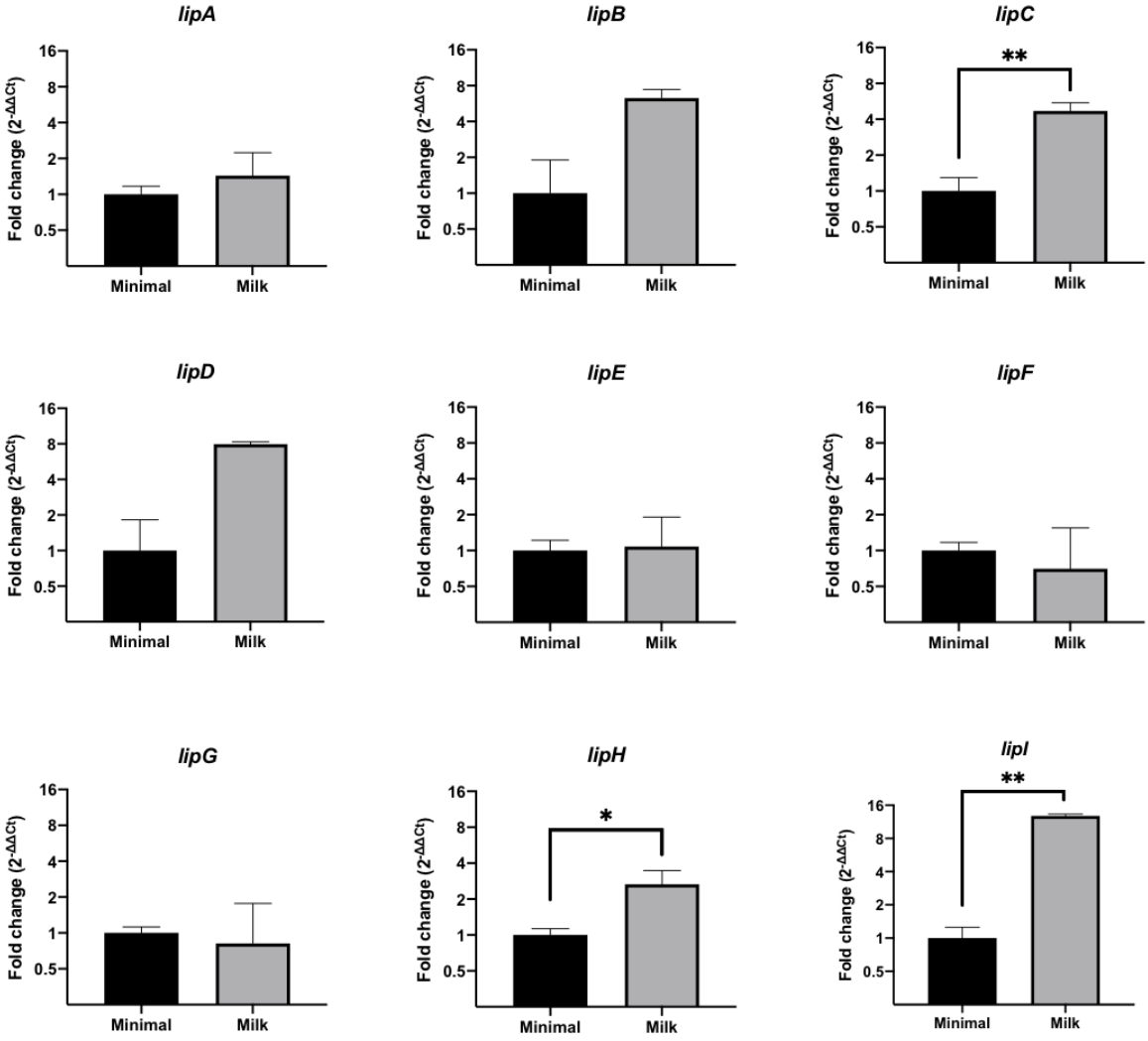

Figure 4.4. *Relative expression of lipase genes of* P. roqueforti *in minimal and milk media. * = p < 0.05. ** = p < 0.01. Error bars represent SEM. n = 3*

4.3.4 Deletion of *pyrG* gene causes uridine and uracil auxotrophy

Putative *pyrG* knockout strains (in which the resident *pyrG* gene was anticipated to be replaced by the *hph* hygromycin resistance marker gene) were characterised both molecularly, via PCR diagnostic and Southern blot methods, and physiologically by assessing the strains ability or inability to grow on media without uridine and uracil supplementation. One transformant (74-258) (out of 17 Hygromycin resistant transformants) was confirmed via

molecular means to have had *pyrG* deleted and the gene replacement cassette inserted at the correct locus (Figure 4.5b). The gene replacement cassette was also confirmed to have inserted only once into the genome (Figure 4.5c). This transformant was unable to grow on media lacking uridine and uracil supplementation and was therefore confirmed to be an auxotrophic mutant (Figure 4.5d). The growth rate on PDA + 10 mM uridine + 10 mM uracil of the Δ*pyrG*::*hph* transformant was slightly higher than of the parental 74-88 (WT) strain [colony diameter 38.70 mm ± 0.25 (SEM) compared to 36.73 mm ± 0.58 (SEM), respectively] (Student's t-test, T = 3.105, DF = 4, $p < 0.05$) (Figure 4.6).

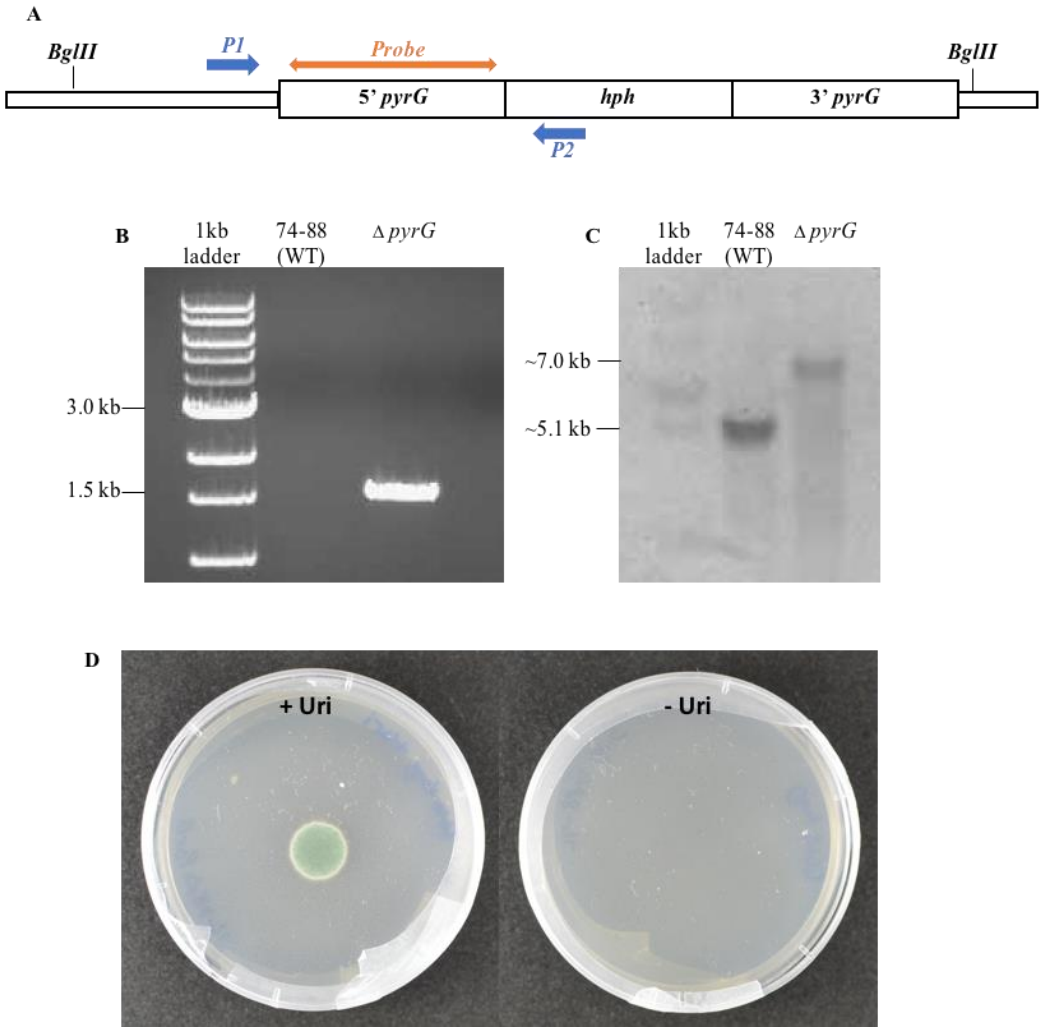

***Figure 4.5**. Characterisation of ΔpyrG::hph. A) Schematic ΔpyrG locus with location of primers for external PCR diagnostic and probe for Southern blot. P1 = pyrG_ex_F, P2 = hph_pos_R. B) External PCR diagnostic confirming insertion of replacement cassette at correct locus in ΔpyrG::hph. C) Southern blot confirming single correct integration of replacement cassette in ΔpyrG::hph. Southern probe hybridised*

to an expected 5.1 kb fragment in the parental isolate and a 7.0 kb fragment in ΔpyrG::hph. D) ΔpyrG::hph is unable to grow on minimal media (GG10) without uridine and uracil supplementation.

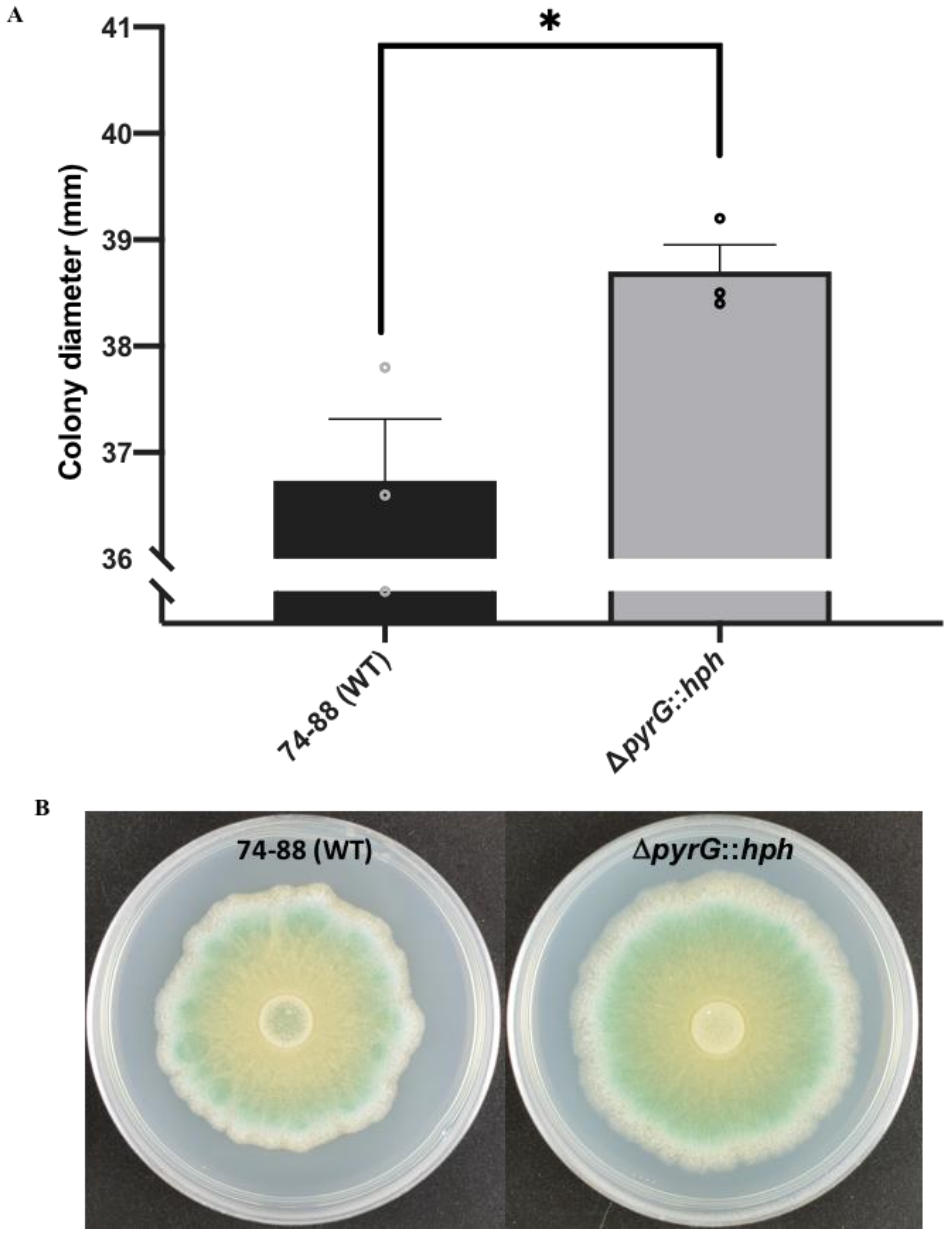

***Figure 4.6**. Growth rate and colony morphology of ΔpyrG::hph. A) Growth rate of ΔpyrG::hph is higher than WT. * = p < 0.05. Error bars represent SEM. n = 3. B) Colony morphology is visually unaffected by pyrG deletion.*

4.3.5 Deletion of *ku70* improves gene targeting efficiency

The Δ*pyrG*::*hph* strain was used as a parental strain in attempts to delete *ku70* using the *pyrG*-blaster. The generation of multiple transformants which were able to grow on media without uridine and uracil supplementation would confirm that the *pyrG*-blaster can restore prototrophy and complement the *pyrG* deletion.

53 Putative *ku70* knockout strains were obtained from transformations. Transformants were then characterised molecularly via PCR diagnostic and Southern blot methods, and 5 were confirmed to have had *ku70* deleted and the gene replacement cassette inserted at the correct locus (Figure 4.7b). The gene replacement cassette was present as a single integration in three of these transformants (Figure 4.7c). Δ*ku70*::*pyrG*-1 [henceforth referred to as Δ*ku70*::*pyrG* (74-259)] was selected for further experimentation.

Genetic transformations were undertaken using both a *ku70*$^{+}$ strain (Δ*pyrG*::*hph*) and a *ku70*$^{-}$ strain (Δ*ku70*::*pyrG*) to assess whether gene targeting efficiency had been altered due to the *ku70* deletion. Gene targeting success was 7.7 % ± 1.8 (SEM) (average based on 17 and 53 screened transformants from individual transformations screened for *pyrG* and *ku70* knockouts, respectively) in the *ku70*$^{+}$ strain but increased to 59.6 % ± 1.9 (SEM) in the *ku70*$^{-}$ strain (average based on 23, 27 and 27 screened transformants from individual transformations screened for *lipA*, *lipB* and *lipD* knockouts, respectively) representing a statistically significant increase in gene targeting efficiency using the latter strain (Student's t-test, T = 18.84, DF = 3, $p < 0.001$) (Figure 4.7d). Deletion of *ku70* did not affect growth rate, which was statistically the same as the parental isolate Δ*pyrG*::*hph* (Student's t-test, T = 0.239, DF = 4, $p > 0.05$) (Figure 4.8).

