Heterokaryon Incompatibility in *Aspergillus fumigatus*

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Abstract

Invasive aspergillosis (IA) is associated with high mortality rates and can be difficult and expensive to treat with current drugs. The drugs used to treat IA are also associated with undesirable, and often severe, side-effects of the patient. The main causative agent of this disease is the opportunistic pathogen *Aspergillus fumigatus*. This study identifies genes which play a role in a fungal-specific type of programmed cell death (PCD) in *A. fumigatus*, known as heterokaryon incompatibility. The development of drugs specifically targeting the products of these genes could lead to fewer side-effects than those arising from currently available anti-fungal drugs. The drug amphotericin B is currently used to treat IA and has been shown to induce an apoptotic-like phenotype in *A. fumigatus*; however, the sterols targeted are present in both fungal and mammalian cell membranes. HI is a fungal-specific self/non-self recognition system that results in rapid compartmentalisation and cell death of hyphal fusion sites if the two fusing fungi are not genetically compatible. The HI system could be exploited as a novel drug target against invasive fungal pathogens through targeting a component of the molecular pathway to induce cell death. In contrast to current drugs, novel drugs could target HI components to induce PCD without affecting non-desirable targets that cause side-effects.

The non-self recognition systems used by *Neurospora crassa*, *Aspergillus Nidulans* and *Podospora anserina* are the well characterised, and they each differ significantly in their modes of action. BLAST searches found 30 homologues of HI genes from other the systems of characterised species in

A. fumigatus, with 8 containing the fungal-specific *het* domain. The first assay used to determine whether disruption of *het* genes could affect HI was to observe the barrage phenotype between incompatible *A. fumigatus* individuals. However, there was no barrage visible as the leading edge of colonies stopped growing when in close proximity to another colony. Instead, nitrate non-utilising (Nit) *A. fumigatus* mutant strains were generated using chlorate and pair-wise crosses of 46 environmentally and clinically isolates on nitrate-containing media resulted in the formation of 16 viable heterokaryons. All of the heterokaryons fell into exclusive compatibility groups where no intergroup crossing was possible.

Homologous recombination was used to disrupt five of the identified *het* domain genes with gene replacement cassettes, generated through fusion-PCR, in an *akuB*$^{(KU80\Delta)}$ *A. fumigatus* strain. The mutant strains displayed both detrimental growth on standard agar growth media and reduced ability to recognise non-self strains. Full and partial heterokaryons were formed during intergroup pair-wise compatibility crosses using the mutants and strains that the *akuB*$^{(KU80\Delta)}$ parent strain was previously incompatible with. This was followed with a non-bias approach of gene disruption using the *Fusarium oxysporum impala160* transposable element in a Nit *A. fumigatus* mutant. Inducing transposon mutagenesis through exposure to low temperature generated a mutant library of spores in which the transposon had disrupted different open reading frames at different locations across the *A. fumigatus* genome. The mutant spore library was also screened for the ability to form viable intergroup heterokaryons with strains belonging to different

compatibility groups. PCR recovery and DNA sequencing was able to identify the locus of *impala160* in three isolates able to form viable heterokaryons. The sequences revealed the transposable element had disrupted the same gene, AFUA_2G05070, in each of the three isolates. This gene encodes an uncharacterised conserved hypothetical protein which may be a critical component for non-self recognition in *A. fumigatus* HI, and a potential target for novel anti-fungal drugs to induce PCD.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

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Thesis Structure

This thesis has been presented in the traditional format. No chapters have been submitted for publication. Abbreviations, table references, figure references and section references are used throughout to form a coherent document with little repetition between figures and tables. The thesis has been written in British English except where proper nouns are in other languages. Internet addresses are presented as hyperlinks, underlined and in a blue font. There is a single reference list followed by an appendix section containing all supplementary material provided at the end of the thesis.

List of Abbreviations, Units and Symbols

Unless using accession codes, genes are in written in italicised lower case (*het*), whereas proteins and protein domains are either fully written in upper case (HET) or start with an upper case letter (HetS). A suffix of -p on a gene name indicates a transcription promoter (*gpdAp*), and a -t suffix on a gene name indicates a transcription terminator (*trpCt*).

Units of measurement

°C	Degrees Celsius
(v/v)	Volume of solute per volume of solvent (as mL dissolved in 100 millilitres)
(w/v)	Weight of solute per volume of solvent (as grammes dissolved in 100 millilitres)
cfu	Colony forming units
bp	Base-pairs
g	Gramme(s)
g_F	Force (m/s^2)
L	Litre(s)
m	Metre(s)
M	Molar
min	Minute(s)
pH	Power of hydrogen (measure of acidity and alkalinity)
rpm	Revolutions per min
s	Second(s)
$T_{\#}$	Time (subscript number indicates time elapsed in hours)

U	Arbitrary unit (described in manufacturer's instructions)
X	Stock solution dilution factor for working concentration

Measurement unit prefixes

µ	Micro (10^{-6})
k	Kilo (10^{3})
m	Milli (10^{-3})
n	Nano (10^{-9})

Abbreviations

ABPA	Allergic bronchopulmonary aspergillosis
Amp	Ampicillin
Amp^{R}	Ampicillin resistance
Ank	Ankyrin repeats (protein domains)
ATP	Adenosine triphosphate
BLAST	Basic local alignment sequence tool (BLASTP and BLASTn are protein and nucleotide alignment tools)
CADRE	The Central Aspergillus Data REpository
CAT	Conidial anastomosis tube
Cat No.	Category number
cbhB	Cellobiohydrolase B
*cbhB*p	Cellobiohydrolase B promoter
CMC	Carboxymethyl cellulose
CNS	Central nervous system
cnx	Group of genes which are required for functional nitrate

	reductase and xanthine dehydrogenase proteins
cont.	Continued
CytC	Cytochrome C
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddH_2O	Double-distilled water
DED	Death effector domain
DEPC-H_2O	Diethylpyrocarbonate-treated water
dGTP	Deoxyguanosine phosphate
DISC	Death-inducing signalling complex
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EEL	Evans electroselenium
et al.	and others
ETC	Electron transport chain
FADD	Fas-associated death domain
FasL	Fas-ligand
FDA	Fluorescein diacetate
FGSC	Fungal Genetic Stock Center
FP	Fusion primer

gDNA	Genomic DNA
GFP	Green fluorescent protein
het	Genes involved in heterokaryon incompatibility
HET	Proteins transcribed from *het* genes
HI	Heterokaryon incompatibility
HmB	Hygromycin B
HmB^R	Hygromycin B resistance
hph	Hygromycin phosphotransferase gene
HR	Hypersensitive response (a type of plant PCD)
Hyp	Hypoxanthine
IA	Invasive aspergillosis
IPA	Isopropyl alcohol
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITS	Internal transcribed spacer
KCl	Potassium chloride
$KClO_3$	Potassium chlorate
KO	Knockout (in reference to a gene)
LacZ	β-galactosidase (encoded within the *lac* operon)
LB	Lysogeny broth
LRT	Lower respiratory tract
MOMP	Mitochondrial outer membrane permeabilisation
MM	Minimal media
MRCM	Mycology Reference Centre Manchester
N	Nitrogen
NACHT	NTPase protein domain

NAP	Nitrogen assimilatory pathway
NHEJ	Non-homologous end joining
niaD	Nitrate reductase gene
nirA	Nitrite reductase gene
Nit	Nitrate non-utilising (confers chlorate-resistance)
NTPase	Nucleoside-triphosphatase
$OD_{\#}$	Optical density (subscript number indicates wavelength measured in nm)
OMP	Orotidine-5'-phosphate
ORF	Open reading frame
PARP	Poly (ADP-ribose) polymerase
PB	Protoplasting buffer
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PEG	Polyethylene glycol
PES	Polyethersulphone
Pfs	Nucleoside phosphorylase (protein domain)
PTB	PEG transformation buffer
rDNA	Ribosomal deoxyribonucleic acid
RE	Restriction enzyme
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROS	Reactive oxygen species

RT	Room temperature
RT-PCR	Real-time polymerase chain reaction
S	Sulphur
sB	sulphate permease (gene)
SB	Sodium borate (gel electrophoresis buffer)
sC	ATPsase (gene)
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SDS	Sodium dodecyl sulphate
SeO	Selenate
SeO^R	Selenate resistance
SF	Screening forward (*het* gene PCR primer)
Sit	Sulphate non-utilising (confers SeO^R)
SOC	Super optimal broth with catabolite repression
SR	Screening reverse (*het* gene PCR primer)
ST-PCR	Semi-random, two-step-PCR
TAE	Tris-acetate-EDTA (gel electrophoresis buffer)
TB	Transformation buffer
TPR	Tetratricopeptide repeats (protein domains)
TRP	Transposon rescue primer
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UV	Ultraviolet
v	Volume (measured in litres)
VC	Vegetative compatibility (typically referring to phenotype)

VCG	Vegetative compatibility group
Vc	Viable count (measured in cfu/mL)
w	Weight (measured in grammes)
WD	WD40 beta-transducin repeats (protein domains)
WT	Wild-type
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside
ZZZ	ZZ-type zinc finger (protein domain)

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Chapter 1: General Introduction

1.1 The Fungal Kingdom

Fungi are unicellular or multicellular heterotrophic eukaryotic organisms that play vital roles in modern society, such as in medicine development (Gutiérrez *et al.*, 1999) and as model organisms in various areas of biological research (Altmann *et al.*, 2007). Fungi are involved in nutrient recycling in the environment as saprophytes (Tekaia and Latge, 2005); organisms that utilise dead or decaying matter, detritus, as a nutrient source. Degrading detritus can require specialised enzymes, such as glycosyl-hydrolases to degrade polymers like lignin and cellulose within the plant cell wall (de Vries and Visser, 2001). Fungi also interact closely with many plants in mutualistic relationships. Mycorrhizal associations of fungi with roots of certain plant species aids nutrient uptake of plants from the soil (Selosse *et al.*, 2006). Whilst the fungi provides a large surface area for increased uptake of nutrients (Li *et al.*, 2006) and water, the plant provides glucose to the fungi through translocation of glucose from leaves to roots (Harrison, 2005). Relationships between fungi and plants are not always so balanced though. Myco-heterotrophy describes the use of fungal-associations by plants to derive nutrients, rather than through photosynthesis (Leake, 2005). Non-photosynthetic plants, such as certain orchid species, are full myco-heterotrophs at certain stages of their life cycles (Ogura-Tsujita *et al.*, 2008). Fungi can be harmful to plants too, however, and affect many commercially produced crops worldwide. *Puccinia triticina*, the causative agent of wheat leaf rust, is an obligate parasitic rust fungus that affects rye and wheat crops, and can lead to a yield-loss of crops up to 20% (Bolton *et al.*, 2008).

Fungi are often found in close relationships with other organisms, such as aiding digestion in the stomach of ruminant mammals (Nam and Garnsworthy, 2007), ant-fungus mutualism where leafcutter ants nurture fungal gardens (Augustin and Santos, 2008), and also as lichens (Westberg *et al.*, 2007). Lichens are an example of a symbiotic relationship; the fungi (generally ascomycota) associate with a photosynthetic partner (usually green algae).

The fungal subkingdom dikarya comprises of yeast and filamentous fungi either belonging to the ascomycota or basidomycota phyla (Hibbett *et al.*, 2007). These are mostly filamentous fungi; multicellular sporogenous species with chitinous cell walls, which grow primarily through their hyphae. Hyphae are long tubular chains of cells (or a single elongated cell) surrounded by a cell wall, and are collectively named mycelium. Ascomycota and basidiomycota are able to construct fruiting bodies to produce the small reproductive structures used for dispersal of the organism. The basidiomycota include bracket fungi, mushrooms and rusts (Elbert *et al.*, 2007). Basidiocarps are the fruiting bodies of basidiomycota, where spores are produced. Spores are often released from basidia, located on gills (Elbert *et al.*, 2006), either as ballistospores or statismospores, depending on whether or not they are actively discharged. Ballistospores are thought to be fired with an acceleration over 10,000 g_F due to a immediate change their centre of mass (Pringle *et al.*, 2005). The Buller's Drop (accumulated fluid) generates surface tension as the drop rapidly moves across the spore surface, causing the spores to be ejected (Stolze-Rybczynski *et al.*, 2009).

Puffball basidiomycota internally liberate statismospores, and these are released in clouds when the fruiting body bursts.

The ascomycota subkingdom includes yeasts as well as filamentous fungi, which produce spores in cells, known as asci, during sexual reproduction, if the fungus is capable of sexual reproduction (Hibbett *et al.*, 2007). Yeasts are typically unicellular organisms, but some, such as *S. cerevisiae*, exhibit dimorphism and are able to also grow in a multicellular filamentous fashion (Edgington *et al.*, 1999). The ascomycota includes several species important to research. *Neurospora crassa* and *Podospora anserina* are used as model organisms in circadian clock and aging studies amongst other areas of research (Kaldi *et al.*, 2006; Lorin *et al.*, 2006). A haploid stage in the life cycle allows for simple genetic analysis through mutagenesis (de Serres *et al.*, 1980). Both *P anserina* and *N. crassa* have been fully sequenced (Galagan *et al.*, 2003; Espagne *et al.*, 2008). The ascomycota also contain several species pathogenic to humans. *Candida albicans*, the predominant causative agent of candidiasis (Scales *et al.*, 1956; Sobel, 2006), is typically a yeast, but the opportunistic pathogen demonstrates dimorphic behaviour, with hyphal growth being linked to virulence (Kumamoto and Vinces, 2005). The main causative agent of invasive aspergillosis (IA) is the opportunistic pathogen *Aspergillus fumigatus* (Latge, 1999), and is also responsible for inducing allergic responses (Ronning *et al.*, 2005). *A. fumigatus* predominantly reproduces asexually, although relatively recently the sexual cycle of this fungus was observed (O'Gorman *et al.*, 2008).

Pier Antonio Micheli catalogued the *Aspergilli* in 1729, naming the genus *Aspergillus* as under a microscope the fungi resembled an aspergillum; a perforated ball at the end of a short handle used to sprinkle holy water. Around 200 defined species of these filamentous fungi are known of (Robson *et al.*, 2005), and are present worldwide (Rydholm *et al.*, 2006). *A. fumigatus* is an opportunistic pathogen, but other *Aspergilli* are used in the food industry, such as the use of *Aspergillus oryzae* to ferment starch-rich ingredients like rice in the production of sake (Archer *et al.*, 2008), as well as for enzyme production (Archer *et al.*, 1994). *Aspergillus nidulans* has long been used as a model organism in research (Dean and Timberlake, 1989; Paoletti *et al.*, 2007b), and had its genome sequenced alongside *A. fumigatus*, *A. oryzae* and *Aspergillus niger* in recent years (Archer and Dyer, 2004). The complex genome of *A. nidulans*, coupled with haploid stages in its life cycle, allow for simple genetic manipulation for investigating the eukaryotic cell life cycle, gene regulation and DNA repair mechanisms (Felenbok *et al.*, 2001; Goldman and Kafer, 2004), and is often more appropriate than using yeast for model research (Miskei *et al.*, 2008).

Conidia, asexual and non-motile spores released from *Aspergilli* are airborne and ever-present in the environment (Ronning *et al.*, 2005), with conidia of *A. fumigatus* being in abundance (Mullins *et al.*, 1984). Immunocompromised individuals who inhale the conidia may develop pulmonary aspergillosis (Denning, 1998; Latge, 1999). *A. fumigatus* is a thermophile found on decaying material (Moody *et al.*, 1999), therefore higher numbers of spores are prevalent in compost heaps and near composting facilities (Fischer *et al.*,

1999). *A. fumigatus* grows in temperatures up to 50°C, surviving those of 70°C (Robson *et al.*, 2005), and is able to cause aspergillosis in animals other than humans (Eder *et al.*, 2000).

Aspergillosis is associated with severe symptoms and high mortality rates. High fevers, chest pains, coughing and organ failure are general symptoms (Latge, 1999). Untreated cerebral aspergillosis has a mortality rate of 99%, and pulmonary around 86% (Denning, 1996). Infection of the central nervous system (CNS) by *Aspergilli* can occur via delivery of the fungi from the blood after infection of the lungs, or directly through the nasal passage (Nadkarni and Goel, 2005). There are several forms of pulmonary aspergillosis. Allergic bronchopulmonary aspergillosis (ABPA) is a severe pulmonary disease caused by *Aspergilli* in atopic asthma sufferers and occurs as a hypersensitive response to *A. fumigatus* antigens (Soubani and Chandrasekar, 2002). ABPA affects around 7% of cystic fibrosis patients (Greenberger, 2003), and between 1 and 2% of asthma sufferers (Basich *et al.*, 1981) with around 15% sensitised to *A. fumigatus*. Aspergilloma is a potentially fatal disease that can affect those with pulmonary cavities from previous ailments, mainly tuberculosis sufferers (Soubani and Chandrasekar, 2002). Formation of a sporulating spherical mass of hyphae can be asymptomatic, but often results in internal bleeding and haemoptysis (Faulkner *et al.*, 1978; Latge, 1999). The fungal-balls are usually stable, and tend not to increase in size, often being found to reduce in size instead, with asymptomatic patients not usually receiving much treatment (Soubani and Chandrasekar, 2002).

IA presents a major complication in modern medicine. This invasive disease is associated with affecting cancer and AIDS patients due to their compromised immune systems (Denning *et al.*, 1991; Bodey *et al.*, 1992), as well as up to a quarter of organ recipients, mainly in heart and lung transplants (Latge, 1999), or bone marrow transplantations (Soubani and Chandrasekar, 2002). Neutropenia is a condition involving a reduction in neutrophils due to use of immunosuppressants, and patients affected by this disease are the largest risk group for IA with 7.5% of patients affected following induction of the treatment (Gerson *et al.*, 1984). Typical symptoms for a lower respiratory tract (LRT) infection are associated with IA, but pleuritic chest pains and haemoptysis may distinguish IA from other LRT infections (Soubani and Chandrasekar, 2002). Treatment of IA is still associated with high mortality rates.

1.1.1 Invasive Aspergillosis and Resistance to Treatment

Drug treatment of aspergillosis includes caspofungin, flucytosine, itraconazole, amphotericin B, and voriconazole (Herbrecht *et al.*, 2002). Caspofungin is an echinocandin, an inhibitor of glucan synthesis, effective against *Aspergillus* and *Candida* via inhibition of β(1,3)-D-Glucan synthase, which is encoded for by the *fsk1* gene (Ha *et al.*, 2006). Resistance to caspofungin has been observed in *Fusarium solani* however, transformation of *A. fumigatus* with the *F. solani fsk1* gene does not significantly raise resistance to caspofungin (Ha *et al.*, 2006). Despite the sensitivity of *A. fumigatus* to caspofungin, co-infection of *A. fumigatus* and *C. albincans* has been observed in a patient being treated with caspofungin solely for a *C.*

albicans infection (Arendrup *et al.*, 2008). The *C. albicans* strain causing infection in the patient had a single point mutation in *fsk1* that conferred increased resistance to caspofungin, but the isolated *A. fumigatus* strain did not have any *fsk1* point mutations associated with caspofungin resistance in other species.

The synthetic drug flucytosine (5FC) is effective against *C. albicans*, but has only shown limited effectiveness against *A. fumigatus* (Verweij *et al.*, 2008). Whilst 5FC is not inherently an anti-fungal, internal conversion to 5-fluorouracil (5FU) by fungi is followed by the production of metabolites that inhibit both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis (Vermes *et al.*, 2000). Resistance is present in *C. albicans* isolated post-treatment, and it has been shown that a single nucleotide change in the uracil phosphoribosyltransferase gene (*fur1*) at position 301 can confer resistance (Dodgson *et al.*, 2004).

The synthesis of ergosterol, a vitamin D_2 precursor, is inhibited by itraconazole. Cytochrome P450-dependent 14 alpha-demethylase of *A. fumigatus* is bound and inhibited by itraconazole with high affinity (Ballard *et al.*, 1990). Ergosterol is a component in fungal cell membranes, but is not present in human cell membranes, making it a suitable target for drugs (Marichal and Vanden Bossche, 1995). Resistance to itraconazole, however, rises through several mechanisms. Mutations involving enzymes of the ergosterol biosynthesis pathway, such as enzyme Δ5-6 desaturase, increase resistance (Marichal and Vanden Bossche, 1995). Increased production of

cytochrome P450-dependent 14 alpha-demethylase (Marichal and Vanden Bossche, 1995) and enhanced drug efflux also increase resistance (Sanglard *et al.*, 1998). Ergosterol is also a target for the anti-fungal drug amphotericin B. The association of amphotericin B and ergosterol leads to pore-formation in the fungal membrane, followed by electrolyte leakage (Andreas Zumbuehl, 2004). Exposure of *A. fumigatus* to amphotericin B is also associated with apoptotic markers (Mousavi and Robson, 2004). However, amphotericin B can induce severe side-effects in a patient, such as renal damage, fever and hypotension (Ringden *et al.*, 1998; Furebring *et al.*, 2000). Voriconazole is a triazole much like itraconazole, and interacts with cytochrome P-450. Survival rates of patients 12 weeks after infection of IA improved significantly when using voriconazole in place of amphotericin B; 70.8% survived with voriconazole treatment compared to 57.9% using amphotericin B (Herbrecht *et al.*, 2002). Voriconazole treatment is also associated with lower rates of side-effects, although patients may experience transient blurred vision (Herbrecht *et al.*, 2002). Although voriconazole is useful for treating invasive *Aspergillus terreus* infections (Sutton *et al.*, 1999), resistance has been observed in the ascomycetes *C. albicans* and *A. fumigatus* (Manavathu *et al.*, 2003; Manavathu *et al.*, 2004; Wakiec *et al.*, 2007).

The range of treatments available to treat aspergillosis are expensive and cause a range of undesirable side-effects in patients. With the emergence of drug resistance to the most effective anti-fungal drugs, there is a need to characterise potential novel drug targets and to develop new drugs.

1.1.2 Recognition Systems in Filamentous Fungi

There are two general recognition systems used by filamentous Ascomycota; a sexual recognition system (Coppin *et al.*, 1997) and a self/non-self recognition system (Glass and Dementhon, 2006). Genotypic self/non-self recognition of basidiomycota is known as heterokaryon incompatibility (HI) (Kauserud *et al.*, 2006). A heterokaryon is a cell containing genetically distinct nuclei, and is the result of two compatible ascomycota hyphae fusing. HI between two incompatible filamentous fungi results in the compartmentalisation of the hyphal tips, followed by rapid programmed cell death (PCD). PCD can be triggered in *Aspergilli* by antifungal agents, sporulation, entry into the stationary phase as well as HI (Mousavi and Robson, 2003; Krishnan *et al.*, 2005). PCD is also used by *Cryphonectria parasitica*, an ascomycota, as a defence mechanism against infection (Cortesi *et al.*, 2001; Biella *et al.*, 2002). PCD components may be viable novel drug targets to treat IA as there is few similarities with the upstream PCD targets of humans and *Aspergilli* (Fedorova *et al.*, 2005), potentially leading to less symptoms. *Aspergilli* have been found to share some PCD homologues with the yeast *S. cerevisiae* with upstream components (Fedorova *et al.*, 2005), whereas the downstream targets are more similar to those of metazoan species. Both yeast and filamentous fungi lack upstream apoptotic regulator homologues of p53 and Bcl-2 family members (Fedorova *et al.*, 2005). PCD pathway core components tend to be conserved amongst *Aspergilli* (Fedorova *et al.*, 2005) which may leave less scope for effective drug resistance to emerge through spontaneous mutations to drugs targeting these components.

1.2 Programmed Cell Death Functions and Characteristics

Many cell types from a large variety of organisms can undergo a form of organised cell death termed PCD. PCD is usually associated with multicellular eukaryotic organisms, but has also been found to occur in prokaryotes and unicellular eukaryotes (Engelberg-Kulka *et al.*, 2006). Mammals use PCD for a multitude of reasons such as in the development of digits in the embryo and in a process known as anoikis in which epithelial cells are prevented from proliferating after detachment from the extracellular matrix (ECM) (Chiarugi and Giannoni, 2008; Kimura *et al.*, 2011). Another example is where the same 131 cells of 1090 cells generated in a developing *Caenorhabditis elegans* hermaphrodite always undergo PCD (Hengartner, 1999), forming a mature *C. elegans* of a defined growth form and of precisely 959 somatic cells.

A damaged mammalian cell, or one under stress, is likely to enter a form of PCD known as apoptosis. Stresses include DNA damage (from ionising radiation or toxic chemicals) and nutrient starvation. PCD is used to prevent the inheritance of damaged DNA in daughter cells as DNA damage can lead to oncogeneic mutations being replicated during cell proliferation, which may eventually result in malignant tumour formation in mammals.

Plant cells prevent the spread of infection using the hypersensitive response (HR), a form of PCD. Reasons for PCD within a population of single cellular organisms, however, are less clear. It has been suggested that infected

organisms in a population may undergo PCD to prevent the spread of a virus (Hazan and Engelberg-Kulka, 2004).

The filamentous ascomycete fungi use PCD as a self/non-self recognition mechanism to remain genetically distinct from other strains. HI groups control whether fusion of hyphae between two strains of *A. fumigatus* is allowed to occur, which would result in the formation of a heterokaryon (Saupe, 2000). Heteroallelism at heterokaryon gene (*het*) loci prevents heterokaryon formation (Fedorova *et al.*, 2005).

PCD is distinguished from necrotic cell death by a number of factors. Necrotic cells lose membrane integrity whilst internal cell structures initially remain intact during PCD, keeping the apoptotic cell compartmentalised. Late stages of PCD can be used as assays for PCD. Fragmentation of the DNA and membrane compartmentalisation releases small apoptotic bodies, and is an active process (Madeo *et al.*, 1999). Apoptotic body vesicles isolate immunogenic and toxic cellular contents during PCD. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) detects consequential DNA-fragmentation from PCD. The enzyme terminal deoxynucleotidyl transferase adds fluorescent deoxyuridine triphosphate (dUTP) molecules to fragmented DNA. TUNEL has been criticised as intact DNA may be stained inaccurately (Charriaut-Marlangue and Ben-Ari, 1995; Labat-Moleur *et al.*, 1998), but reworked techniques allow this relatively simple staining method to remain useful as a stain for apoptotic cells (Labat-Moleur *et al.*, 1998). Another marker of PCD is the flipping of

phosphatidylserine to the outer leaflet of the membrane from the inner leaflet (Mousavi and Robson, 2003). Annexin A5 binds to phosphatidylserine exposed on the outer leaflet. When labelled with FITC, fluorescing annexin V-FITC bound to the cytoplasmic membrane indicates the exposed phosphatidylserine apoptotic marker. The PI staining is coupled with fluorescein diacetate (FDA) staining to discriminate between necrotic and apoptotic cells. Red fluorescence of PI increases when bound to DNA, but PI is membrane impermeable, so only enters cells with a loss of membrane integrity (i.e. necrotic cells). FDA is hydrolysed to the green-fluorescing compound fluorescein in the cytosol of metabolically-active cells. Cells which do not fluoresce either red or green (PI^-/FDA^-) are apoptotic (Salinas *et al.*, 2007), whereas FDA^-/PA^+ staining indicates necrotic cells. Staining techniques detected apoptotic markers in *A. fumigatus* during stationary phase of glucose minimal media broth cultures, and also in response to exposure of *A. fumigatus* to toxic levels of amphoteracin B (Mousavi and Robson, 2003; Mousavi and Robson, 2004). The rapid loss of viability of these filamentous fungi was attributed to PCD after analysing the cultures with several dyes, including TUNEL and annexin V-FITC.

1.2.1. PCD in Mammalian Cells

The signalling pathways of PCD in mammalian cells are tightly regulated complex sets of molecular interactions. The mammalian PCD systems have been characterised more extensively than PCD pathways of fungi.

1.2.1.1 Stress and DNA Damage

Apoptosis induced by DNA damage is regulated by the tumour-suppressor transcription factor p53 in mammalian cells. p53 is also able to regulate DNA repair by halting the cell cycle at the G_1/S-phase checkpoint (Giono and Manfredi, 2006) and to maintain arrest at G2 if DNA damage is detected (Bunz *et al.*, 1998). The removal of damaged and stressed cells allows a mammalian body to maintain homeostasis through constant replacement with healthy daughter cells. p53 is known as a tumour suppressor as PCD is triggered to prevent damaged DNA from being inherited by daughter cells during mitosis. Bcl-2 family proteins target the mitochondria to initiate PCD through altering the balance of the mitochondrial outer membrane permeabilisation (MOMP). The anti-apoptotic Bcl-2 proper is activated via phosphorylation as a result of p53 initiating the Cdc42/JNK1 pathway (Thomas *et al.*, 2000). Pro-apoptotic Bax & Bak promote calcium signalling to mitochondria (Suen *et al.*, 2008), whereas anti-apoptotic Bcl-2 & Bcl-X_L inhibit calcium signalling (Rong and Distelhorst, 2008). The pro-apoptotic signalling of Bax & Bak increases release of Ca^{2+} from the endoplasmic reticulum (ER) (Rong and Distelhorst, 2008) through IP_3R (Ray, 2006), and is followed by increased uptake of Ca^{2+} by mitochondria, promoting Cytochrome C (CytC) release (Breckenridge *et al.*, 2003), and apoptosis.

Oxidative stress is caused by the accumulation of reactive oxygen species (ROS) which damage the cell, and the mitochondria in particular, through lipid peroxidation (Asumendi *et al.*, 2002; Shih *et al.*, 2004). ROS are also generated by the cell to trigger apoptotic pathways (Zhang *et al.*, 2008).

1.2.1.2 Survival Signals

Growth factors and cytokines are exogenous survival signals for cells, and these signals are transduced via Akt phosphorylating Bad, inhibiting stimulatory effects Bad has on Bax & Bak. Bad is inactive and located in the cytoplasm bound to scaffolding proteins, in the presence of survival signals. This also inhibits the p53-dependant stress pathway (Datta *et al.*, 1997). When the cell receives no survival signals, dephosphorylation of Bad releases it from the scaffolding proteins and Bad localises to the mitochondrial membrane and initiates apoptotic signalling pathways. Caspase 3 is also able to cleave Bad to produce a truncated protein more efficient at promoting apoptosis through binding Bcl-X_L (Condorelli *et al.*, 2001). Caspases are aspartate-specific cysteine proteases (Cheng *et al.*, 2008) which are involved in the 'caspase cascade' of apoptotic signalling. The caspase cascade involves caspases promoting their own multimerisation and activating further effectors through their protease mode of action (Steller, 1998).

1.2.1.3 Extrinsic Pathway

Ligand binding to transmembrane death receptors, such as Fas-ligand (FasL) binding to Fas, cause the death-inducing signalling complex (DISC) and procaspases 8 and 10, to be recruited on the inner membrane to the Fas-associated death domain (FADD) (Ramaswamy *et al.*, 2004). Procaspases 8 and 10 are autocatalytic and self-cleave once associated with FADD. Once released from the DISC as caspases, caspases 8 and 10 cleave and activate caspases 3, 6 and 7 (Ashkenazi, 2008). The caspase

cascade of caspases 3, 6 and 7 enter the cell into apoptosis, whereas caspase 8 also cleaves Bid to tBid, which localises to the mitochondrial membrane, acting as a pro-apoptotic signal. Caspase 8 therefore feeds into the intrinsic pathway through Bid, as tBid promotes Bax and Bak activity (Ashkenazi, 2008) whilst also inhibiting the anti-apoptotic Bcl-X_L.

1.2.1.4 Intrinsic Pathway

The intrinsic pathway is fed into by stress signals, survival signalling and the extrinsic apoptotic pathways. CytC is located in the inner membrane of mitochondria, but is released in the presence of pro-apoptotic signals. Bad and Bim inhibit Bcl-2 and Bcl-X_L from preventing CytC release to the cytosol (Adrain *et al.*, 2001). CytC binds to Apaf-1 and procaspase 9, cleaving the inactive procaspase to the active form, caspase 9, and forming the apoptosome. The apoptosome then acts in a similar manner to initiator caspases 8 and 10 from the extrinsic pathway by cleaving and activating the effector caspases 3, 6 and 7 to trigger apoptosis. Caspase-independent apoptosis arises when the mitochondria generates ROS via the electron transport chain (ETC) (Shih *et al.*, 2004). These ROS are released through the MPTP, and can damage the mitochondrial membrane through lipid peroxidation, resulting in CytC release followed by caspase activation (Asumendi *et al.*, 2002).