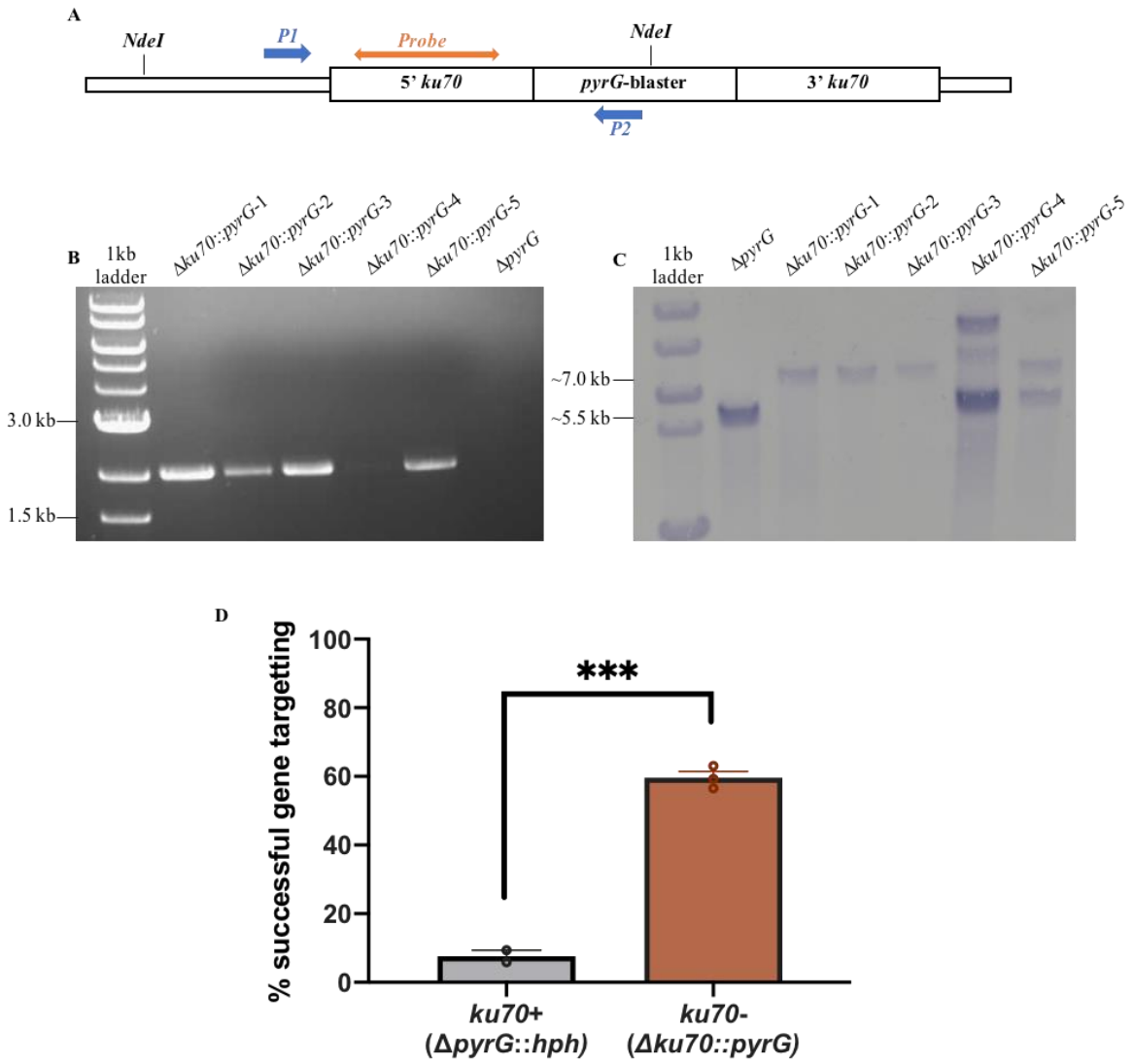

Figure 4.7*. Characterisation of Δku70::pyrG. A) Schematic Δku70 locus with location of primers for external PCR diagnostic and probe for Southern blot. P1 = ku70_ex_F, P2 = ura_pos_R. B) External PCR diagnostic confirming insertion of replacement cassette at correct locus in Δku70::pyrG strains. C) Southern blot confirming single correct integration of replacement cassette in Δku70::pyrG-1, Δku70::pyrG-2 and Δku70::pyrG-3. Southern probe hybridised to an expected 5.5 kb fragment in the parental isolate and a 7.0 kb fragment in Δku70::pyrG. D) Gene targeting was more efficient in Δku70::pyrG compared to the parental isolate [based on attempted transformations with either* pyrG *and* ku70 *(*ku70+*) or* lipA, lipB *and* lipD *(*ku70-*)]). *** = p < 0.001. Error bars represent SEM.* Ku70+ *n = 2,* ku70- *n = 3.*

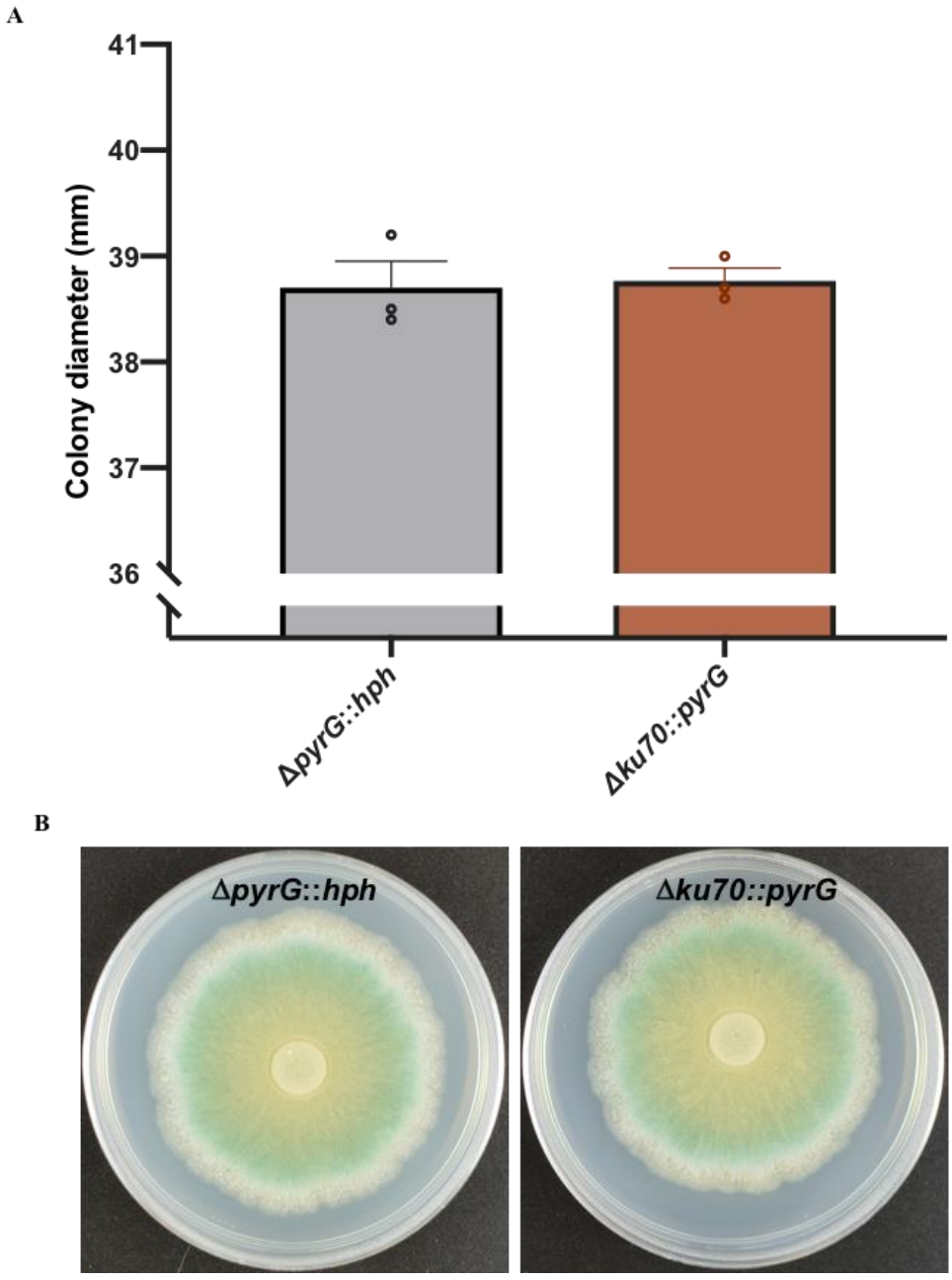

Figure 4.8. *Growth rate and colony morphology of Δku70::pyrG. A) Growth rate of Δku70::pyrG is the same as parental isolate ΔpyrG::hph. Error bars represent SEM. n = 3. B) Colony morphology is visually unaffected by ku70 deletion.*

4.3.6 Iterative gene deletions are possible using the *pyrG*-blaster

In order to generate multiple gene replacements in the same genetic background strain, an attempt was made to screen and select for fungal colonies which no longer possessed a functional *pyrG*. Δ*ku70*::*pyrG* was grown on GG10 supplemented with 1.0 mg mL^{-1} 5-FOA and after 5 days several healthy colonies began to appear (Figure 4.9). This indicted that in these

colonies, the functional *pyrG* was most likely to have been excised and uridine and uracil auxotophy had been restored. A number of these colonies were sub-cultured and one, Δ*pyrG*Δ*ku70* (74-272), was chosen as a representative for use in further experimentation. This strain was used to successfully generate lipase gene knockouts as described in the following Section 4.3.7.

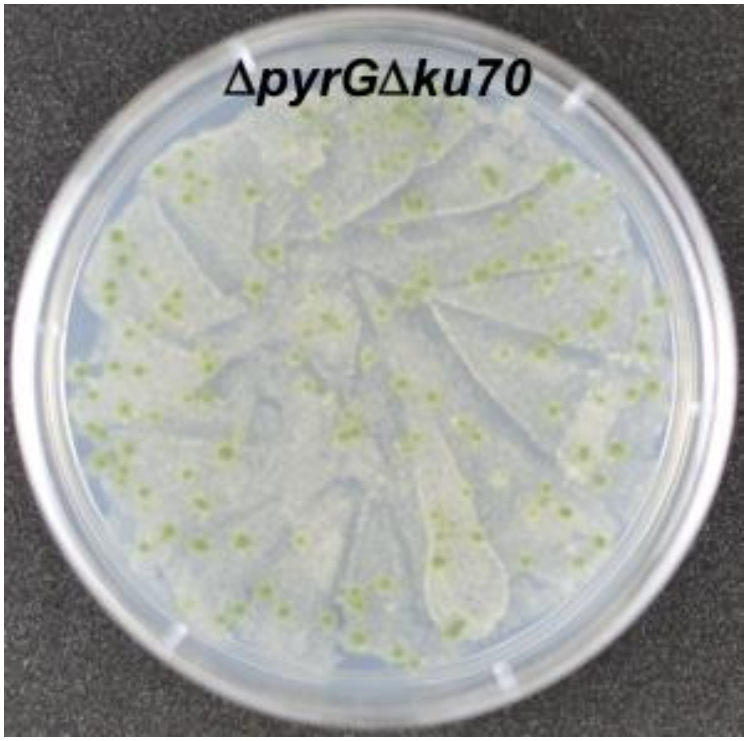

Figure 4.9*. Growth of Δku70::pyrG on 5-FOA selection plate. Appearance of colonies is consistent with the pyrG-blaster having been excised in growing colonies, and the genotype of these colonies has been altered to Δ*pyrGΔku70*.*

4.3.7 *lipD* and *lipI* both contribute to the production of the key blue cheese-associated volatile compounds, 2-heptanone and 2-nonanone

All lipase genes identified *in silico* (*lipA to lipI*; Section 4.3.1) were successfully deleted by replacement of the gene with the *pyrG*-blaster, as confirmed by appropriate PCR diagnostic tests (Figure 4.10). All transformations generated multiple entopic transformants, therefore a representative knockout strain for each gene deletion was chosen for further experimentation. Growth rates on PDA were unaffected by lipase gene deletions (Welch's ANOVA, W (DFn, DFd) = 0.3851 (9.00, 8.04), $p > 0.05$), and neither were colony morphologies (Figure 4.11).

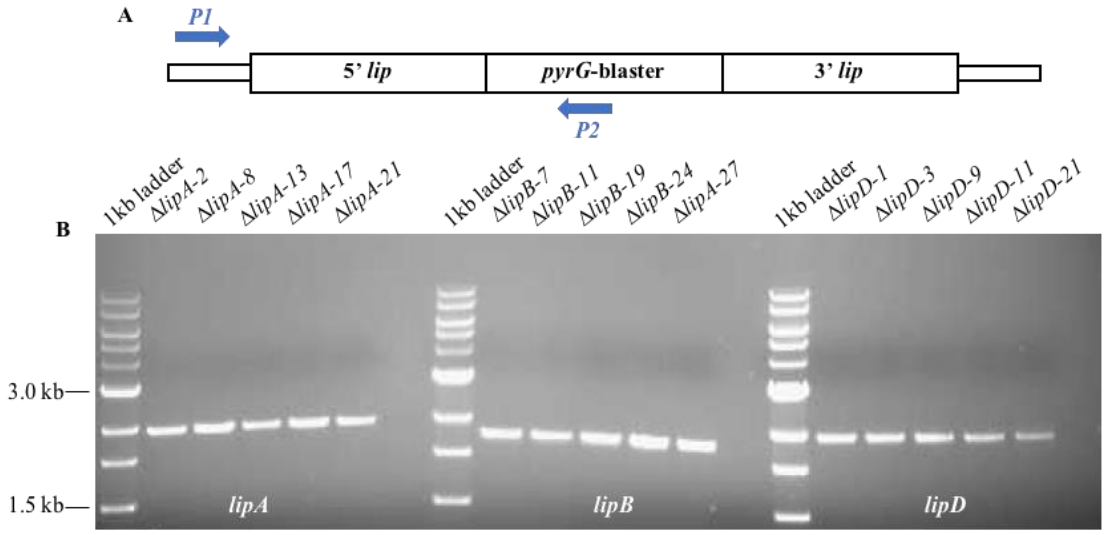

***Figure 4.10**. Representative genotyping of lipase transformants. A) Schematic Δlipase locus with location of primers for external PCR diagnostic P1 = lipX_ex_F, P2 = ura_pos_R. B) External PCR diagnostic confirming insertion of replacement cassette at correct locus in Δlipase strains.*