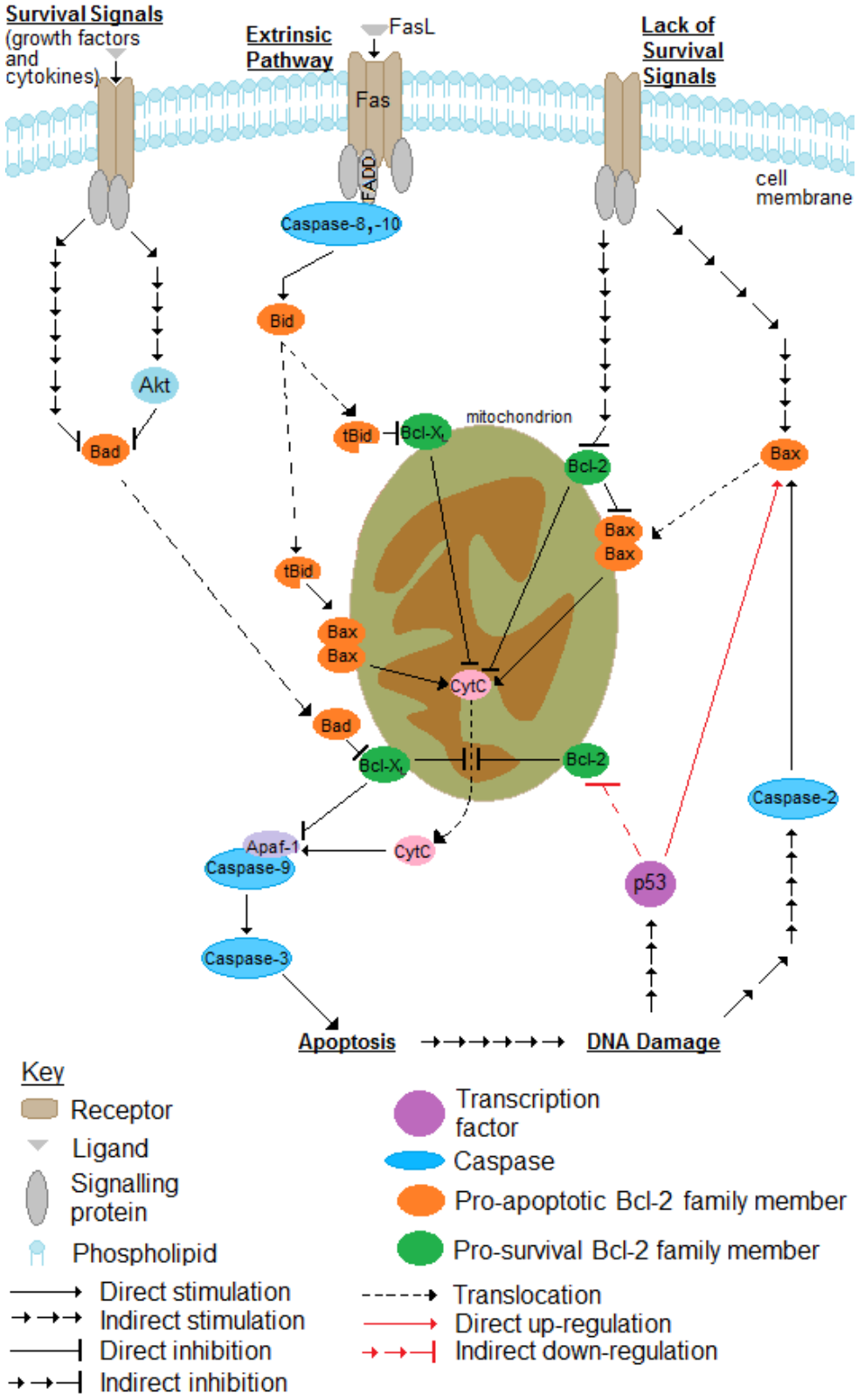


Figure 1.1 Mammalian Cell Apoptosis Cell Signalling Pathways

This figure shows the roles of caspases and Bcl-2 family proteins in the apoptotic pathways of mammalian cells. The simplified signalling pathways

Figure 1.1 *cont*.

include pro-apoptotic signalling of in response to transcription factor p53 activity, and anti-apoptotic signalling of Bcl-2 proper and Bcl-X_L.Cessation of survival signals, activation of the Fas receptor by FasL, and DNA damage initiate early pro-apoptotic signalling. Bad is released from scaffolding proteins and relocates to mitochondrial membrane when survival signals are not present, as the presence of these survival signal stimulates Akt activity. CytC release allows Apaf-1and CytC to form the early apoptosome which then recruits and activates caspase 9. Interaction of the apoptosome with caspase 3 induces apoptosis which stimulates a positive feedback loop through caspase 2 activity (Cell Signalling Technology, 2003).

1.2.2 PCD as a Method of Controlling the Spread of Infection

PCD is used to control the spread of infectious material in the mammalian immune system, including cancerous cells (Ashkenazi, 2008) as well as other species infected with intracellular parasites such as bacteria or viruses. It could be that defence against infection for single cell populations played a major role in the initial evolution of cell suicide prior to the driving force of a developmental role observed in multicellular organisms. Unicellular organisms also utilise PCD to inhibit the spread of infectious agents to the neighbouring population (Hazan and Engelberg-Kulka, 2004). *E. coli* are able to self-terminate and prevent spread of the bacteriophage P1 to the surrounding population using the toxin-antitoxin *mazEF* system (Hazan and Engelberg-Kulka, 2004). The *mazEF* system consists of a stable toxin, MazF, and a labile antitoxin, MazE (Hazan and Engelberg-Kulka, 2004). Expression of the antitoxic MazE is required for cellular survival as it prevents the toxic activity of MazF in *E. coli*. Stresses, such as amino-acid deprivation, alter the expression of *mazE* which encourages PCD (Aizenman *et al.*, 1996). *E. coli* expressing non-functional Δ*mazEF* were found to generate higher levels of bacteriophage P1 when the culture was infected. Infected cells were also unable to undergo PCD to prevent phage replication (Hazan and Engelberg-Kulka, 2004), threatening a higher proportion of the population with infection.

1.2.2.1 Plant Hypersensitive Response

As an aid against from infection, plant species use a form of cell suicide known as the HR. Cells located near a recognised infection all die rapidly as

a primary response, slowly followed by a gain of systemic immunity to the threat (Pontier *et al.*, 1998). The death of surrounding cells prevents the spread of infection. The HR is driven by plant *R* genes, highly polymorphic genes that continually adapt to recognise infections (Bergelson *et al.*, 2001). The R proteins generally have conserved domains such as the TIR domain, NBS or a PK domain (Liu *et al.*, 2007). The *R* genes function by adapting alongside pathogenic avirulence (*Avr*) genes, in a gene-for-gene relationship, to constantly recognise and confer resistance to new variations (Bakker *et al.*, 2006). *Avr* genes share no common traits other than that they are mostly secreted, and may play a role in infection of plant species without appropriate *R* proteins to confer resistance (Ellis *et al.*, 2007). The membrane potential of plant cells is affected by *R* genes, and the first phase of HR is for activated *R* proteins to trigger a huge mobilisation of ions (Ellis *et al.*, 2000). The ion flux involves hydroxide and potassium building outside the cell, with a calcium and hydrogen ion influx. Cyclic nucleotide-gated channels facilitate the rapid Ca^{2+} influx to plant cells upon host recognition of a pathogen, through the *R* and *Avr* gene-for-gene relationship, with downstream signalling inducing both ROS generation and further activation of Ca^{2+} transporters (Ma and Berkowitz, 2007). Plant cell death occurs during this event, and lesions form containing anti-microbial agents around the infection site. Cells then produce ROS to generate an oxidative burst that causes membrane damage (Baker *et al.*, 1993); the damage is targeted at the plant cells (Pontier *et al.*, 1998). The generation of ROS causes lignin deposition which forms a barrier around the site of infection, sealing the site and preventing the spread of the infectious agent (Pontier *et al.*, 1998).

1.3 Programmed Cell Death in Fungi

1.3.1 PCD in Yeast

Over the last decade evidence has increased showing yeasts *Saccharomyces cerevisiae* and *C. albicans* use apoptotic pathways under oxidative stress, DNA damage and as a response to infection to viruses to protect the population and prevent generation of damaged daughter cells (Ivanovska and Hardwick, 2005). Apoptotic behaviour was identified recently in a *CDC48 S. cerevisiae* mutant through annexin V and TUNEL staining techniques (Madeo *et al.*, 1997). Late and early apoptotic behavioural markers associated with mammalian cells are present in the yeast *S. cerevisiae* (Khan *et al.*, 2005), even though some orthologs are not (Madeo *et al.*, 1997). PCD can be observed in *S. cerevisiae* during oxidative stress, upon receiving DNA damage, and also after failed mating attempts (Severin and Hyman, 2002). The characteristics of PCD in yeast appear similar to that of mammalian cells: DNA fragments (Madeo *et al.*, 1997), CytC escapes the mitochondria (Manon *et al.*, 1997) and phosphatidylserine is exposed on the outer leaflet of the membrane (Madeo *et al.*, 1997). The apoptotic behaviour of *S. cerevisiae*, induced by salt exposure, could be inhibited when excess mammalian Bcl-2 is supplied (Huh *et al.*, 2002); indicating that yeast and mammalian PCD pathways may share similarities. Mutagenesis allowing expression of mammalian Bax in *S. cerevisiae* encourages apoptosis as seen by the increased generation of ROS (Madeo *et al.*, 1999). Bax promotes calcium signalling to mitochondria (Suen *et al.*, 2008). The generation of ROS also alters the composition of the mitochondrial membrane through lipid peroxidation, which aids insertion of Bax into the

mitochondrial membrane (Manon, 2004). CytC is able to escape the mitochondria in this situation of *S. cerevisiae*. Some molecules in yeast apoptotic pathways can therefore be substituted by mammalian functional equivalents in knockout yeast mutants to restore an apoptotic phenotype.

Yeast and filamentous fungi cells also have homologous cysteine proteases to mammalian caspases, known as metacaspases. Metacaspases are arginine-/lysine-specific, rather than aspartate-specific as caspases are, but still play a role in PCD (Madeo *et al.*, 2002). The apoptotic signals in *S. cerevisiae* affect the activity of the metacaspase YCA1, which self-cleaves to activate, before conveying pro-apoptotic signals targeting the mitochondria, as mammalian cells do (Madeo *et al.*, 2004). Apoptotic behaviour can be prevented by deletion of the yeast metacaspase gene *YCA1* (Madeo *et al.*, 2002). A homologous metacaspase to YCA1, CaMCA1, is present in the opportunistic pathogen *C. albicans*. CaMCA1 drives the apoptotic pathways of *C. albicans* during oxidative stress as YCA1 does in *S. cerevisiae* (Cao *et al.*, 2008). *YCA1* mutants lack the ability to undergo PCD, and CaMCA1 mutants are unable to respond in an regulated apoptotic-like manner to H_2O_2 exposure (Cao *et al.*, 2008). Yeast PCD is an active process requiring protein synthesis as it is prevented by cycloheximide treatment. The addition of oxygen radical scavengers to remove ROS also inhibits yeast PCD (Madeo *et al.*, 1999).

1.3.2 Heterokaryon Incompatibility of Filamentous Ascomycota

Ascomycota hyphae in close contact have to ability to fuse resulting in the formation of a heterokaryon; a cell where more than one type of nucleus is present in the cytoplasm (Saupe, 2000). Although potential benefits of this include genetic exchange during the parasexual cycle (Pontecorvo, 1956; Glass and Kaneko, 2003), and increased hyphal-network size for obtaining resources, virtually no heterokaryosis is thought to actually take place in the environment (Mylyk, 1976). Ascomycota may use HI to prevent genetically different individuals (non-self) from accessing nutrients within the fungal cells (Debets and Griffiths, 1998). The filamentous ascomycete *C. parasitica* was found to use *vic* genes to prevent horizontal transfer of viruses by encouraging PCD upon hyphal contact (Cortesi *et al.*, 2001; Biella *et al.*, 2002). Increased levels of cell death was associated with a lower transfer rate of viruses (Biella *et al.*, 2002). Whether or not hyphal fusion can produce a viable heterokaryon is genetically dependent on the *het* genes of the species (Glass *et al.*, 2000). If co-expression of *het* alleles of the two fungi are different at the same locus, the contacting cells initiate allelic HI (Glass and Kaneko, 2003), with non-allelic HI being triggered by incompatible alleles at different loci. It was found in *P. anserina* that a single amino-acid difference in the HetS protein can be enough to prevent heterokaryon formation (Deleu *et al.*, 1993). When two incompatible filamentous fungal hyphae contact it results in the compartmentalisation and PCD of the local area.

The number of *het* genes in each ascomycota species can differ considerably (Galagan *et al.*, 2003; Nierman *et al.*, 2005b; Espagne *et al.*, 2008), as demonstrated by the *Aspergilli* that range from between 7 and 43 *het* genes (Galagan *et al.*, 2005). The *het* genes encode proteins involved in HI that share similarities in the type of domains they contain, but many of these proteins also do not have the HET protein domain which is specific to filamentous fungi (Paoletti and Clave, 2007). Amongst the *Aspergilli* there are different numbers of *het* genes suggesting that there has been a positive selection of *het* genes at some evolutionary stage, leading to gene duplications (Saupe, 2000), followed by rapid evolutionary divergence (Fedorova *et al.*, 2005). *A. fumigatus*, *Aspergillus clavatus*, *A. nidulans* and *Neosartorya fischeri* all contain less than 12 *het* loci, whereas *A. niger*, *A. terreus*, *Aspergillus flavus* and *A. oryzae* all contain over 30 *het* loci. The HET domain can be found in various ascomycete proteins; HET-6 & TOL of *N. crassa*, and HET-D & HET-E of *P. anserina* also contain the HET domain (Saupe, 2000). The HET domain is characterised by a length of approximately 200 amino-acids, with three intermittent conserved short sequences of around 15-20 amino-acids in length (Smith *et al.*, 2000). *N. crassa het* genes HET-*6*, *tol* and *pin-c* expression requires the expression of *vib-1* (Dementhon *et al.*, 2006). VIB-1 is a transcription factor active during both hyphal growth and HI, which controls not only HET-*6*, *tol* and *pin-c* expression, but also downstream effectors of HI (Dementhon *et al.*, 2006).

Basic local alignment sequence tool protein (BLASTP) queries of the *Aspergilli het* genes reveal various protein domains frequently found in HET

proteins, such as ankyrin repeat domains (Ank), WD40 beta-transducin repeat domains (WD) and nucleoside-triphosphatase domains (NACHT). Less common domains such as nucleoside phosphorylase (Pfs), pyruvate kinase (P-kinase) and LipA are also present (Leipe *et al.*, 2004). The NACHT NTPase domain is a variation of STAND NTPases. STAND NTPases are complex signalling peptides. The C-terminal helical bundle undergoes conformational changes caused from NTP hydrolysis to enforce a conformational change of the effector domains (Leipe *et al.*, 2004). These NACHT domain proteins are often coupled with Ank or WD40 protein-binding domains and associated with apoptosis proteins (Koonin and Aravind, 2000). The STAND family proteins N-terminus are linked with PCD in metazoa and plants, as these proteins are often associated with a death effector domain (DED) (Leipe *et al.*, 2004). Considering the abundance of related NACHT domains in *het* genes of *Aspergilli*, it is possible that the HI system uses the conserved death signalling role of NACHT as an early stage apoptotic signal. HET-C2 shows similarity to genes from many species ranging from *P. anserina* to GLTP from *Homo sapiens* which is involved in glycosphingolipid metabolism. HET-C2 of *P. anserina* HET-C2 is highly similar to that of the *Aspergilli*, and therefore it is thought that their roles are relatively similar. HET-C2 in *P. anserina* is thought to trigger HI through heterocomplex formation (Sarkar *et al.*, 2002) as well being involved in glycolipid transfer, and being a glycolipid metabolite sensor (Mattjus *et al.*, 2003).

het-c genes in *Aspergilli* have are orthologs of those in *N. crassa* (Saupe *et al.*, 1996). Evidence from TinC, a HET-C homologue, in *A. nidulans* suggests

a role of HET-C in control of the cell cycle through nuclear membrane fission (Davies *et al.*, 2004). However the *N. crassa* HET-C heterocomplex is associated with HI (Sarkar *et al.*, 2002). The HET-C heterocomplexes in incompatible heterokaryons localise to the plasma membrane (PM), followed by impaired growth and hyphal compartmentalisation & death (Sarkar *et al.*, 2002). A predicted signalling peptide from HET-C expressed in *N. crassa* formed HET-C heterocomplexes in the cytosol, coinciding with growth arrest (Sarkar *et al.*, 2002). The open reading frame (ORF) of the *P. anserina het-c* gene of shows greater variability than the intron and flanking sequences (Saupe *et al.*, 1995). The *P. anserina* similar *het-d* and *het-e* genes interact with the unrelated *het-c* gene. The HET domain proteins HET-D and HET-E both have a GTP-binding domain and require ten or more WD40 repeats to actively play a part in HI (Espagne *et al.*, 1997; Espagne *et al.*, 2002). WD40 repeats and GTP-binding domains are present in the individual peptides of trimeric G-proteins which are involved in signalling (Espagne *et al.*, 1997), implying a role of signal transduction in both HET-D and HET-E. Only experimental alteration of WD40 domains produces non-functional proteins involved in HI (Espagne *et al.*, 2002), and WD40 sequence is thought to be responsible for HET-E binding specificity (Espagne *et al.*, 2002). HET-D and HET-E belong to a 10-member gene family that exhibit concerted evolution and all contain WD repeat domains which are also subject to concerted evolution (Paoletti *et al.*, 2007a). Four residues of the repeat units located on the surface of the WD repeat domain protein/protein site are under further positive selection for variation (Paoletti *et al.*, 2007a). These factors encourage mutation and variability in the HET-D and HET-E proteins of *P.*

anserina, which discourages heterokaryon formation due to the specificity of the non-self recognition system.

The downstream metacaspase-dependent signalling of PCD in fungi shows similarities to metazoan cells. Caspases and metacaspases both share dependency on a conserved cysteine residue to act as functional proteases (Madeo *et al.*, 2002), and *S. cerevisiae* MCA1 also requires cleavage to an active form as effector caspases do (Mazzoni and Falcone, 2008). Caspase 3- and 8-like activity was monitored during sporulation autolysis in *A. nidulans*, as well poly (ADP-ribose) polymerase (PARP) activity (Thrane *et al.*, 2004). PARP family proteins usually detect DNA damage and then signal to, and recruit, DNA repairing enzymes, such as DNA ligase. PARP activity utilises adenosine triphosphate (ATP), which can act as a pro-apoptotic signal. PARP members are known metazoan caspase targets and are also broken down by *A. nidulans* metacaspase activity, indicating similarities of apoptotic caspase cascade signalling in mammalian cells & filamentous fungi. These similarities make targeting this pathway component for drug development less desirable, however, metacaspases were found to be unnecessary for HI and PCD in *N. crassa* indicating a novel PCD system may be in use by ascomycota (Hutchison *et al.*, 2009). Sequencing of *A. fumigatus* found distinct *het* genes that shared common protein domains to those of *N. crassa* and *P. anserina* (Fedorova *et al.*, 2008).

Methods used to investigate compatibility of filamentous fungi include observation of a barrage phenotype, identifying conidial anastomosis tubes

(CATs) and the use of selectable media which encouraged growth of heterokaryons comprising of two auxotrophic strains that alone would be unable to grow on the media. Barrages are visible zones of disruptions of the growth of fungi and consist of either dead cells or thick raised mycelium from HI reactions where two incompatible species or strains have come into contact. This rapid method has been successfully used to determine vegetative compatibility (VC) groups (VCGs) of *Fusarium graminearum* (McCallum *et al.*, 2004). Vegetative compatibility typically refers to the phenotypic differences between members of a species, rather than genotypic variation which is more commonly known as HI (Burgess *et al.*, 2009).

CATs are short, thin structures that emerge from germinating conidia but do not branch like germ tubes. The structures are density-dependent and grow towards other emerging CATs. CATs pass nuclei and organelles through connected hyphae (Roca *et al.*, 2005b), effectively forming a heterokaryon. CAT fusion between incompatible mating types in *N. crassa* have success rates of just over half (22%) compared to that of compatible mating types (38-42%) (Roca *et al.*, 2005a). Heterokaryon formation between compatible strains can also be detected through use of selectable media. Chlorate-resistance has been used to generate nitrate non-utilising (Nit) mutants (Cove, 1976). Several different individual mutations of the nitrogen assimilatory pathway (NAP) can incur the Nit phenotype, and crossing two Nit strains with different mutations will generate a heterokaryon if the strains are compatible. Compatible strains that form heterokaryons can grow on nitrate media due to complementing genotypes.

1.4 Aims and Objectives

This study aims to begin characterisation of the HI system in the opportunistic pathogen *A. fumigatus* to identify potential proteins or pathways suitable for use as novel drug targets. The filamentous fungi-specific non-self recognition system and cell death pathway, HI, has been characterised in other species, but differs in the mode of action in each. Compatibility in *N. crassa* is governed mainly through allelic HI, whereas *P. anserina* compatibility is dependent on non-allelic HI. Although the *A. fumigatus* genome sequences show the *het* genes are distinct to those of *N. crassa* and *P. anserina*, the proteins do share similar domains including the ascomycota HET domain. Expression of the HET domain in *P. anserina* induces an apoptotic phenotype, indicating focus on the HET domain of *A. fumigatus* could also allow manipulation of the PCD pathway. Targeted methods of triggering HI, and therefore PCD, in *A. fumigatus* could lead to anti-fungal drug treatments for IA with less severe side effects if they specifically target proteins not native to the patient. Amphotericin B has been shown to induce apoptotic markers in *A. fumigatus* indicating that PCD is a viable drug target.

The initial approach is to link Aspergilli genomic data on *het* genes available in public resources to build a picture on which genes show variation amongst the Aspergilli and which domains are commonly encoded for by *het* genes, and are therefore likely to be involved in non-self recognition.

As seen with *A. nidulans*, characterisation of HI systems has traditionally included establishment of VCGs (Croft and Jinks, 1977; Dales *et al.*, 1993). Comparing the pair-wise compatibility grouping of *A. fumigatus* sourced from different backgrounds will identify whether the VCGs are discrete or whether viable heterokaryons can form between there are certain groups. The presence of barrage phenotypes between strains can be used to identify VGCs however, inoculating selective media with complementary Nit mutants will both identify VCGs and also show whether crosses can result in partial heterokaryons if the strains are not fully compatible or incompatible.

Manipulation of *A. fumigatus het* genes using polymerase chain reaction (PCR) techniques such as fusion-PCR to knockout (KO) *het* genes will be used to assess whether specific disruptions alter the ability of individuals to effectively utilise the self/non-self recognition pathway. This assessment will be achieved through pair-wise crosses of genetically modified strains with other *A. fumigatus* strains that the parent strain of the mutants were incompatible with. If the mutant strains have impaired ability to recognise other strains as non-self then the disrupted gene is responsible for acting in the HI pathway. Identifying genes that have an active role in *A. fumigatus* HI would lead to further work on expression of those genes.

Another technique to characterise *A. fumigatus het* genes is through utilisation of transposon mutagenesis to randomly disrupt the genome. The mobilisation of a transposable element to disrupt random loci, followed by pair-wise crosses, will be used to provide a broader approach identifying

genes that play a role in HI. Expressional analysis of *A. fumigatus* cultures under environmental stresses that induce PCD, such as stationary phase in broth culture, will allow a whole-genome approach to identify which genes are switched on or up-regulated during PCD.

Chapter 2: Materials and Methods

All culture media, solutions and buffers were dissolved in double-distilled water (ddH_2O) and sterilised after preparation via a steam autoclave at 121°C for 15 min unless specifically mentioned otherwise. Percentages used in media composition are in weight per volume (w/v) format as g of solute dissolved in 100 mL of solvent, or volume per volume (v/v) format as mL mixed in 100 mL. For filter sterilisation, solutions were passed through disposable 0.22 µm polyethersulphone (PES) membrane filters which were factory sterilised using gamma-radiation.

2.1 Materials

2.1.1 Strains

A list of *A. fumigatus* strains used in this study is available in Appendix 1. This list includes *A. fumigatus* mutants generated throughout this study validated through PCR screening (sections 2.2.3.3.1 and 2.2.3.5). Nit mutants and sulphate non-utilising (Sit) mutants used for compatibility grouping are listed in Table 3.3.

2.1.1.1 Fungal Strains

A selection of environmentally and clinically isolated *A. fumigatus* isolates was gathered for compatibility grouping. Strains were confirmed by genomic sequencing of the 18S ribosomal DNA (rDNA) internal transcribed spacer (ITS) region using primers ITS1 and ITS4 (White *et al.*, 1990). Two clinically isolated strains of *Aspergillus fumigatus* with annotated genomes were used for studies: wild-type strain Af293, also labelled as A110 by the Fungal

Genetic Stock Center (FGSC), and strain A1163 (as known by the FGSC). The ectopic insertion of *A. niger pyrG* gene in A1163 was identified post sequencing (Fedorova *et al.*, 2008) and therefore A1163 is not the same strain as CEA10. A1163 owes lineage to CEA17 which itself a uracil auxotrophic derivative strain of CEA10. The *pyrG* gene encodes orotidine-5'-phosphate (OMP) decarboxylase, conferring the uracil autotrophy phenotype. Strains Af293, A1163, AF24 and AF71 were provided by Dr Peter Warn at the University of Manchester, UK.

The ten RB11-20 *A. fumigatus* strains were isolated through environmental air sampling in Dublin by Prof. Hubert Fuller at University College Dublin, Ireland. The air sampling took place at Belfield campus of University College Dublin on the 19th November 2005. The isolates were identified as *A. fumigatus* through microscopic observations. The following twelve *A. fumigatus* strains were isolated through environmental air sampling outside Michael Smith Building (University of Manchester) by Ph.D. student Fadwa Alshareef in 2009: Ap33; Ap61; Ap63; Ma3; Ma4; Ma5; J4; J10; J13; Au9; Au10; Au14. The strains were labelled according to the month they were isolated; April (Ap#), May (Ma#), June (J#) and August (Au#).

Clinically isolated *A. fumigatus* strains were provided by Dr Caroline Moore from the Mycology Reference Centre Manchester (MRCM) at Wythenshawe Hospital (Manchester, UK). The twenty strains used in this thesis are: 15562; 15819; 16795; 16916; 16975; 17318; 17406; 17768; 17796; 17835; 17871; 17882; 18565; 19164; 19258; 20395; 21522; 21705; 22178; 22577. A

detailed list of all the clinical isolates supplied by Dr Caroline Moore is available as provided by Patrycja Kent of the MRCM (Appendix 2).

The CEA17 derivative non-homologous end joining (NHEJ) deficient (*akuB*$^{(KU80\Delta)}$) strain A1160 (da Silva Ferreira *et al.*, 2006) and D1-4 strain containing the *impala160::pyrG* transposon mutagenesis machinery (Firon *et al.*, 2003; Carr *et al.*, 2010) were provided by Dr Michael Bromley at the University of Manchester.

2.1.1.2 Bacterial Strain

Transformation of bacteria with plasmid vectors was performed using Bioline α-select bronze efficiency *E. coli* competent cells (Cat No. BIO-85025) with the following genotype: F$^-$ *deo*R *end*A1 *rec*A1 *rel*A1 *gyr*A96 *hsd*R17(r_k^-, m_k^+) *sup*E44 *thi*-1 *pho*A Δ(*lac*ZYA-*arg*F)U169 Φ80*lac*ZΔM15λ$^-$. The transformed bacterial strains were used as a means both to store and also amplify plasmids containing material for fungal transformation.

2.1.2 Culture Media

2.1.2.1 Media for Fungal Culturing

Potato dextrose agar (PDA) and potato dextrose broth (PDB) from ForMedium^{TM}, United Kingdom, were used for general culturing of wild-type (WT) *A. fumigatus* in solid and broth culture media respectively. Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) media containing 4% glucose and 1% peptone at pH 5.6 were used when less sporulation of

A. fumigatus colonies was desired compared to PDA and PDB (Pashley *et al.*, 2012). Agar concentration in SDA was 1.5%.

Minimal media (MM) agar was prepared by adding 50 X Vogel's salts stock solution to autoclaved ddH_2O with 1% glucose and 1.5% agar (Vogel, 1956). 50 X nitrogen (N) and sulphur (S) free Vogel's salts stock solution was used in media for controlling N and S sources in minimal media; these two Vogel's salt stock solutions are outlined in Table 2.1. MM agar allowed for specific supplementation of nitrogen and sulphur sources when working with nitrate and sulphate non-utilising spontaneously generated mutants (Nit and Sit, respectively).

Four filter sterilised 100 X N stocks were made for supplementing N- and S-free MM agar: 20% $NaNO_3$ (N3S and N3T); 2% $NaNO_2$ (N2S and N2T); 2% hypoxanthine (Hyp) dissolved in 1 M NaOH (HS and HT); 20% CH_4N_2O (US and UT). Two filter sterilised 100 X S stocks used to supplement N- and S-free MM agar: 0.02 g/L $MgSO_4$ (N3S, N2S, HS and US), or 1 M $Na_2S_2O_3$ (N3T, N2T, HT and UT). Table 2.2 is a reference for the nomenclature of media with nitrogen and sulphur supplementation used to isolate and screen Nit and Sit *A. fumigatus* mutants.

Media used to culture uracil auxotrophic (*pyrG*$^-$) strains of *A. fumigatus* were supplemented with filter sterilised 5 mM uracil and 5 mM uridine (Xue *et al.*, 2004). Media used as negative control for *pyrG*$^-$ strains were not supplemented with any nutrients.

<u>Table 2.1</u> Composition of Vogel's salts and N- and S-free Vogel's salts stock solutions for Minimal Media Agar

	Vogel's salts (50 X)		**N- and S-free Vogel's salts (50 X)**	
	Chemical or Solution	Quantity	Chemical or Solution	Quantity
1 L stock solution (50 X)	$CaCl_2.2H_2O$	5 g	$CaCl_2.2H_2O$	5 g
	$Na_3C_6H_5O_7$	125 g	$Na_3C_6H_5O_7$	125 g
	KH_2PO_4	250 g	KH_2PO_4	250 g
	$MgSO_4.7H_2O$	10 g	$MgCl_2.6H_2O$	8.248 g
	NH_4NO_3	100 g		
	Trace elements stock	5 mL	N- and S-free trace stock	5 mL
	Biotin solution	2.5 mL	Biotin solution	2.5 mL
Biotin solution	$C_{10}H_{16}N_2O_3S$	0.1 mg/mL	$C_{10}H_{16}N_2O_3S$	0.1 mg/mL
100 ml trace elements stock	$C_6H_8O_7.1H_2O$	5 g	$C_6H_8O_7.1H_2O$	5 g
	$ZnSO_4.7H_2O$	5 g	ZnCl2	2.37 g
	$Fe(NH_4)_2(SO_4)_2.6H_2O$	1 g	$FeCl_2.4H_2O$	0.51 g
	$CuSO_4.5H_2O$	0.25 g	$CuCl_2.1H_2O$	0.17 g
	$MnSO_4.1H_2O$	0.05 g	$MnCl_2.4H_20$	0.058 g
	H_3BO_3	0.05 g	H_3BO_3	0.05 g
	$Na_2MoO_4.2H_2O$	0.05 g	$Na_2MoO_4.2H_2O$	0.05 g

Table 2.2 Nitrogen and Sulpher Sources used in Minimal Media Agar

Medium	Nitrogen Source	Sulphur Source
N3S	Sodium nitrate ($NaNO_3$)	Magnesium sulphate ($MgSO_4$)
N3T	Sodium nitrate	Thiosulphate ($Na_2S_2O_3$)
N2S	Sodium nitrite ($NaNO_2$)	Magnesium sulphate
N2T	Sodium nitrite	Thiosulphate
HS	Hypoxanthine ($C_5H_4N_4O$)	Magnesium sulphate
HT	Hypoxanthine	Thiosulphate
US	Urea (CH_4N_2O)	Magnesium sulphate
UT	Urea	Thiosulphate

2.1.2.2 Media for Measurement of Fungal Growth

Germinating *A. fumigatus* spores aggregate in broth media, preventing reliable optical density (OD) and viable count (Vc) measurements, but addition of Junlon PW 110 or 111 to media aids dispersal of colonies (Trinci, 1983; Jones *et al.*, 1988). Junlon PW 110 MM broth was prepared using filter sterilised 50 X Vogel's salts stock solution, autoclaved 5 X glucose solution (5%) and autoclaved 2 X Junlon PW 110 stock solution. The 2 X Junlon PW 110 stock solution consisted of 3 g/L Junlon PW 110 (Honeywell and Stein, UK) brought to pH 5.5 through slow addition of NaOH during vigorous stirring. Junlon PW 110 MM broth was used for growth rates and stationary curve measurements (section 2.2.2.4).