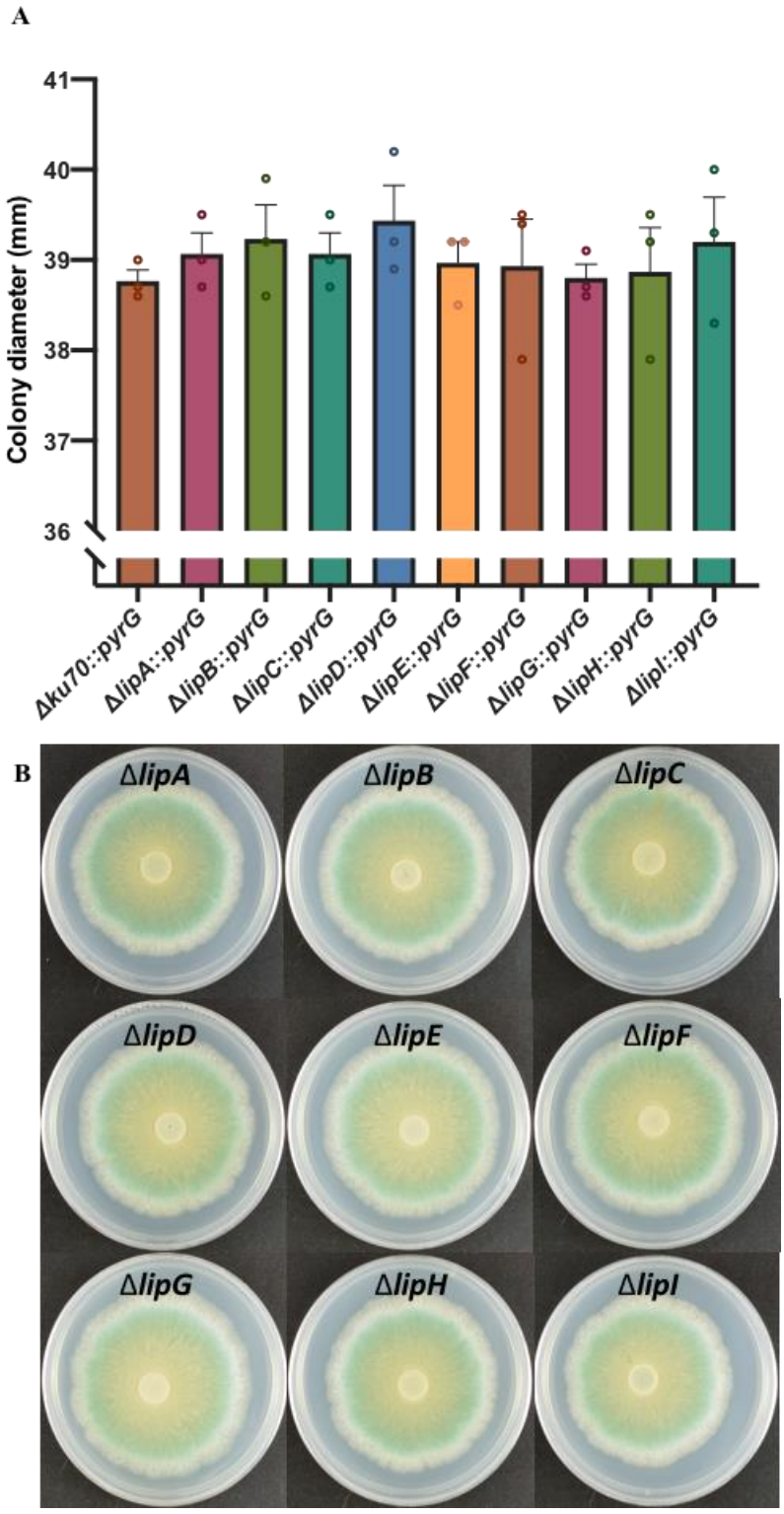

Figure 4.11*. Growth rate and colony morphology of lipase knockouts of* P. roqueforti *on PDA medium. A) Growth rates of all lipase knockout strains are similar to parental isolate Δku70::pyrG. Error bars represent SEM. n = 3. B) Colony morphology is visually unaffected by lipase deletions.*

Each individual lipase knockout strain was assayed for total lipolytic activity based on depth of clearance in a tributyrin assay (Figure 4.12). A Welch's

ANOVA revealed significant differences in total lipolytic activity between strains (Welch's ANOVA, W (DFn, DFd) = 69.59 (9.00, 8.07), $p < 0.0001$). No individual lipase knockout strain except Δ*lipD::pyrG* had a statistically significant difference in total lipolytic activity compared to the parental strain (Δ*ku70*::*pyrG*). Total lipolytic activity of Δ*lipD::pyrG* was 70.4 ± 0.7% (SEM) of the parental strain (Dunnett's T3 multiple comparisons test, T = 15.56, DF = 2.616, $p < 0.01$).

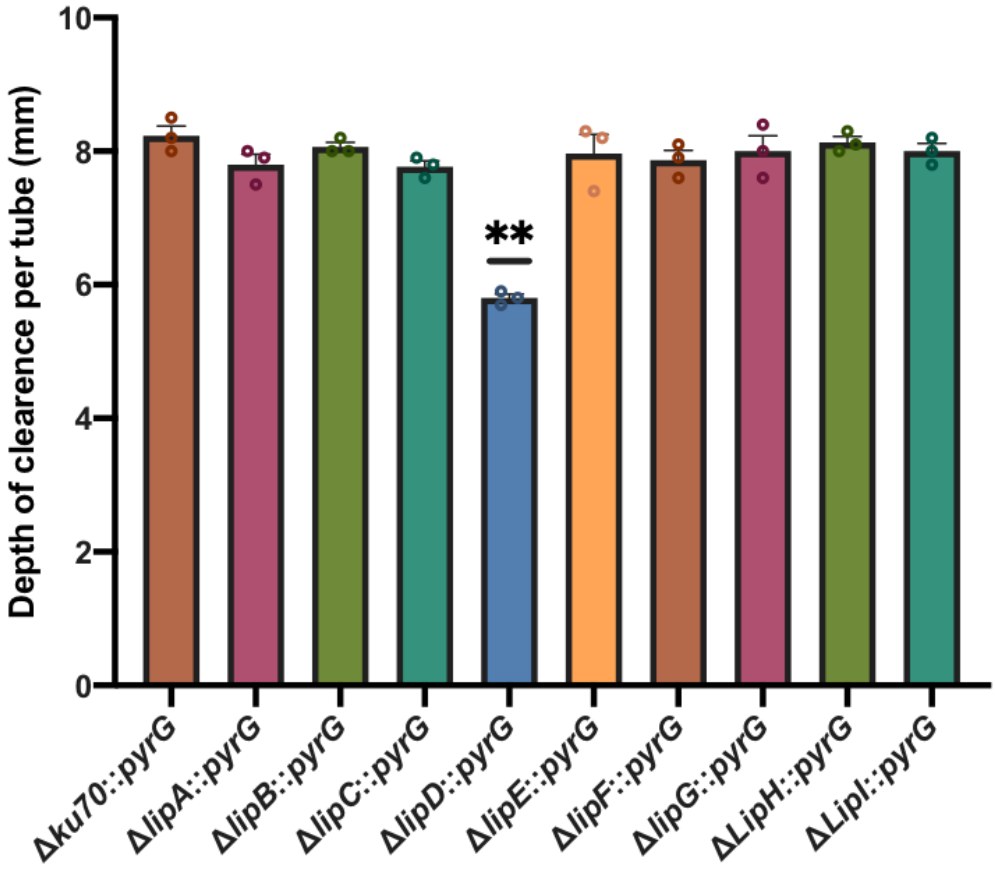

Figure 4.12. *Total lipolytic activity of parental isolate Δku70::pyrG and lipase knockout strains. ΔlipD::pyrG had significantly lower totally lipolytic activity to parental isolate. ** = p < 0.01. Error bars represent SEM. n = 3.*

SPME-GCMS profiling was undertaken to assess the levels of volatile compounds produced by each lipase gene knockout strain, after 7 days' growth in milk. Specific analysis was undertaken for certain volatile compounds which are formed as a result of lipolysis and which are thought to

be essential for blue cheese flavour and aroma, namely 2-heptanone and 2-nonanone (Gkatzionis et al., 2009).

There were significant differences in 2-heptanone levels between strains (Welch's ANOVA, W (DFn, DFd) = 8.368 (9.00, 11.54), $p < 0.001$) (Figure 4.13a). Of note, the level of 2-heptanone produced by two knockout strains, Δ*lipD::pyrG* and Δ*lipI::pyrG*, was reduced to 14.5 ± 3.7 % (SEM) (Dunnett's T3 multiple comparisons test, T =4.636, DF = 3.248, $p = 0.08$) and 20.8 ± 2.1 % (Dunnett's T3 multiple comparisons test, T = 4.355, DF = 3.085, $p = 0.10$) that of the parental strain, respectively (Figure 4.13a). There were also significant differences in 2-nonanone levels between strains (Welch's ANOVA, W (DFn, DFd) = 7.188 (9.00, 19.93), $p < 0.01$) (Figure 4.13b). The level of 2-nonanone produced by Δ*lipD::pyrG* and Δ*lipI::pyrG* was again of note, with Δ*lipD::pyrG* producing 14.5 ± 5.2 % (SEM) (Dunnett's T3 multiple comparisons test, T = 2.418, DF = 3.134, $p = 0.37$) and Δ*lipI::pyrG* producing 13.2 ± 1.9 % (SEM) (Dunnett's T3 multiple comparisons test, T = 2.477, DF = 3.018, $p = 0.36$) that of the parental strain (Figure 4.13b). These results were each verified with a separate individual *lipD* or *lipI* knockout strain (results not shown). Although the reductions in methyl ketone production were not statistically significant (according to Dunnett's T3 multiple comparisons test), the marked decreases warranted further investigation.

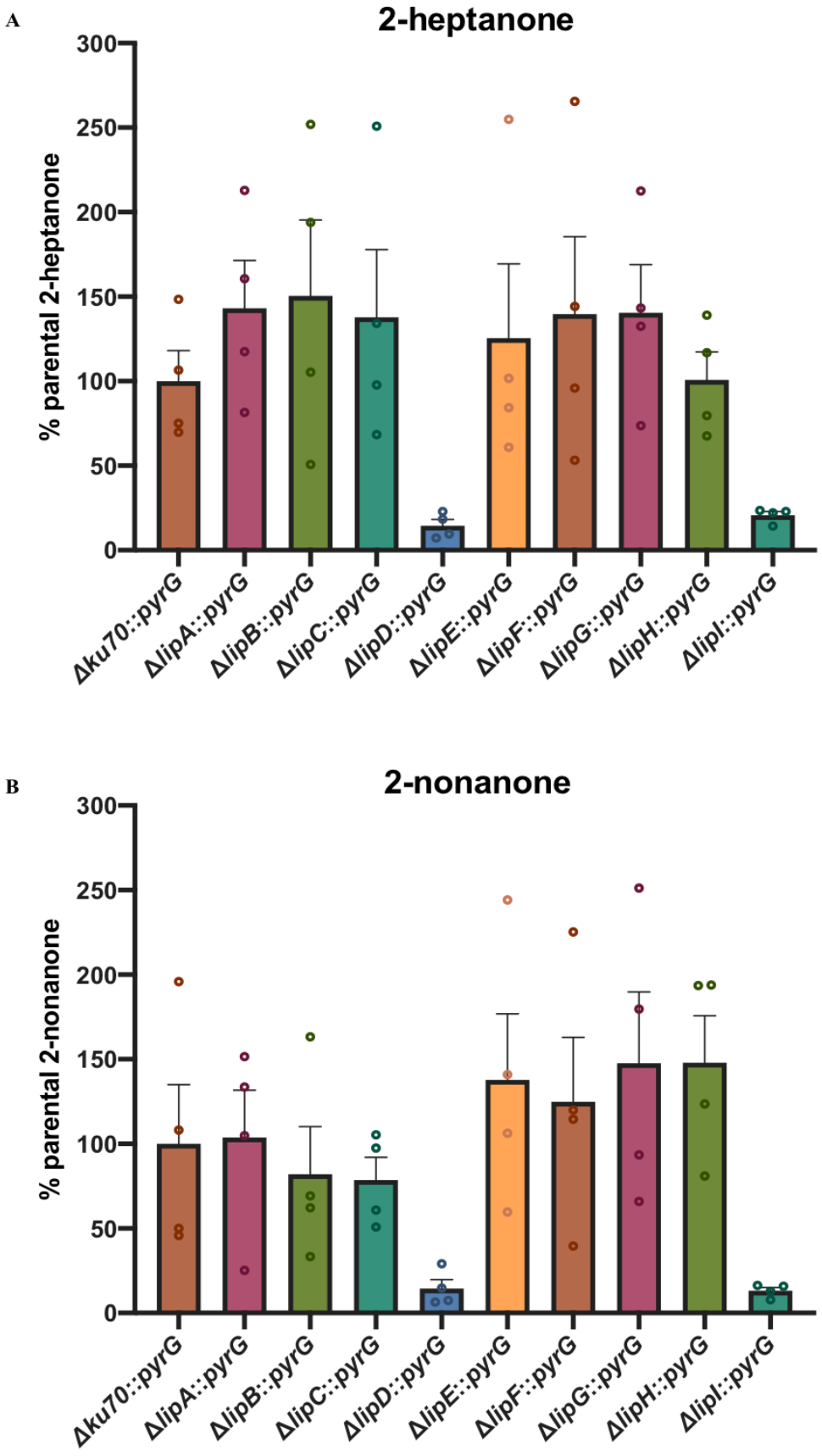

Figure 4.13. *Methyl ketone production levels of lipase knockout strains, expressed as percentage of parental isolate (Δku70::pyrG). A) 2-heptanone. B) 2-nonanone. Error bars represent SEM. n = 4.*

Δ*lipD::pyrG* and Δ*lipI::pyrG* were transformed with complementation cassettes containing the full open reading frame plus regulatory sequences of the corresponding deleted lipase gene. The cassettes were confirmed to have inserted at the correct loci via positional PCR. Representative transformants were then used to assess methyl ketone production levels in new, individual experiments.

2-heptanone production by Δ*lipD::pyrG* was 16.5 ± 6.5 % (SEM) that of the parental isolate (Student's t-test, T = 5.542, DF = 2, $p < 0.05$). Addition of *lipD* to Δ*lipD::pyrG* (creating Δ*lipD::pyrG* + LIPD) restored 2-heptanone levels to 117.6 ± 28.1 % (SEM) that of the parental isolate, which was not a statistically significant difference from the parent (Student's t-test, T = 0.5629, DF = 2, $p > 0.05$) (Figure 4.14a). Similarly, 2-nonanone production which was 15.8 ± 7.6 % (SEM) that of the parental isolate in Δ*lipD::pyrG* (Student's t-test, T = 4.008, DF = 2, $p = 0.057$) was restored to 256.0 ± 34.1 % (SEM) that of the parental isolate in Δ*lipD::pyrG* + LIPD, which was not a statistically significant difference from the parent (Student's t-test, T = 4.065, DF = 2, $p > 0.05$) (Figure 4.14a).