2.1.2.3 Media for Fungal Transformation

Transformed protoplasts (sections 2.2.3.6.2 and 2.2.3.6.3) were cultured on autoclaved TransAspA; 1.2 M sorbitol, 1% glucose and 1% agar media supplemented with pre-autoclaved stock solutions of AspA+N, $CaCl_2$, $MgSO_4$ and trace elements to 1 X final concentrations). 50 X concentration stock solution of AspA+N consisted of 300 g/L $NaNO_3$, 26 g/L KCl and 76 g/L KH_2PO_4. AspA+N was adjusted to pH 5.0 using KOH. The 100 mM $CaCl_2$ stock solution was 1000 X concentration, and the 1 M $MgSO_4$ stock solution was 500 X concentrated. The 1000 X trace element stock solution was adjusted to pH 5.0 using KOH after addition of the following solutes: 50 g/L $Na_2EDTA.2H_2O$; 22 g/L $ZnSO_4.7H_2O$; 11 g/L H_3BO_3; 5 g/L $MnCl_2.4H_2O$; 5 g/L $FeSO_4.7H_2O$; 1.7 g/L $CoCl_2.6H_2O$; 1.6 g/L $CuSO_4.5H_2O$ and 1.5 g/L $Na_2MoO_4.2H_2O$.

Selective media for hygromycin phosphotransferase (*hph*) transformants with hygromycin B (HmB) resistance (HmB^R) were supplemented with 200 µg/mL HmB (Duchefa Biochemie BV, The Netherlands) after media had cooled to 50°C post-autoclaving. Selective media for mutants with transformation DNA conferring uracil autotrophy (*pyrG*$^+$) were not supplemented with uracil and uridine.

2.1.2.4 Media for Fungal Spontaneous Mutagenesis

Chlorate-PDA was used to generate Nit mutants (Cove, 1976). The 750 mM potassium chlorate ($KClO_3$), required to generate *A. fumigatus* spontaneous Nit mutants, would only dissolve readily in media above 70°C, and was therefore added directly as a solid into the PDA upon removal from the autoclave. The medium had to be kept warm at around 37°C after solidifying to reduce crystallisation of $KClO_3$. Spare media was prepared as a negative control to reassure that the addition of unsterilised $KClO_3$ had not introduced contaminants. N3S, N2S, HS and US agar media were used to characterise Nit mutants generated from the chlorate-PDA medium.

Selenate-PDA media was used to generate mutants with selenate (SeO) resistance (SeO^R). SeO^R also confers a sulphate non-utilising (Sit) phenotype. Selenate-PDA was standard PDA supplemented with filter sterilised 0.4 mM Na_2SeO_4 and 10 mM $Na_2S_2O_3$ after autoclaving (Buxton *et al.*, 1989). N3T, UT and US agar were used to characterise Sit mutants generated selenate-PDA medium.

2.1.2.5 Media for Bacterial Culturing and Transformation

Lysogeny broth (LB) used for propagation of *E. coli* cells contained 1% tryptone, 0.5% yeast extract and 0.5% NaCl at pH 7.0. 1.5% agar was added for solid media. 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) and filter sterilised ampicillin (Amp) and 5-bromo-4-chloro-indolyl-β-D-galactopyranoside, Isopropyl β-D-1-thiogalactopyranoside (IPTG) and were added to the LB agar when screening for *E. coli* containing plasmid vectors. When the agar had cooled to 50°C after autoclaving, Amp was added to a final concentration of 100 µg/mL. 40 µL of each of IPTG and X-gal stock solutions were spread evenly over the top of LB agar media in Petri dishes after solidification. The IPTG stock solution was 0.1 M. The X-gal stock solution was 20 mg/mL, dissolved in dimethyl sulphoxide (DMSO), and not sterilised prior to addition to media.

Super optimal broth with catabolite repression (SOC) media used for bacterial transformation consisted of 2% tryptone; 0.5% yeast extract; 0.4% glucose; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$ and 10 mM $MgSO_4$. SOC is supplemented NaOH with until pH 7.0. If required, filter sterilised Amp was added to a final concentration of 100 µg/mL.

2.1.3 Plasmids

The commercial plasmid pGEM®-T Easy (Promega, USA) was used for storage of PCR-fusion cassettes in *E. coli*. The plasmid allowed for blue/white colony screening of bacterial transformants due to the insertion site within *lac*Z, and also conferred Amp resistance (Amp^R). The plasmid

map with suitable restriction sites is available from the Promega website. pGEM®-T Easy is supplied as a linearised vector with a single 3' T-overhang on each DNA strand; suitable for ligation with PCR products with a 3' A-overhang.

The *E. coli* HmB phosphotransferase gene (*hph*) was amplified by PCR for generation of fusion-cassettes from pAN7-1 (Figure 2.1). *hph* was used as a marker for *A. fumigatus* transformants (Punt *et al.*, 1987). A digest pattern using 1 µg of pAN7-1 and the restriction enzyme (RE) BstZI was run by agarose gel electrophoresis to confirm the plasmid by comparing the expected pattern and fragment sizes to gel.

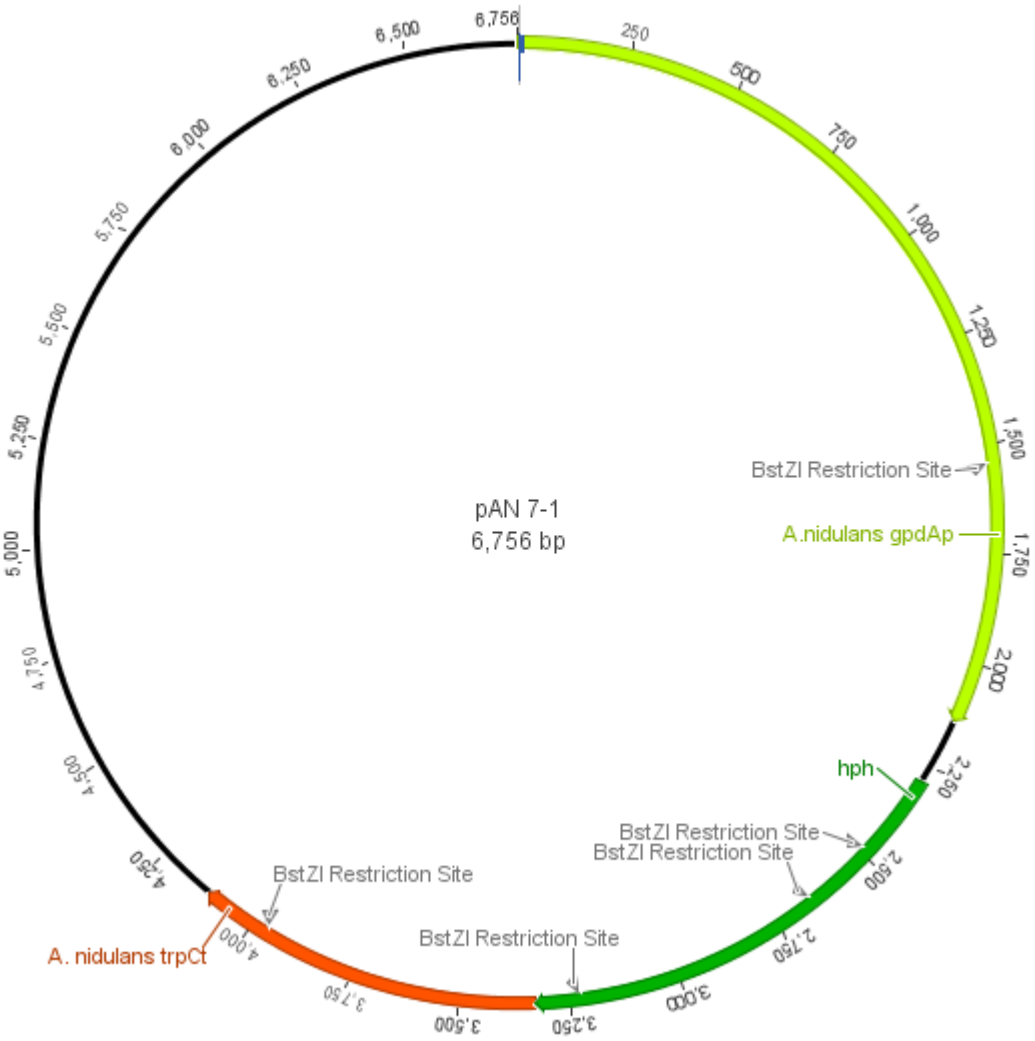


Figure 2.1 pAN7-1 Map

The *hph* gene used as a marker for fungal transformations is present in pAN7-1, flanked either side by *A. nidulans gpd*Ap and *trp*Ct. The light green region represents a promoter region (*gpdA*p), the dark green region marks a gene (*hph*), and the orange region marks a terminator region (*trpC*t). The promoter and terminator regions of pAN7-1 were sourced from *A. nidulans*. The DNA sequence of pAN7-1 is provided in Appendix 3.

2.1.4 Oligonucleotide Primers

Primers for PCR were designed using the two annotated *A. fumigatus* genomes to determine suitable target sites (Nierman *et al.*, 2005b; Fedorova *et al.*, 2008) and were stored at -20°C. Most primers in this study were designed within the target sites to have approximate 50% GC-content, 55°C melting temperature, and 18-20 nucleotides in length. The large fusion primers (FP) labelled as FP2, FP3, FP4 and FP5, were designed to have overlapping complementary regions and were 50 nucleotides in length (detailed in section 2.2.3.6.1). These primers were larger than other standard primers, and therefore had varying melting temperatures and GC-contents.

A full list of primers used in this study for for validation, fusion-PCR and transposable element rescue is available in Table 2.3. Primers for specific *het* genes contain "-#" suffixes in the primer names. For example, FP4-5 is fusion primer 4 (FP4) specific for the gene *het5* (-5), and SR-3 is the sequencing reverse (SR) primer for *het3* (-3).

Table 2.3 Primers for PCR and DNA Sequencing Reactions

Primer	Sequence (5'-3')
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC
FP1-1	TGGGACATCTACAGCAGCTA
FP1N-1	TCTCATGATAGGTGGGTGTG
FP2-1	GCGCCCACTCCACATCTCCACTCGAGTCAGTATCGAGCTGCCAACTGTCA
FP3-1	TGACAGTTGGCAGCTCGATACTGACTCGAGTGGAGATGTGGAGTGGGCGC
FP4-1	CCTATAGGACAAGTCGGCGACTGCTCTATAGAATCATCCTTATTCGTTGA
FP5-1	TCAACGAATAAGGATGATTCTATAGAGCAGTCGCCGACTTGTCCTATAGG
FP6-1	CTGTTACCAATGCAATCAGC
FP6N-1	GGCCTTCTTACTTCCACAGA
FP1-3	CTCGCGCAGTGTCTCTC
FP1N-3	CGCCGGTCTACTTCATGT
FP2-3	GCGCCCACTCCACATCTCCACTCGATTTGCGGCTTCGCTTGGGGGCACGG
FP3-3	CCGTGCCCCCAAGCGAAGCCGCAAATCGAGTGGAGATGTGGAGTGGGCGC
FP4-3	ATCAAAGTCTGCGCAGTCATTGCATCTATAGAATCATCCTTATTCGTTGA
FP5-3	TCAACGAATAAGGATGATTCTATAGATGCAATGACTGCGCAGACTTTGAT
FP6-3	TACACCGTCGACAACACG
FP6N-3	ACAGCGCCAGTATCCCTA
FP1-4	GATGGTCAGCACGGACTT
FP1N-4	CGAAGGAGACTGCCATTG
FP2-4	GCGCCCACTCCACATCTCCACTCGACCTATTGCGTGTGGTGATGATGGCT
FP3-4	AGCCATCATCACCACACGCAATAGGTCGAGTGGAGATGTGGAGTGGGCGC
FP4-4	TTTGTGTGGAATATATCCAATCTTGCTATAGAATCATCCTTATTCGTTGA
FP5-4	TCAACGAATAAGGATGATTCTATAGCAAGATTGGATATATTCCACACAAA
FP6-4	ATGTGCCAAAGTGGAAGC
FP6N-4	CACCTGAAGCACGAACCT
FP1-5	AGTATCCTTGCCGCGTTA
FP1N-5	GACATGCCCTCTTCCAGA
FP2-5	GCGCCCACTCCACATCTCCACTCGACAAAGTGGGAGCGCTTCAAACATTG
FP3-5	CAATGTTTGAAGCGCTCCCACTTTGTCGAGTGGAGATGTGGAGTGGGCGC
FP4-5	ACCATCCCATATAACATATCCGTATCTATAGAATCATCCTTATTCGTTGA
FP5-5	TCAACGAATAAGGATGATTCTATAGATACGGATATGTTATATGGGATGGT
FP6-5	TCGGGATGTCATTTAGGG
FP6N-5	GCTAAGCTGGATGGCAAG

Table 2.3 *cont.*

FP1-6	TGTTCGAAGGTGCAGTTG
FP1N-6	CAGGCAGTTGCAGCATTA
FP2-6	GCGCCCACTCCACATCTCCACTCGATCGGGCAGACAGGATCGCTAATATG
FP3-6	CATATTAGCGATCCTGTCTGCCCGATCGAGTGGAGATGTGGAGTGGGCGC
FP4-6	TCATCACAGGCTACAACCATTACTACTATAGAATCATCCTTATTCGTTGA
FP5-6	TCAACGAATAAGGATGATTCTATAGTAGTAATGGTTGTAGCCTGTGATGA
FP6-6	CGAAGAAGGCACTCCAAG
FP6N-6	TGAGGCTGCTGAAAGGTT
FP1-7	AGGGCAACGGTGCTATTA
FP1N-7	AAAAGCGAACGAGGATCA
FP2-7	GCGCCCACTCCACATCTCCACTCGACTTGCGGTCAAAATAAGAATGCCCT
FP3-7	AGGGCATTCTTATTTTGACCGCAAGTCGAGTGGAGATGTGGAGTGGGCGC
FP4-7	CAATGGGAGTATGCTGGATGTCGATCTATAGAATCATCCTTATTCGTTGA
FP5-7	TCAACGAATAAGGATGATTCTATAGATCGACATCCAGCATACTCCCATTG
FP6-7	GCGACAATGACTTGCAGA
FP6N-7	GATCTCACGGTCCTGGAA
FP1-8	TATGAGGGTTGGGTCAGG
FP1N-8	TCAAAACTGGAGCCCATC
FP2-8	GCGCCCACTCCACATCTCCACTCGAAGTCGTTCTGAACTATTACCTGTCA
FP3-8	TGACAGGTAATAGTTCAGAACGACTTCGAGTGGAGATGTGGAGTGGGCGC
FP4-8	TATCTGCTTCCTTACATTCTTAGCACTATAGAATCATCCTTATTCGTTGA
FP5-8	TCAACGAATAAGGATGATTCTATAGTGCTAAGAATGTAAGGAAGCAGATA
FP6-8	CTTATCCCCGTTCCACAG
FP6N-8	ATGCAAGAATGCGAAAGG
TRP1	ATGGAAGGCGTAAGTTCCTTGC
TRP2	GGCCACGGTGGACTAGTACNNNNNNNNNNGATAT
TRP3	GGCCACGGTGGACTAGTACNNNNNNNNNNACGTC
TRP4	GTGTGGAGGAGGAAGAAAGAGC
TRP5	GGCCACGGTGGACTAGTAC
SF-1	AGCCAGTCTATCATGGCACTT
SR-1	CCGACACCTCACTGGCTAGA
SF-3	AGTTCGACTAGCCACGGACT
SR-3	CCTTGACACGGTCTCACGAA
SF-4	AGACCTCGGTCTCTTTCCCC
SR-4	TTGCGTGGGGGCTTTATCAC
SF-6	TCGAGCCACATGGTCAAAGC
SR-6	GGAGCCAGGGCATCTGTTTC
SF-7	ACCAATAGGCAAAGCACCGT
SR-7	AATCGGGAGCTCGGCAGATA
SF-8	AGTTGCCGTAGCCATTCACG
SR-8	TGCGTTTTTGTCTCCTCTTCCA

2.2 Methods

2.2.1 Phylogenetic Trees and Sequence Alignments

For alignments of DNA and protein sequences, and for inferring phylogenetic trees, version 5.05 of the MEGA5 software was used in this study (Tamura *et al.*, 2011). The MEGA5 software utilised the the ClustalW2 algorithm for multiple sequence alignments (Larkin *et al.*, 2007). Tree building for Figure 3.2 was performed using a maximum likelihood method and boostrap phylogeny test (with 200 replications).

2.2.2 Culturing Techniques

2.2.2.1 Harvesting Fungal Spores

PDA was inoculated with an *A. fumigatus* strain and supplemented with any required nutrients for auxotrophs. The PDA was then incubated at 37°C for 4 days, or until a lawn of spores was observed. The surface was flooded with 0.01% Tween® 20 and spores were dislodged by gentle agitation with an L-spreader or sterile loop. The spores were filtered through a double layer of sterile lens tissue to remove mycelia. *A. fumigatus* spores germinate when suspended in Tween® 20; therefore spores were washed twice through centrifuging the suspension at 3000 g_F for 10 min, removing the supernatant and suspending the spores in sterile ddH_2O.

2.2.2.2 Fungal Spore Storage, Quantification and Viability

Spores were stored suspended in sterile ddH_2O at 4°C for short-term storage. Strain D1-4, and any mutants generated from the strain, could not be chilled as it would encourage transposon mutagenesis. D1-4 spores were

therefore kept at room temperature (RT) for short-term storage. For long-term storage of spores from all strains, 0.5 mL of the short-term suspension was mixed with 0.75 mL of sterile 75% glycerol and immediately stored at -80°C.

Spore count was quantified using an improved Neubauer haemocytometer and optical microscope. Spore viabilities were assessed by comparing the expected colony count to the actual colony count from germinated spores of triplicate serial dilutions on PDA after 2 days. The number of expected colonies was calculated assuming all spores in the quantified stock would each produce a single germinating colony.

2.2.2.3 Broth Media for Fungi

Broth media for general growth purposes was inoculated to a final concentration of $1x10^6$ spores/mL and shaken at 200 revolutions per minute (rpm) to discourage formation of large mycelial aggregates.

2.2.2.4 Fungal Growth Rate in Junlon Broth Media

Previous studies showed *A. fumigatus* underwent PCD during the stationary phase of a standard growth curve (Mousavi and Robson, 2003). In order to extract RNA from log phase cultures and stationary phase cultures of *A. fumigatus* for gene expression analysis, broth cultures were set up to determine the timing of these growth phases in the desired medium. Addition of Junlon PW 110 to broth media (recipe in section 2.1.2.2) ensured filamentous fungi are dispersed rather than forming biomass aggregates

(Jones *et al.*, 1988). Junlon PW 110 MM broth was inoculated with Af293 to a final concentration of $5x10^6$ spores/mL after preliminary results suggested this concentration was consistent for stationary growth curves. Preliminary tests also indicated the log phase initiated around 16 hours after inoculation. The media was pre-warmed to 37°C prior to inoculation, and was kept at 37°C at 200 rpm for 32 hours after inoculation. OD_{550} readings were taken at T_0 and every hour from T_{16} to T_{32} using an EEL Colorimeter with a green filter (Evans Electroselenium Ltd, Essex, United Kingdom). Dilution series of the cultures were taken at timed intervals and spread onto MM agar to calculate the viable count of fungi in the Junlon PW 110 MM broth. The results of the growth curves are described in section 3.2.1 and Figure 3.4. However, there was no analysis of gene expression of *A. fumigatus* stationary phase cultures in this study due to time constraints.

2.2.2.5 Barrage Formation

Two *A. fumigatus* strain spore suspensions were spread in parallel thin lines on PDA approximately 20 mm apart and incubated at 37°C for 3 days determine whether compatible strains would fuse hyphae, and whether visible contact zones that resulted in cell death, known as barrages, would form between incompatible strains. Results of pair-wise crosses to observe barrage formation are in section 3.2.2.1.

2.2.2.6 Spontaneous Nit and Sit Mutant Generation and Screening

Nit mutants were generated by spreading *A. fumigatus* spores onto chlorate-PDA and incubating at 37°C for 3 days (Cove, 1976). Spontaneous mutants were identified as strong growing colonies compared to background growth. Spores from individual chlorate-resistant colonies were isolated through the single spore technique (Figure 2.2). Spores from colonies isolated through single sporing were cultured on the four Nit screening MM agar and incubated at 37°C for 3 days to identify the Nit genotype. Xanthine dehydrogenase (*cnx*) mutants (*cnx*$^-$) were identified by those that showed weak growth on N3S and HS agar; nitrite reductase (*nirA*) mutants (*nirA*$^-$) showed poor growth on N3S and N2S agar; nitrate reductase (*niaD*) mutants (*niaD*$^-$) grew weakly on N3S agar. All three mutant genotypes grew strongly on US agar, whereas isolates able to grow on N3S agar were rejected as revertants to a nitrate-utilising phenotype.

Strain D1-4 was screened for HI against other *A. fumigatus* strains after transposon mutagenesis (section 2.2.3.7), but the *impala160* transposable element resided within the *nia*D gene prior to mutagenesis and incorporated *pyr*G within the transposon machinery (Carr *et al.*, 2010). Sulphate utilisation was selected as a marker for compatibility testing in place of Nit and *pyr*G. SeO^R can be acquired through spontaneous mutations leading to a non-functional sulphate permease (*sB*$^-$) or ATPsase (*sC*$^-$) which lead to the inability of *A. fumigatus* to use sulphate as a sole S source. (Buxton *et al.*, 1989). SeO^R mutants were generated through spreading D1-4 spores onto selenate-PDA medium (described in section 2.1.2.4) and incubating at 37°C

for 3 days. The optimum concentration of selenate for generation of SeO^R mutants was identified initially using a dilution series of concentrations of Na_2SeO_4 in selenate-PDA and spreading Af293 spores onto the medium.

Six recovered colonies (D1-4A, D1-4B, D1-4C, D1-4D, D1-4E and D1-4F) were screened on several media to ensure transposon mutagenesis had not occurred and restored nitrate-utilising phenotype. N3S, US, N3T, N2T, HT and US agar were used to screen the SeO^R mutants for Sit and *nia*D^- phenotypes. Nit and Sit mutant generation results are in section 3.2.2.2.

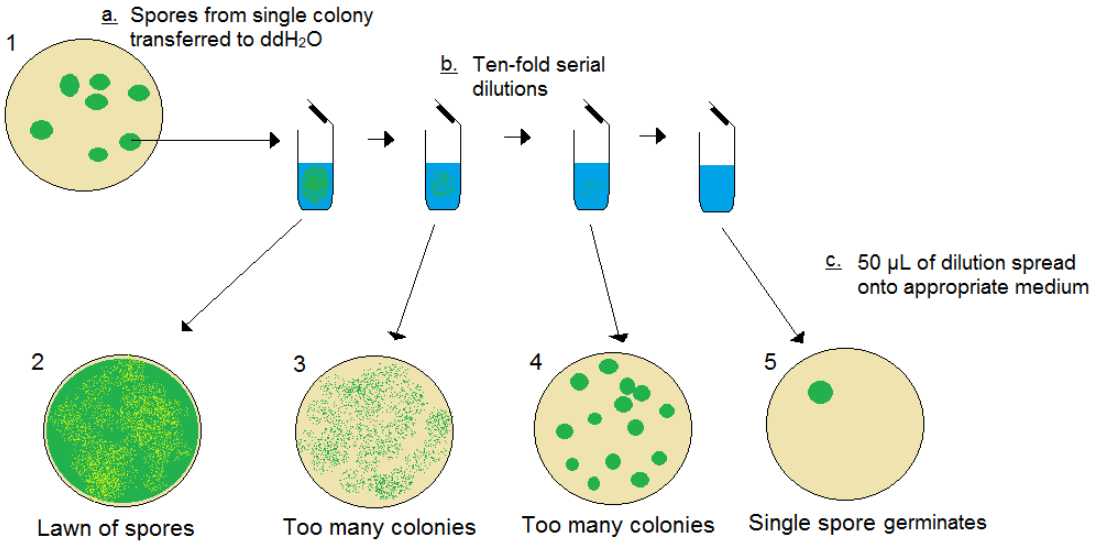


Figure 2.2 Single Sporing of *A. fumigatus* Colonies

When multiple different strains or mutants of *A. fumigatus* are sporulating on a single medium the spores can dislodge and contaminate other colonies. To ensure subcultured colonies consist of a single genotype the spores used for subculturing were single spored. Single sporing involved transferring a small quantity of spores from a single colony into ddH_2O for serial dilution as described in steps a, b and c above. Triplicates of 50 µL from each serial dilution were spread onto appropriate media for the strain or mutant being isolated, followed by incubation at 37°C for 2-3 days. Ideally a single colony would grow on an agar plate (shown on plate 5). Single *A. fumigatus* colonies could also be subcultured individually from a more densely populated agar plate, such as plate 4, if isolated before any colonies on the medium were sporulating.

2.2.2.7 Pair-Wise Compatibility Grouping

The compatibility grouping of *A. fumigatus* strains was to act as a foundation for testing how genetic manipulation of *het* genes could alter HI specificity. To determine the compatibility groups of *A. fumigatus*, spores were harvested from Nit and Sit spontaneous mutants and then mixed in pairs. Three drops of 10 µL from each pair spore mix was used to inoculate a separate N3S agar plate, as shown in Figure 2.3. Plates were incubated at 37°C for around 96 hours. Pair-wise crosses were performed between mutants with different genotypes in order for positive heterokaryons to rescue the nitrate-utilising phenotype. All mutants generated and used in pair-wise crosses in this study are displayed in Table 3.3. The results of the compatibility grouping are shown in section 3.2.2.3, Table 3.4 and Table 3.5

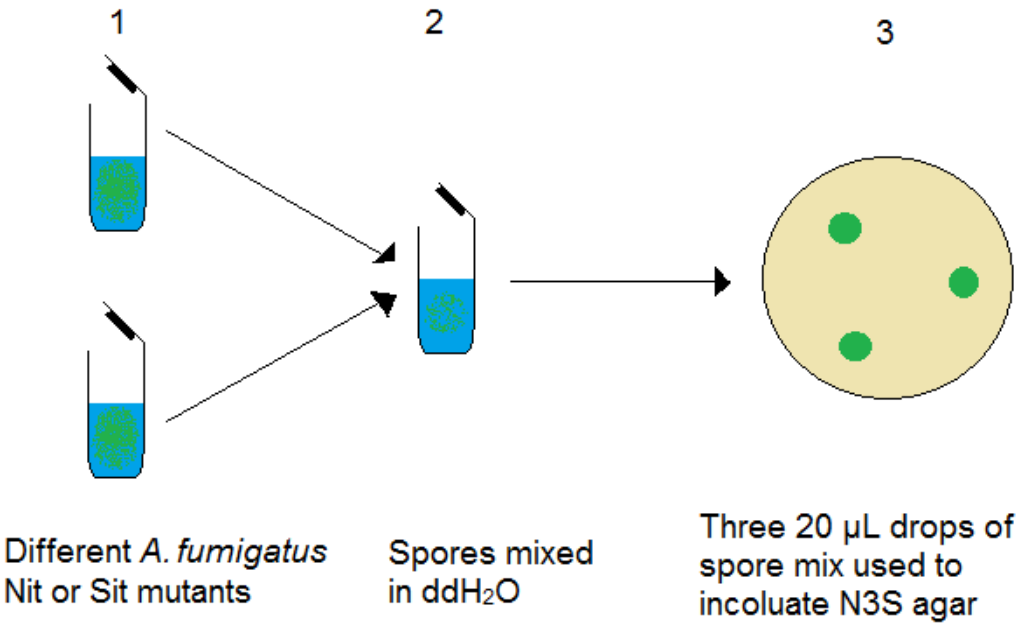


Figure 2.3 Pair-Wise Crosses on N3S Agar Media

N3S agar media were inoculated with pair-wise crosses of *A. fumigatus* Nit mutants spore mixes. Strong growth indicated that the mixed spores germinated and the colonies fused to form a positive heterokaryon. Lack of growth indicated that no viable heterokaryon was formed between the mixed spores.

2.2.3 Molecular Genetics Methods

2.2.3.1 DNA Extraction and Quantification

2.2.3.1.1 gDNA Extraction from Fungal Tissue

Fungal genomic DNA (gDNA) was initially extracted using the DNeasy Plant Mini Kit (QIAGEN, United Kingdom) after crushing the frozen fungal biomass frozen using liquid nitrogen, and a mortar and pestle. Fungi were cultured in PDB for this method.

A sodium dodecyl sulphate (SDS) lysis method was used to extract fungal gDNA after it was published (Feng *et al.*, 2010). This method was comparatively inexpensive and provided higher yields of gDNA than the DNeasy Plant Mini Kit. 20 mg of fungal mycelia and spores were collected from PDA and suspended in 2 ml tubes containing sterile 0.7 ml lysis buffer (100 mM Tris-HCl; 50 mM EDTA; 1% SDS) and autoclaved 0.5 mm diameter glass beads (Cat No. 11079105; BioSpec Products, USA). 1 M Tris-HCl, pH 8.0 and 0.5 M EDTA, pH 8.0, and 10% SDS stock solutions were used to make the lysis buffer. In each sample 10 µL of 10 µg/mL RNaseA was added before homogenising the samples twice for 30 s at 5000 rpm using a Precellys®24 homogeniser (Bertin Technologies, France). The samples were centrifuged at 13000 rpm for 2 min, and the supernatant was removed and mixed with 100 µL of 3 M potassium acetate, pH 5.5 (CH_3CO_2K). After centrifugation at 13000 rpm for 2 min, 0.4 ml of the supernatant was removed and mixed with 0.4 ml isopropyl alcohol (IPA) and the samples were centrifuged at 13000 rpm for 5 min. The supernatant was discarded and the pellets were washed with 0.8 ml 70% ethanol by centrifuging at

13000 rpm for 30 s. The ethanol was discarded and pellets were left to air-dry in a fume cupboard for 1 hour before they were suspended in 30 µL diethylpyrocarbonate-treated water (DEPC-H_2O).

2.2.3.1.2 Bacterial DNA Extraction

A quick DNA extraction method, followed by PCR, was used to check whether *E. coli* colonies had taken up a plasmid of the correct size. Individual colonies cultured on appropriate selective LB agar overnight were transferred to 20 µL sterile ddH_2O and boiled for 10 min. The samples were immediately transferred to -80°C for 10 min, and were then centrifuged at 13000 rpm for 1 min after defrosting. 2 µL of the supernatant was used as template material for PCR reactions.

2.2.3.1.3 Transformation and Screening of *E. coli*

Competent *E. coli* cells were used to store and amplify plasmids all plasmids in this study as per manufacturer's instructions. Culture media supplemented with Amp when the bacteria were transformed with plasmids that conferred resistance. The surface of agar was spread with X-gal and IPTG (section 2.1.2.5) for bacterial transformations of pGEM®-T Easy vectors with fusion cassettes insertions (section 2.2.3.1.3). As ligated fusion cassette inserts disrupted the *lac*Z gene of the vector, β-galactosidase (LacZ) was not produced by *E. coli* expressing the ligated vectors, leading to colonies unable to break down X-gal. Colonies able to metabolise X-gal were blue in appearance, therefore white colonies were selected as the *E. coli* had taken up the pGEM®-T Easy vector with an insert disrupting *lacZ* site.