2-heptanone production by Δ*lipI::pyrG* was 2.5 ± 0.2 % (SEM) that of the parental isolate (Student's t-test, T = 4.938, DF = 2, $p < 0.05$). Addition of *lipI* to Δ*lipI::pyrG* (creating Δ*lipI::pyrG* + LIPI) only partially restored 2-heptanone levels to 11.6 ± 0.4 % (SEM) that of the parental isolate, which was significantly different (Student's t-test, T = 4.475, DF = 2, $p < 0.05$) (Figure 4.14b). Similarly, 2-nonanone production which was 1.4 ± 0.1 % (SEM) that of the parental isolate in Δ*lipI::pyrG* (Student's t-test, T = 24.10, DF = 2, $p < 0.01$) was again only partially restored to 8.3 ± 0.2 % (SEM) that of the parental isolate in Δ*lipI::pyrG* + LIPI, which was significantly different (Student's t-test, T = 22.39, DF = 2, $p < 0.01$) (Figure 4.14b).

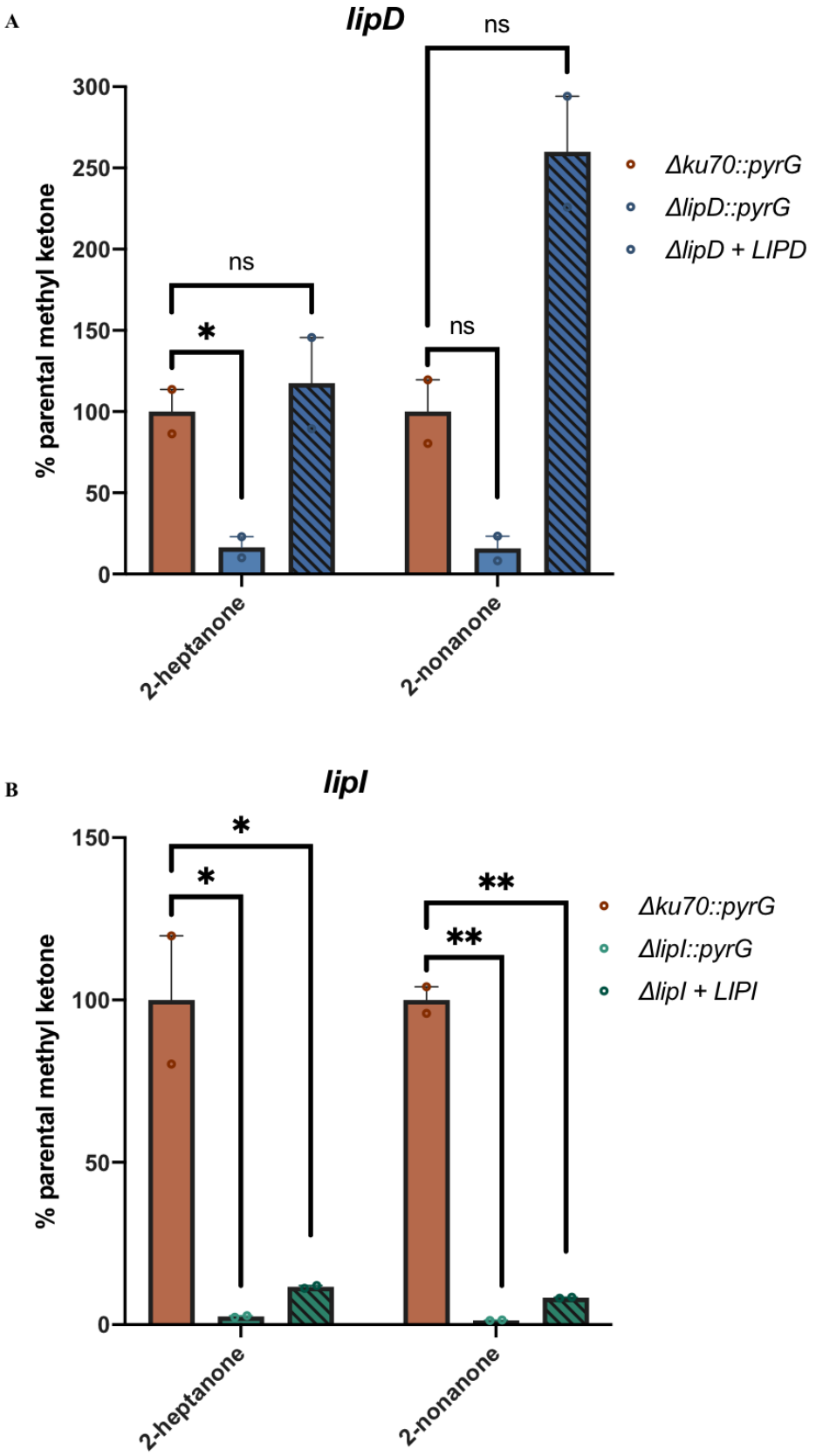

***Figure 4.14**. Methyl ketone production levels of lipase knockout strains and complemented strains, expressed as percentage of parental isolate (Δku70::pyrG). A) lipD. B) lipI. * = p < 0.05. ** = p < 0.01. Error bars represent SEM. n = 2.*

4.3.7 LipD and LipI are predicted to function efficiently in differing pH conditions

Previous studies have found that *P. roqueforti* produces two extracellular lipases, one which operates more efficiently in acidic conditions and the other in alkaline conditions (Lamberet and Menassa, 1983b; Mase et al., 1995; Menassa and Lamberet, 1982). AcalPred and NCBI BLAST were therefore used to gain insight into the potential pH optimuma for LipD and LipI. LipD was predicted by AcalPred to be an alkaline enzyme, with a probability of 0.64. In addition, LipD has 85.5 % sequence identity to a triacylglycerol lipase from *P. expansum* (accession number AGO44976) which has been shown experimentally to have an optimum of pH 8, with activity only slightly reduced at pH 9 and pH 10 (Mohammed et al., 2013). Further, LipD has 81.6 % sequence identity to an "alkaline lipase" from *P. cyclopium* (accession number AF274320). Conversely, LipI was predicted by AcalPred to be an acidic enzyme, with a probability of 0.63. Results from BLAST analysis revealed homology with many predicted lipases from closely related species (e.g. *Penicillium spp., Aspergillus spp.*), however no proteins with experimental data were evident.

Several closely related fungal species produce lipases which specifically hydrolyse di- and monoacylglycerides and cannot efficiently hydrolyse triacylglycerides (Shotaro et al., 1991; Tsuchiya et al., 1996; Xu et al., 2015). Three of these lipases (MdlA from *P. camemberti*, accession number BAA14345; MgMDL2 from *Malessezia globosa*, accession number XP_002732206; and MdlB from *Aspergillus oryzae*, BAA12912) were used to screen for homology to LipD and LipI, however none was found. Interestingly, some homology to these lipases was found for LipB and LipG (between 25 – 44 % sequence identity).

4.3.8 Sexual reproduction can lead to novel expression profiles of *lipD* and *lipI*

Expression of *lipD* and *lipI* in milk was determined for parental isolates 74-130 and 74-144 and sexual progeny from cross between these parental isolates,

using expression of *sac*7 as a reference gene (Figure 4.15). Expression of both *lipD* (One-way ANOVA, F (DFn, DFd) = 58.46 (6, 7), $p < 0.0001$) and *lipI* (One-way ANOVA, F (DFn, DFd) = 69.50 (6, 7), $p < 0.0001$) was significantly different between isolates. Novel (i.e. significantly different to both parental isolates, denoted by "a,b") *lipD* expression was found in progeny A7, A18 and A22 (Figure 4.15a). By contrast, none of the progeny were found to exhibit novel levels of *lipI* expression, although all progeny had significantly different *lipI* expression to 74-130 (Figure 4.15b). A22 had the highest relative expression of *lipD* out of the isolates, being 28.6-fold higher than 74-130 (Dunnett's multiple comparisons test, Q = 15.18, DF = 7, $p < 0.0001$) and 8.7-fold higher than 74-144 (Dunnett's multiple comparisons test, Q = 9.792, DF = 7, $p < 0.001$). A7 had the highest relative expression of *lipI* out of the isolates, being 69.2-fold higher than 74-130 (Dunnett's multiple comparisons test, Q = 17.38, DF = 7, $p < 0.0001$) and 1.9-fold higher than 74-144 (Dunnett's multiple comparisons test, Q = 2.545, DF = 7, $p = 0.1416$).

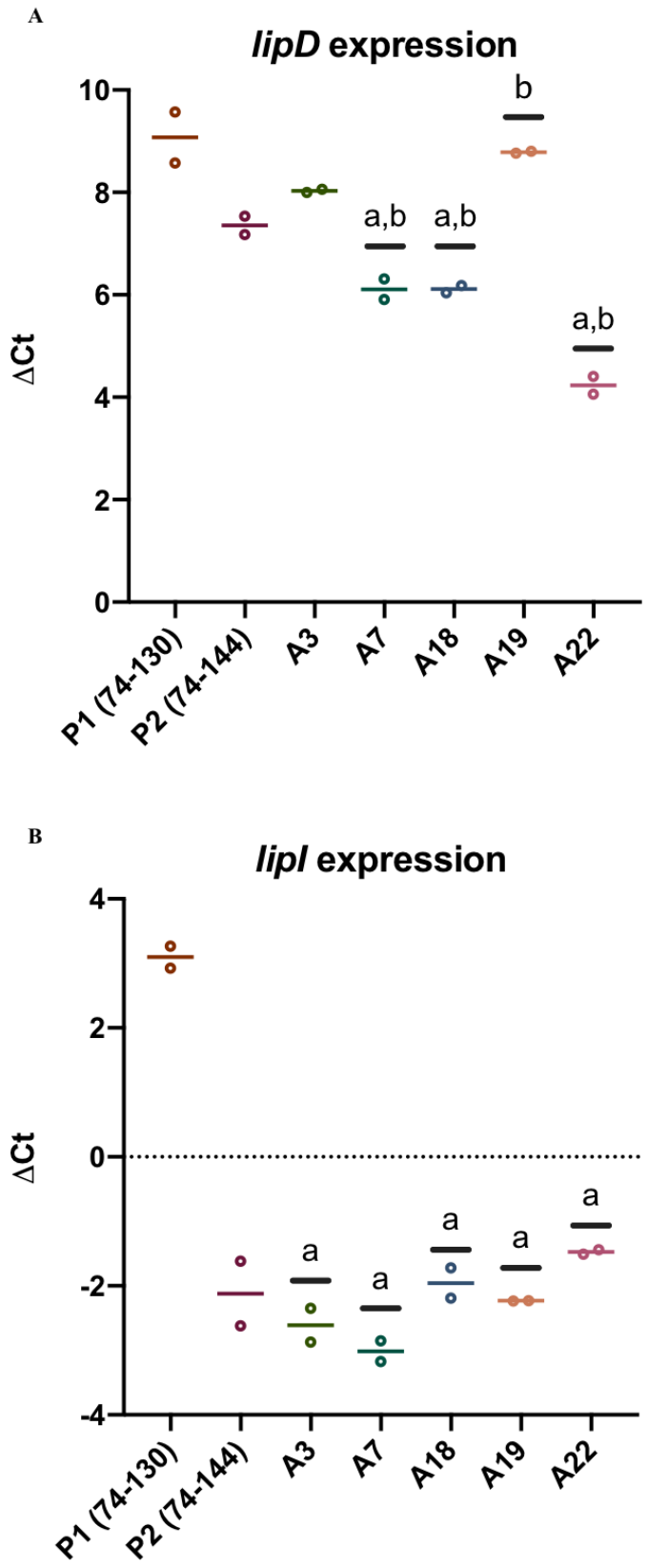

Figure 4.15. Expression of lipase genes in milk for parental isolates 74-130 and 74-144 and sexual progeny relative to expression of sac7 as a reference gene A) lipD expression. B) lipI expression. ***a*** *= significantly different to P1 (Dunnett's multiple comparisons test, $p < 0.05$).* ***b*** *= significantly different to P2 (Dunnett's multiple comparisons test, $p < 0.05$).* ***a,b*** *= significantly different to P1 and P2 (Dunnett's multiple comparisons test, $p < 0.05$). Error bars represent SEM. n = 2*

4.4 Discussion

The lipolytic system of *Penicillium roqueforti* is the most important factor in determining the flavour of blue cheese, but until now the particular genes which constitute this system have remained elusive. This may be partially because genetic manipulation of this fungus is difficult, and genetic tools commonly used in other filamentous fungi to make manipulation more efficient are unavailable. In this study, a "laboratory strain" which is highly efficient for use in gene deletions was created by identifying and deleting two genes, *pyrG* and *ku70*. Concurrently, several lipase genes were identified by bioinformatic analysis and some were found to be upregulated in response to growth in milk, suggestive of their potential importance for maturation and flavour production in blue cheese. The newly created laboratory strain was then used to generate lipase knockout strains which led to the identification of two genes, *lipD* and *lipI*, which encode for lipases that likely constitute the main extracellular lipolytic activity of *P. roqueforti* when grown in milk medium. These enzymes have a key role in the formation of the most important blue cheese-associated compounds, 2-heptanone and 2-nonanone, and are therefore critical for blue cheese maturation and flavour production. Finally, the expression of these key genes in sexual progeny of *P. roqueforti* was determined and novel expression profiles were found, suggesting that sexual reproduction can lead to variation in lipolytic activity by generating new patterns of gene expression.

4.4.1 Creation of a laboratory model strain of *Penicillium roqueforti*

Gene replacement in *P. roqueforti* by homologous recombination has been reported to be "very challenging" (Gillot et al., 2017a). Therefore, this study set out to create a strain which was more efficiently able to be manipulated in this way.

The first stage of creating the laboratory strain was to delete the putative *P. roqueforti pyrG* gene in order to establish a marker recycling system via the

use of a *pyrG*-blaster (Alani et al., 1987; Brock et al., 2007). As expected, deletion of *pyrG* in isolate 74-88, via hygromycin selection, resulted in uridine/uracil auxotrophy in this fungus. This auxotrophy was subsequently complemented with a *pyrG*-blaster. The successful restoration of prototrophy confirms that the *Aspergillus nidulans pyrG* gene contained within the blaster is functional in *P. roqueforti*. In a previous study, the *pyrG* gene of *P. camemberti* was successfully used to restore uridine/uracil prototrophy in an *A. nidulans ⊿pyr*G strain (Navarrete et al., 2009). However, the present study represents the first report of use of a heterologous *pyrG* to restore prototrophy in a fungus used in cheese production. It was found that *pyrG* was successfully excised after growth on media containing 5-FOA, which confirms that the *pyrG*-blaster system is suitable for multiple rounds of gene deletions in *P. roqueforti*. Surprisingly, the growth rate of *Δku70::pyrG* was slightly higher than the wild-type isolate. This could be because the *A. nidulans* PyrG is more active than the native PyrG. Alternatively, the expression of *A. nidulans pyrG* native may be higher than the native *pyrG* because of chromosome positional effects (Akhtar et al., 2013; Bok et al., 2009). No other morphological differences were apparent, however it is conceivable that the deletion could have undesired effects on other biological processes which were not tested in this study, such as sexual reproduction (Robellet et al., 2010). It is also noted that initial efforts to produce a laboratory model strain using a different isolate of *P. roqueforti* (74-170) were not successful, suggesting some genetic variation in *P. roqueforti* as to the amenability of strains for laboratory manipulation.