2.2.3.1.4 Plasmid Extraction

To isolate pure plasmid samples from bacterial cultures confirmed to contain desired plasmids, the Plasmid Mini Kit (QIAGEN, United Kingdom) was used according to the manufacturer's instructions.

2.2.3.1.5 DNA Quantification

DNA concentrations and sample purity of gDNA, plasmids and PCR products were quantified at OD_{260} using a NanoDrop 1000 and ND-1000 software (Thermo Fisher Scientific, USA).

2.2.3.2 Agarose Gel Electrophoresis

Agarose gels were used to visualise plasmid DNA, restriction digest products, PCR products, and gDNA. All 1% agarose gels were made and run using sodium borate (SB) buffer except where Tris-acetate-EDTA (TAE) buffer is specifically mentioned (Brody *et al.*, 2004; Brody and Kern, 2004a; Brody and Kern, 2004b). SB buffer used for gels and as a conductive medium in the gel tank was prepared from filter sterilised 20 X SB buffer stock (8 g/L NaOH; 45 g/L H_3BO_3). DNA was visualised in agarose gels using nucleic acid dye SafeView (NBS Biologicals Ltd., United Kingdom) under ultraviolet (UV) illumination.

2.2.3.3 PCR Conditions, Routines and Purification

Concentrations of buffer, polymerase, dNTPs and primers in PCR mixes were prepared as per polymerase manufacturer's instructions unless otherwise stated. Denaturation and extension PCR conditions varied

depending on the polymerase used. Most primers were designed with a melting temperature of 55°C. dNTP mixes were equal concentrations of dATP, dCTP, dGTP and dTTP. PCR was used for validation of strains as *A. fumigatus* through rDNA amplification, amplification of material contained in plasmid DNA, fusion-PCR and screening of mutants and plasmids to confirm sequences.

2.2.3.3.1 PCR Routine for Amplification of 18S rDNA

ITS regions were amplified using fungal universal primers ITS1and ITS4 and BIOTAQ™DNA polymerase (Bioline, United Kingdom) to generate strain-specific sequences approximately 500 bp in length. The sequences were used to validate fungal strains as *A. fumigatus*. Reaction conditions were as follows: Initial denaturation of 94°C for 1 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s. Purified PCR samples were sequenced as described in section 2.2.3.5.

2.2.3.3.2 General PCR Routine for Generation of Products from Plasmid DNA and gDNA

Phusion® High-Fidelity DNA Polymerase (New England Biolabs, USA) was used to amplify regions of plasmids in place of *Taq* polymerase due to a lower error rate. Reactions were set up using Buffer HF without any DMSO, with other reaction concentrations as suggested by manufacturer. Primers were specific to the plasmid template. Initial denaturation of 98°C for 30 s was followed by 35 cycles of denaturation at 98°C for 10 s, annealing at

56°C for 30 s and extension at 72°C for 20 s/kb. The reactions ended with a final extension step of 72°C for 1 min. Products amplified for screening were purified and sequenced as described in sections 2.2.3.3.6 and 2.2.3.5 respectively.

2.2.3.3.3 Step 1 and Step 2 Fusion-PCR Routines

Fusion-PCR is used to generate a synthetic PCR cassette for fungal transformation using complementary base overhangs on PCR products (Szewczyk *et al.*, 2007). Step 1 amplified the components for each fusion cassette, and step 2 fused these components together. All primers used in fusion PCR were specific to the target gene.

Fusion-PCR step 1 was performed using Phusion® High-Fidelity DNA Polymerase as the enzyme leaves the blunt ended PCR products required for step 2. For each fusion cassette, three step 1 reaction tubes were mixed. Tube 1 contained gDNA as a template and primers FP1 and FP2. Tube 2 contained pAN7-1 as the template with primers FP3 and FP4. Primers FP5 and FP6 were in tube 3 with gDNA as template material. All reactions were set up using Buffer HF without any DMSO, with other reaction concentrations as suggested by manufacturer. The fusion-PCR step 1 routine is shown in Table 2.4. The elongation phase in step 1 fusion-PCR varied in length depending on the predicted size of the desired PCR products. The three generated PCR products per target gene (upstream of target, *hph* marker and downstream of target) were purified by gel extraction before use as template material in fusion-PCR step 2.

AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen / Life Technologies, USA) was selected for generating the fusion cassette in step 2 due to a combination of high accuracy requirement and the terminal transferase activity that leaves a single 3' A-overhang on PCR products. The A-overhang was for use in TA cloning for storage of generated cassettes in a plasmid vector. Each reaction contained buffer 1, primers FP1N and FP6N, and 10-15 ng template material from each the three associated products from step 1 fusion-PCR. The three products were to fuse through the PCR to produce a single amalgamated PCR product. Concentrations of the other PCR reagents in the reactions were as suggested by manufacturer. The fusion-PCR step 2 routine is shown in Table 2.4. The second and third elongation phases of the step 2 fusion-PCR extended in length each cycle to accommodate for loss of activity of AccuPrime™ *Taq* DNA Polymerase over the course of the PCR routine. AccuPrime™ *Taq* DNA Polymerase is more active at 72°C than 68°C, although denaturation of the protein occurs at a higher rate. Therefore elongation at 72°C was used only in the final phase of step 2 fusion-PCR reactions. Ramp rates were used in step 2 fusion-PCR as they increased the efficiency of the step 1 products associating at the complementary overhang regions. Step 2 products were purified through gel extraction for ligation into the pGEM®-T Easy vector, as fusion-PCR generated undesirable PCR products. Ligated vectors were used to transform *E. coli* for storage of fusion cassettes (section 2.2.3.1.3). Fusion cassette generation and screening results are shown in section 3.3.1.1.

Table 2.4 Step 1 and Step 2 Fusion-PCR Routines

Fusion-PCR	Phase	Cycles	Final Temperature (°C)	Ramp From Temperature (°C)	Ramp Rate (°C/s)	Time (s)
Step 1	Initial Denaturation	1	98	-	-	30
	Denaturation	35	98	-	-	10
	Annealing		55	-	-	30
	Elongation		72	-	-	20 (s/kb)
	Final Elongation	1	72	-	-	60
Step 2	Initial Denaturation	1	94	-	-	360
	Denaturation	10	94	-	-	30
	Annealing		55	70	0.1	30
	Elongation		68	55	0.2	360
	Denaturation	5	94	-	-	20
	Annealing		55	70	0.1	30
	Elongation		68	55	0.2	360 + 5 s/cycle
	Denaturation	10	94	-	-	30
	Annealing		55	70	0.1	30
	Elongation		68	55	0.2	360 + 20 s/cycle
	Final Elongation	1	72	-	-	600

2.2.3.3.4 PCR Screening of Fungal *het* KO Transformants

Prior to sequencing *A. fumigatus het* KO mutants to check whether the target gene had been replaced by the *hph* marker the gDNA was amplified. gDNA extracted from transformed *A. fumigatus* on selective media was amplified in the same manner as amplifying regions of plasmids described in section 2.2.3.3.2. Phusion® High-Fidelity DNA Polymerase was used to amplify the homologous recombination sites using primer pairs FP1 and FP6, and FP1N and FP6N, for the corresponding transformation cassette. Reactions were set up using Buffer HF without any DMSO, with other reaction concentrations as suggested by manufacturer. The accession codes for *het* genes were assigned short reference names, shown in Table 2.5 and summarised in Table 3.1. There were no fusion cassettes for *het2* and *het5* due to problems in step 2 fusion-PCR preventing cassette amplification.

Initial denaturation of 98°C for 30 s was followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s and extension at 72°C for 20 s/kb. Reactions finished ended with a final extension step of 72°C for 2 min. PCR products were visualised by 1% agarose gel electrophoresis and sequenced. Successful transformations were confirmed through comparing screening PCR product sizes of the FP1N and FP6N products to the predicted lengths of the *het* genes and fusion cassette lengths. If product matched that of the fusion cassette, the products from the FP1 and FP6 PCR reactions were used as templates for PCR reactions under the same conditions, using primers screening forward (SF) and screening reverse (SR)

for the corresponding fusion cassette. Products of these PCRs were sent for DNA sequencing (results section 3.3.1.2).

Table 2.5 Fusion Cassette and *het* Gene Sizes

Target *het* Gene	Gene Size (bp)	Fusion Cassette Size (bp)
het1 (AFUA_1G17550)	3549	3975
het2 (AFUA_2G12280)	3532	N/A
het3 (AFUA_2G12700)	3598	4540
het4 (AFUA_3G01620)	4053	4398
het5 (AFUA_3G03140)	4306	N/A
het6 (AFUA_6G12090)	3572	4523
het7 (AFUA_8G01020)	3786	4536
het8 (AFUA_8G07340)	3947	4488

2.2.3.3.5 *impala160* Rescue: ST-PCR

The rescue PCR to determine the position of the transposable element was adapted from semi-random, two-step-PCR (ST-PCR) (Chun *et al.*, 1997; Firon *et al.*, 2003; Carr *et al.*, 2010). In the first rescue step, the template material was gDNA extracted from strains in which the *impala160::pyrG* element had mobilised. The target site of transposon rescue primer (TRP) TRP4 was within the *impala160* transposable element, whereas primer TRP3 has a specific 19 nucleotide sequence followed by 10 bases of degenerate sequence with a common 5-base anchor. The anchor and degenerate sequence allowed TRP3 to act as a semi-random forward primer (Figure 2.4). Reaction mixes containing Extensor Hi-Fidelity PCR Master Mix were made to manufacturer's instructions. The PCR protocol was as follows: initial denaturation of 94°C for 2 min was followed by 5 cycles of denaturation at 94°C for 30 s, annealing at 30°C for 30 s and extension at 68°C for 2 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 30 s and extension at 68°C for 2 min; final extension step of 68°C for 2 min.

Strong products from step 1 were gel purified and amplified in step 2 using primers TRP4 and TRP5. TRP5 was a truncated version of TRP3 primer, missing the degenerate and anchor sequences to increase specific binding. Reaction mixes containing were made to the same specifications as step 1.
PCR protocol: initial denaturation of 94°C for 2 min was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 68°C for 2 min; final extension step of 68°C for 2 min. Purified products were sent for unidirectional sequencing using TRP4.

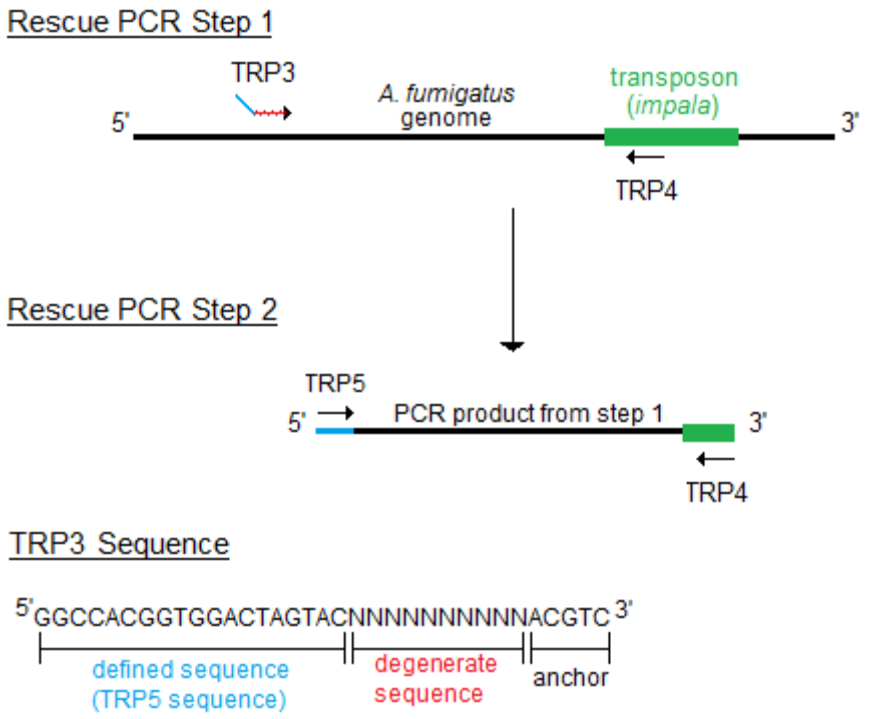


Figure 2.4 ***impala160*** **Rescue through ST-PCR**

ST-PCR uses a primer with a degenerate sequence and a common anchor to bind upstream of the transposon locus (TRP3). The primer pool of TRP3 contains a mix of primers with all the possible sequence combinations in the degenerate sequence section. The anchor is a common sequence expected approximately every few hundred bases in the *A. fumigatus* genome and encourages the primer to bind the gDNA. The PCR product of TRP3 and TRP4 contains the defined sequence at the 5' end of the amplicon, providing a specific target for TRP5 in the second ST-PCR step. The products of the second PCR step were sent for unidirectional sequencing (section 2.2.3.5).

2.2.3.3.6 PCR Purification and Gel Extraction

PCR samples were purified using QIAquick PCR Purification Kit (QIAGEN, United Kingdom) if one PCR product was visible when the sample was visualised on by agarose gel. If more than one product was present in a sample, the products were separated using gel electrophoresis with TAE buffer agarose gels and TAE buffer in the gel tank. The desired fragments were gel purified by excision of the band and then purified using the QIAquick Gel Extraction Kit (QIAGEN, United Kingdom).

2.2.3.4 Restriction Digests

Restriction digest patterns of DNA were used to identify successful ligation of fusion-cassettes into pGEM®-T Easy. Other digests were performed to excise fragments from plasmids for transformation or linearise plasmids. 1 µg of pGEM®-T Easy vectors ligated with transformation fusion-cassettes were subject to a one hour digestion at 50°C by 1 U of the RE BstZI (Promega, USA) according to manufacturer's instructions. There are two BstZI recognition sequences on pGEM®-T Easy, but each fusion-cassette contained multiple individual BstZI recognition sequences that resulted in specific expected restriction digest patterns. Fusion-cassettes were excised from pGEM®-T Easy using NotI-HF™ and ApaI (New England Biolabs, USA). Large quantities of DNA were required for transformations, therefore double-digest conditions of 10 µg plasmid and incubation at 25°C overnight in the presence of 2 U both NotI-HF™ and ApaI in NEBuffer 4 were used. The recognition sites were immediately either side of the insertion site. Digests

were run on an agarose gel in order for the desired fragments to be gel purified.

2.2.3.5 DNA Sequencing

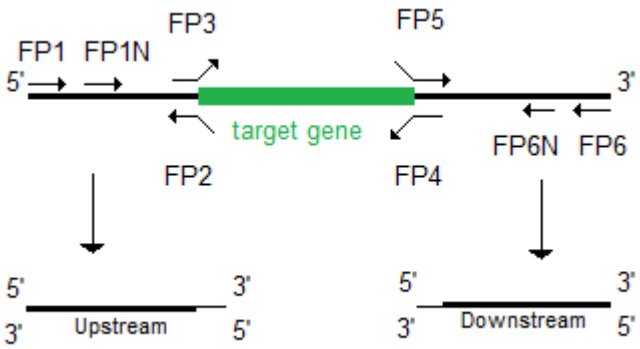
Purified gDNA samples were sent to The University of Manchester in-house DNA sequencing facility using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems Inc., USA). Sequence files were viewed using FinchTV 1.4.0 (Geospiza Inc., USA) to identify signal strength and reliability. DNA sequences are available in the appendices.

2.2.3.6 Fungal Transformation

2.2.3.6.1 Generation of Fusion Cassettes

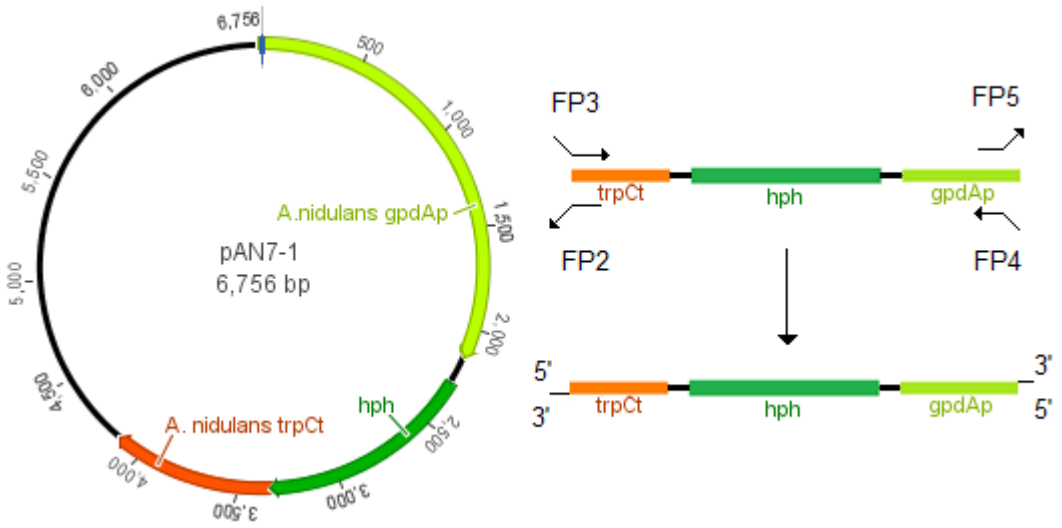
The *het* domain genes of A1163 were identified as targets for deletion due to the roles the *het* domain plays in HI in other species (Espagne *et al.*, 2002; Sarkar *et al.*, 2002; van Diepeningen *et al.*, 2009). Fusion cassettes for five *het* domain genes were generated using fusion-PCR. The 50 bp primers would leave complementary overhangs at the 3' ends of the PCR products. The binding sites of the eight primers designed for a target gene are shown in Figure 2.5. Amplification of the *hph* marker is shown in Figure 2.6. Fusion-PCR step 2 is shown in Figure 2.7, where the desired product is amplified through the three step 1 components binding to form a single DNA template. PCR reaction conditions for steps 1 and 2 of fusion-PCR were as described in section 2.2.3.3.3.

Gel purified fusion cassettes generated from step 2 fusion-PCR were ligated into pGEM®-T Easy using T4 DNA Ligase in an overnight reaction at 4°C. *E. coli* competent cells were transformed with the ligation reaction products as described in section 2.2.3.1.3, and incubated overnight at 37°C on LB agar with Amp, X-gal and IPTG. DNA was extracted from white colonies and screened for the presence of the fusion cassette using primers FP1N and FP6N for the corresponding target gene. Colonies that were confirmed for the presence of the target gene in the PCR screening reaction were cultured for plasmid purification using the Plasmid Mini Kit. Recovered plasmids were subject to a restriction digest by BstZI, as described in section 2.2.3.4, to ensure the correct digest pattern was observed when run by agarose gel electrophoresis. As culturing *E. coli* and using the Plasmid Mini Kit provided high yields of genomic material, the linearised fusion cassettes for transformation of *A. fumigatus* were obtained through a double-digest of plasmids rather than using the plasmids as template for a PCR reaction. The REs NotI-HF™ and ApaI did not have recognition sites within the fusion cassettes, but did each have one recognition site either side of the pGEM®-T Easy insertion site. The fusion cassettes were gel purified after double-digests.



<u>Figure 2.5</u> Fusion-PCR Step 1: Specificity of the Fusion-PCR Primers to the Target Gene

Primers were designed to amplify upstream (FP1 and FP2) and downstream (FP5 and FP6) of the target gene, leaving complementary flanking regions to the amplified *hph* marker (FP3 and FP4). Primers FP1N and FP6N are used in fusion-PCR step 2. The *hph* gene is marked in green.



<u>Figure 2.6</u> Fusion-PCR Step 1: Specificity of the Fusion-PCR Primers to pAN7-1

FP3 and FP4 were used as primers to amplify the *hph* marker with a promoter and terminator from pAN7-1 in fusion-PCR step 1. The 25 bp overhang of the 50 bp primers results in extension of the marker PCR product to include complementary sequences to the PCR products of the upstream and downstream regions of the target gene (Figure 2.5). FP2 and FP5 are also 50 bp primers and were used to amplify the upstream and downstream regions of the target gene, leaving complementary sequences to FP3 and FP4 PCR products. The light green region represents a promoter region (*gpdAp*), the dark green region marks a gene (*hph*), and the orange region marks a terminator region (*trpCt*). The promoter and terminator regions of pAN7-1 were sourced from *A. nidulans*.

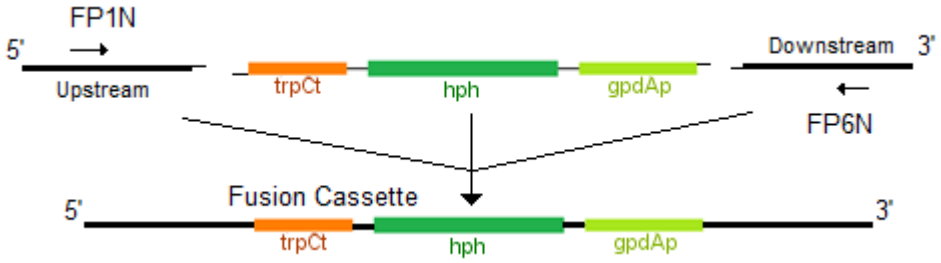


Figure 2.7 Fusion-PCR Step 2: Generation of Fusion Cassette

The 3' complementary overhangs of the three fragments associate during the slow temperature changes in step 2 fusion-PCR to generate a single fusion cassette template. The nested primers FP1N and FP6N bind the upstream and downstream fragments regions, inside the binding sites of FP1 and FP6, resulting in amplification of the fusion cassette by AccuPrime™ *Taq* DNA Polymerase. The light green region represents a promoter region (*gpdA*p), the dark green region marks a gene (*hph*), and the orange region marks a terminator region (*trpC*t). The promoter and terminator regions of pAN7-1 were sourced from *A. nidulans*.

2.2.3.6.2 Protoplast Generation

Protoplasts for *het* gene replacement were generated from *A. fumigatus* strain A1160 (*aku*B$^{(ku80\Delta)}$; *pyr*G^{-}). Protoplasting allowed transformation of A1160 cells using the fusion-PCR cassettes (section 2.2.3.6.1). SDB supplemented with 5 mM uracil and 5 mM uridine was inoculated with A1160 spores to a final concentration of 1x10^{6} spores/mL. Six Petri dishes containing only enough SDB media to form a thin liquid layer that covered the bases were used. The Petri dishes were incubated at 37°C for 14 hours, static, so a layer of mycelia formed on the SDB. SDB was filtered away from mycelia by pouring the liquid through triple-layered autoclaved lens tissue. The mycelia were washed with ice-cold autoclaved transformation buffer (TB). TB (0.6 M KCl; 50 mM $CaCl_2$; pH 8.0) was kept on ice for the duration of the protoplasting and transformation procedures. Mycelia was transferred to 20 mL freshly filter sterilised protoplasting buffer (PB) to lyse the cell wall (Wiebe *et al.*, 1997). PB consisted of 0.6 M KCl; 50 mM $CaCl_2$; pH 8.0; 5% Glucanex. The PB was mixed thoroughly to disperse mycelial biomass, and then incubated at 30°C for 3 hours at 100 rpm. PB was then filtered through sterile lens tissue to remove mycelia, with the flow-through containing protoplasts being collected. The filtered PB and protoplasts were centrifuged at 3000 rpm and 4°C for 10 min, the supernatant was removed, and the pellet was resuspended in 1 mL of the ice-cold TB. The protoplasts were counted using an improved Neubauer haemocytometer and diluted to 1x10^{7} protoplasts/mL using TB for optimum transformation efficiency.

2.2.3.6.3 Transformation of *A. fumigatus* Protoplasts with Fusion Cassettes

Polyethylene glycol (PEG) 6000 was added in TB to produce the PEG transformation buffer (PTB). PTB (0.6 M KCl; 50 mM $CaCl_2$; pH 8.0; 60% PEG-6000) was warmed to 60°C to facilitate pipetting, and 200 µL was transferred to separate tubes for each transformation. PTB was allowed to cool to RT before use. 5-10 µg of fusion-PCR cassette transformation DNA (section 2.2.3.6.1) was gently mixed with 100 µL of the $1x10^7$ protoplasts/mL TB suspension and put on ice for 20 min. The transformation DNA was designed specifically to replace the target gene via homologous recombination (Figure 2.8). The protoplast-DNA mixes were then gently transferred to the corresponding 200 µL PTB tubes and left at RT for 5 min. The protoplast-DNA-PEG mixes were spread on TransAspA selective media (section 2.1.2.3) with any appropriate supplements stored at RT for 1 hour before transferring the media to 37°C for 2-3 days.

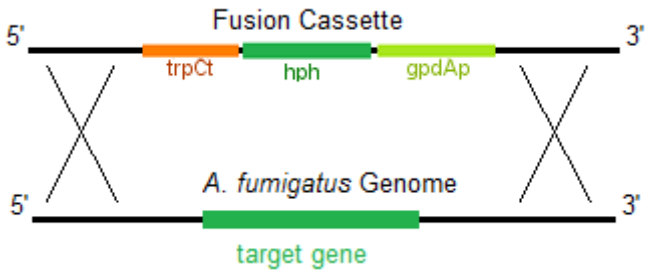


Figure 2.8 Homologous Recombination of *A. fumigatus* Target Gene with *hph* Marker Fusion Cassette

The linearised fusion cassette has identical flanking sequences to the target gene, allowing for homologous recombination to replace the target gene with the marker. Non-homologous recombination frequency is more likely than homologous recombination in WT strains, and therefore strain A1160 was used for transformations as the strain is deficient in NHEJ. Transformants expressing the fusion cassette acquired HmB^R from the *hph* marker. Mutant isolates recovered from selective HmB media were screened via PCR to ensure recovered isolates were not spontaneous resistant mutants, or were non-homologous recombinants in spite of NHEJ deficiency.

2.2.3.6.4 Screening Transformants

Recovered fusion-cassette transformants were screened by PCR, as outlined in section 2.2.3.3.4, to determine their genotype. If two DNA fragments were visualised through agarose gel electrophoresis of a product amplified from transformant gDNA using primers FP1N and FP6N, then the fusion-cassette integrated into the genome through non-homologous means despite transformation with the NHEJ-deficient strain, A1160 (da Silva Ferreira *et al.*, 2006). The second fragment would be an intact *het* gene within the genome. If a single band of the predicted size of the fusion cassette was visualised, then purified products from PCR reactions using FP1 and FP6 primers were used as templates for PCR using primers SF and SR. This method provided PCR products that were of sufficient length for DNA sequencing to determine the absence of the *het* gene, and presence of the central portion of the fusion cassette (Figure 2.7 and Figure 2.8).

2.2.3.7 Transposon Mutagenesis

2.2.3.7.1 D1-4 Strain Culturing Techniques

The *impala*160*::pyr*G strain D1-4 was handled carefully as stress-inducing conditions increased the rate of transposon mutagenesis, and the spores could not be stored at 4°C (Carr *et al.*, 2010). Spores were frozen at -80°C, and for every separate study of D1-4, the strain was cultured on the Nit screening Vogel's agar to ensure *nia*D was still non-functional. Any stocks of D1-4 removed from -80°C were autoclaved rather than frozen again, and new spores were harvested for storage. Six Sit mutants were generated (D1-

4A, D1-4B, D1-4C, D1-4D, D1-4E and D1-4F) by culturing D1-4 on PDA supplemented with 0.4 mM sodium selenate for 3 days at 37°C.

2.2.3.7.2 Gene Disruption of D1-4 Sit Mutant Strains

Transposon mutagenesis was used to disrupt genes in the *A. fumigatus* genome in a non-specific manner to determine whether any single gene disruption could influence the ability of strains to form heterokaryons. Large quantities of spores were harvested for Sit strain D1-4E. 200 ml culture dishes were used for transposon mutagenesis. Autoclaved N3T agar was cooled to 50°C, and spores were added directly into the media to a final concentration of $2x10^6$ spores/mL of molten agar. After the agar had solidified, the culture dishes were incubated at 40°C for 24 hours to encourage germination, followed by 4°C for 4 days to induce the *impala160* mutagenesis. The culture dishes were then moved to 37°C for 4 days to encourage growth and sporulation of the mutants on the nitrate media. The spores were harvested from the entire culture dish as a transposon mutant spore library.

2.2.3.7.3 Compatibility Screening of the Transposon Mutant Spore Library

A transposon mutant spore library from a culture dish was mixed in equal quantities to spores from a Nit mutant that D1-4 was previously unable to form a viable heterokaryon with. 1 mL of spore mix was spread onto the surface of N3S agar in a 200 mL culture dish and incubated at 37°C for 4

days. Colonies exhibiting strong growth were subcultured individually onto N3T agar.

2.2.3.7.4 Rescue of the *impala160* Locus in Compatibility Mutants

ST-PCR (section 2.2.2.3.5) was used to amplify the neighbouring region of the mobilised *impala160* transposable element using the degenerate sequence of TRP3 to act as a semi-random forward primer and TRP4 as a common reverse primer. The PCR products of the second ST-PCR step were sent for unidirectional DNA sequencing using primer TRP4, the results of which are in section 3.3.2.2.

Chapter 3: Results

3.1 Phylogenetics of *A. fumigatus het* Genes

Many fungal genomes are now publically available, with more currently being sequenced and annotated. Web-based tools were used to collate a *het* gene database to identify HI-associated genes of interest in *A. fumigatus*. This approach focused on establishing any similarities between the *A. fumigatus het* genes and those of species where HI has been characterised to act as a framework for further studies to characterise the HI reaction in the opportunistic pathogen *A. fumigatus*.

The majority of the HI homologues between the Af293 and A1163 genomes are identical or have little variance, with eight *het* genes encoded in each genome (Table 3.1). The predicted *het* genes of both sequenced *A. fumigatus* genomes are listed alongside their corresponding predicted proteins as UniProt accession codes, with blank areas indicating no available corresponding predicted protein for the gene in the UniProt database. Although the gene homology between the two *het4* genes is near 100%, it is the lowest of the eight pairs. *het4* is also found at a different locus in the A1163 genome to that of Af293.

Variation of a single base difference in certain compatibility genes can induce HI reaction between fungal colonies (Deleu *et al.*, 1993), and only a single predicted *het* gene was of 100% identity between Af293 and A1163. Table 3.2 contains Af293 HI gene accession numbers, protein domains,

percentage homology to A1163 proteins, and similar proteins from three other species. The *het* domain genes are highlighted in yellow.

The HET domain plays a key role in the HI reaction of *P. anserina* and *N. crassa* systems, but these two species contain 146 and 69 *het*-containing DNA sequences respectively. There are few genes with the *het* domain in each *A. clavatus* (7), *A. nidulans* (11), *A. fumigatus* (8), and *N. fischeri* (9). However, other Aspergilli have more *het* domain genes: *A. niger* (32); *A. oryzae* (43); *A. terreus* (42); *A. flavus* (53). A divergence event in the evolutionary history of the Aspergilli appears to have caused multiple duplications of *het* genes (Figure 3.1). This is evident when the genes of these species are aligned on a phylogenetic tree (Figure 3.2). Many of the *het* genes shown in Table 3.2 share domains to HI genes of *N. crassa* and *P. anserina* genes, despite the differences in VC mechanisms between the two characterised fungi (Galagan *et al.*, 2003; Espagne *et al.*, 2008). Seven of the *het* domain genes of *A. fumigatus* do not contain domains associated with HI genes from other species (Table 3.2), setting the *het* genes of *A. fumigatus* apart from the characterised systems of *N. crassa* and *P. anserina*. Figure 3.3 illustrates the positions of the domains in *A. fumigatus* HET domain proteins using sequence data from CADRE (Mabey Gilsenan *et al.*, 2012). HI proteins in other fungal species associate additional protein domains with HET, such as the NACHT and WD domains. However, in *A. fumigatus* these domains are frequently associated with other domains, such as Pfs (Fedorova *et al.*, 2005).