The second stage of creating the laboratory strain was to delete the putative *ku70* gene to disrupt non-homologous end joining and hence increase the rates of entopic integration of gene replacement cassettes (Kück and Hoff, 2010). Although the *P. roqueforti ku70* gene has been identified in a previous study (Ropars et al., 2012), this was the first reported attempt to characterise its function by gene deletion. The rate of successful gene targeting in transformations increased from 7.7 ± 1.8 % in the *ku70*$^{+}$ isolate (*Δpyr*G*::hph)*

to 59.6 ± 1.9 % in the *ku70*$^{-}$ (Δ*ku70::pyrG*) strain, indicating that the latter can be used to significantly increase the percentage of entopic transformants and reduce the time required for screening. This level of gene targeting efficiency is similar to that achieved in other filamentous fungi in which the respective *ku70* or *ku80* homologue had been deleted (Qiao et al., 2019). In agreement with previous studies, growth rate was unaffected by *ku70* deletion (Qiao et al., 2019). No obvious abnormal phenotype was observed in the Δ*ku70::pyrG* strain. However, *ku* disruption has been shown to cause mild unintended phenotypes in other *Penicillium spp*., such as genomic instability (Bugeja et al., 2012) or reduced fitness when co-cultured with wild-type strains (Snoek et al., 2009). Therefore, further characterisation of the Δ*ku70::pyrG* strain is required to assess whether this may be the case for *P. roqueforti*.

4.4.2 Identification and characterisation of lipase genes in *P. roqueforti*

Nine putative lipase genes were identified from the genome of *P. roqueforti* based on bioinformatic analysis, these being termed *lipA*-*lipI.* Further *in silico* analysis revealed that seven of the genes (*lipA*-*D, LipF, LipG and LipI*) were predicted to be exported extracellularly. The role of the putative lipase genes was investigated by both gene knock out and expression studies.

The newly developed *P. roqueforti ΔpyrGΔku70* strain was successfully used to generate deletion strains for each of the individual *lipA*-*I* genes. In initial experiments the activity of these deletion strains was investigated using a tributyrin assay system (Larsen and Jensen, 1999). This revealed that a significant loss of lipolytic ability was only seen following deletion of *lipD.* However, the tributyrin assay was considered not sufficiently representative of the complex range of lipids found in milk and cheese. Therefore additional experiments using milk as a substate were undertaken.

RT-qPCR was used to measure and compare expression levels of the putative lipase genes in minimal media and milk. All of the lipase genes were found to

be constitutively expressed but most genes had relatively low expression. The genes *lipB and lipI* had the overall highest levels of gene expression in milk. Meanwhile, it was theorised that genes which are more upregulated during growth in milk are more likely to be involved with *P. roqueforti* lipolysis during blue cheese maturation. Overall, 5 out of 9 genes were more than 2-fold upregulated in milk compared to minimal media, of which *lipD* (7.9-fold increase) and *lipI* (12.8-fold increase) were the most upregulated. A limit of this experiment was that expression was only assessed at a single time point, and therefore it is unclear whether a certain lipase might be differentially expressed during other stages of growth in milk. RNA should be extracted at additional time points to explore this further. Although upregulation in milk suggests an importance for the encoded enzymes, further evidence was needed and therefore additional functional characterisations were undertaken.

The phenotypes of several lipase knockout strains were characterised specifically in the context of volatile compound production. It was found that deletion of *lipD* or *lipI* reduced (albeit not significantly) the levels of key blue cheese flavour-associated compounds (2-heptanone and 2-nonanone) produced from milk compared to the parental isolate (Δ*ku70*::*pyrG*). By contrast, deletion of *lipB,* the gene with the highest overall expression in milk, had no significant effect on production of 2-heptanone and 2-nonanone. In initial screening experiments, the reduction in methyl ketone levels seen in the Δ*lipD* and Δ*lipI* strains was not statistically significant. This is likely because of experimental limitations in that the homogeneity of the UHT milk used could not be fully controlled. This may have meant there were differing total fat contents or initial methyl ketone levels present in the milk used for different biological replicates which might have affected final measurements (Liu et al., 2020; Vazquez-Landaverde et al., 2005). In more directed experiments, assessing the complementation strains, experiments were performed using milk from a single carton to limit experimental variation and statistical

significance between the parental isolate and the lipase knockout strains was achieved.

Previous biochemical studies have found that *P. roqueforti* produces two extracellular lipases, one which is more adapted to alkaline conditions and the other acidic conditions (Lamberet and Menassa, 1983b; Mase et al., 1995; Menassa and Lamberet, 1982). It is possible that LipD and LipI correspond with these enzymes and therefore evidence for predicted pH optimums was sought. The free online software AcalPred was employed, which has been found to accurately predict the pH adaptions (alkaline or acidic) of 96.3 % of acidic enzymes and 97.1 % of alkaline enzymes (Lin et al., 2013). Interestingly, LipD is predicted to be an alkaline enzyme and LipI an acidic enzyme, although the probability values were not strong. To reinforce the prediction for LipD as an alkaline lipase, significant sequence identity was found to be shared with an experimentally validated alkaline lipase from *Penicillium expansum* (Mohammed et al., 2013) and a lipase from *P. cyclopium* that is annotated as "alkaline lipase". Another observation that may allude to LipD being an alkaline lipase is that the Δ*lipD* strain was the only lipase knockout strain to have reduced total lipolytic activity as shown in the tributyrin assay. The media used for the assay is alkaline and therefore loss of an alkaline lipase is more likely to cause a noticeable phenotype in these conditions (Larsen and Jensen, 1999). Although predictive tools and homology screening can be useful, future studies should aim to purify LipD and LipI and undertake biochemical analysis to experimentally determine the pH optimuma of these lipases.

It was perhaps surprising that deletion of both individual *lipD* and *lipI* genes resulted in decreases (albeit not significant) in levels of methyl ketones, as it might have been expected that there would be some level of functional redundancy in *P. roqueforti* given the number of lipase genes - and hence one lipase might compensate for the other (Amich and Krappmann, 2012; Gu et al., 2003; Kitami and Nadeau, 2002). However, observations from the current study suggest that both LipD and LipI are required for full lipolysis. An

explanation for this might be that the lipases have differing affinities for particular fatty acid positions (*sn*-positions) on the glycerol backbone of triacylglycerides (TAGs) found in milk (Eichmann et al., 2012). Alternatively, either LipD or LipI function might be reliant on the enzymatic action of the other lipase (Lass et al., 2011). Several closely related species produce lipases which have high affinities for diacylglycerides (DAGs) or monacylglycerides (MAGs), but are unable to effectively hydrolyse TAGs because of steric hindrances caused by bulky side chains of amino acid residues found within the lipase binding site (Shotaro et al., 1991; Tan et al., 2014; Tsuchiya et al., 1996; Xu et al., 2015). DAG/MAG lipases therefore depend on the release of the DAG substrate by TAG lipases in order to begin to release fatty acids (Tan et al., 2014). LipD and LipI were screened for homology to known DAG/MAG lipases but no homology was found. However, this does not rule out the possibility of either of the lipases showing low affinity for TAGs, as DAG/MAG lipases have been shown to share less than 20 % sequence identity (Xu et al., 2015). Owing to the greater reduction in methyl ketone production in the Δ*lipI* knockout strain (97.5 % 2-heptanone reduction) compared to the Δ*lipD* knockout strain (83.5 % 2-heptanone reduction), it is more likely that LipD is reliant on the action of LipI to produce DAGs, than the inverse. Higher methyl ketone production in the Δ*lipD* knockout (presumably possessing a functional LipI) compared to the Δ*lipI* knockout might be because of the biogenesis of methyl ketones from the fatty acids released via TAG → DAG hydrolysis by LipI. Further lipolysis by LipI may be limited because of low affinity for DAGs such as that seen in adipose triglyceride lipase in mammals (Zimmermann et al., 2004) Further characterisation of the exact substrate affinities of both LipD and LipI is required to validate these explanations. If one lipase is indeed found to be unable to hydrolyse TAGs, it would be intriguing to solve the crystal structure of this enzyme and determine whether the cause of this inability is the same as in known DAG/MAG lipases (Liu et al., 2013). Interestingly, both LipB and LipG were found to share some sequence identity with known

DAG/MAG lipases, but the functional characterisation done in this study suggests that they are not important for methyl ketone production in milk.

As expected, complementation of Δ*lipD::pyrG* with *lipD* restored 2-heptanone and 2-nonanone production to parental levels. Surprisingly, complementation of Δ*lipI::pyrG* with *lipI* only partially restored methyl ketone production, increasing from an average of 2 % that of the parental isolate in the knockout strain to 10 % that of the parental isolate in the complemented strain. One possible explanation for this is that through genetic manipulation, the locus has been altered either genetically (e.g. frameshifts, loss of regulatory elements) or epigenetically (e.g. change of chromatin structure) so that *lipI* expression has been reduced. An alternative explanation is that the initial deletion of *lipI* may have reduced the expression of *lipD*, which remains altered in the complemented strain. In both cases, this is likely to result in less protein being produced and hence less overall lipolysis. Determining expression of both lipase genes in the complemented strains will be important in testing these theories.

4.4.3 Variation in *lip* gene expression in sexual progeny

Total lipolytic activity has been found to vary significantly between strains of *P. roqueforti* (Dumas et al., 2020; Gillot et al., 2017a; Larsen and Jensen, 1999). In addition, results from Chapter 3 suggest that novel total lipolytic activity can result from sexual reproduction. Expression of *lipD* and *lipI* was therefore determined for a pair of parental isolates and sexual progeny resulting from a cross between these parents, with the aim of establishing whether novel lipase gene expression could result from sexual reproduction.

Three progeny were found with novel *lipD* expression, with exciting implications for strain development. Indeed, isolate A22, which had the highest relative expression of *lipD* out of isolates in the present study, is being developed commercially following positive feedback from public and professional taste trials with cheeses made with this strain (PS Dyer *pers.*

comm; Myconeos.com). By contrast, no progeny were found with novel *lipI* expression. *lipI* expression did vary between sexual progeny though, which suggests that novel expression may be found by screening extra progeny. Although this data provides evidence that novel lipase gene expression can be generated via sexual reproduction, it is unclear how well expression of the genes correlates to protein content and actual lipolytic activity (Gygi et al., 1999). To complicate matters further, abundance of a particular lipase may not be as influential on total lipolysis as expected, as one lipase may be more active than the other. It will be useful to correlate *lipD* and *lipI* expression with methyl ketone levels to determine how far expression can be used as a predictor of methyl ketone production. This would also be useful in determining whether expression changes in one of the two lipase genes are more important for lipolytic activity than expression changes in the other.

4.4.4 Limitations of current study

The model system used in this study involved establishing pure *P. roqueforti* cultures in UHT whole milk and assessing the levels of blue cheese-associated volatile compounds produced by the fungus from the milk, using SPME-GCMS. While this system has the benefit of being simple to set up and work with, one could argue that it does not accurately represent the complex environment found within blue cheese itself (Cantor et al., 2017). However, almost all volatile compounds detected from blue cheese in previous studies (Gallois and Langlois, 1990; Gkatzionis et al., 2009) could be detected using our system. Nevertheless, it cannot be ruled out that differences between the UHT milk model system used in the present study and 'real' blue cheese could influence the *P. roqueforti* lipolytic system. The microbiome of blue cheese, for example, is complex and microbial interactions have been shown to influence *P. roqueforti* growth and sporulation (Hansen and Jakobsen, 1997). Differences in physicochemical factors between the liquid UHT milk model system and solid (curd-based) blue cheese, such as pH and salt concentration, may also influence the relative importance of a particular lipase. It is noted that during the 7 day growth period in the UHT milk that some precipitation was evident,

consistent with curd formation due to proteins becoming insoluble. Characterising the lipase knockout strains in a variety of conditions encountered during the blue cheese maturation process will be needed to confirm whether LipD and LipI are the most important lipases for the production of key volatile compounds in blue cheese. To conclusively determine this, a blue cheese could be produced using the Δ*lipD* and Δ*lipI* knockout strains. Currently, this is unfeasible in the UK and the rest of Europe owing to strict regulations regarding the use of GM organisms in food production sites, and general public opinion surrounding the use of genetically modified organisms in food products (https://www.food.gov.uk/safety-hygiene/genetically-modified-foods). Therefore, it can be argued at a practical level that the UHT milk system used provides a sufficiently representative and workable model in which to study volatile compound production of genetically modified *P. roqueforti* strains.

A further limitation of the present study is that the identified lipase genes were deleted in only one background strain. In the strain used in this study, and under the conditions of our model system, LipD and LipI appear to be the key lipases responsible for the production of 2-heptanone and 2-nonanone. It is possible though that in other strains, other lipases may have a more active role. Determining the effect of *lipD* and *lipI* deletion on blue cheese-associated volatile flavour production in other strains, particularly in Roquefort lineage isolates which are thought to be significantly different to all other cheese production isolates (Dumas et al., 2020), could test the hypothesis that these genes are important across the entire species.

4.4.5 Concluding remarks

In conclusion, a "laboratory strain" of *Penicillium roqueforti* has been created which is NHEJ-deficient and can be used for multiple rounds of gene deletion using the *pyrG*-blaster system. This will dramatically improve the ease of which gene knockout by homologous recombination can be undertaken in *P. roqueforti* in the future, and can circumvent the negative aspects of RNAi

knockdown which has recently been the preferred method of functional gene characterisation in this fungus (Gillot et al., 2017a; Rojas-Aedo et al., 2017). The laboratory Δ*ku70*::*pyrG* strain (and subsequent 5-FOA selected Δ*pyrG*Δ*ku70* variant) has been used to characterise two genes, *lipD* and *lipI*, which encode for lipases which appear to be important for the production of the key blue cheese-associated compounds 2-heptanone and 2-nonanone during blue cheese maturation. This represents the first functional characterisation of *P. roqueforti* genes involved with lipolysis in blue cheese, and improves the overall understanding of this key process. Knowledge of *lipD* and *lipI* will allow more specified screening for strains which show altered expression of or mutations within these genes, and could therefore be of interest to blue cheese producers.

Chapter 5 - General Discussion

5.1 Fungal sex for disease control and strain improvement

The overarching theme of this thesis was to explore if and how sexual reproduction in fungi could be used as a tool for disease control and strain improvement. Work focussed on two fungal species in particular, but it was anticipated that knowledge gained from investigations would be broadly applicable to other fungi and also provide insights into sexual reproduction in general. On balance, by expanding on the current understanding of the sexual machinery in *Pyrenopeziza brassicae*, a good deal of progress was made towards future exploitation of the sexual cycle for disease control. In addition, investigations of sexual progeny of *Penicillium roqueforti* provided evidence that sex can indeed be used for strain improvement, and that this process is likely underpinned by changes in gene expression. Overall, I believe the work presented in this thesis has contributed to our fundamental understanding of fungal sex as well as exploring how this process can be practically applied.

5.1.1 Use of fungal sex hormones for disease control

The first aim of this body of work, detailed in Chapter 2, was to explore whether a hormonal compound(s) (SF) involved in the sexual cycle could be used as a novel form of disease control. Identifying novel methods to control the spread of fungal pathogens is important because of the emergence of resistance to common fungicides (Carter et al., 2014). Chapter 2 expanded on previous research which had provided evidence for the existence of a hormonal compound in crude extracts of sexual cultures of *P. brassicae* (Ilott et al., 1986; Siddiq et al., 1990).

Initial investigations validated the findings of Ilott et al. (1986) and Siddiq *et al.* (1990) and subsequently, 16-(β-D-Glucopyranosyloxy)-2,15-dihydroxyhexadecanoic acid was identified as a putative candidate for being the active component of crude SF. However, it should be noted that further

work is needed to definitively prove that 16-(β-D-Glucopyranosyloxy)-2,15-dihydroxyhexadecanoic acid is the compound in question. This might involve further purification and use of nuclear magnetic resonance (NMR) to provide structural information. It also remains possible that several complementary bioactive components are present in SF, as seen in the closely related *Psi* factors of *A. nidulans* (Champe and el-Zayat, 1989; Mazur et al., 1991, 1990). Nevertheless, significant progress has been made which will facilitate future identification attempts. Identification is a vital step in allowing future chemical work to artificially synthesise the compound(s), which will likely be needed for potential future wide-scale application as a disease control agent. This is because the time needed to extract and isolate the compound from sexual cultures, not to mention the resulting relatively low yield, makes this process non-practical.

Unfortunately, the ongoing efforts of Nottingham DeepSeq to sequence COVID-19 variants meant that planned transcriptomic experiments were not completed before the thesis deadline, although results are expected sometime in 2021. The knowledge gained from this study may be important for identifying novel antifungal targets in the future. Use of endogenous compounds to elicit a developmental response, combined with RNA-sequencing to identify genes involved with response, draws parallels with a recent study by Niu *et al.* (2020) in which transcriptomic changes elicited by exposure to *psi*Cα (5,8-dihydroxyoctadecadienoic acid) were mapped in *A. fumigatus.* Future work could mimic the latter study further, by assessing the *P. brassicae* transcriptome at different time points after exposure to SF.

Although this study has provided key fundamental insights into sexual hormones in *P. brassicae*, more work is required in order to assess the viability of using such hormones in disease control. Future studies should determine whether SF can repress asexual sporulation *in planta*, and importantly test that is not toxic to plant and animal cells. If this proves to be the case, full field trials should be undertaken to investigate how long SF activity lasts and whether any

resistance develops. In addition, it should be noted that SF can increase the rate of sexual reproduction *in vitro* (Siddiq et al., 1990; Chamberlain et al., 1996), and therefore it is possible that the benefits of repressing asexual sporulation may be counterbalanced by an increased risk of spread of disease via sexual sporulation. However, sexual reproduction appears to be dependent on mild temperatures and plant senescence signals (Gilles et al., 2001b; McCartney and Lacey, 1990; Singh and Ashby, 1998), and therefore the ability of SF to promote sexual reproduction may not matter during large parts of the cropping season where these conditions are not met. Also SF might only promote 'futile sex' in the absence of a complementary partner of opposite mating type. Future disease control could involve a dual approach of seasonal SF treatment to limit asexual reproduction when natural rates of sexual reproduction are low, combined with removal of crop debris to limit sexual reproduction in summer. In practice it would also be likely that SF treatment would be used as part of an overall disease management strategy that would include the use of classical fungicidal sprays. The use of such mixed treatment with different modes of action could also slow down the rate of possible evolution of resistance to the antifungal treatment.

Looking forward, it will be of great interest to characterise hormonal compounds in other fungal species. Not only would this be of fundamental importance, by expanding current knowledge regarding hormonal control of fungal sex, it could also have practical implications. If hormones were characterised in industrial species such as *P. roqueforti*, then hormones which promote sex could be used to boost the fertility of crosses and thereby increase the rate of production of novel sexual progeny.

5.1.2 Use of fungal sex for strain improvement

The second aim of this thesis, detailed in Chapter 3, was to investigate whether sexual reproduction could be used for strain improvement, using *P. roqueforti* and phenotypic traits relevant to blue cheese production as a model. Many sexual progeny had already been generated using the newly discovered sexual

cycle in this species, and it was of interest to determine whether any of these progeny had novel desirable characteristics. If so, this may have implications for commercial producers of blue cheese who may benefit from an increased variety of production strains.

Promisingly, a good percentage of tested progeny had novel lipolytic and/or proteolytic activity, as determined using the tributyrin and casein model assays, respectively. Owing to the fact that *P. roqueforti* enzymatic activity is the main driver of blue cheese flavour and texture (Coghill, 1979), this finding suggests that it may be possible to use these progeny to produce novel blue cheeses. However, the only true way of knowing would be to use some of the novel progeny in blue cheese production and assess the characteristics of the final product. The model assays used probably do not accurately represent the complex environment found within blue cheese (Cantor et al., 2017; Hansen and Jakobsen, 1997), and therefore further tests are needed to determine how closely the activities shown by the model assays correlate with activities within blue cheese. Of great promise are the results of ongoing commercial work by a University of Nottingham spin-out company, Myconeos, which have shown that trial batches of blue cheese made with some of the novel progeny have rated very highly in taste tests with both professional and public taste panels (https://bbsrc.ukri.org/research/impact/new-fungi-for-tastier-cheese/).

None of the sexual progeny tested had novel levels of roquefortine C production compared to the parental strains, although there was significant variation between progeny which indicates the potential for novel capacity to be generated by sex. Generating sexual progeny which produce lower amounts of roquefortine C may be of interest to blue cheese producers. In addition, *P. roqueforti* also produces secondary metabolites which have potential health benefits, such as mycophenolic acid and andrastin A. Therefore, future work could assess whether sex can generate progeny which produce higher amounts of these compounds.

Whilst the data presented in Chapter 3 provided evidence that sexual reproduction could lead to the generation of progeny with novel lipolytic activities, it was of interest to delve deeper and study whether changes in gene expression were the underlying cause for these novel activities. The gene encoding the key protease (*aspA*) has been known for some time (Gente et al., 1997), and previous data generated in the Nottingham Fungal Biology and Genetic group has shown that expression of this gene varied in sexual progeny (Michaela Novodvorska and Matthew Kokolski, unpubl. results). However, the genes encoding the key lipases were not known and thus it was not possible to conduct expression studies until these genes were identified. Therefore, work presented in Chapter 4 was undertaken to genetically characterise parts of the lipolytic system of *P. roqueforti*.

Several lipases were identified *in silico* from the genome of *P. roqueforti* and it was found that deletion of two lipase encoding genes, *lipD* and *lipI*, lead to an apparent marked decrease (albeit non-significant) in the production of both 2-heptanone and 2-nonanone, the most important volatile compounds contributing to blue cheese flavour (Gallois and Langlois, 1990; Gkatzionis et al., 2009). These genes are therefore predicted to constitute the main extracellular lipolytic activity of *P. roqueforti*. The function of *lipD* was confirmed by gene complementation, although further complementation work is ideally required to confirm the role of *lipI*. Interestingly, some of the tested progeny had novel expression levels of *lipD*, which suggests that changes in phenotypic traits in progeny compared to parental isolates may be underpinned by changes in gene expression. It is speculated that this may be owed to chromosomal rearrangement of the loci containing *lipD*, which may have potentially moved into a more highly expressed region (i.e. more in the euchromatin state). Alternatively, genetic rearrangement may have caused the parental promoter to be replaced by a more active promoter from elsewhere in the genome. Characterisation of the key lipases and investigation of their expression contributes to the overall mechanistic understanding of how *P. roqueforti* influences blue cheese flavour, and could

be of interest to blue cheese producers because it may allow for more exact strain selection.

Unexpectedly, lipase expression profiles did not always match up with the total lipolytic activities of the isolates, determined in Chapter 3. For example, A7 had the lowest activity as determined by the tributyrin assay, but relatively high expression of both key lipase genes. This potentially brings into question the relevance of the tributyrin assay in assessing actual lipolytic activity in blue cheese. However, it should be noted that it is currently unknown how accurate an indicator lipase gene expression is of actual secreted protein activity, and therefore the expression data rather than the data generated from the tributyrin assay may be inaccurate. In hindsight, a more relevant model may be to compare the levels of methyl ketones being produced by isolates, although this is far more expensive and labour intensive than the tributyrin assay. A subsequent experiment may then be to test whether there is a correlation between *lipD* and *lipI* expression and methyl ketone levels.

In the future, it might be interesting to determine the levels of secondary alcohols in the Δ*lipD* and Δ*lipI* strains, as these are also products of lipolysis and themselves contribute to the flavour of blue cheese (Gkatzionis et al., 2009). Other investigations could use the newly created "laboratory strain" to make multiple lipase strain knockouts, for example Δ*lipD*Δ*lipI* to investigate impact on lipase activity and possible functional redundancy of genes. Indeed, the URA-blaster system was employed partly to facilitate such studies. The newly created Δ*ku70* strain could also be used to characterise other genes involved in the production of methyl ketones and secondary alcohols, such as thiohydrolase, β-ketoacyldecarboxylase and reductase (Thierry et al., 2017). Expression studies could then be undertaken to determine whether sex also leads to novel expression profiles of these genes in sexual progeny. It would also be intriguing to conduct transcriptomic studies by extracting RNA at different time points during fungal growth within blue cheese, to determine at

what stages of blue cheese-maturation the genes are expressed. Together, these investigations would give a more complete picture of the lipolytic system in *P. roqueforti.*

Additional work could also be undertaken with the newly available Δ*ku70* strain to more fully characterise the suite of protease genes in *P. roqueforti.* There are also further possibilities such as gene overexpression work with *lipD* and *lipI* to assess the impact on lipase activity and subsequent production of flavour volatiles and taste.

5.2 Concluding remarks

In conclusion, this thesis has contributed to the overall understanding of sexual reproduction in fungi and explored how fungal sex can be used as a tool for disease control and strain improvement. The fungal kingdom contains numerous important species which are involved in pathogenicity, industrial chemical production and food production, and future study of sexual reproduction in this kingdom is vital in working towards a complete understanding of their biology. Improved knowledge of this complex and fascinating process will have far reaching implications for controlling devastating fungal diseases and improving industrial processes that we rely on.

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Appendix

1 *Penicillium roqueforti* gene sequences

1.1 *PyrG* gene sequence

orotidine 5-phosphate decarboxylase pyrG-Penicillium chrysogenum. CDM27009.1.

```
2225521 aaagcatcaa gttgcaaaaa ggttatatag cacgtcatca aatatagaat tttcattatg
2225581 tatgtacata cagtggaacg tggactgcac actctattgc gcacccacac gggccagata
2225641 cgcctcccag ccctgctgct ggtactgttt aacagcttca acagggtccg cagcggcata
2225701 gataccacga ccagagataa tgaagtcggc accgcggcca atagccgatt gcggtgtctg
2225761 gtactgctgg ccaagcttat cgcctttaga cgagaggttg acgccagtgg tgaagacgac
2225821 aaagtcctcg tcctgcgcgg gcgcctccgt agactcgacc tcgcccagcg accgggtcga
2225881 gacaaagccg agcacaaagc tggggtactt gcgcgcgata tcgacagagg cggaggtgta
2225941 ggcgcctgtt gcgagggatc ccttcgaggt catctcggcg aggatgagga ggccgcgctc
2226001 agagccgtag gggaaatcag tagcctgggc agtttgcgcg aggcatcgac tatgccctcg
2226061 ccgggtagga tggagcagtt gattatgtgg gcccattcgg agatacggag ggtgccgttg
2226121 tggtactgtt tctggacagt gttgccgatg tcgatgaatt tgcggtcttc gaagatgagg
2226181 aagttgtgtt tttgggctag ggcattcagg ccatcgattg ttgcttggct gaagtcggag
2226241 aggatgtcaa tgtgtgtttt tatgacggcg atgtagggac caagacctgt gaatgggtta
2226301 gtaggctgtt tcgtcggagg ggtggtggct gggtttactt acggtcggcg aggtccagga
2226361 gctcctttgt tgtggtcaca tcagcggaga cggtaacatt gctcttcttt tcttccgcga
2226421 cttggaaaag cttgcgtgcg aggggattgg ggtgcgattg ggcgcggacg ctataggtca
2226481 attgcgactt ggacgacatg gcggggttgg cggggtgggt tgatatgtat ttgtatgtga
2226541 ttttgaggtt caaacgataa gtgaatcaaa ttgaaactgg ctgataaatg cctttagaat
2226601 acaattctct tttttgaggt ccgaaagcag aataatagaa gcgaagttca agtcgaagac
2226661 cttcggggaa ctttttctgc cacggtggac ccacgggcca aaccttcccg gaggctggaa
2226721 ctgtggcg
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CDS

```
2225581 tatgtacata cagtggaacg tggactgcac actctattgc gcacccacac gggccagata
2225641 cgcctcccag ccctgctgct ggtactgttt aacagcttca acagggtccg cagcggcata
2225701 gataccacga ccagagataa tgaagtcggc accgcggcca atagccgatt gcggtgtctg
2225761 gtactgctgg ccaagcttat cgcctttaga cgagaggttg acgccagtgg tgaagacgac
2225821 aaagtcctcg tcctgcgcgg gcgcctccgt agactcgacc tcgcccagcg accgggtcga
2225881 gacaaagccg agcacaaagc tggggtactt gcgcgcgata tcgacagagg cggaggtgta
2225941 ggcgcctgtt gcgagggatc ccttcgaggt catctcggcg aggatgagga ggccgcgctc
2226001 agagccgtag gggaaatcag tagcctgggc agtttgcgcg aggcatcgac tatgccctcg
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2226181 aagttgtgtt tttgggctag ggcattcagg ccatcgattg ttgcttggct gaagtcggag
2226241 aggatgtcaa tgtgtgtttt tatgacggcg atgtagggac caagacctgt gaatgggtta
2226301 gtaggctgtt tcgtcggagg ggtggtggct gggtttactt acggtcggcg aggtccagga
2226361 gctcctttgt tgtggtcaca tcagcggaga cggtaacatt gctcttcttt tcttccgcga
2226421 cttggaaaag cttgcgtgcg aggggattgg ggtgcgattg ggcgcggacg ctataggtca
2226481 attgcgactt ggacgacatg gcggggttgg cggggtgggt tgatatgtat ttgtatgtga
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1.2 *Ku90* gene sequence