Table 3.1 HET Domain Proteins of *A. fumigatus* strains Af293 and A1163

	Af293 Accession Codes		A1163 Accession Codes		Gene
Name	**Gene**	**Protein**	**Gene**	**Protein**	**Homology (%)**
het1	AFUA_1G17550	Q4WR49_ASPFU	AFUB_016930	B0XPD2_ASPFC	100
het2	AFUA_2G12280	Q4X0U3_ASPFU	AFUB_027950		99.9
het3	AFUA_2G12700	Q4X0Q2_ASPFU	AFUB_028340	B0XSZ6_ASPFC	99.9
het4	AFUA_3G01620	Q4WFN2_ASPFU	AFUB_046770	B0XWN9_ASPFC	99.7
het5	AFUA_3G03140		AFUB_045110		99.9
het6	AFUA_6G12090	Q4WLW4_ASPFU	AFUB_078080	B0Y8P6_ASPFC	99.8
het7	AFUA_8G01020	Q4WB46_ASPFU	AFUB_085570	B0YAR5_ASPFC	99.9
het8	AFUA_8G07340	Q4WBN7_ASPFU	AFUB_080400	B0Y9B2_ASPFC	99.9

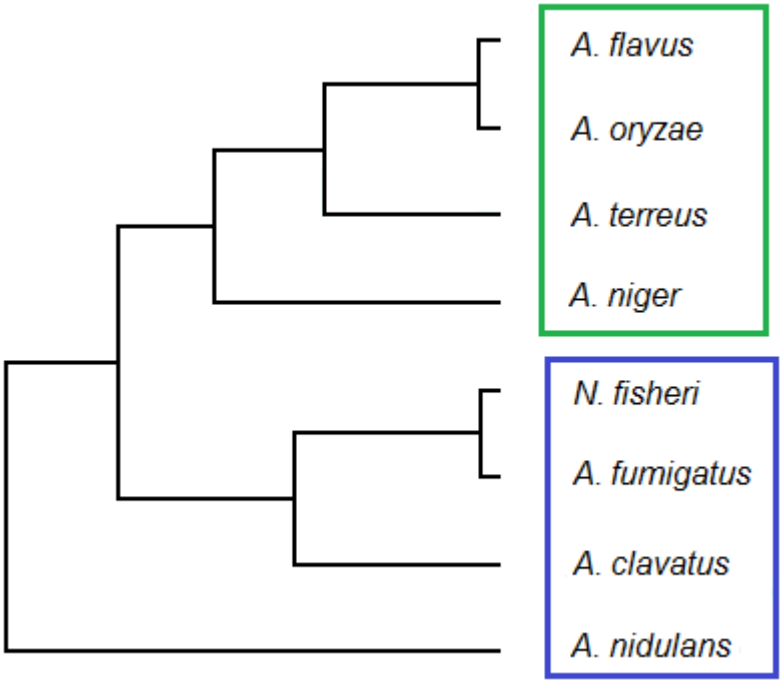


Figure 3.1 Phylogenetic Tree of the Aspergilli

An unknown evolutionary pressure encouraged the duplication of the Aspergilli *het* genes. This coincides with the emergence of *A. flavus*, *A. oryzae*, *A. terreus* and *A. niger* in a separate clade (in green) to that of *A. nidulans*, *A. clavatus*, *A. fumigatus* and *N. fisheri* (in blue) from a common ancestor. Phylogenetic tree not to scale.

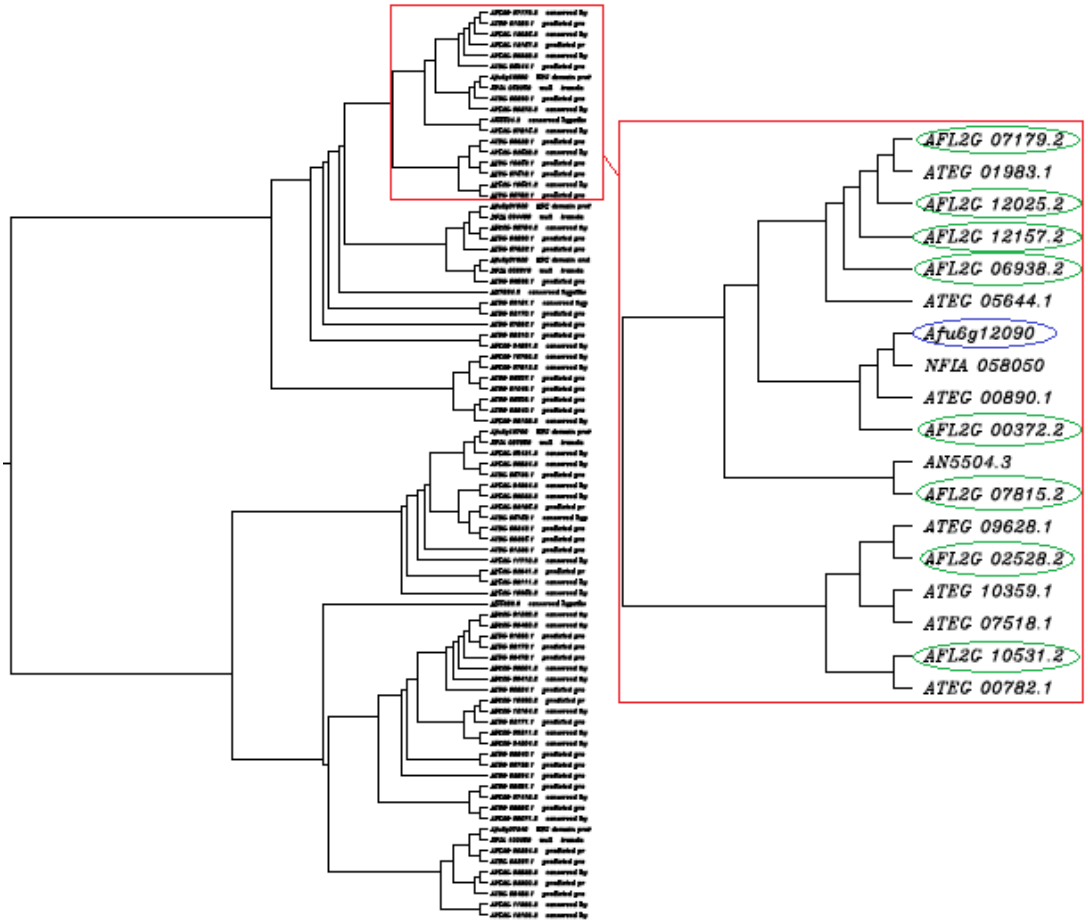


Figure 3.2 Phylogenetic Tree of Aspergilli *het* Genes

The *het* domain genes of some Aspergilli are aligned in the phylogenetic tree and appear to fall into loosely related clades. The clades show similar patterns to that of the enlarged section where there may only be one *A. fumigatus het* gene (AFUA_6G12090, circled in blue) related to multiple *het* genes of other species. Eight *het* genes of *A. flavus* (AFL2G accession codes, circled in green) are closely related to the single *A. fumigatus* het gene AFUA_6G12090.

Table 3.2 Related *A. fumigatus het* Genes

Af293 Accession Codes	Similar Proteins in Other Fungal Species				Protein Domains Present	A1163 Accession Codes and Homology
3g01620	HET-6	NC			ANK, HET	046770 (99%)
6g03160	HET-E2C*40	PA			NACHT, P-Kinase	095130 (99%)
8g01020	HET-6	NC			HET	085570 (99%)
7g02570	HET-C	PA	Het-COR	NC	HET-C	n/a
7g00550	HET-D2Y	PA			NACHT, WD40	087140 (99%)
7g00530	HET-E1	PA			NACHT, ANK, P-Kinase	087120 (99%)
6g12090	HET-6	NC			HET	078080 (99%)
6g07030	HET-eN	NH			NACHT, ANK	072950 (72%)
8g05780	HET-E1	PA			NACHT, ANK	081770 (96%)
8g06400	HET-eN	NH			Pfs, ANK	079240 (99%)
2g00960	HET-E1	PA			NACHT	018030 (97%)
2g01760	HET-eN	NH			NACHT	018830 (99%)
2g12700	HET-E1	PA			HET, ZZZ	028340 (99%)
2g15840	HET-C	PA	Het-C	NC	HET-C	031500 (99%)
2g17230	HET-E2C*40	PA			NACHT	032880 (99%)
3g07460	HET-E	PA			NACHT, WD40	041630 (99%)
3g13820	HET-C	PA			HET-C2	n/a
5g14582	HET-E1	PA			NACHT, ANK, P-Kinase	062260 (99%)
4g09460	HET-E1	PA			NACHT	066580 (99%)
1g01020	HET-eN	NH	HET-E1	PA	NACHT, ANK, Pfs	n/a
1g01490	HET-E1	PA			NACHT	079300 (99%)
7g08500	HET-E2C	PA			NACHT, WD40	098000 (87%)
7g07100	HET-E2C*40	PA			NACHT, ANK, Pfs	n/a
7g07030	HET-E1	PA			WD40, NACHT	075420 (74-61%)
7g06670	HET-E4s	PA			NACHT, TPR	092240 (100%)
7g06290	HET-E4s	PA			NACHT, ANK, Pfs	091840 (99%)
8g07340	HET	NC			HET	080400 (99%)
1g17550					HET	016930 (100%)
2g12280	HET	NC			HET	027950 (99%)
3g03140					HET	045110 (99%)

Species: NC - *N. crassa*; PA - *P. anserina*; NH - *Nectria Haematococca*.

Domains: HET - heterokaryon incompatibility effector domain; NACHT - NTPase domain; Ank - ankyrin repeat domain; Pfs - nucleoside phosphorylase; ZZZ - ZZ-type zinc finger; WD40 - protein binding repeat domain; TPR - tetratricopeptide repeat domain.

Accession code suffixes: AFUA_ (Af293 genes); AFUB_ (A1163 genes).

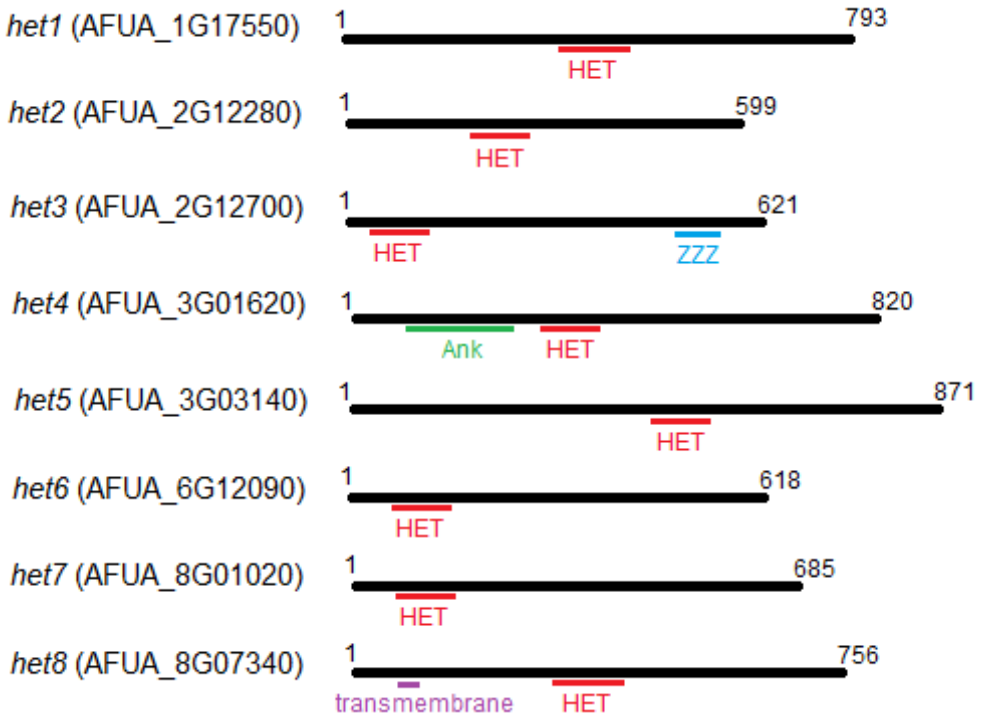


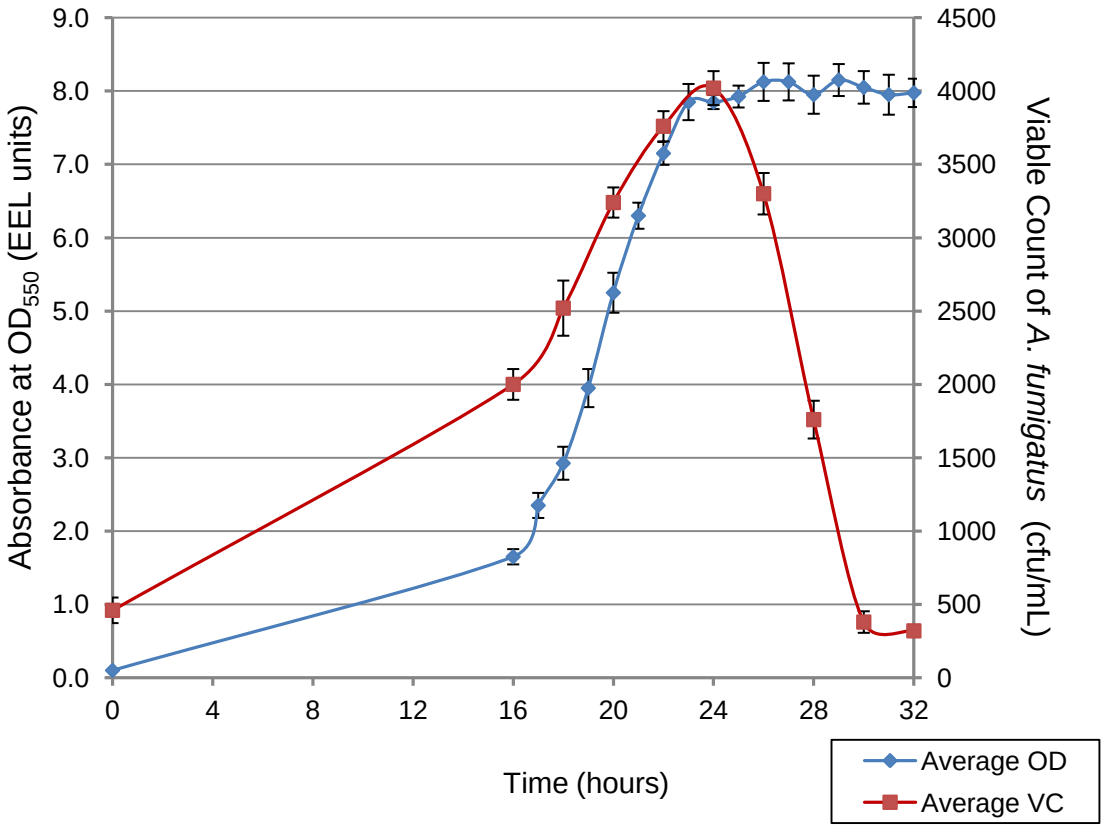
Figure 3.3 ***A. fumigatus*** **HET Protein Domains**

Five of the eight HET domain proteins of A. fumigatus contain no other protein domains. *het8* has a transmembrane domain of 20 amino-acids in length near the N-terminus, *het4* has an Ank domain, and *het3* has a ZZZ domain. The HET domain is positioned either near to the N-terminus or towards the center of the proteins.

3.2 Fungal Cultures

3.2.1 Growth Rate in Broth Media and Stationary Curves

Growth curves of Af293 in Junlon PW 110 MM broth provided the timing of log and stationary phases of *A. fumigatus* (section 2.2.2.4). Junlon PW 110 was included in the culture media to discourage clumping of fungal cells in broth media (section 2.1.2.2). The timing of the log and stationary phases were required for analysis comparing gene expression during growth of a culture to that of a culture where the conditions encourage PCD (section 1.1.2). Af293 growth was in log phase for four hours between the T_{18} and T_{22}, as indicated by the steepest gradients of both the OD_{550} and viable count (Vc) data sets, shown in Figure 3.4. Stationary phase was marked by a rapid decline in Vc from T_{24} to T_{30}. The OD_{550} also began to plateau from T_{23} and remained stable until time points were no longer taken (T_{32}). The stable OD_{550}. readings after T_{23} indicate Af293 growth had slowed or halted, while the drop in Vc indicated that fungal cells were losing viability.



<u>Figure 3.4</u> Mean averages of absorbance and viable count of *A. fumigatus* in Junlon PW 110 MM broth culture over time

Junlon PW 110 MM broth was inoculated with Af293 spores to a final concentration of $5x10^6$ spores/mL and shaken at 200 rpm for 32 hours at 37°C. OD_{550} readings were taken every hour from T_{16} and samples were removed for Vc measurements once every two hours from T_{16}. Dilution series of the Vc samples were spread onto a medium with the same recipe as Junlon PW 110 MM broth, except there was 1.5% agar and 0% Junlon PW 110. As shown by the graph, Junlon PW 110 did not completely stop spores from aggregating as the initial Vc is far lower than the inoculation concentration. The OD_{550} readings plateau and the Vc begins to drop at T_{23}, marking entry of Af293 into stationary phase.

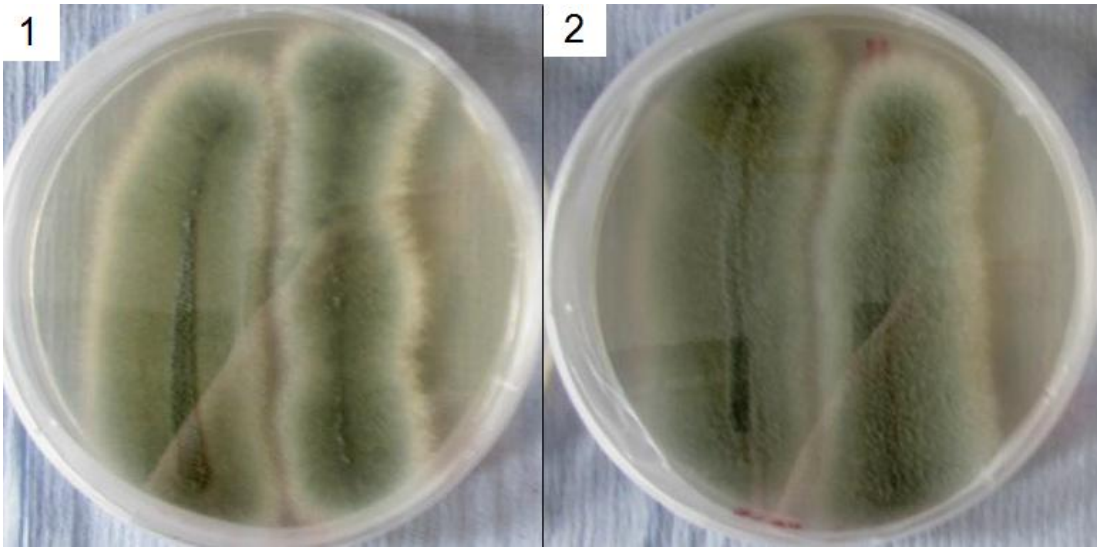
3.2.2 Compatibility Grouping

VCGs of the available clinical and environmental *A. fumigatus* strains were determined (section 3.2.2.3) so that compatibility changes resulting from genetic manipulation could be observed (section 3.3.1.3). The formation of macroscopic barrage zones has been noted in other fungal species, such as *F. graminearum* (McCallum *et al.*, 2004). However, incompatibile *A. fumigatus* strains did not form visible barrages when grown in close proximity (section 3.2.2.1), so a pair-wise compatibility method requiring Nit mutants of each strain was used (section 3.2.2.2) which has been previously utilised to establish compatibility groups in *Fusarium oxysporum* (Correll *et al.*, 1987).

3.2.2.1 Barrages

Barrages are visible zones of cell death at the site of hyphal contact between vegetative incompatible fungal strains of the same species. Visible barrages are a relatively quick method for observing VC between strains of a fungus. Spores from pairs of *A. fumigatus* strains were streaked on PDA roughly 30 mm apart and incubated at 37°C for 3 days before the plates were checked for signs of barrages. Barrages were not visible on media streaked with the same *A. fumigatus* strain twice or on media with two different strains (Figure 3.5). In both examples shown the colonies halted growth along the leading edge facing another colony whether the opposite strain was a compatible or incompatible strain of *A. fumigatus*. Incubating the media for another 2 days at 37°C did not result in the gaps at the leading edges between the colonies being bridged. As no barrages phenotype was observed, an alternate

method using spontaneously generated mutants of the nitrate-utilisation pathway was used to determine compatibility grouping.



<u>Figure 3.5</u> No Visible Barrage Phenotype between Compatible or Incompatible *A. fumigatus* Strains

These images each shows two colonies of *A. fumigatus* streaked on PDA after 3 days of incubation at 37°C. Plate 1 was a positive control where both colonies are strain A1163 that were inoculated onto the medium from the same spore suspension. Plate 2 was inoculated with colony of RB18, left, streaked alongside a colony of RB13, right. RB18 and RB13 are shown as they are an example of incompatible strains of *A. fumigatus* (see Table 3.4).

3.2.2.2 Spontaneous Nit and Sit Mutant Generation and Screening

Nit mutants of all the *A. fumigatus* strains used in this study were required for compatibility grouping. The Nit mutant *A. fumigatus* strains were spontaneously generated by spreading spore solutions onto chlorate-PDA and incubating for 4 days at 37°C. The Nit phenotype also conveys resistance to the toxic compound chlorate (Figure 3.6). Plate 1 of Figure 3.6 shows several colonies sporulating and growing stronger than the background growth of strains with no resistance to chlorate, whereas plate 2 shows colonies were able to germinate and grow strongly in the absence of chlorate. Larger sporulating colonies were individually subcultured as described in section 2.2.2.6 using the single spore technique (Figure 2.2), and then screened on selective media to determine individual Nit phenotypes.

Colonies were counted on ten chlorate-PDA plates where each plate was inoculated with 100 µL of a spore solution of $1x10^7$ spore/mL concentration. There was a mean average of 11.1 strong growing colonies on each plate, and therefore an average of 11.1 spontaneous Nit mutants were generated per $1x10^6$ spores over a 72 hour incubation period at 37°C (Figure 3.6). However, each *A. fumigatus* strain had a different susceptibility to chlorate, and actual Nit mutants recovered from strains varied greatly, with some strains able to readily grow on chlorate-PDA without acquiring the Nit phenotype. Six Nit mutants were recovered from each chlorate-PDA Petri dish and each subcultured on the six screening media (Figure 3.7). On each plate in the figure are the following four isolates, clockwise from top right

quadrant, as labelled according to section 2.2.2.6: WT (positive control), *niaD*$^{-}$, *nirA*$^{-}$, *cnx* $^{-}$. Each of the three genotypes that conferred chlorate-resistance exhibited different growth patterns on the selective media: *cnx* $^{-}$ grew weakly on N3S and HS agar; *nirA*$^{-}$ mutants showed poor growth on N3S and N2S agar; *niaD*$^{-}$ grew weakly on N3S agar. Two different genotype mutants that conferred chlorate-resistance were required to be able to use a strain for pair-wise compatibility crosses. The two Nit mutants of each of the 46 strains used for compatibility grouping are shown in Table 3.3 alongside the mutation sites that conferred their chlorate-resistance. The strain labelled as D1-4ET was a spore library of different Sit mutant spores recovered after inducing *impala160* transposon mutagenesis in the SeO^{R} D1-4 strain, D1-4E.

Sit mutants of the *A. fumigatus* strain D1-4 were used to determine whether random gene interruption could alter the compatibility grouping of the strain. The *F. oxysporum* transposable element, *impala160*, was present in strain D1-4, with transposon mutagenesis of *impala160* encouraged through stress factors, such as low temperature (section 2.2.2.6). The *impala160* transposable element interrupts the *niaD* gene in *A. fumigatus* strain D1-4 and transposon mutagenesis restores the strain D1-4 to a nitrate-utilising phenotype as the relocation of *impala160* restores *niaD* functionality. As environmental stresses could induce transposon mutagenesis, it was desirable to introduce a nutrient auxotrophic phenotype prior to mutagenesis. Using a Sit phenotype would allow the mutants to be screened for the novel auxotrophy and Nit phenotype prior to transposon mutagensis. Selenate (SeO) is toxic to *A. fumigatus*, but spontaneous resistance also confers the

Sit phenotype where the fungus is unable to utilise $MgSO_4$ but can acquire sulphur from thiosulphate. SeO resistant (SeO^R) spontaneous mutants recovered from selenate-PDA (recipe in section 2.1.2.4) were screened on selective media to ensure the Sit phenotype was present alongside the Nit phenotype, which indicated Seo^R was not acquired through transposon mutagenesis (Figure 3.8). Five D1-4 isolates with both Sit and Nit phenotypes were screened, labelled D1-4A, D1-4B, D1-4C, D1-4D and D1-4E. The Sit strain D1-4E was used for transposon mutagenesis as more spores were harvested from this strain than the other Sit mutant strains.

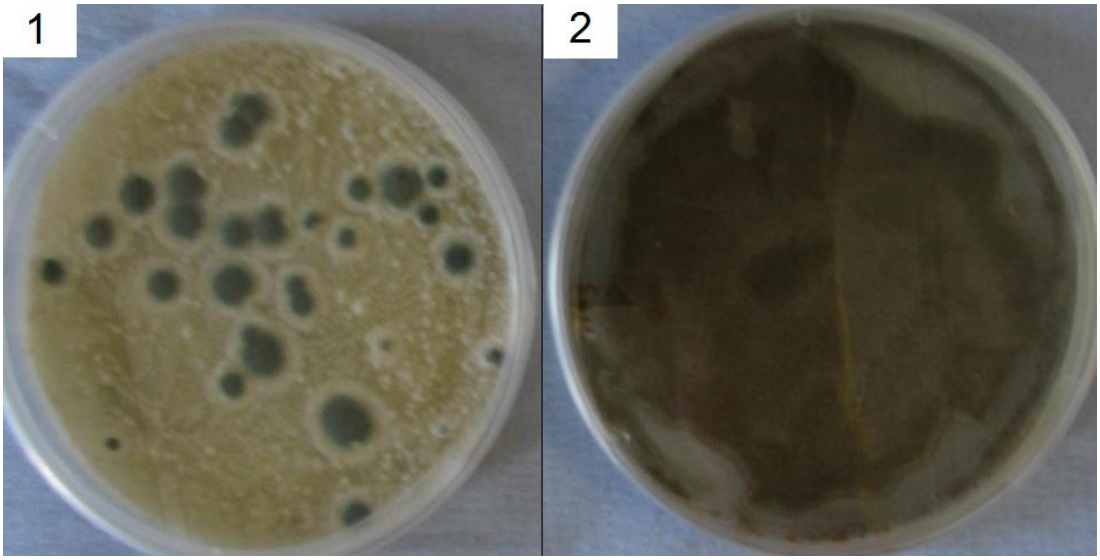


Figure 3.6 Generation of Chlorate-Resistant *A. fumigatus* Spontaneous Mutants on Media Containing Potassium Chlorate

Chlorate-PDA (recipe in section 2.1.2.4) was used to generate *A. fumigatus* Nit mutants. Both plates were inoculated with Af293 spores and incubated for 3 days at 37°C. Plate 1 is PDA containing 750 mM $KClO_3$ and plate 2 is standard PDA medium.

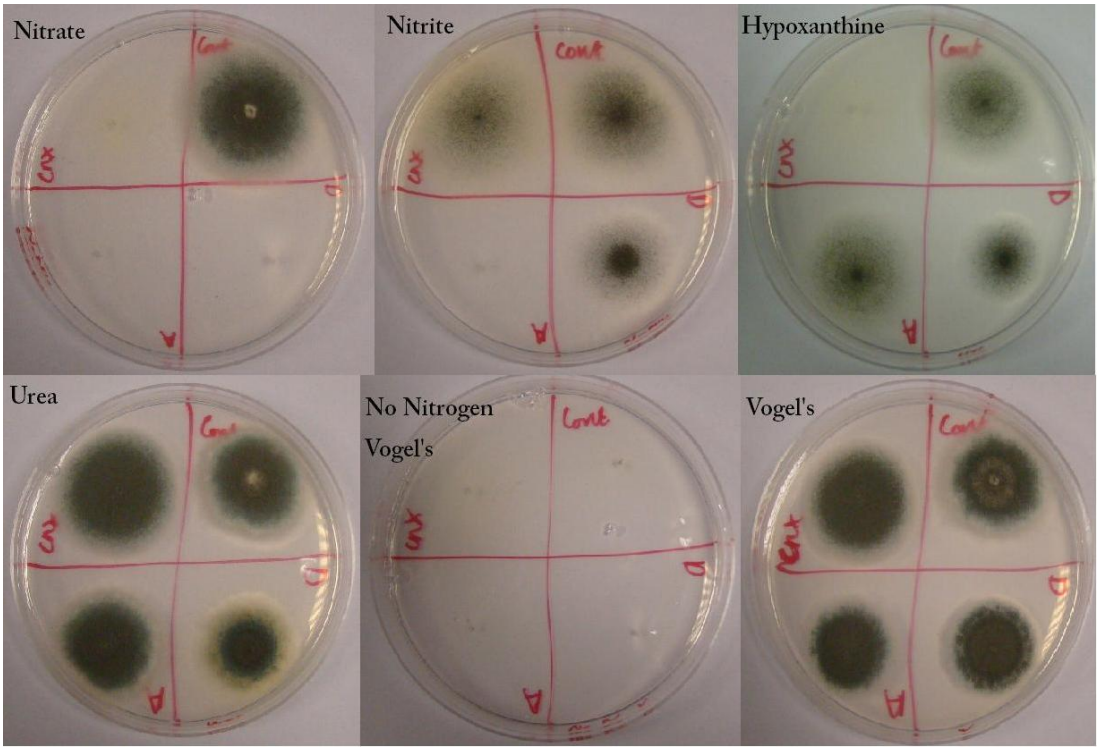


Figure 3.7 Screening of *A. fumigatus* Nit Mutant Phenotypes on Various Selective Minimal Media

Six selective media were used to describe the phenotype of Nit mutants recovered from chlorate-PDA. Each recovered potential Nit mutant from chlorate-PDA was screened on the six selective media described in section 2.1.2.1 and Table 2.2. The top three and the bottom left media are labelled according to the sole nitrogen (N) source they contain. The bottom centre plate contained no N source and the bottom right media was made using standard Vogel's stock solution that contains ammonium nitrate (Table 2.1). The strains in this example are *A. fumigatus* strain A1163, and three Nit mutants derived from A1163.

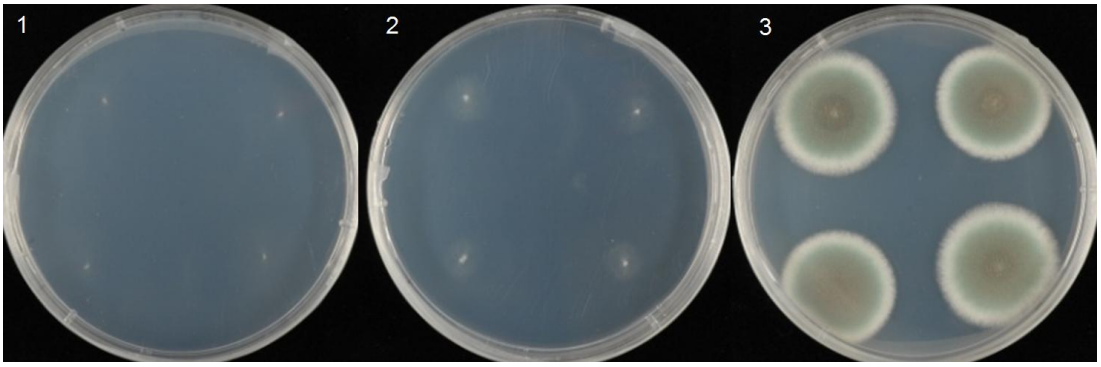


Figure 3.8 Screening of Sit Mutants from *A. fumigatus* strain D1-4 on Selective Media

Four selenate resistant (SeO^R) *A. fumigatus* D1-4 spontaneous mutants recovered from selenate-PDA were screened for the Sit phenotype. The strains, clockwise from the top left of each plate, were D1-4B, D1-4C, D1-4D and D1-4E. Plate 1 was N3T agar medium and plate 2 was US agar medium. The mutants grew weakly on these two media. All four mutants grew strongly on plate 3, which was composed of UT agar medium. These growth patterns show the D1-4 mutants all had the Sit and Nit phenotype, indicating that the SeO^R was not generated from transposon mutagenesis as that would have restored a nitrate-utilising phenotype. The four SeO^R mutants with the Sit phenotype were therefore generated through spontaneous mutagenesis. The Sit phenotype was required for pair-wise compatibility crosses of D1-4 as the *impala160* transposon locus incorporated a gene involved in the nitrate-utilisation pathway, and therefore the Nit phenotype could not be used. N3T, US and UT media were made as described in section 2.1.2.1, Table 2.1 and Table 2.2.

Table 3.3 List of Nit Mutation Sites of the Nit Mutants Used for VCG Determination through Pair-Wise Compatibility Grouping

Strain	Nit Reference	Mutation Site	Nit Reference	Mutation Site
Af293	1	*cnx*	2	*niaD*
A1163	11	*niaD*	12	*cnx*
Af24	21	*cnx*	22	*niaD*
Af71	31	*niaD*	32	*cnx*
RB11	41	*cnx*	42	*nirA*
RB12	51	*cnx*	52	*nirA*
RB13	61	*cnx*	62	*nirA*
RB14	71	*cnx*	72	*nirA*
RB15	81	*cnx*	82	*niaD*
RB16	91	*niaD*	93	*cnx*
RB17	101	*nirA*	103	*cnx*
RB18	111	*nirA*	112	*cnx*
RB19	121	*cnx*	122	*nirA*
RB20	131	*cnx*	132	*nirA*
Ap33	141	*cnx*	142	*niaD*
Ap61	151	*niaD*	153	*cnx*
Ap63	161	*cnx*	162	*niaD*
Ma3	181	*cnx*	182	*niaD*
Ma4	191	*cnx*	192	*nirA*
Ma5	201	*cnx*	202	*nirA*
J4	211	*niaD*	213	*cnx*
J10	231	*nirA*	232	*niaD*
J13	241	*niaD*	242	*cnx*
Au9	251	*niaD*	252	*cnx*
Au10	261	*nirA*	262	*cnx*
Au14	271	*nirA*	273	*cnx*
15562	281	*nirA*	282	*cnx*
15819	291	*nirA*	293	*cnx*
16795	311	*cnx*	312	*niaD*
16916	321	*cnx*	322	*nirA*
16975	331	*cnx*	332	*nirA*
17318	341	*cnx*	342	*nirA*
17406	351	*cnx*	352	*niaD*
17768	371	*cnx*	372	*niaD*
17796	381	*cnx*	382	*niaD*
17835	391	*cnx*	392	*nirA*
17871	401	*cnx*	402	*niaD*
17882	411	*nirA*	412	*cnx*
18565	421	*niaD*	422	*cnx*

Table 3.3 *cont.*

19164	441	*niaD*	442	*cnx*
19258	451	*nirA*	452	*cnx*
20395	501	*niaD*	502	*cnx*
21522	531	*nirA*	532	*cnx*
21705	551	*cnx*	552	*niaD*
22178	561	*nirA*	562	*cnx*
22577	581	*nirA*	582	*cnx*
3-1	601	*nirA*	605	*niaD*
3-2	612	*nirA*	613	*cnx*
3-4	621	*niaD*	622	*nirA*
3-6	631	*niaD*	632	*nirA*
4-3	663	*nirA*	664	*niaD*
4-4	673	*nirA*	674	*niaD*
6-2	711	*niaD*	712	*nirA*
7-1	723	*nirA*	727	*niaD*
D1-4	D1-4	*niaD*	D1-4ET	*sB* or *sC*
A1160	732	*nirA*	734	*niaD*

3.2.2.3 Pair-Wise Compatibility Grouping

In order to determine compatibility grouping, *A. fumigatus* strains were cultured in pairs on PDA to observe barrages. Barrages were not visible between strains of *A. fumigatus* (section 3.2.2.1) and therefore the more complex pair-wise compatibility grouping system of exploiting Nit mutants was used. This system involved generating chlorate-resistant mutants of all *A. fumigatus* strains to be tested and then inoculating selective media plates with the spores of these Nit mutants in a pair-wise cross system. Nit mutants had to be crossed with a different type of Nit mutant to ensure a heterokaryon would regain a fully functional nitrate-utilisation pathway. Strong growth on these plates indicated the presence of a viable heterokaryon and that the pair used to inoculate the medium were compatible. Weak growth indicated the strain pair were unable to form a positive heterokaryon and were incompatible (Figure 3.9).

Nit mutants from 46 *A. fumigatus* strains (Table 3.3) were used for pair-wise compatibility crosses on N3S agar (Table 3.4). Strong positive crosses are highlighted in yellow in the table, while negative crosses are not highlighted. There were no weak or partial crosses observed. A fully constructed image of the full table is in the appendix 4, labelled with the corresponding section numbers from this table (sections 1-15). The strains used consisted of various environmental and clinical isolates of *A. fumigatus*, including the two genotyped strains Af293 and A1163. All 46 strains formed viable heterokaryons with a rescued nitrate-utilising phenotype when self-crossed with a different type of Nit mutant of the same strain. There were 16 positive

crosses, not including self-crosses, which formed six discrete VCGs. A VCG was defined as a discrete compatibility group with more than one member strain. There were no partial heterokaryons with all the crosses being polarised towards either strong growing heterokaryons or weak growth after germinating spores failed to form heterokaryons. The six VCGs were distinct groups, in that members of a VCG could only form a positive heterokaryon with any member of the same VCG (Table 3.5). As there were 16 strains that formed the six VCGs, the remaining 30 strains used for pair-wise crosses were each in separate single-member compatibility groups. It cannot be determined whether there was any bias in grouping of strains into VCGs, such as clinical strains being more likely to belong in the same VCG as other clinical strains, as the groups were both too small and too few. VCG 1 consists of four strains all isolated from the same location (Belfield campus of University College Dublin, section 2.1.1.1), which could indicate either some grouping bias or that the strains were clonal isolates.

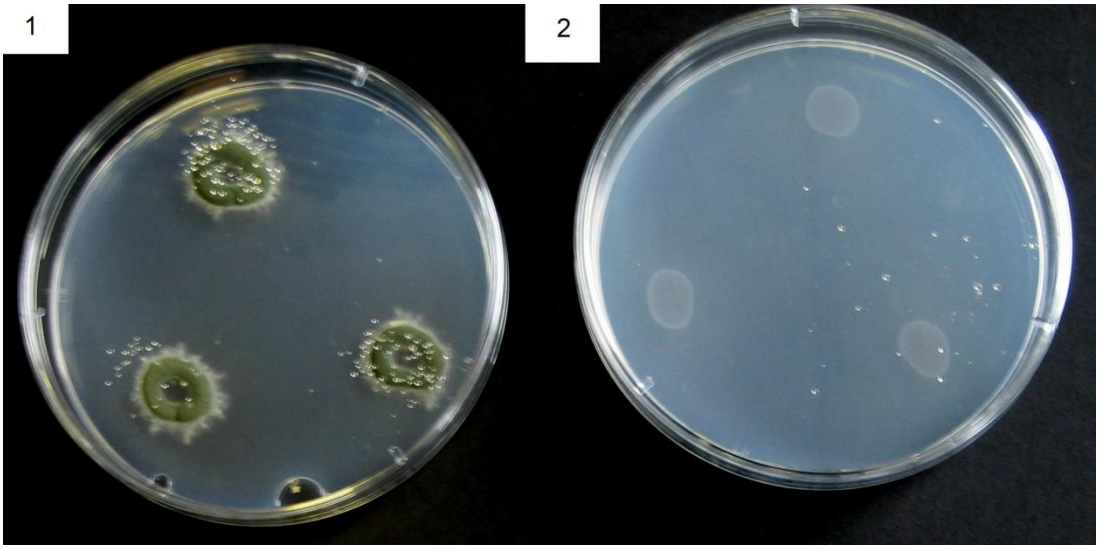


Figure 3.9 Compatible and Incompatible Pair-Wise Crosses of *A. fumigatus* Nit Mutants

Heterokaryons exhibit strong growth on N3S agar which neither strain can independently utilise. Plate 1 shows heterokaryon formation between *A. fumigatus* strains Ma3 (environmental isolate, mutant 181, *cnx*$^-$) and 17768 (clinical isolate, mutant 372, *nia*D$^-$). Heterokaryon formation allowed growth when $NaNO_3$ was the only available nitrogen source. Plate 2 shows an incompatible cross resulting in the germination of spores being followed by weak growth by two *A. fumigatus* strains (Ma3 mutant 181, *cnx*$^-$; A1163 mutant 11, *nia*D$^-$). Both plates shown were incubated at 37°C for 3 days.

Table 3.4 Pair-Wise Compatibility Grouping of *A. fumigatus* Nit Mutants with Highlighted Viable Heterokaryons

1.

Af293	A1163	Af24	Af71	RB11	RB12	RB13	RB14	RB15	RB16	
+	-	-	-	-	-	-	-	-	-	Af293
	+	-	-	-	-	-	-	-	-	A1163
		+	-	-	-	-	-	-	-	Af24
			+	-	-	-	-	-	-	Af71
				+	+	-	+	-	-	RB11
					+	-	+	-	-	RB12
						+	-	-	-	RB13
							+	-	-	RB14
								+	-	RB15
									+	RB16

2.

RB17	RB18	RB19	RB20	Ap33	Ap61	Ap63	Ma3	Ma4	Ma5	
-	-	-	-	-	-	-	-	-	-	Af293
-	-	-	-	-	-	-	-	-	-	A1163
-	-	-	-	-	-	-	-	-	-	Af24
-	-	-	-	-	-	-	-	-	-	Af71
-	-	-	+	-	-	-	-	-	-	RB11
-	-	-	+	-	-	-	-	-	-	RB12
-	-	-	-	-	-	-	-	-	-	RB13
-	-	-	+	-	-	-	-	-	-	RB14
-	-	-	-	-	-	-	-	-	-	RB15
-	-	-	-	-	-	-	-	-	-	RB16

3.

J4	J10	J13	Au9	Au10	Au14	15562	15819	16795	16916	
-	-	-	-	-	-	-	-	-	-	Af293
-	-	-	-	-	-	-	-	-	-	A1163
-	-	-	-	-	-	-	+	-	-	Af24
-	-	-	-	-	-	-	-	-	-	Af71
-	-	-	-	-	-	-	-	-	-	RB11
-	-	-	-	-	-	-	-	-	-	RB12
-	-	-	-	-	-	-	-	-	-	RB13
-	-	-	-	-	-	-	-	-	-	RB14
-	-	-	-	-	-	-	-	-	-	RB15
-	-	-	-	-	-	-	-	-	-	RB16

Table 3.4 *cont.*

4.

16975	17318	17406	17768	17796	17835	17871	17882	18565	19164	
-	-	-	-	-	-	-	-	-	-	**Af293**
-	-	-	-	-	-	-	-	-	-	**A1163**
-	-	-	-	-	-	-	-	-	-	**Af24**
-	-	-	-	-	-	-	-	-	-	**Af71**
-	-	-	-	-	-	-	-	-	-	**RB11**
-	-	-	-	-	-	-	-	-	-	**RB12**
-	-	-	-	-	-	-	-	-	-	**RB13**
-	-	-	-	-	-	-	-	-	-	**RB14**
-	-	-	-	-	-	-	-	-	-	**RB15**
-	-	-	-	-	-	-	-	-	-	**RB16**

5.

19258	20395	21522	21705	22178	22577	
-	-	-	-	-	-	**Af293**
-	-	-	-	-	-	**A1163**
-	-	-	-	-	-	**Af24**
-	-	-	-	-	-	**Af71**
-	-	-	-	-	-	**RB11**
-	-	-	-	-	-	**RB12**
-	-	-	-	-	-	**RB13**
-	-	-	-	-	-	**RB14**
-	-	-	-	-	-	**RB15**
-	-	-	-	-	-	**RB16**

6.

RB17	RB18	RB19	RB20	Ap33	Ap61	Ap63	Ma3	Ma4	Ma5	
+	-	-	-	-	-	-	-	-	-	**RB17**
	+	-	-	-	-	-	-	-	-	**RB18**
		+	-	-	-	-	-	-	-	**RB19**
			+	-	-	-	-	-	-	**RB20**
				+	-	-	-	-	-	**Ap33**
					+	-	-	-	-	**Ap61**
						+	-	-	-	**Ap63**
							+	-	-	**Ma3**
								+	-	**Ma4**
									+	**Ma5**

Table 3.4 *cont.*

7.

J4	J10	J13	Au9	Au10	Au14	15562	15819	16795	16916	
-	-	-	-	-	-	-	-	-	-	**RB17**
-	-	+	-	-	-	-	-	-	-	**RB18**
-	-	-	-	-	-	-	-	-	-	**RB19**
-	-	-	-	-	-	-	-	-	-	**RB20**
-	-	-	-	-	-	+	-	-	-	**Ap33**
-	-	-	-	-	-	-	-	-	-	**Ap61**
-	-	-	-	-	-	-	-	-	-	**Ap63**
-	-	-	-	-	-	-	-	-	-	**Ma3**
-	-	-	-	-	-	-	-	-	-	**Ma4**
-	-	-	-	-	-	-	-	-	-	**Ma5**

8.

16975	17318	17406	17768	17796	17835	17871	17882	18565	19164	
-	-	-	-	-	-	-	-	-	-	**RB17**
-	-	-	-	-	-	-	-	-	-	**RB18**
-	-	-	-	-	-	-	-	-	-	**RB19**
-	-	-	-	-	-	-	-	-	-	**RB20**
-	-	-	-	-	-	-	-	-	-	**Ap33**
-	-	-	-	-	-	-	-	-	-	**Ap61**
-	-	-	-	-	-	-	-	-	-	**Ap63**
-	-	-	+	-	-	-	-	-	-	**Ma3**
-	-	-	-	-	-	-	-	-	-	**Ma4**
-	-	-	-	-	-	-	-	-	-	**Ma5**

9.

19258	20395	21522	21705	22178	22577	
-	-	-	-	-	-	**RB17**
-	-	-	-	-	-	**RB18**
-	-	-	-	-	-	**RB19**
-	-	-	-	-	-	**RB20**
-	-	+	+	-	-	**Ap33**
-	-	-	-	-	-	**Ap61**
-	-	-	-	-	-	**Ap63**
-	-	-	-	-	-	**Ma3**
-	-	-	-	-	-	**Ma4**
-	-	-	-	-	-	**Ma5**

Table 3.4 *cont.*

10.

J4	J10	J13	Au9	Au10	Au14	15562	15819	16795	16916	
+	-	-	-	-	-	-	-	-	-	J4
	+	-	-	-	-	-	-	-	-	J10
		+	-	-	-	-	-	-	-	J13
			+	-	-	-	-	-	-	Au9
				+	-	-	-	-	-	Au10
					+	-	-	-	-	Au14
						+	-	-	-	15562
							+	-	-	15819
								+	-	16795
									+	16916

11.

16975	17318	17406	17768	17796	17835	17871	17882	18565	19164	
-	-	-	-	-	-	-	-	-	-	J4
-	-	-	-	-	-	-	-	-	-	J10
-	-	-	-	-	-	-	-	-	-	J13
-	-	-	-	-	-	-	-	-	-	Au9
-	-	-	-	-	-	-	-	-	-	Au10
-	-	-	-	-	-	-	-	-	-	Au14
-	-	-	-	-	-	-	-	-	-	15562
-	-	-	-	-	-	-	-	-	-	15819
-	-	-	-	-	-	-	-	-	-	16795
-	-	-	-	-	-	-	-	-	-	16916

12.

19258	20395	21522	21705	22178	22577	
-	-	-	-	-	-	J4
-	-	-	-	-	-	J10
-	-	-	-	-	-	J13
-	-	-	-	-	-	Au9
-	-	-	-	-	-	Au10
-	-	-	-	-	-	Au14
-	-	+	+	-	-	15562
-	-	-	-	-	-	15819
-	-	-	-	-	-	16795
-	-	-	-	-	-	16916

Table 3.4 *cont.*

13.

16975	17318	17406	17768	17796	17835	17871	17882	18565	19164	
+	-	-	-	-	-	-	-	-	-	**16975**
	+	-	-	-	-	-	-	-	-	**17318**
		+	-	-	-	-	-	-	-	**17406**
			+	-	-	-	-	-	-	**17768**
				+	-	-	-	-	-	**17796**
					+	-	-	-	-	**17835**
						+	-	-	-	**17871**
							+	-	-	**17882**
								+	-	**18565**
									+	**19164**

14.

19258	20395	21522	21705	22178	22577	
-	-	-	-	-	-	**17318**
-	-	-	-	-	-	**17406**
-	-	-	-	-	-	**17768**
-	-	-	-	-	-	**17796**
-	-	-	-	-	+	**17835**
-	-	-	-	-	-	**17871**
-	-	-	-	-	-	**17882**
-	-	-	-	-	-	**18565**
-	-	-	-	-	-	**19164**

15.

19258	20395	21522	21705	22178	22577	
+	-	-	-	-	-	**19258**
	+	-	-	-	-	**20395**
		+	+	-	-	**21522**
			+	-	-	**21705**
				+	-	**22178**
					+	**22577**

Table 3.5 **Discrete VCGs of *A. fumigatus* Strains from Pair-Wise Compatibility Grouping of Nit Mutant Strains**

VCG	Strains			
1	RB11	RB12	RB14	RB20
2	RB18	J13		
3	Af24	15819		
4	Ap33	15562	21522	21705
5	Ma3	17768		
6	17835	22577		

3.3 Gene Disruption

HI is controlled by *het* genes in other fungal species. The *het* genes of *A. fumigatus* were manipulated to observe whether this affects compatibility grouping. Specific *het* genes were replaced by fusion-PCR cassettes with the *hph* marker using homologous recombination, and a non-specific gene interruption approach was used with the *A. fumigatus* strain D1-4.

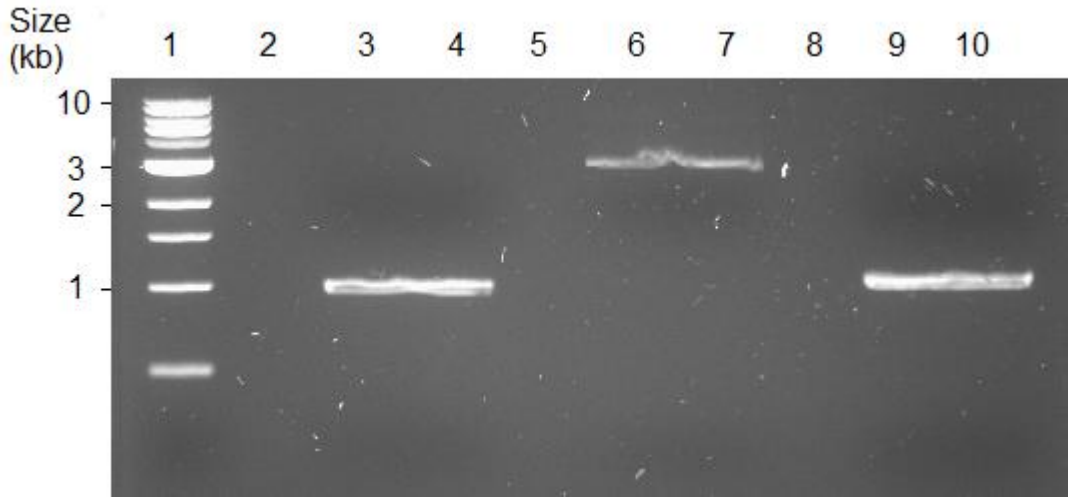
3.3.1 *het* Gene Replacement of *A. fumigatus*

3.3.1.1 Fusion-PCR Cassette Generation

The KO of *het* genes in *A. fumigatus* involved generating fusion cassettes through fusion-PCR, protoplasting the *akuB*$^{(KU80\Delta)}$ strain A1160 for transformation, transformation of the fungal protoplasts via homologous recombination and screening transformants through selective media, PCR and DNA sequencing. Cassettes containing the *hph* marker gene for transformation of *A. fumigatus* were generated through two steps of fusion-PCR (described in 2.2.2.5.1). Step 1 involved the generation of three separate PCR products to be used as template in step 2 (Figure 3.10).

Gel extraction was required for purification of the desired step 2 fusion-PCR products as the PCR routine generates a large volume of non-specific products. An example of a successful step 2 fusion-PCR is shown in Figure 3.11, as the desired products for the majority of step 2 fusion-PCRs were too faint to be picked up by the camera without over saturation of the lanes by the non-specific products. AccuPrime™ *Taq* DNA Polymerase High Fidelity used for step 2 fusion-PCR left a single 3' A-overhang on generated PCR

products which allowed for ligation into a T-overhang vector as described in section 2.2.3.6.1. Plasmids containing fusion-cassettes were extracted and subject to restriction digests using the RE BstZ1 (Figure 3.12). The predicted restriction digest patterns were visualised for the *het1*, *het3*, *het4*, *het6*, *het7* and *het8* fusion cassettes. The *het5* fusion cassette did not match the predicted pattern. The six *het* fusion-cassettes that matched the predicted restriction digest pattern were sequenced as described in section 2.2.3.5 using primers FP1N and FP6N. A graphical representation of the fusion cassette sequences aligned with the full predicted sequences of the fusion cassettes is shown in Figure 3.13. The predicted fusion cassette DNA sequences are in Appendix 5, and the bidirectional DNA sequencing of the six *het* fusion cassettes are available in Appendix 6.



<u>Figure 3.10</u> Agarose Gel Electrophoresis of Step 1 Fusion-PCR Products

Products of step 1 fusion PCR specific to target gene *het3* visualised on a 1% agarose gel. Fusion-PCR products were run in double lanes in order to load more of the PCR product in the gel for gel extraction. Lane 1 is 1 kb DNA Ladder (New England Biolabs, USA). Lanes 3-4 are the upstream flanking region of *het*3. Lanes 6-7 are the *hph* marker amplified from pAN7-1. Lanes 9-10 are the downstream flanking region of *het*3. Lanes 2, 5 and 8 were left empty to allow for easier gel extraction of the PCR products. The products were used as template material in step 2 fusion-PCR after being purified by gel extraction.

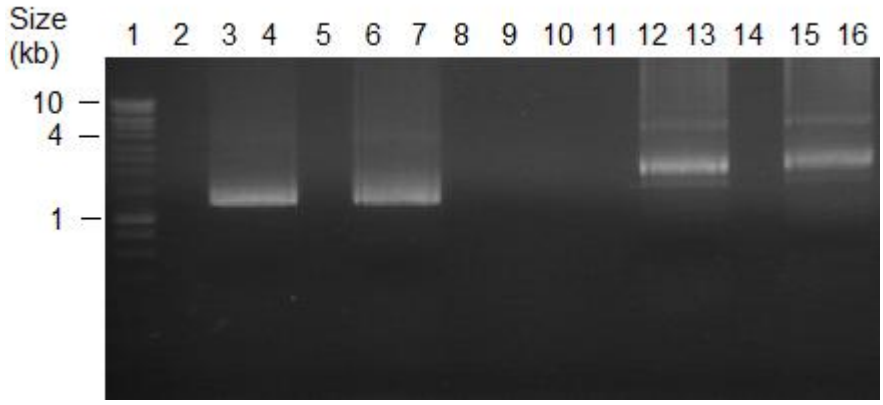
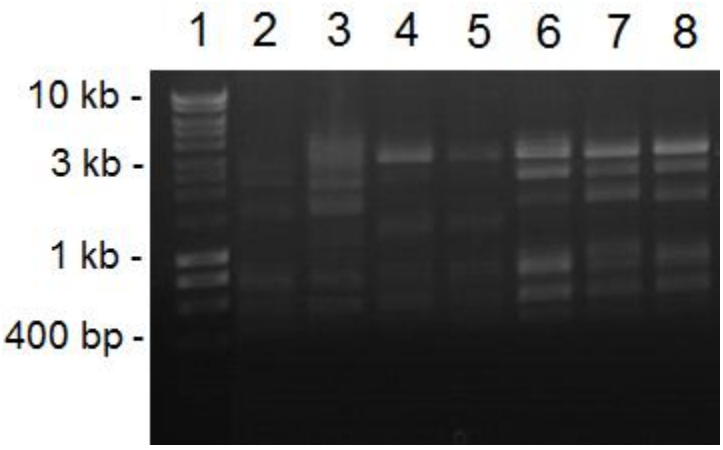


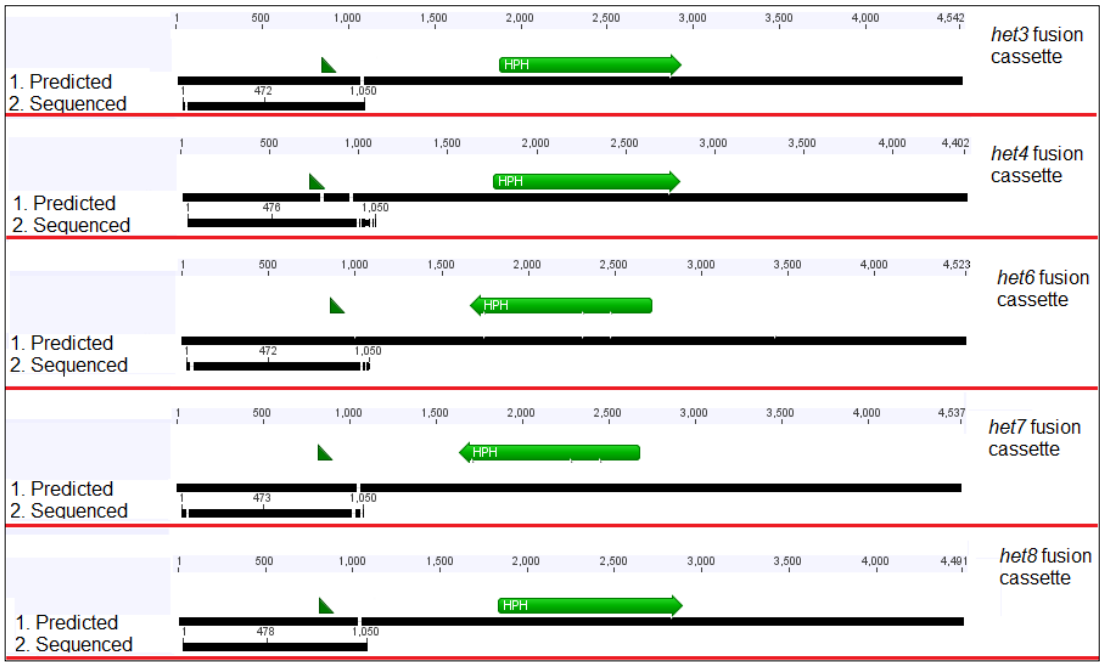
Figure 3.11 Agarose Gel Electrophoresis of *het1* and *het3* Fusion Cassettes and Undesirable Products from Step 2 Fusion-PCR

PCR fusion cassettes for gene replacement in *A. fumigatus* were run in 1% agarose gel in double lanes for gel extraction. Lane 1 contains Hyperladder I (Bioline, United Kingdom). Lane pairs 3-4 and 6-7 contain 4 kb fusion cassettes specific for *het*1. Lane pairs 12-13 and 15-16 show strong 4.5 kb fusion cassette specific for *het*3. All four lane pairs contain undesirable fragments which were discarded following gel extraction of desired fusion cassette bands. Lane pairs 3-4 and 6-7 highlight the inefficiency of step 2 fusion-PCR. The double lanes all have products matching the predicted fusion cassette sizes that are barely visible over the background non-specific products. Lanes 2, 5, 8-11 and 14 were empty.



<u>Figure 3.12</u> Restriction Digest of pGEM®-T Easy Vectors after Ligation with Fusion-PCR Cassettes

Restriction digests of fusion-PCR cassettes ligated into pGEM®-T Easy vectors were run on a 1% agarose gel. The ligated vectors were used to transform competent *E. coli* cells (section 2.2.3.1.3). Plasmids were extracted from *E. coli* colonies and then subject to a restricition digest using the RE BstZ1. Bands of approximately 750 bp, 580 bp and 170 bp indicated the presence of the *hph* marker gene in the vector. Lane 1 is the DNA marker hyperladder I, lane 2 is a restriction digest of pGEM®-T Easy ligated with the *het*1 specific fusion-cassette and lanes 3-8 are restriction digests of pGEM®-T Easy vectors ligated with specific fusion-cassettes for het genes *het3-het8* (as named in Table 3.1). All lanes matched the expected digest pattern except the product contained in lane 5 that did not match the predicted sequence for the *het5* fusion cassette.



<u>Figure 3.13</u> Graphical Representation of Alignments Between Predicted Sequence and Actual Sequence of Fusion Cassettes

Five fusion cassettes were used for *het* gene KO in *A. fumigatus* via homologous recombination. The sequences of the fusion-cassettes, labelled 2 in each alignment, were around 1 kb in length, with the signal being weaker and less reliable at each end of the sequence. To confirm the fusion cassettes were composed of the correct sequence, the sequences were aligned against the corresponding predicted sequences (labelled 1) using ClustalW2. If the sequence of the fusion cassette matched that of the predicted sequence around the flanking regions then the cassette was verified as being composed of the correct sequence in the centre. The dark green triangles represent the binding sites of either FP3 or FP4 (described in section 2.1.4 and Table 2.3) and the long green arrow represents the position and orientation of the *hph* marker gene. The 50 bp primers, FP3 and FP4, mark the site of the flanking regions of the step 1 fusion-PCR products.

Figure 3.13 *cont*.

The black bars represent matching fusion-PCR products. The black bars represent matching nucleotides, with gaps representing where the alignments do not match. Sequences of the predicted and actual *het* fusion cassettes are in Appendix 5 and Appendix 6 respectively.

3.3.1.2 Transformation of *A. fumigatus* strain A1160 and Screening of Recovered Mutants

Protoplasts were obtained through exposure of strain A1160 to Glucanex, described in section 2.2.3.6.2, to digest the fungal cell wall. The protoplasts were transformed using fusion-PCR cassette DNA to replace the target *het* gene via homologous recombination. Transformants for five *het* genes (*het3*, *het4*, *het6*, *het7* and *het8*) were recovered from transformation media after 2-3 days of incubation at 37°C (section 2.2.3.6.3) and gDNA was extracted from the isolates for PCR to amplify and sequence the target region. Four of the *het* gene KO genotypes formed noticeably smaller colonies to Af293 and the untransformed A1160 strain (Figure 3.14). The spores in the frozen stock for long-term storage of the only confirmed *het8* $^{-}$ transformant were not viable. The *het8* $^{-}$ strain was recovered from a pure culture Petri dish, however, there was no further study of the mutant due to time restraints. The DNA sequences of screening PCR (section 2.2.3.6.4), using forward and reverse screening primers (SF and SR) of the recovered *het* gene KO transformants are in Appendix 7. There were four *het3* KO strains (3-1, 3-2, 3-4 and 3-6), two *het4* KO strains (4-3 and 4-4), one of *het6* KO strain (6-2), one *het7* KO strain (7-1) and a single *het8* $^{-}$ KO strain (8-2) were verified through PCR screening. There were minor mismatches in these sequences to the predicted sequences near each end of the sequence data. However, sequence data is less reliable at both the start and end of the read, and these mismatches were not observed at the fusion primer target sites of the overlapping regions (FP2, FP3, FP4 and FP5 binding sites).