ATP-dependent DNA helicase II subunit 1. CDM35978.1.

```
2077801 ttgcagcacc ttggataaaa atattgatct ttcgaaatag ctctggccat aagtaaacag
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2077921 atgatcctaa gcaaattgga tcagtgttta aagcttttgc tcaaaatact gctcaactcg
2077981 ctctacaaga tctgctttct tccctgctgt ggcacgccca tgcgaatgca agaactcctt
2078041 cagcacgacc atcgtaagct gaacgaggta ttagtttggt caattacccg agaggattcg
2078101 aatgctcacc ttggaaacag tgcctttgtc aaagcacttt tttacttcat cttccactcc
2078161 aggggcgctg tcttcaatct taactcgctt tggtgggtgg tcgctagcag gctcggtctt
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2078341 cattcggtca gtgcatttcc gcaatttcta agagcatgga agcagcaacc ttatgaacct
2078401 gtcggtactt gggcacagtc ttatcatctg gtgattctgg gaaatcttcg tccaaggcga
2078461 tagcctgtaa gatacgatag tgccattgaa gcgctgcaac ttggtcagat catttccaac
2078521 aaatcacagt aatgaggctc acaagggttc ggatattttg aagggtcgta tatagccttg
2078581 ggaagctgca attggcgaat aacatcccgc atggcattat tcaatgcgtc ccctgctcgg
2078641 ttaatcttac tatctggatt ttggcgcaca tcatctgcga aaggaagcgc gataatccac
2078701 atcccaggtg gaattttctg cacgccattc tcgtcgatct tttcagctcc agcaatcata
2078761 gcagctaaga ccggtgaggc atttctgcgg gggataaacc aaaccaaagc cagtttctcg
2078821 gaatcgagaa gtttctggtg caatgcagaa aagacccgtg tggagccaat gtaaccctct
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2079061 tcgatctcct gtccggtctc gtcggacatt agagttgtga caccttgagc aatctgagca
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2079181 tatccattga cggatatctt gaaatcaggt ccgatctcaa gtggcacatt tgagaacagc
2079241 gatcggcggg gtacggatct cgagttgatg ctagacaata gagaattaag caatgtgagt
2079301 ccgtcacctt taccagtcga tgcgttggtg tccggctgta ggtatgccgg ggcatcacca
2079361 tcactgggcg atgttttgta gatgatgtcc tgctgattgt gagtataccg ctatacaatt
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CDS

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2078521 aaatcacagt aatgaggctc acaagggttc ggatattttg aagggtcgta tatagccttg
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2078761 gcagctaaga ccggtgaggc atttctgcgg gggataaacc aaaccaaagc cagtttctcg
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2079841 gaacttggat aatttcgttc cgtagagcag aacgccaatc atatcatgag ggttggagac
2079901 gatgcgctgt tgcatcagat ggtatgcaca cttcaaggct gcagacgcgg gtgattcgtc
2079961 gccatgtttc ttaggatcag gtgatggacg aggcgtgagc atcgagctgc tgatgtctat
2080021 ggcaaacagc accgcatctt tgactgattt gaagctctat gtaggcaaca gtcaacatcg
2080081 gtaactatat ctccggcttt tgatactcac ggtctggtcg agctcttcgt cctcgtaatt
2080141 atcctcctcg cgcgtgtagt aatcttcagc catggttgtt acaaatatga gcttgacaga
```

1.3 Lipase gene sequences

Lipase, class 3. CDM30754.1 [LIPASE A]

```
2093401 gctccaccaa tgcctccaaa tttgatctat ttcgtcgatc attgtgtgga actgtgctga
2093461 attccctcgt cacggagact agtatcgtat ttttatcata tgccgttgtt tattggttcc
2093521 cccgacagga aatggcttgc gcaagtgtgg aacccattca ggaacatact gtatgaattt
2093581 gccaggggggg atcgaacata taaagccgtg ggtccccctg gcctcaatta ggcaccgcga
2093641 ttgacaacaa gccatcatgc gtttctcttt attcacagcg ctatctgcag cagcatcgtt
2093701 gggtcatgcc ctccccggtc aactgcaagc tcgaggtacc atgccacaaa cattgatcag
2093761 tgcatcttga ccacctcgct cacacccggt tagatgtttc gactagcgaa ctgaaccagt
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2094361 tgcctcgcct cgggtggcga acgtgccttt ggctaagcat atcacggctc aggggaacaa
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2094541 tgatatcaag gttattgacg gagatatctc atttgctgga aacactggaa ctggtctccc
2094601 atcattggag gatttcgagg cgcataagtg gtactttatg aagactgatg caggaaagaa
2094661 tgatgggagg ccgttcaaga gggtttgatt ttcttgtcga attttgttta atagattcta
2094721 gaattgctgg ctgacattaa tatgtataat aaagaataat aatggata
```

CDS

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2094241 ctcccagaac cccggttacg agctggccgt agtgggccac agtctcggtg ccgctgtcgc
2094301 aacccttgcg gtggctgatc tccggggcaa aggctacccg tcggctaagc tgtatgcgta
2094361 tgcctcgcct cgggtggcga acgtgccttt ggctaagcat atcacggctc aggggaacaa
2094421 ctaccgcttc actcacaccg atgacccggt gcctaagctg ccgttgctgg ccatgggcta
2094481 tgttcatatc agccctgagt attggatcac ctcgcctaac aatgtgactg tcggtgcttc
2094541 tgatatcaag gttattgacg gagatatctc atttgctgga aacactggaa ctggtctccc
2094601 atcattggag gatttcgagg cgcataagtg gtactttatg aagactgatg caggaaagaa
2094661 tgatgggagg ccgttcaaga gggtttgatt ttcttgtcga attttgttta atagattcta
```

Lipase CDM29794.1 [LIPASE B]

```
8481841 tagcaactgt acagctataa cggttggaac atataaaaac aagcatattt tcaaattatt
8481901 tagaatgcat atattgatgt atttgatatg atagattgaa ttgatttaac cacgatatat
8481961 acatctgacc ggtagatatt gtctttcccc caggacatac tacctacata tccatagatg
8482021 tattcaccct atttgcatgc atcaatatgt cccaagtacc aattgtgtgc tgcagtatcc
8482081 ggattcaacg tccccgcatt tccagacttc gagcccacgc cgacaatgac atcaatatca
8482141 gacgaggtca ccgtcgtatt gtcgccacta gtaatccagt actcaggact tgcgtggcta
8482201 aagccaacag tcgcgggtgg caccttaggc accaagtcgt ctgtatgtgt cactcgccag
8482261 agactgccct ggtctgtgat atattgagct agagccttgt tacccacccg tggctggcca
8482321 taagtatact agtctcattg tgagtataca ctagcaatta aggcagattt ccaaacatgg
8482381 tacttaccac acttggttca tatccgccat ttcgaagcac aatacctccc aacgtggcaa
8482441 gggctccgcc atagctgtgg cctgtcacga caagagtgta gcccgagtat gttgcctttg
8482501 cggccttgat cttggatgtc agatcagctg cgacagcctc ccaggcttcc aagaacccgc
```

```
8482561 cgtgggcttc gcaaccgctg cagatactac tagcatcggt gaagccaaag ttgatgttcg
8482621 cgatccagtt actcagtgtg cgactgcctc ggaatgagac aacaagtagc ttgtttgtct
8482681 tgtcaaccgc gaggaagccg gcaacatcac catagctgga ggtcctgaaa ggtgtcactg
8482741 tattagcgaa cgacttgtga atgacattgg aagttggtct catactcgtc aaactcgtat
8482801 agagtagttg tgtcagcgga ctccaccaag gggcagttgc cttgctcgca agataccca
8482861 tcgccggtgg agttgctatt gttcgtgcag tatgatgctg cagcccactg cgagaacagg
8482921 gtcaattgac cgagcacatc agccgacaca tctattgctt gcgtcagttg gtcaatcatc
8482981 ggatggcatg aacaaaaggc aaacgcatcc atacctcgag gaacagaacg agaaggagca
8483041 gcaaccgtca gcgccgcaag agcagcgata ctccaaagac gaccaaaaag aagcatgatg
8483101 aggatctctg gagtagggct ttctcaacgg gtgtgcatac acgtcaacac aaccacgcag
8483161 acacagctcc cttatgtatg ttcggcgagt ccaaatggat cccgtataga tcccgaagtc
8483221 cttcacaaat caggacagct tgaggggcgg ggtatagcgc ccgttctctc gtatattaaa
8483281 ttgttgccgg actcagctca atctccgggc ttatggcgca tatttcacgc gtgaccggtg
```

CDS

```
8482021 tattcaccct atttgcatgc atcaatatgt cccaagtacc aattgtgtgc tgcagtatcc
8482081 ggattcaacg tccccgcatt tccagacttc gagcccacgc cgacaatgac atcaatatca
8482141 gacgaggtca ccgtcgtatt gtcgccacta gtaatccagt actcaggact tgcgtggcta
8482201 aagccaacag tcgcgggtgg caccttaggc accaagtcgt ctgtatgtgt cactcgccag
8482261 agactgccct ggtctgtgat atattgagct agagccttgt tacccacccg tggctggcca
8482321 taagtatact agtctcattg tgagtataca ctagcaatta aggcagattt ccaaacatgg
8482381 tacttaccac acttggttca tatccgccat ttcgaagcac aatacctccc aacgtggcaa
8482441 gggctccgcc atagctgtgg cctgtcacga caagagtgta gcccgagtat gttgcctttg
8482501 cggccttgat cttggatgtc agatcagctg cgacagcctc ccaggcttcc aagaacccgc
8482561 cgtgggcttc gcaaccgctg cagatactac tagcatcggt gaagccaaag ttgatgttcg
8482621 cgatccagtt actcagtgtg cgactgcctc ggaatgagac aacaagtagc ttgtttgtct
8482681 tgtcaaccgc gaggaagccg gcaacatcac catagctgga ggtcctgaaa ggtgtcactg
8482741 tattagcgaa cgacttgtga atgacattgg aagttggtct catactcgtc aaactcgtat
8482801 agagtagttg tgtcagcgga ctccaccaag gggcagttgc cttgctcgca agataccca
8482861 tcgccggtgg agttgctatt gttcgtgcag tatgatgctg cagcccactg cgagaacagg
8482921 gtcaattgac cgagcacatc agccgacaca tctattgctt gcgtcagttg gtcaatcatc
8482981 ggatggcatg aacaaaaggc aaacgcatcc atacctcgag gaacagaacg agaaggagca
8483041 gcaaccgtca gcgccgcaag agcagcgata ctccaaagac gaccaaaaag aagcatgatg
```

Lipase, secreted CDM34589.1 gene [LIPASE C]

```
3049741 ctgcttctac caatcggtct tgtatcctat tccaaacctc acttctgatt aagctccaca
3049801 cttaccaaaa ggggaagtgt catctgcctc ccaacatctc tggaattttg tctcgacaga
3049861 atgaagctga aaagtgcaac ccaagtcctt tcatgtgaag cgccaatcca aagttagcgt
3049921 cagcatctta tgcaagagcc tgctttttgc aagatatcct ccgcatcttg aggggtaacg
3049981 ctaagacaat gccggggtag agccctcatg cgcaactaat tgggtatata cggatgtagg
3050041 atatggagct aaacgtgctt actctggctc gagtcgtatt cactacgtat ctgtcgcaga
3050101 agaggtttct ccgatacaaa tgtctcaata atcagcctcg gatattggat atcatgcccc
3050161 ccagacggga acaaaaagaa actttgtgcg gggttttggc gccgatcttc cacgagtttg
3050221 aaattttgtt aggccccaac ttctatttag atgctgaagg aaaaggaagg taaagaggtg
3050281 agtctatcga taagtgtatt tgcatatcga cctgctaaat gtgttcacgc ttctgtaggc
3050341 agaagatatc aaacaaacct catatcaggg agtctgaaag gcatcagaca gaccatgtat
3050401 tgctccgaat ggtttatgca cggagtatcc cccgttgcca tgaacaggtg atgatcgtcg
3050461 cactgttcac cggcctcggt aatctatatc aatcacattc ttagaagttg tttaagtata
3050521 taatatgaga atcgtcccgc tctatcatgt agaataacgg ccttgaatcc cttatataga
3050581 gcaggaaaat ggcattgtcc cttgcgctca tggtgcgcct agccctggtt ggcctcgtgg
3050641 tattcacttc aattgcctca gccctgccgg cgcctcaagt ccctcgcagc acggttcagg
3050701 accccgacac tgatccgttc tatcagccac ctgctggctt cgcatccgag gcgcctggga
3050761 caatcctgag ccaacgcaac atcactgccg cgttctttgg tctcgtcccg gttgacgtgg
3050821 aagcctacca gctgctttac cgcacgactg caatcaatgg ctcggctatt gcaacagtca
3050881 ccacggtgtt taagccgaag gacgctaagc ttgatcgttt cgtttccttc gccacggcct
3050941 acgatagctc tgcgacgaaa tgtcagccta gctacaccta ccagcttggt tcgccacagg
3051001 acagtttgat agcttcggtc gagctattga tcattgatat ctaccttgct cttggttaca
3051061 ccgtcgcttc tcctgactat gagggacccg aggctgcctt tggacctggt cgcctcgcag
3051121 gtatgggtgt attggatggc atccgtgccg tgaagagctt caagacgttg agtcttactg
3051181 atgaccccat ggttgtcggt ataggctatt caggtggtgc cattgctacc ggatgggcag
3051241 cctctctgca acccctctac gcgtcagagc tgaacatcaa gggctgggcg cagggtggaa
3051301 ctcccgcgaa cgtcactggc acaatgtatc agcttgacaa cacagccttc agtggcttac
3051361 tccccccagc tattgctggt ctcataaagc caagcgccta tggtgcttcg ctgggccctt
3051421 tcatcagcga gatcataacg gctgaaggcc aacagatact cgacgccgcc gttgccgaat
3051481 gcgccactgc ggatatcgcg gcgttcttcg aacggtcaat cttcgacact agcttccaaa
3051541 cgctaggcga aaattttatt ttcgacccga tcgctcagtc ggtactaaag cagaacacca
3051601 tggctgccaa cgaggacgag accccgacgg cgccaacctt tgtgtaccac gccacagacg
3051661 acgaagtcat tccttatgcc gatgccaaga ctatggtcaa ttcctggtgt aactggagtg
3051721 caaatgtcaa gtttactact tatgccagcg gtggtcatgc gaccactgaa atcattgcca
3051781 ttcccgatgt catcaatttt gtccaggatg cctttgctgg cacgaccgca agtggctgca
3051841 ccacgaatac cgagcttggt agtattctta acccacttgc tcttggtgtg gatcttgagc
3051901 caattcttgt ggagctcatt gatgcgctgt tcaatctggg cgatcaggac tcgaaggtca
3051961 agaacgaccc cgttggagtg ctcggcacaa gcctttaagc gaatgctgac atctattatc
```

```
3052021 attggtcctg atgagcgttg agggtttgag ccgcaactgt ttctgatatg cacatggtta
3052081 ttcgctctgc caccttggt gtctttgttt cctttggcta ggctttggta tcctttgtgg
3052141 gacattataa ccttttctgt cagcacataa tgtttctcac caacaatgag aaggcagact
3052201 tccgagtcga gcttgtgttg atcaatggaa tgaagtccac aggatatgac gttggatcag
3052261 ataacggtgt gacctgccaa aatatagtat ggggaaatct tggaagatgt caaagtactc
3052321 agatataaag gatccatcag ctgcctatac ctggctgtct gtagcctcca agtagaacgt
```

CDS