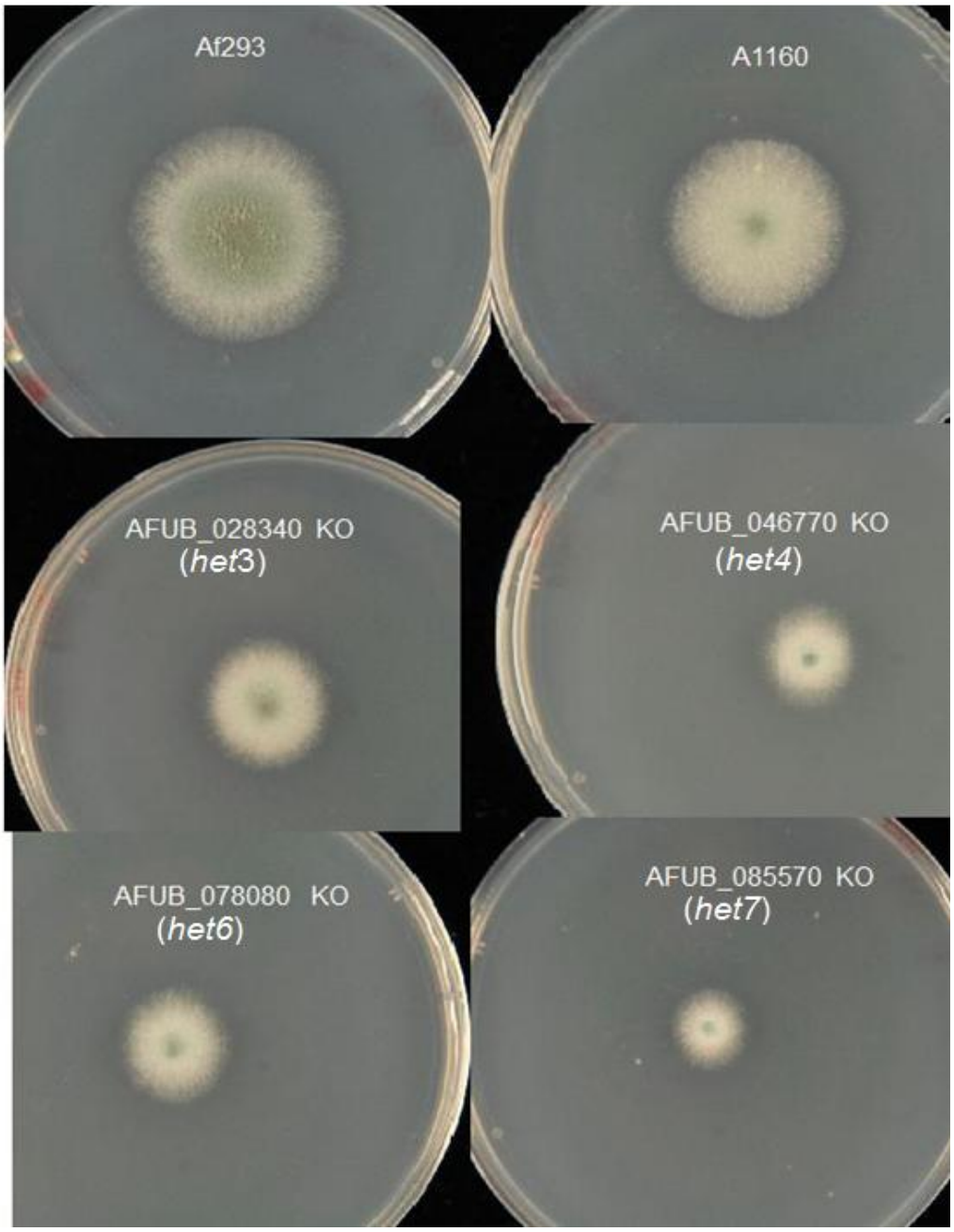


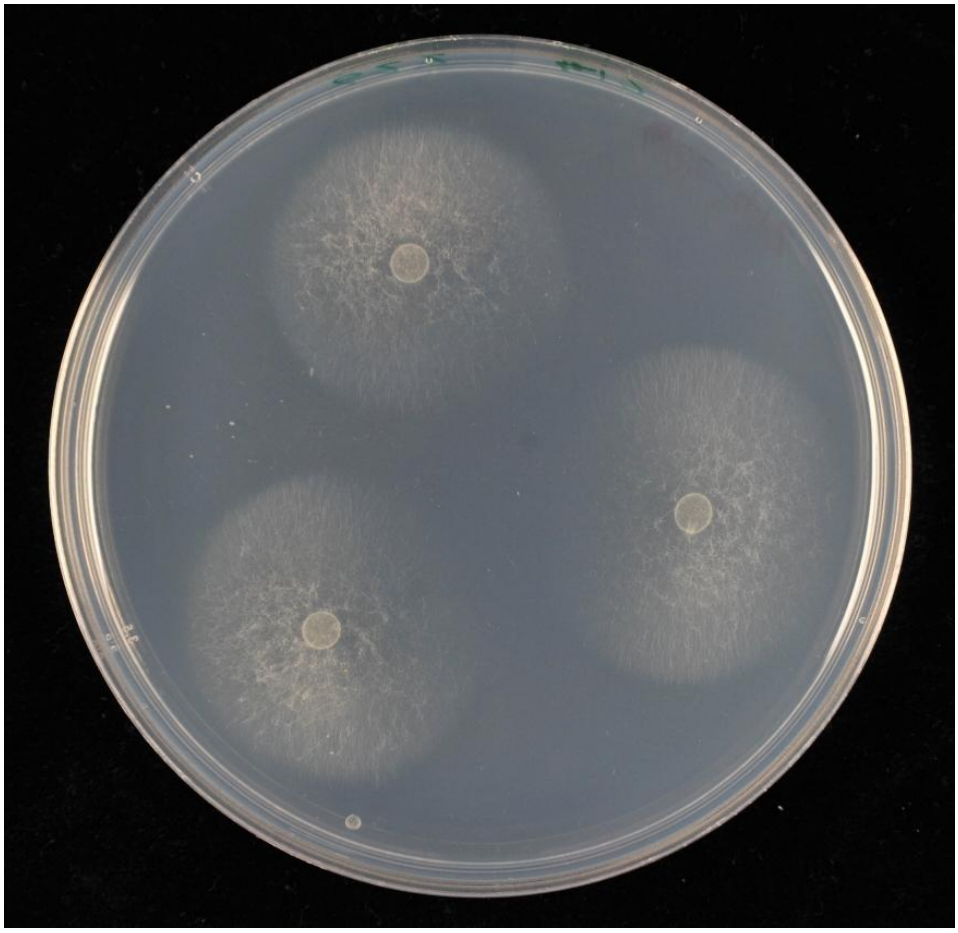
Figure 3.14 Af293, A1160 and Four *het* KO Strains on PDA Supplemented with 5 mM Uridine and 5 mM Uracil

Af293 is the strongest growing of these six *A. fumigatus* strains, but A1160 is noticeably larger than the four KO strains derived from it. Agar plates were incubated at 37°C after inoculation, with the images being taken after 60 hours. The single recovered *het8*$^{-}$ mutant did not grow using spores from the frozen spore stock to inoculate the same media.

3.3.1.3 Compatibility Grouping of *het* KO Transformants

The *het* gene KO strains that had the presence of the fusion cassette verified through DNA sequencing were subject to a selection of pair-wise crosses for compatibility grouping on N3S agar (section 2.2.2.7). The media was supplemented with 5 mM uridine and 5 mM uracil for crosses involving A1160 and *het* KO strains. Spores from *het* gene KO strains, and strains D1-4 and A1160, were mixed in pair-wise crosses with spores of clinical and environmental isolates that represented the distinct compatibility groups (section 3.2.2.3 and Table 3.5) as well as strains Af293, A1163 and A1160. In many crosses, stronger growth was observed on the N3S agar than would be expected if the paired strains were incompatible (Figure 3.15 and Table 3.6). The *het* gene KO strains did not all form a strong positive heterokaryon in self-crosses, or with strain A1160 from which all the *het* gene KO strains were derived from.

In Table 3.6, the self-cross of strain D1-4 was a compatibility test of D1-4 with the SeO^R strain after transposon mutagenesis (D1-4ET) on N3S agar. There are four *het3* KO strains (3-1, 3-2, 3-4 and 3-6), two *het4* KO strains (4-3 and 4-4), one of *het6* KO strain (6-2) and one *het7* KO strain (7-1). Strong positive crosses are highlighted in yellow, negative crosses are not highlighted, and weak crosses (moderate growth) are denoted as a question mark and highlighted in a pale orange. The single recovered *het8* $^-$ (8-2) mutant did not grow using spores from the frozen spore stock, and was not used in the pair-wise crosses.



<u>Figure 3.15</u> Moderate Growth of Pair-wise Cross between *A. fumigatus A1160 het3* KO Mutant Strain and Clinical Isolate Strain 17882

Pair-wise crosses between Nit mutants of *het3* KO mutant and clinical isolate 17882 show relatively strong growth. The growth is not as strong as a full positive heterokaryon shown in Figure 3.9. The N3S media was kept at 37°C until the image was taken 4 days after inoculation.

Table 3.6 Nit Mutants Pair-Wise Crosses of *het* KO strains with Environmental and Clinical Strains

D1-4	3-1	3-2	3-4	3-6	4-3	4-4	6-2	7-1	A1160	
	+	+	+	+	?	?	+	+	?	3-2
	?	?	?	+	?	?	?	?	?	4-4
	+	?	+	+	?	?	+	+	?	6-2
	?	?	?	+	?	?	?	?	?	7-1
-	?	?	?	?	?	?	?	?	-	Af293
-	+	+	+	+	+	+	+	+	+	A1163
-	?	?	?	?	?	?	?	?	-	RB20
-	?	?	?	?	?	?	?	?	-	Ap33
-	?	?	?	?	?	?	?	?	-	Ma3
-	?	?	?	?	?	?	?	?	-	J13
-	?	?	?	?	?	?	?	?	-	15819
-	?	?	?	?	?	?	-	-	-	17882
-	?	?	?	?	?	?	?	?	-	A1160
+			+	+	+				+	Self

3.3.2 Transposon Mutagenesis

A non-specific gene interruption technique was employed to try and disrupt HI in *A. fumigatus*. Exposure to low temperatures induces transposon mutagenesis in the strain D1-4, causing the *impala160* transposable element to disrupt a different locus within the genome.

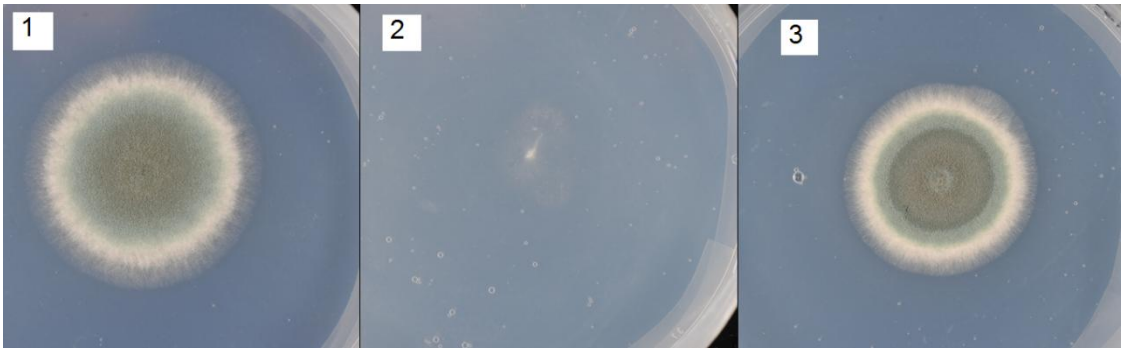
3.3.2.1 Induction of *impala160* Transposon Mutagenesis

The *impala160::pyrG* transposable element interrupts the *niaD* gene in the *A. fumigatus* strain D1-4, and as such D1-4 has a Nit phenotype prior to transposon mutagenesis but is able to utilise nitrate after mutagenesis. The Sit strain D1-4E was used in place of D1-4 (section 2.2.3.7.1). This ensured recovered mutagenesis mutants would still have a suitable nutrient auxotrophy for compatibility screening. Once inoculated, the N3T agar culture dishes were incubated at 40°C for 24 hours to encourage germination of the D1-4E spores. *impala160* mutagenesis was encouraged by moving the culture dishes to a 4°C room for four days. A further four days incubation at 37°C encouraged growth of colonies which had undergone transposon mutagenesis (collectively called D1-4ET). All the D1-4ET spores were harvested from the culture dish and stored together at room temperature as a D1-4ET spore library.

Highly variable colony counts were recovered on the large culture dishes when generating the spore library; ranging between 150 and 1000 colonies per 200 mL dish. The mean average colony count of the ten 200 mL culture dishes was 217.56 colonies per dish. The transposon mutagenesis rate while

the culture dishes were in the cold room at 4°C was 0.272 transpositions mL^{-1} day^{-1}. With 9926 genes in the *A. fumigatus* genome (Nierman *et al.*, 2005b), at least 46 culture dishes were required to generate enough transposon mutants to interrupt every gene. As the transposon was likely to interrupt non-coding regions, enough plates were set up to generate approximately 15000 transposon mutants. More mutants could not be generated due to time constraints.

Spores from the D1-4ET spore library were screened for Sit and nitrate-utilising phenotypes (Figure 3.16). The nitrate-utilising phenotype indicated transposon mutagenesis had occurred. The Sit phenotype was required for compatibility grouping of the spore library to other *A. fumigatus* strains representing distinct VCGs (Table 3.4 and Table 3.5).



<u>Figure 3.16</u> Sit and Nit Screening of the Transposon Mutagenesis D1-4ET Spore Library

D1-4ET spore libraries were screened on selective media to ensure the presence of the Sit phenotype and absence of the Nit phenotype. Plate 1 is N3T agar and plate 2 is US agar. Plate 3 is UT agar supplemented with1 mM selenate. Growth on plate 1 showed the D1-4ET spore library was composed of spores with a nitrate-utilising phenotype. Plates 2 and 3 showed the D1-4ET spore library was composed of spores with the selenate-resistant Sit phenotype. Using these selective media showed that the *impala160* transposable element has mobilised in the D1-4ET spore library mutants, restoring the *niaD* gene function, and that the Sit phenotype was still present for compatibility screening.

3.3.2.2 *impala160* Rescue of D1-4ET Colonies

D1-4ET spore libraries, mixed spores of Sit mutant D1-4 strains after transposon mutagenesis induced by low temperature (section 2.2.3.7.2), were mixed in equal concentrations in pair-wise crosses with spores of nine *A. fumigatus* strains incompatible with strain D1-4 (Table 3.6). The strains used for pair-wise crosses with D1-4ET spore libraries were Af293, A1163, RB20, Ap33, Ma3, J13, 15819 and 17882. 1 mL of spore mix was spread onto the surface of N3S agar in a 200 mL culture dish and incubated for 4 days at 37°C. D1-4ET strains could not grow strongly on N3S media as the Sit phenotype prevented utilisation of $MgSO_4$. The Nit phenotype strains were unable to utilise the $NaNO_3$. Heterokaryon formation between a D1-4ET strain and a Nit mutant strain restored both sulphate-utilising and nitrate-utilising phenotypes and allowed growth on N3S media. A total of 50 colonies grew on the 16 N3S culture dishes and were subcultured to N3T agar.

A two-step PCR reaction using rescue primers, ST-PCR, was used to determine the locus of the *impala160* transposable element in recovered colonies (2.2.3.3.5). In step one of ST-PCR, TRP3 acted as a semi-random forward primer, and TRP4 was used as a specific reverse primer (Figure 3.17). Products from step 1 ST-PCR were gel purified and then amplified in step 2 ST-PCR using primers TRP4 and TRP5 (Figure 3.18). TRP5 was the core sequence as TRP3 without the degenerate and anchor sequences. Reaction mixes containing were made to the same specifications as step 1. Purified products from step 2 ST-PCR were sent for unidirectional sequencing using the primer TRP4.

All of the DNA sequences of the step 2 ST-PCR products were contaminated with background signal (Figure 3.19). BLASTn queries of the majority of sequences returned no matches. Two recovered strains provided PCR products that matched the same short sections (~60 bp) of the *A. fumigatus* Af293 gene AFUA_2G05070 and the A1163 gene AFUB_022100, and one strain matched the same gene over a 200 bp section. The sequences of transposon mutagenesis strains 1, 21 and 31, which had DNA segments matching AFUA_2G05070, also had background contaminating signal that prevented correct identification of the full sequences. AFUA_2G05070 is annotated as a conserved hypothetical protein on the Central Aspergillus Data REpository (CADRE) database (Mabey Gilsenan *et al.*, 2012). The A1163 gene AFUB_022100 is of the same length as AFUA_2G05070, with a single base difference in position 1517 where a cytosine base has been replaced by a thymine base. The DNA sequence of AFUB_022100, and of the three ST-PCR step 2 sequences of the matching strains are in Appendix 8 and Appendix 9 respectively.

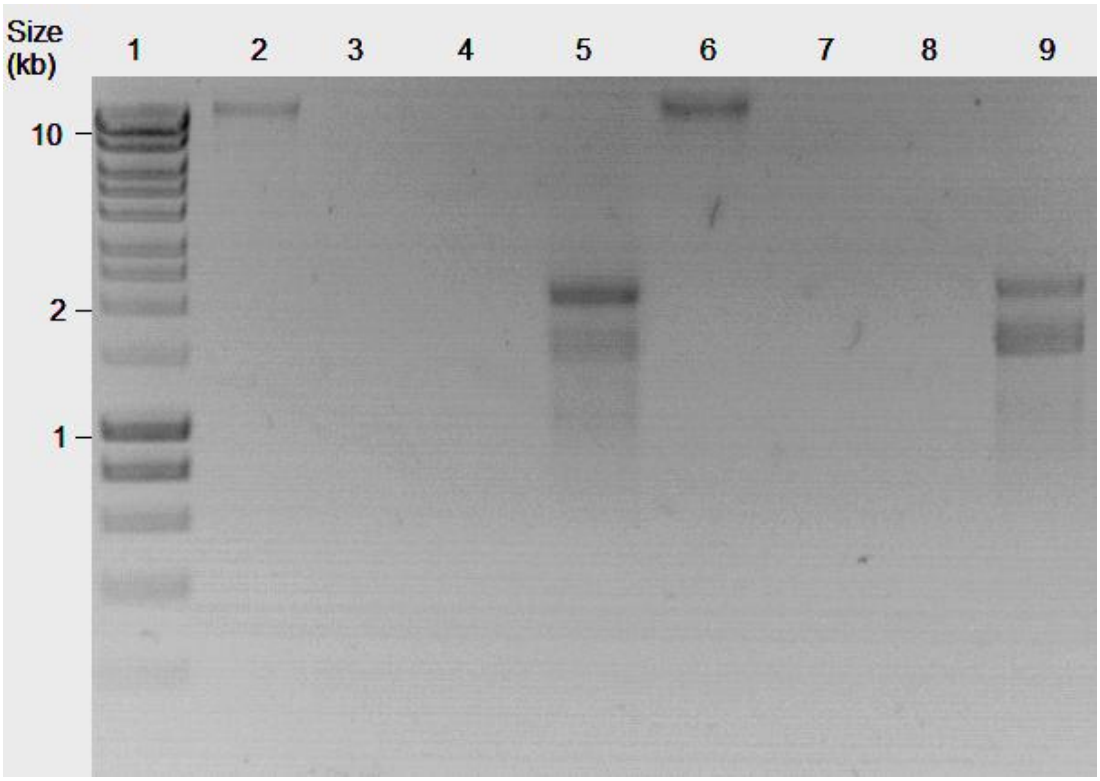


Figure 3.17 ST-PCR Step 1 of D1-4ET Transposon Mutants

The gDNA of recovered D1-4ET heterokaryon strains and step 1 ST-PCR products were visualised on 1% agarose gel. Lane 1 is Hyperladder I. Lanes 2 and 6 contain gDNA of two *impala160* transposon mutants used as template material for step 1 ST-PCR; D1-4ET heterokaryon recovered strains 1 and 41. The rescue PCRs in lanes 3 and 7 using primers TRP1 and TRP2 failed to generate a product, as did the rescue PCRs in lanes 4 and 8 using primers TRP1 and TRP3. The rescue PCRs using primers TRP3 and TRP4 yielded fragments of around 2.5 kb, shown in lanes 5 and 9. The image colours are inverted to improve visibility of all DNA bands in lanes 1, 2, 5, 6 and 9.

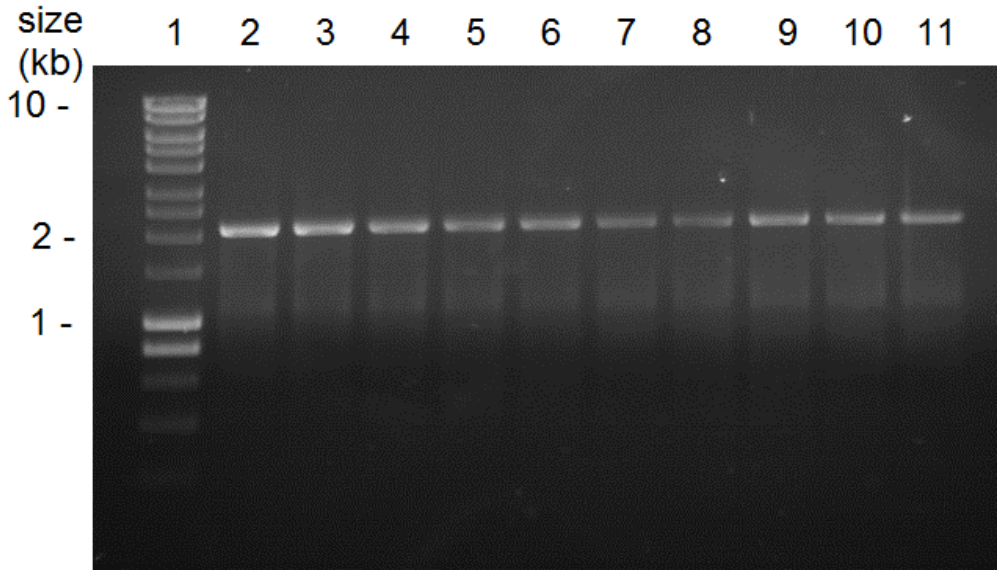


Figure 3.18 ST-PCR Step 2 of Products Purified from Step 1

Step 2 ST-PCR products were visualised on 1% agarose gel. Lane 1 is DNA marker Hyperladder I, and lanes 2-11 are step 2 ST-PCR products generated using primers TRP4 and TRP5 to amplify gel purified products of step 1 ST-PCR. The DNA templates for lanes 2-11 were generated from step 1 ST-PCR using gDNA from D1-4ET heterokaryon strains 1, 5, 11, 15, 21, 25, 31, 35, 41 and 45 respectively. All the generated products of step 2 ST-PCR matched the sizes of the template fragments used and were gel purified for DNA sequencing.

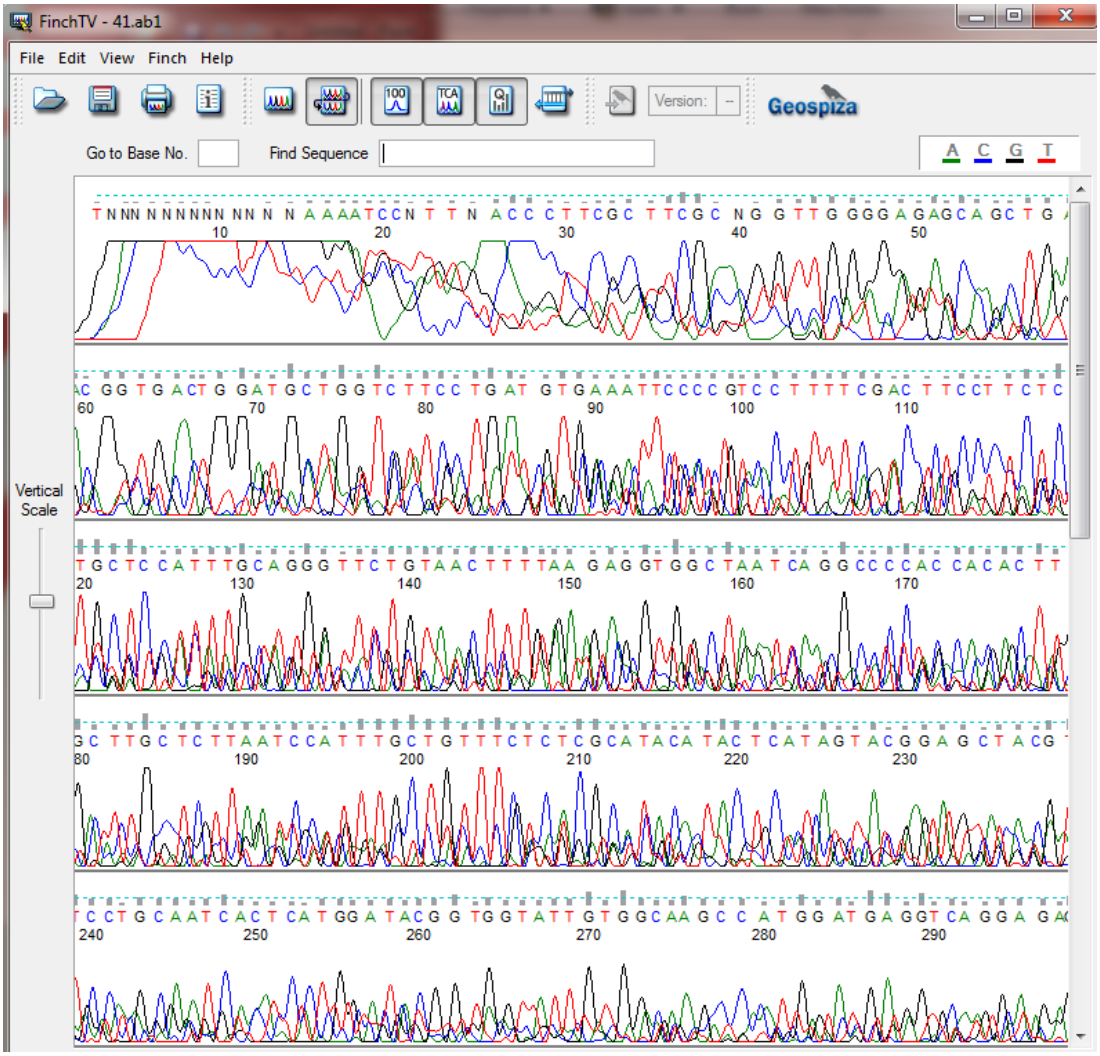


Figure 3.19 DNA Sequencing Signal of Step 2 ST-PCR Product

The recovered DNA sequence files of the D1-4ET strains that had formed heterokaryons were contaminated with background signals as shown by the shown signal from recovered strain 41. All DNA sequences of the ST-PCR step 2 fragments contained background signals that disrupted the sequence of the desired products and prevented reliable alignments. The background signals are visible as extra signal peaks that in the image when there should be only single peaks for each nucleotide position (every tenth position is marked in the image).

Chapter 4: Discussion

4.1 *het* Genes Phylogenetics

As this study has progressed, so has the field of research on *A. fumigatus*. Multiple strains of *A. fumigatus* are currently being sequenced and this will provide a large database with information on *het* genes. Looking for variations in *het* genes across multiple genomes would yield more information than using only the two currently available sequences of Af293 and A1163 (Nierman *et al.*, 2005b; Fedorova *et al.*, 2008). Table 3.1 shows *het* domain gene homology is relatively high, but still lower than expected when compared to the mean average gene homology of the entire genome between strains Af293 and A1163 (Fedorova *et al.*, 2008). Newly sequenced *A. fumigatus* genome data will expand upon whether the homology of *het* genes is significantly lower when compared to all *A. fumigatus* genes across multiple strains.

The *A. fumigatus* genome contains eight *het* domain genes (Table 3.2), which is a relatively low number when compared to other filamentous fungi (Galagan *et al.*, 2003; Espagne *et al.*, 2008). The HI reaction requires genetic variation to function as a non-self recognition system, and although the homologues may be highly similar, variation of as little as a single base difference in certain compatibility genes can be responsible for a HI reaction between fungal colonies (Deleu *et al.*, 1993). Of the eight *het* genes, only one *het* gene was identical between Af293 and A1163 (Table 3.2). Of the eight *A. fumigatus het* domain genes, seven are not associated with other protein domains commonly found in HI proteins from other species (Table

3.2), setting the *het* genes of *A. fumigatus* apart from the characterised systems of *N. crassa* and *P. anserina* (Fedorova *et al.*, 2005). The low number of *het* genes in *A. fumigatus*, and the lack of domains accompanying the HET domain in proteins, suggest the HI reaction of *A. fumigatus* may be mediated through alternative means to *P. anserina* and *N. crassa*. The *A. fumigatus* HET DED could play a lesser role in HI than in other fungal species (Fedorova *et al.*, 2005). New sequence data may also yield clues as to what evolutionary pressure encouraged some *Aspergillus* species to undergo *het* gene duplication events, whereas other Aspergilli, such as *A. fumigatus*, appear not to have experienced the same selective pressure (Figure 3.1 and Figure 3.2).

A. fumigatus are also able to undergo sexual reproduction, as shown in a recent study (O'Gorman *et al.*, 2008). The interactions between heterokaryon formation and sexual reproduction have been investigated in other filamentoius fungi (Glass and Kaneko, 2003), and it has been observed in *A. nidulans* that nuclear recognition during hybrid diploid formation favours nuclei that are closely matched at HI loci, whereas nuclei that differ at multiple alleles form separate asci (Hoffmann *et al.*, 2001). The research avenue for sexual reproduction in *A. fumigatus*, and how it can affect HI, was not explored in this study but could be investigated more fully with other *A. fumigatus* genomes currently being sequenced.

4.2 Culturing Techniques and VCGs of *A. fumigatus*

4.2.1 Stationary Phase

Entry of *A. fumigatus* broth cultures into stationary phase has previously been associated with PCD (Mousavi and Robson, 2003). The timings of *A. fumigatus* broth cultures entry into log and stationary phases were required for gene expression analysis to compare growth phase with stationary phase and the early PCD markers associated with it. Viable count and optical density measurements of Af293 in broth media (section 3.2.1) showed the same growth patterns to those previously published (Mousavi and Robson, 2003). A suitable time point for log phase was observed at T_{24} (Figure3.3). A rapid decrease of viable count of *A. fumigatus* occurred around the same time point (T_{24}) that the optical density of the broth culture began to plateau. Gene expressional analysis was not performed in this study due to time constraints, however, further study of *A. fumigatus* PCD in broth cultures could utilise microarray, real-time PCR (RT-PCR), or RNA sequencing (RNA-seq) techniques to investigate the expressional changes that occur between log and stationary phases (Nierman *et al.*, 2005a; Noodeh *et al.*, 2012; Rokas *et al.*, 2012).

4.2.2 Compatibility Grouping

4.2.2.1 Barrage Zones

To investigate how het genes affected HI in *A. fumigatus*, a method of identifying compatibility groups was required. Once this was established, it would be possible to observe any behavioural changes in compatibility reactions following manipulation of the *A. fumigatus* genome.

Observation of barrage zones between fungal colonies has been used with other fungal species to visualise HI (McCallum *et al.*, 2004), although no barrage zones were visible when pairs of both clonal isolates and distinct isolates of *A. fumigatus* were grown on the same media (Figure 3.5). Non-self recognition prior to hyphal fusion has been noted in *N. crassa* and could explain the prevention of growth of *A. fumigatus* hyphae towards another strain (Roca *et al.*, 2005a). However, clonal isolates of *A. fumigatus* behaved the same as non-clonal isolates, indicating the lack of growth was not related to non-self recognition. Sensing nutrient deprivation in the environmental also impairs colony expansion of *A. fumigatus* (Gehrke *et al.*, 2010). Despite using a nutrient rich media (section 2.2.2.5), nutrients may have depleted in the zone between paired colonies due to *A. fumigatus* being an adept species at extracellular nutrient degradation and absorption (Richie *et al.*, 2011). A possible test to determine whether nutrient deprivation was responsible for cessation of growth would be to add filter sterilised Vogel's salts stock (section 2.1.2.1) to the zone between colonies over a range of different frequencies and observe whether growth is encouraged within the zone. As *A. fumigatus* formed no visible barrage zones (section 3.2.2.1), a different approach to determine VCGs was used where spore density of inocula encouraged germination and growth of *A. fumigatus* colonies (Mowat *et al.*, 2007).

4.2.2.2 Nit Mutants

Chlorate resistance in *A. fumigatus* is characterised by the Nit phenotype (Cove, 1976). Growth of *A. fumigatus* on chlorate-PDA generated

spontaneous mutants with the Nit phenotype that were subject to pair-wise crosses on selective media (sections 2.2.2.6 and 2.2.2.7). In practice, each individual *A. fumigatus* strain had a different native susceptibility to chlorate, with some strains able to readily grow on chlorate-PDA without acquiring the Nit phenotype. Nit mutants of some strains also readily reverted to a nitrate-utilising phenotype when grown on media without chlorate. The 46 strains used for pair-wise crosses (Table 3.3) were those that readily generated a variety of Nit mutant genotypes and did not readily revert to a nitrate-utilising phenotype when screened using N3S agar selective media (Table 2.2 and Figure 3.7).