```
3050581 gcaggaaaat ggcattgtcc cttgcgctca tggtgcgcct agccctggtt ggcctcgtgg
3050641 tattcacttc aattgcctca gccctgccgg cgcctcaagt ccctcgcagc acggttcagg
3050701 accccgacac tgatccgttc tatcagccac ctgctggctt cgcatccgag gcgcctggga
3050761 caatcctgag ccaacgcaac atcactgccg cgttctttgg tctcgtcccg gttgacgtgg
3050821 aagcctacca gctgctttac cgcacgactg caatcaatgg ctcggctatt gcaacagtca
3050881 ccacggtgtt taagccgaag gacgctaagc ttgatcgttt cgtttccttc gccacggcct
3050941 acgatagctc tgcgacgaaa tgtcagccta gctacaccta ccagcttggt tcgccacagg
3051001 acagtttgat agcttcggtc gagctattga tcattgatat ctaccttgct cttggttaca
3051061 ccgtcgcttc tcctgactat gagggacccg aggctgcctt tggacctggt cgcctcgcag
3051121 gtatgggtgt attggatggc atccgtgccg tgaagagctt caagacgttg agtcttactg
3051181 atgaccccat ggttgtcggt ataggctatt caggtggtgc cattgctacc ggatgggcag
3051241 cctctctgca acccctctac gcgtcagagc tgaacatcaa gggctgggcg cagggtggaa
3051301 ctcccgcgaa cgtcactggc acaatgtatc agcttgacaa cacagccttc agtggcttac
3051361 tccccccagc tattgctggt ctcataaagc caagcgccta tggtgcttcg ctgggccctt
3051421 tcatcagcga gatcataacg gctgaaggcc aacagatact cgacgccgcc gttgccgaat
3051481 gcgccactgc ggatatcgcg gcgttcttcg aacggtcaat cttcgacact agcttccaaa
3051541 cgctaggcga aaatttttatt ttcgacccga tcgctcagtc ggtactaaag cagaacacca
3051601 tggctgccaa cgaggacgag accccgacgg cgccaacctt tgtgtaccac gccacagacg
3051661 acgaagtcat tccttatgcc gatgccaaga ctatggtcaa ttcctggtgt aactggagtg
3051721 caaatgtcaa gtttactact tatgccagcg gtggtcatgc gaccactgaa atcattgcca
3051781 ttcccgatgt catcaatttt gtccaggatg cctttgctgg cacgaccgca agtggctgca
3051841 ccacgaatac cgagcttggt agtattctta acccacttgc tcttggtgtg gatcttgagc
3051901 caattcttgt ggagctcatt gatgcgctgt tcaatctggg cgatcaggac tcgaaggtca
3051961 agaacgaccc cgttggagtg ctcggcacaa gcctttaagc gaatgctgac atctattatc
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Lipase, class 3 CDM28427.1 [LIPASE D]

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5392681 aaatcgaaat acaccaatat cgcttcacta tgcgtaatga gttcctggct ccgtgctctg
5392741 gatttcctat ttgggaccat ccccgatgta acccacctgg cgcaattcca actcctccgc
5392801 ggcataacta tagtgtgaaa atatagtgac agtgagtacg gggaaaggtt tgaggggtag
5392861 ttgaagcatt atttaaagag caaccaaggc agtttttctt ttttctttca tccaaaacag
5392921 cgctcaagca gccttgctaa atatttcaac aacgttctac aaaatacttg aatattgttc
5392981 caacatgttg ttcaacagtc aatccttgct tgtagggata tccttgattt ctcaagccct
5393041 ttctgcacct cttttggagt cgagggcaac tgctggtgtg tcaatccacc agcccatctc
5393101 tgttagctag gcagctctaa cctcatctca agccagcgct gccttccctg atttgcaccg
5393161 tgcagcacag ctttcttccg ctgcttacac tggttgtatc ggaaaagcct tcgatgtcac
5393221 tattaccaag aggatttatg acctcctgac cgacaccaat gtaggcgtgg tatttgcttt
5393281 ttacgctagc aaatgactaa ttccaatagg gattcgttgg atactccacg gagaagaaga
5393341 agatatcggt tatcatgagg ggctcgacta ccagtatgga caaattattg attttaatac
5393401 taggtcacct tgctaattaa tccagtcgcc gacatcttga atgacattga tattaccctt
5393461 gtcactccct cgctctcggg cgtgaccttc ccttccgacg tgaagatcat gagaggtatt
5393521 aacagacctt ggtcagctgt acacgacaca gtgattgccg aagtcaaatc actcatcgcg
5393581 aagtacccgg attactcttt ggaggcagtc ggacattcct tgggtggttc cctcacatac
5393641 attgcccacg ttgcactggc tcagaacttc ccggggaagg cactcaccag caacgcactt
5393701 gctgcttttc ccatcggcaa cgaagcgtgg gccaactttg ctagttctca gcccggtact
5393761 ttgaaccgtg gaaataacat tcttgatggt gttccagtaa gacatggttg aactttagat
5393821 gatgcaatga agtggctaac cgtagcagaa catgtacgtg agcgggctta tcaacttcaa
5393881 gcactatgga accgtgagtg atcctatcga gggcttttca tactaatatc caatcgctta
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5394001 gcgcagggga acgagacctg gcctgctctg ctggcaatgg catgtacgct gtcactatcg
5394061 gtcacttctc gagctttggc attacgatgc ttactgctgg ttgtggcctg ttttgagtac
5394121 cagaagtgga cacaactggg caatatatga tatagatcgc ttattttggg gacaaagcga
5394181 ttttggatga ttacttcctc gctggcgagg gatgacagaa atatgtcaaa taacctaaca
5394241 tgaccagtct caatatatat atcatttgac gtcaatcaca ctctactatg gcttg
```

CDS

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5392981 caacatgttg ttcaacagtc aatccttgct tgtagggata tccttgattt ctcaagccct
5393041 ttctgcacct cttttggagt cgagggcaac tgctggtgtg tcaatccacc agcccatctc
5393101 tgttagctag gcagctctaa cctcatctca agccagcgct gccttccctg atttgcaccg
5393161 tgcagcacag ctttcttccg ctgcttacac tggttgtatc ggaaaagcct tcgatgtcac
5393221 tattaccaag aggatttatg acctcctgac cgacaccaat gtaggcgtgg tatttgcttt
```

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5393281 ttacgctagc aaatgactaa ttccaatagg gattcgttgg atactccacg gagaagaaga
5393341 agatatcggt tatcatgagg ggctcgacta ccagtatgga caaattattg attttaatac
5393401 taggtcacct tgctaattaa tccagtcgcc gacatcttga atgacattga tattacccct
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5393521 aacagacctt ggtcagctgt acacgacaca gtgattgccg aagtcaaatc actcatcgcg
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5393641 attgcccacg ttgcactggc tcagaacttc ccggggaagg cactcaccag caacgcactt
5393701 gctgcttttc ccatcggcaa cgaagcgtgg gccaactttg ctagttctca gcccggtact
5393761 ttgaaccgtg gaaataacat tcttgatggt gttccagtaa gacatggttg aactttagat
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5393881 gcactatgga accgtgagtg atcctatcga gggcttttca tactaatatc caatcgctta
5393941 ccgaccgtga ccatacatag gaatactaca gcgctggctc gggggccacc tgcgtaaagt
5394001 gcgcagggga acgagacctg gcctgctctg ctggcaatgg catgtacgct gtcactatcg
5394061 gtcacttctc gagctttggc attacgatgc ttactgctgg ttgtggcctg ttttga
```

Lipase, class 3 CDM27260.1 [Lipase E]

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2827801 ggccactgta aatgtaaaat ttccatggct tgaatattct cattcttgat atgtttacaa
2827861 aatccaatga ccggggaaaa cctgatatcg agacgaacct cgactatgcg cacgctcaca
2827921 ttccagcctc aggatcaact ctgctcccgc gaccagtcgc gtcgttgata tctttgatta
2827981 cacaatcaac ctcgctatcg ttgcgactag gtacattctt tggcggggta gccctggatg
2828041 gcgcgcgagc tacgactttg accagcttag aactaagccg ggctttggtc gaggggatct
2828101 tgacgagggc aggaagagat gtcgccattc gtagtggcga tgaacacggt aggacagaag
2828161 ccgagtctct cctcgagcga agtgtatgtt cctaaatcct ccaaggctgt cgacttagtc
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2828461 cgagaatatt ggtgtcggag acctcctcat tggctcccta ggcttcgcgc tattgcagcg
2828521 ttggggcaga aagaataccg aaagacatct ccggcagaat ggcggggatg aaattgtttg
2828581 ggatgttgtc atcctagaca acggcgcacg agcagacgtg atcggatccc atcagctgca
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2828701 tggaaatggc gagaaagcat ttgatgtaat tcgacggtcg agcgtgtctg attcggttgg
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2828941 tggaacgaaa ctgatagagg agagattcca ccatgatcat gattatgagg ataccaatac
2829001 gtccgatcgt cagcgacggt gtcccaagca tacagtggtg tttaggacag ccttcaacaa
2829061 atcccagagc actcaactgc gatcccacga cggctctgga aattttgccc tacctgagtc
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2829181 tcatggtccg tcacgttccc atctggatac cacgcagagc attgccccca agccgcgtgg
2829241 gacaactccc gccttgccga atgaacatcc caagcctgca cttactaaag gctcattcgc
2829301 gaaatttgca caaaaggtta agccagctgt tcttgaaaaa aatgaggcca aacggccaca
2829361 gggaaagaag ccatctatag actcatctgc gccctcgggc attccaagtc ttcgtctacc
2829421 tcctaagcct acaaaaggaa cagcgtctgt gaaggaagcg acggcgcgag aggtctctag
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2829541 cccgaaccgg ggtttacgaa ggtccccctt cccaaactcc ccccaaagac aatcgtcaga
2829601 gtcgccaacg aaacacgccc tatctcacga aatgcacgcc tctggaggct cacgaggggg
2829661 ccattttacg gtgcgagaga acagccacga gtcgctcctc actcaaaccg acacatttct
2829721 ttcacgccgt agtgctggta tgcgttctgg atcgcctgcc gcggtccaga ctcacgttcg
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2829901 ctccctagcg actgcagggt ctgagacgtc actgctgctt gcgcaccggc ctcgcaaaag
2829961 tgcatatgaa gacatgaaaa ccatacaggc actcaatcgc gatggctttg ttccaggtat
2830021 atttccagag agacacttcg tgcaaaacat tcgccggttc tcccggttct cctctgcttc
2830081 atacggctcg aatgcgctca cggtcatggg tgttcctagt ggctcgaaga atatacctag
2830141 taccaagtca gacagccaca agcatagtgc attctcagag aatgcaggcc ttccaccctc
2830201 tacgatcctc ctatcttcat tcgtcgatcc agccggtggc tcgaatgcag ctggtgagac
2830261 agagacaggc ttcccattgg tccactatct ttctatcgac catgactcca aagccgtggt
2830321 tctgacttta cgaggaacat ggggcttcga ggacatcctc acagacatga cctgcgatta
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CDS

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2827801 ggccactgta aatgtaaaat ttccatggct tgaatattct cattcttgat atgtttacaa
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2827921 ttccagcctc aggatcaact ctgctcccgc gaccagtcgc gtcgttgata tctttgatta
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2828101 tgacgagggc aggaagagat gtcgccattc gtagtggcga tgaacacggt aggacagaag
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Lipase, class 3 CDM34977.1 [LIPASE F]

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4072441 gagttacccc ggattctggt cacctctcca catcacgcgg tcatgctctc ttcctttaaa
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4072681 tcatactgcg ttggaacaac gggagtacag cagccatttc agtgcctcag ccgctgcgat
4072741 gagtttcctg atctagaact ggtaaccgtg agtcatatcc ctagtcaact ctaaaagaca
4072801 cacccactaa cctaatccaa cgatcccgta gacctggaac accggcctcc tcctctccga
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4073761 tttttggcgc attggtcttt gtgttccagg gggagatcca actggataaa gtagtatatc
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4073881 tacattatca ggtaccttgc tttacgagaa actcctttct ttctctgata tatatcacct
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CDS

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4072621 aaccatgtct caaacgacct attcaactcc ctggaggagt tatcccggct agtggatatt
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4072801 cacccactaa cctaatccaa cgatcccgta gacctggaac accggcctcc tcctctccga
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Lipase, class 3 CDM29966.1 [LIPASE G]

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 241861 cgttcattgc attgtgaagc tgggatgttg cctgagccgc tacggcgctc cagtaagccc
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 242401 acttcgcaag tacagccaat ctatcaaagg cgcctgttct tttgatctct gggaaatgag
 242461 cttatattta taggtttctt ttcccaccgt caacaacagc ccacctatgg aatgttgcct
 242521 aatagagaag gagtaatggc cgcaggattc aatcccacga tgcaccgtga tgactgtata
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242581 acagctgagt tattggcggc actaaccttg cacccggagg atgattcgat tctagagctg
242641 agagaacaaa tgattatatg ccattgaaac gatcaaaatt ctataatatg atacatacat
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CDS

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241381 acactcatat tcccaaaata ccacccatga ttaggattgg gccacctcaa atctcgatcc
241441 gaaccagcat tccccacctt gctattgatc ccttcaatat actccacccc gtcagtagtg
241501 acctgcaccc cagtcttctg ggtgatccag tattcgggcg aaggctggct ccaatcaaag
241561 aatggcagat tgagcggcac tttcggtatg gggtctgtag tatgtgtggc tcggtatatg
241621 ctgtttggcg cttgttggtt gctgataaac tcggccagct tcaaatttcc tggtttaggg
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241741 ttccaacttg acttacaata tccagactga acccttttgt tcgaagaaca gtccccgcta
241801 acgccgcaat acctccacct agactgtgtc ctacgacact cagcctatag ctcggatacg
241861 cgttcattgc attgtgaagc tgggatgttg cctgagccgc tacggcgctc cagtaagccc
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242041 tccgcgtgtg gtctagggcg atgtagcctg tcgggccgtg atcactgtgg aatgtgtcga
242101 ttacggttgt gttgtcggct gctacgagtg gacagttgcc atagtcgcag gtcaaacttt
242161 ggcctgtgtg attgatgttc tggtcgcagg ccgatagggt ggcgaactgt gagaataggg
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Lipase, class 3 CDM34335.1 [LIPASE H]

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2440621 aaagcatcaa tgcgacgaga atacaagtcc atgaaatgca taacgggatc accaaagacc
2440681 acacctcgga gctcagtgtc tacgatccca caggcatcca cattctcccg cggtggcagt
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2441521 taaacccggc gcgagtactt ggctgctagc tggaggaggg gatatgttgg aggatagctg
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```

CDS

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Lipase, class 2 CDM26301.1 [Lipase I]

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CDS

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