Formation of viable heterokaryons between Nit mutant pairs rescued the nitrate-utilising phenotype as predicted (Cove, 1976; Jo *et al.*, 2007). However, few viable heterokaryons were observed in the initial 1081 crosses between the 46 strains (section 3.2.2.3). Such large diversity of VCGs has been observed in *A. nidulans* and suggests HI in *A. fumigatus* could similarly be controlled by multi-allelic interactions (Dales *et al.*, 1993). Previous studies of *A. nidulans* have found partial heterokaryon formation during crosses due to the additive action of multiple *het* genes (Coenen *et al.*, 1994). However, the pair-wise crosses of Nit *A. fumigatus* mutants resulted in either one of two inflexible outcomes: strong growth of a viable heterokaryon, or the spores germination followed by weak or no growth (Figure 3.9 and Table 3.4). With so 16 of positive crosses form it may be that *A. fumigatus het* genes have highly specific recognition sites similar to *het-s* and *het-S* of P. anserina (Deleu *et al.*, 1993). The six characterised *A.*

fumigatus VCGs were distinct in that all members of a group could form a viable heterokaryon with another member strain, but no strain would be able to do so with a strain outside the group (3.2.2.3). Distinct VCGs are common amongst other fungal species (Croft and Jinks, 1977; Katan and Shabi, 1996), and the genetic variance at *het* loci between groups is thought to prevent most heterokaryosis in the environment (Mylyk, 1976; Pandit and Maheshwari, 1996). With only six *A. fumigatus* VCGs with more than a single member strain were identified in this study, more pair-wise crosses could be performed to expand upon the data and reach more informed conclusions.

4.3 Manipulation of the *A. fumigatus* Genome

4.3.1 *het* Gene KO

The compatibility grouping provided a foundation for further study of the mechanics of *A. fumigatus* HI where altered behaviour of *het* gene KO strains could be observed using pair-wise crosses. The HET domain is specific to filamentous fungi (Paoletti and Clave, 2007) and, therefore, is a desirable possible target for anti-fungal drugs. The eight Af293 *het* domain genes (labelled in Table 3.1) were targeted for deletion using specific gene cassettes to replace the target genes with an antibiotic resistance marker via homologous recombination. The gene cassettes were the product of fusion-PCR (section 2.2.3.6.1), each consisting of three components: upstream of the target gene; the *hph* marker; downstream of the target gene (Figure 2.7). Transformation of Af293 resulted in no homologous recombination KO strains and therefore a NHEJ *A. fumigatus* strain, A1160 (da Silva Ferreira *et al.*, 2006), was used for further attempts. A1160 is a CEA17 derivative strain,

as is the sequenced strain A1163 (Fedorova *et al.*, 2008), and hence have almost identical genomes. Fusion-PCR primers were redesigned using the A1163 genome. Seven fusion cassettes were produced from fusion-PCR, as the three components of the *het5* fusion cassette would not generate a detectable fusion-cassette, despite redesigning the fusion primers. Step 2 fusion-PCR was inefficient and the desired products were seen as faint bands when visualised on agarose gels (Figure 3.11). Gel extraction of the gel at the predicted size of the *het5* fusion cassette did not provide any product for ligation into pGEM®-T Easy vectors. The fusion cassettes for *het1*, *het3*, *het4*, *het6*, *het7* and *het8* were confirmed through restriction digests using BstZI and DNA sequencing (Figure 3.12 and Figure 3.13) and used as transformation material for *het* gene KO in the NHEJ-deficient *akuB*$^{(KU80\Delta)}$ strain A1160 (da Silva Ferreira *et al.*, 2006).

Nine homologous recombination transformants were recovered, covering five of the A1163 *het* genes. Four of the transformants were *het3*$^{-}$ strains, two were *het4*$^{-}$, and one of each *het6*$^{-}$, *het7*$^{-}$ and *het8*$^{-}$ were recovered (listed in Appendix 1). DNA sequencing of the recovered transformants confirmed presence of the central component of the fusion cassettes (*hph* marker) in place of the target gene (Appendix 7). The *het3*$^{-}$, *het4*$^{-}$, *het6*$^{-}$, and *het7*$^{-}$ transformants displayed stunted growth when compared to the parent strain, A1160 (Figure 3.14). This stunted growth is unusual as co-expression of incompatible genes is required in *P. anserina* to arrest of cell growth (Loubradou *et al.*, 1999). It may be that an inability of the *A. fumigatus het* KO mutants to recognise non-self leads to stunted colony growth through an

uncharacterised signalling pathway or that basal levels of expression of *het* genes are involved in progression of the cell cycle.

The Nit mutants of the *het* KO strains also showed more promiscuous tendencies than WT strains in pair-wise crosses with member strains of other VCGs (Table 3.6) as moderate growth was observed on the selectable media (Figure 3.15). The *het* KO strains may have an impaired ability to recognise other strains as non-self if the *het* gene mode of operation is additive, as in *A. nidulans* (Coenen *et al.*, 1994), which could result in partial crosses. However, the *het* KO strains were expected to all belong to the same VCG as all shared the same parent strain, A1160, and therefore should recognise the other *het* mutants as a clonal isolate. Table 3.6 shows the over half of pair-wise crosses between *het* KO strains resulted in moderate growth on the selectable media, rather than the predicted strong growth observed with a viable heterokaryon. The moderate growth could be the result of *het* gene KO removing a component of an additive self-recognition mechanism rather than a non-self recognition mechanism, or the basal levels of expression of disrupted *het* genes may aid progression of the cell cycle. Characterised HI systems in other filamentous fungi indicate HI mechanisms are governed by differences (non-self recognition), suggesting a recognition system based on similarities (self-recognition) to have convergently evolved in only *A. fumigatus* is unlikely (Sarkar *et al.*, 2002; Glass and Dementhon, 2006; Paoletti *et al.*, 2007a). A separate explanation is that observed moderate growths were actually viable heterokaryons,

where the stunted growth of individual *het* KO strains resulted in impaired growth of viable heterokaryon colonies.

Despite numerous transformation attempts, no confirmed A1160 *het1*$^{-}$ strains were recovered. The *het1* gene may be essential for cell survival and could be targeting for disruption using a transposon disruption technique or through use of an inducible promoter to regulate *het1* expression (Firon *et al.*, 2003; Bromley *et al.*, 2006; Hu *et al.*, 2007). These techniques could also be employed to characterise the *het2* and *het5* genes. The *het8*$^{-}$ strain was recovered late in the study after the frozen spore stock of the mutant used to inoculate media resulted in no growth. Viable spores of the strain was recovered from a short-term storage chilled Petri dish and were confirmed through DNA sequencing (section 3.3.1.1 and Appendix 7). Due to time restraints, this strain was not available for the compatibility grouping.

4.3.2 Transposon Mutagenesis

A second technique was used to disrupt the *A. fumigatus* genome and determine how it alters the ability of A. fumigatus to form discrete VCGs (sections 2.2.2.6 and 2.2.2.7). The *A. fumigatus* strain D1-4 has the *F. oxysporum impala160* transposable element which mobilises under environmental stresses, including storage at 4°C. (Firon *et al.*, 2003; Carr *et al.*, 2010). *impala160* resides within the *niaD* gene of D1-4, disrupting the gene prior to mobilisation. As transposon mutagenesis restores *niaD* function, and therefore the nitrate-utilisation phenotype, Sit mutants of D1-4 were generated to allow compatibility screening. Five D1-4 Sit mutants were

recovered from selenate-PDA (section 3.2.2.2) and were screened to ensure the strains were resistant to selenate due to mutation causing defective adenosine triphosphate sulphurylase (Buxton *et al.*, 1989), and that *impala160* mobilisation was not the causative factor of SeO^R (Figure 3.8). Of the five Sit strains, spores were harvested from D1-4E as this strain sporulated most readily. N3T agar inoculated with D1-4E was stored at 4°C to encourage *impala160* mobilisation, and then transferred to 37°C, before spores from the cultures were harvested (section 2.2.3.7.2). Approximately 15000 heterokaryon colonies grew on the media (calculated in section 3.3.2.1) and were harvested and labelled collectively as the D1-4ET spore library. The D1-4ET spore library was divided into nine, and each was used for a pair-wise cross on selective media with spores from one of the nine Nit mutants generated from the *het* KO strains (section 3.3.1.3 and Table 3.6). As the *impala160* transposase will preferentially locate outside of ORFs (Li Destri Nicosia *et al.*, 2001; Firon *et al.*, 2003), generation of more transposon mutants would also be required to continue this work to increase the chances of entire genome coverage through transposon mutagenesis of impala160 in *A. fumigatus* (Nierman *et al.*, 2005b).

50 heterokaryon colonies were identified, subcultured and PCR screened, using ST-PCR, from the selective media (section 3.3.2.2). The sequenced ST-PCR products were contaminated with background signals (Figure 3.19) making identification of disrupted genes, and therefore the *impala160* loci, problematic. The ST-PCR technique requires revision to minimise PCR product contaminants (Chun *et al.*, 1997). Extracted gDNA of mutants may

have been contaminated by gDNA where transposon mutagenesis had occurred again after isolation of the mutants, leading to amplification of multiple PCR products in ST-PCR. Of the 50 strains, three ST-PCR products provided sequences that matched the same *A. fumigatus* gene, while no other sequences matched the *A. fumigatus* genome. Two of the DNA sequences were around 60 bp in length, one was of 200 bp, and all three were homologous to ORF sections of the Af293 gene AFUA_2G05070. This gene is predicted to encode a conserved hypothetical protein on which there is no literature available. It is unlikely that three recovered sequences from a small sample size would match the same gene. The *impala160* transposable element mobilisation is thought not to be entirely random, however this does not explain the frequency of disruption of AFUA_2G05070 as these the most likely relocation of *impala160* is to gene promoter regions (Carr *et al.*, 2010). Further work, such as KO of AFUA_2G05070 through fusion-PCR, and reinsertion of the gene to restore functionality, could identify whether this gene is indeed involved in non-self recognition in *A. fumigatus*.

4.4 General Discussion and Further Study

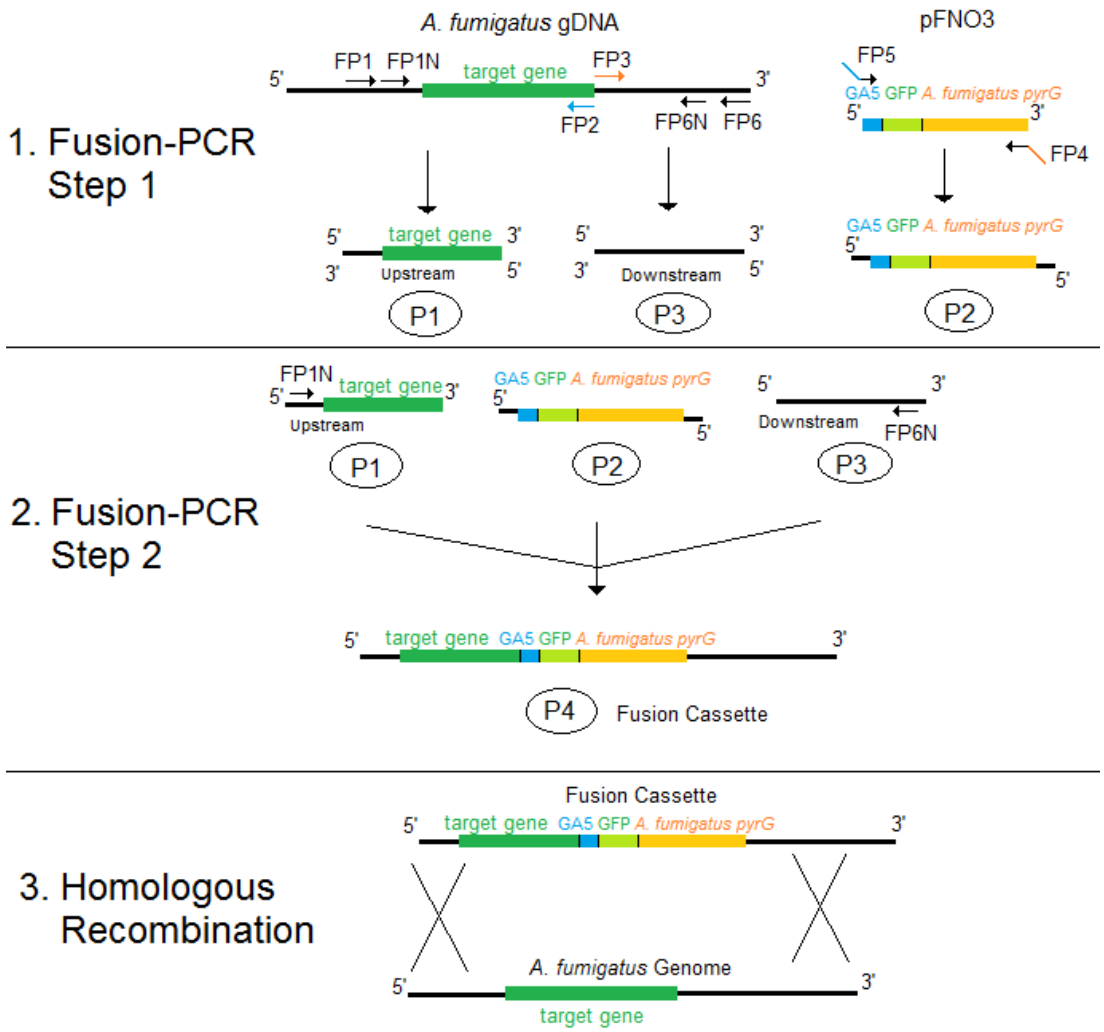
The possibility of targeting the fungal-specific PCD mechanism, HI, to treat aspergillosis may lead to drugs with less severe symptoms than those currently employed. Amphotericin B is know to trigger an apoptotic-like phenotype in *A. fumigatus* (Mousavi and Robson, 2004), showing that the pathway is a viable target. However, amphotericin B targets sterols which are present in both fungal and mammalian cell membranes, which can lead to potentially fatal side-effects in the patient, such renal damage (Ringden *et*

al., 1998; Furebring *et al.*, 2000). This study has provided a framework for further study on the *het* genes of *A. fumigatus* to determine whether the *het* genes could be exploited as a fungal-specific drug target to treat patients suffering from IA.

Due to time constraints, the objectives of using expressional analysis and identifying the *impala160* loci in more mutants to better characterise PCD were not realised. RNA-seq is a powerful tool that has been used to investigate gene expression of *A. fumigatus* (Rokas *et al.*, 2012), and analysis of the log and stationary phases could be provide insight into which genes are up-regulated, or solely expressed, during PCD (section 3.2.1).

Fusion-PCR has been used to design gene cassettes tagging fungal proteins with the green fluorescent protein (GFP) (Yang *et al.*, 2004), and could be used to further manipulate *A. fumigatus het* genes to identify cellular locations (Figure 4.1). The *pyrG* selectable marker is more cost effective than use of *hph* as a marker. However, *hph* was selected due to the initial target strain of this study, Af293, being uridine/uracil autotophic. The targets of the Af293 fusion cassettes were common to A1160 and only minor adjustments were made in the primer design to facilitate using A1160 as the target strain (Nierman *et al.*, 2005b; Fedorova *et al.*, 2008). GFP-tagging of *het* genes would identify the cellular location of HET proteins if base expression levels were detectable. This could highlight which *het* genes are active at different stages of HI and where the proteins are being localised to within the hyphae.

Promoter replacement of *het* genes could also be achieved using fusion-PCR (Figure 4.2). The *A. fumigatus* cellobiohydrolase B (*cbhB*) promoter (*cbhBp*) is induced in the presence of carboxymethyl cellulose (CMC) (Bromley *et al.*, 2006). Inducing *het* genes by addition of CMC in broth media at specific time-points could be used in conjunction with expressional analysis techniques, such as RNA-seq, to identify any changes in gene expression that occur as a result of inducing specifically *cbhBp* tagged *het* genes. Expression of the HET domain in *P. anserina* induces PCD (Paoletti and Clave, 2007), and promoter replacement could identify whether the domain has a similar role in *A. fumigatus*. If expression of the HET domain alone is enough to trigger PCD then there would be more potential for developing novel drugs to treat aspergillosis that act through triggering *A. fumigatus* fungal-specific PCD.

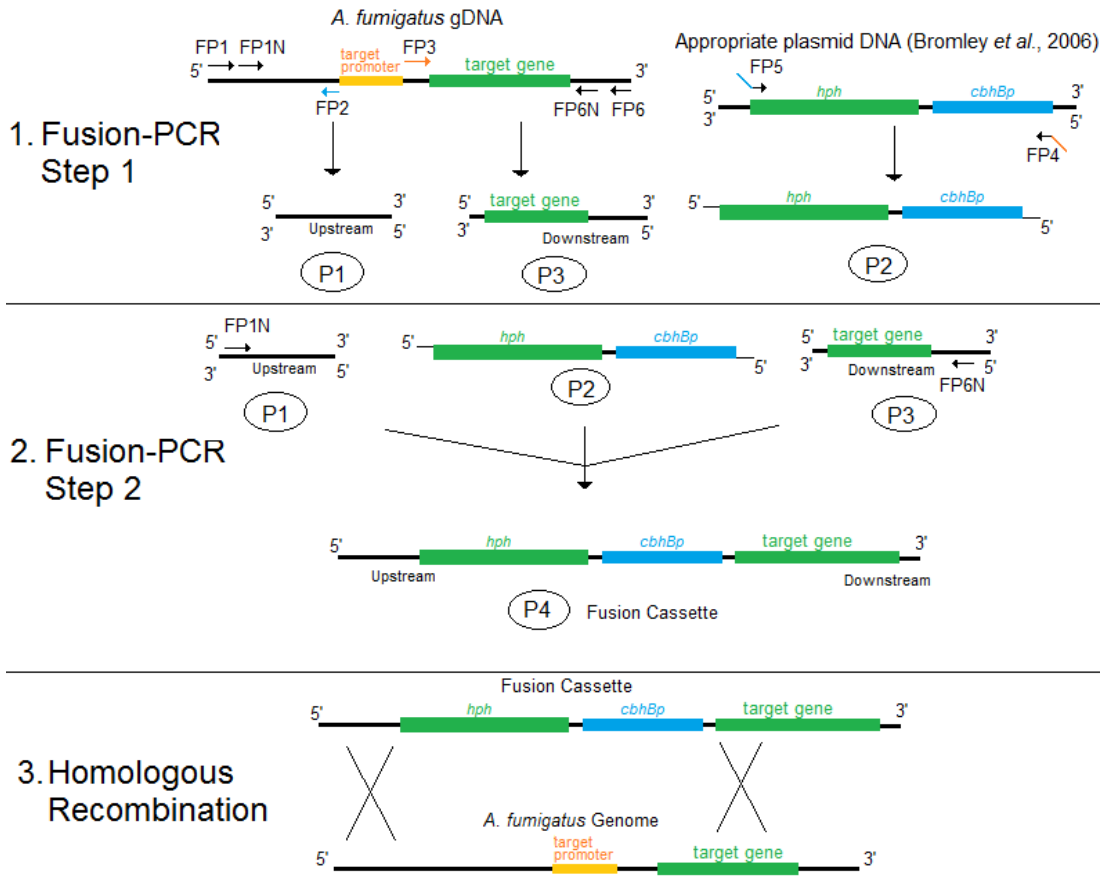


<u>Figure 4.1</u> Fusion-PCR to GFP-tag *A. fumigatus* Genes

A similar principle to fusion-PCR, outlined in Figures 2.4-2.7, can be used to tag proteins with GFP (Yang *et al.*, 2004). Fusion-PCR step 1 generates three PCR products (P1, P2 and P3) to be used as template for step 2. pFNO3 provides suitable template material to generate P2 as the GA-5 linker and *gfp* gene are accompanied by the *pyrG* marker. The GA-5 linker acts as a hinge between the transcribed protein and GFP. In contrast to gene KO fusion-PCR (section 2.2.3.6.1) the fusion primers FP2, FP3, FP4 and FP5 are not 50 bp in length (Table 2.3). FP2 and FP3 are 25-30 bp and FP4 and FP5 are 45-50 bp. The coloured sections of both primers FP4 and FP5 in the

Figure 4.1 *cont*.

fusion-PCR step 1 image are complementary in sequence to the corresponding coloured section of FP3 and FP2 respectively; this causes step 1 PCR to generate a P2 product with 25-30 bp 5' overhangs complimentary to P1 and P3 sections. Fusion cassettes (P4) would be used to transform a *pyrG*$^{-}$ *A. fumigatus* strain via homologous recombination (section 2.2.3.6.3), replacing the target gene with a *pyrG* and the target gene tagged with *gfp* Mutants would be uracil autotrophs due to the downstream insertion of *pyrG*.



<u>Figure 4.2</u> Fusion-PCR Promoter Replacement of *A. fumigatus* Genes

The inducible promoter, *cbhB*p, can be inserted upstream of target genes using fusion-PCR in a similar manner to that in Figures 2.4-2.7. Fusion-PCR step 1 generates three PCR products (P1, P2 and P3) to be used as template for step 2. Plasmids generated in previous studies could be used as template material for P2, or a suitable plasmid containing *cbhBp* and a marker could be designed (Bromley *et al.*, 2006). Primers FP2 and FP3 flank the promoter region of the target gene. In contrast to gene KO fusion-PCR (section 2.2.3.6.1) the fusion primers FP2 and FP3 are not 50 bp in length (Table 2.3). FP2 and FP3 are 25-30 bp, whereas FP4 and FP5 are 45-50 bp. The highlighted sections of primers FP4 and FP5 in the fusion-PCR step 1 image are complementary in their sequence to the corresponding highlighted

Figure 4.2 *cont*.

section sequences to of FP3 and FP2 respectively; this causes step 1 PCR to generate a P2 product with 25-30 bp 5' overhangs complimentary to P1 and P3 sections. Fusion cassettes (P4) would be used to transform *A. fumigatus* via homologous recombination (section 2.2.3.6.3), replacing the target gene and promoter region with *cbhBp*, the target gene and a suitable marker (*hph*, in this example).

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Appendices

All appropriate supplementary material to the thesis has been included in the various appendices.

Appendix 1: *A. fumigatus* Strain List

A list of all *A. fumigatus* strains used in study and notes of the source and genotypes or phenotypes of the strains.

Strain / Reference	Source	Genotype/Phenotype
Af293	WT	WT
A1163	CEA10 / CEA17 derivative strain	*pyrG*$^{+}$
Af24	WT	WT
Af71	WT	WT
RB11	Environmental (Dublin)	WT
RB12	Environmental (Dublin)	WT
RB13	Environmental (Dublin)	WT
RB14	Environmental (Dublin)	WT
RB15	Environmental (Dublin)	WT
RB16	Environmental (Dublin)	WT
RB17	Environmental (Dublin)	WT
RB18	Environmental (Dublin)	WT
RB19	Environmental (Dublin)	WT
RB20	Environmental (Dublin)	WT
Ap33	Environmental (Manchester)	WT
Ap61	Environmental (Manchester)	WT
Ap63	Environmental (Manchester)	WT
Ma3	Environmental (Manchester)	WT
Ma4	Environmental (Manchester)	WT
Ma5	Environmental (Manchester)	WT
J4	Environmental (Manchester)	WT
J10	Environmental (Manchester)	WT
J13	Environmental (Manchester)	WT
Au9	Environmental (Manchester)	WT
Au10	Environmental (Manchester)	WT
Au14	Environmental (Manchester)	WT
15562	Clinical (MRCM)	WT
15819	Clinical (MRCM)	WT
16795	Clinical (MRCM)	WT
16916	Clinical (MRCM)	WT
16975	Clinical (MRCM)	WT

Appendix 1 *cont*.

17318	Clinical (MRCM)	WT
17406	Clinical (MRCM)	WT
17768	Clinical (MRCM)	WT
17796	Clinical (MRCM)	WT
17835	Clinical (MRCM)	WT
17871	Clinical (MRCM)	WT
17882	Clinical (MRCM)	WT
18565	Clinical (MRCM)	WT
19164	Clinical (MRCM)	WT
19258	Clinical (MRCM)	WT
20395	Clinical (MRCM)	WT
21522	Clinical (MRCM)	WT
21705	Clinical (MRCM)	WT
22178	Clinical (MRCM)	WT
22577	Clinical (MRCM)	WT
A1160	da Silva Ferreira *et al*., 2006	*akuB*$^{(KU80\Delta)}$
D1-4	Firon *et al*., 2003; Carr *et al*., 2010	*impala160; niaD*$^{-}$
D1-4A	D1-4 derivative strain	*impala160; niaD*$^{-}$*; Sit*
D1-4B	D1-4 derivative strain	*impala160; niaD*$^{-}$*; Sit*
D1-4C	D1-4 derivative strain	*impala160; niaD*$^{-}$*; Sit*
D1-4D	D1-4 derivative strain	*impala160; niaD*$^{-}$*; Sit*
D1-4E	D1-4 derivative strain	*impala160; niaD*$^{-}$*; Sit*
D1-4ET	Transposon library of D1-4E	*impala160; Sit*
D1-4ET strains 1-50	50 heterokaryon D1-4ET strains	*impala160; Sit*
3-1	A1160 derivative strain	*akuB*$^{(KU80\Delta)}$; *het3*$^{-}$
3-2	A1160 derivative strain	*akuB*$^{(KU80\Delta)}$; *het3*$^{-}$
3-4	A1160 derivative strain	*akuB*$^{(KU80\Delta)}$; *het3*$^{-}$
3-6	A1160 derivative strain	*akuB*$^{(KU80\Delta)}$; *het3*$^{-}$
4-3	A1160 derivative strain	*akuB*$^{(KU80\Delta)}$; *het4*$^{-}$
4-4	A1160 derivative strain	*akuB*$^{(KU80\Delta)}$; *het4*$^{-}$
6-2	A1160 derivative strain	*akuB*$^{(KU80\Delta)}$; *het6*$^{-}$
7-1	A1160 derivative strain	*akuB*$^{(KU80\Delta)}$; *het7*$^{-}$
8-2	A1160 derivative strain	*akuB*$^{(KU80\Delta)}$; *het8*$^{-}$

Appendix 2: Background of Clinically Isolated *A. fumigatus* Strains Provided by MRCM

The geographical and physical isolation sites of all provided clinical *A. fumigatus* strains are outlined in this table exactly as provided by Dr Caroline Moore and Patrycja Kent from the MRCM at Wythenshawe Hospital (Manchester, UK). Strains had been previously validated as *A. fumigatus* via microscopic and macroscopic means. Only the twenty strains confirmed in this study as *A. fumigatus* through PCR and DNA sequencing of the 18S rDNA ITS region were used (listed in Appendix 1).

Strain	Location (Hospital/Ward)	Isolation Site
15562	FREEMAN (MICRO)/27	BAL
15791	AINTREE (MICRO)	RIGHT TEMPORAL AREA
15819	NMGU (MICRO)/ICU	SPUTUM
16258	AHH (MICRO)	SPUTUM
16795	TAMESIDE (MICRO)/GP	SPUTUM
16916	MCH BOOTHALL (MICRO)/BMLI	SPUTUM
16975	RMCH/OP	COUGH SWAB
17318	SHH/A1	SPUTUM
17406	BOOTHALL (MICRO)/BOR	SPUTUM
17597	SALFORD/SCBU	SKIN SCRAPE
17768	SHH(MICRO)/ICU	SPUTUM
17796	ALDER HEY/A3	SPUTUM
17835	BHCH (MICRO)/SIL	SPUTUM
17871	OLDHAM (MICRO)/J4	SPUTUM
17882	KINGSTON(MICRO)/COOMB	LEFT EAR SWAB
18565	TRAFFORD (MICRO)/MAV	SPUTUM
18947	PAPWORTH (MICRO)/OPD	BAL
19164	DONCASTER (MICRO)/18	GROIN SWAB
19258	BOOTHALL (MICRO)/ICH	CHEST FLUID
19414	MRI (MICRO)/HTU	SPUTUM
19446	AHCH (MICRO)/RESP.UNIT	COUGH SWAB
19563	FRENCHAY BRISTOL (MICRO)	PUS
19767	AHH (MICRO)/NMW	BC

Appendix 2 c*ont*.

20395	ALDER HEY (MICRO)/C2	SPUTUM
20548	WGH EDINBURGH/54	SPUTUM
20718	STEPPING HILL/CHEST CLINIC	BRONCHIAL WASHING
21201	HPA BRISTOL	SPUTUM
21407	WYTH.	SPUTUM
21522	WYTH.	SPUTUM
21602	WYTH.	SPUTUM
21636	NORTH DURHAM/ITU	SPUTUM
21705	WYTH.	SPUTUM
21732	ALDER HEY/E3	BRONCHIAL LAVAGE
21932	DURHAM	NASAL SECRETIONS
22020	DURHAM	BRONCHIAL LAVAGE
22178	HOPE/A3	PUS
22216	CHESTER HEALTH PARK/ITU	SPUTUM
22236	LEEDS MYCOLOGY	CYST CAPSULE
22356	MRI/76C	BRONCHIAL LAVAGE
22577	STEPPING HILL/SSSU	TISSUE
22636	SOUTHPORT/ITU	TRACHEAL ASPIRATE
22769	BLACKPOOL	TISSUE
22773	ALDER HEY	BRONCHIAL LAVAGE
22821	KINGSTON GP	SPUTUM
22851	BLACKBURN/38	CHEST CAVITY
23192	MRI	BAL
23364	MRI	ASCITIC FLUID
23440	WYTH.	SPUTUM
23525	WYTH.	SPUTUM
23615	WYTH.	BAL

Appendix 3: pAN7-1 DNA sequence

5'-3' DNA sequence of the 6756 bp pAN7-1 plasmid beginning from the start of the *gpdAp* region as marked in Figure 2.1.

> pAN7-1

GAATTCCCTTGTATCTCTACACACAGGCTCAAATCAATAAGAAGAACGG
TTCGTCTTTTTCGTTTATATCTTGCATCGTCCCAAAGCTATTGGCGGGAT
ATTCTGTTTGCAGTTGGCTGACTTGAAGTAATCTCTGCAGATCTTTCGAC
ACTGAAATACGTCGAGCCTGCTCCGCTTGGAAGCGGCGAGGAGCCTCG
TCCTGTCACAACTACCAACATGGAGTACGATAAGGGCCAGTTCCGCCA
GCTCATTAAGAGCCAGTTCATGGGCGTTGGCATGATGGCCGTCATGCA
TCTGTACTTCAAGTACACCAACGCTCTTCTGATCCAGTCGATCATCCGC
TGAAGGCGCTTTCGAATCTGGTTAAGATCCACGTCTTCGGGAAGCCAG
CGACTGGTGACCTCCAGCGTCCCTTTAAGGCTGCCAACAGCTTTCTCA
GCCAGGGCCAGCCCAAGACCGACAAGGCCTCCCTCCAGAACGCCGAG
AAGAACTGGAGGGGTGGTGTCAAGGAGGAGTAAGCTCCTTATTGAAGT
CGGAGGACGGAGCGGTGTCAAGAGGATATTCTTCGACTCTGTATTATA
GATAAGATGATGAGGAATTGGAGGTAGCATAGCTTCATTTGGATTTGCT
TTCCAGGCTGAGACTCTAGCTTGGAGCATAGAGGGTCCTTTGGCTTTCA
ATATTCTCAAGTATCTCGAGTTTGAACTTATTCCCTGTGAACCTTTTATTC
ACCAATGAGCATTGGAATGAACATGAATCTGAGGACTGCAATCGCCATG
AGGTTTTCGAAATACATCCGGATGTCGAAGGCTTGGGGCACCTGCGTT
GGTTGAATTTAGAACGTGGCACTATTGATCATCCGATAGCTCTGCAAAG
GGCGTTGCACAATGCAAGTCAAACGTTGCTAGCAGTTCCAGGTGGAAT
GTTATGATGAGCATTGTATTAAATCAGGAGATATAGCATGATCTCTAGTT
AGCTCACCACAAAAGTCAGACGGCGTAACCAAAAGTCACACAACACAA
GCTGTAAGGATTTCGGCACGGCTACGGAAGACGGAGAAGCCACCTTCA
GTGGACTCGAGTACCATTTAATTCTATTTGTGTTTGATCGAGACCTAATA
CAGCCCCTACAACGACCATCAAAGTCGTATAGCTACCAGTGAGGAAGT
GGACTCAAATCGACTTCAGCAACATCTCCTGGATAAACTTTAAGCCTAA
ACTATACAGAATAAGATAGGTGGAGAGCTTATACCGAGCTCCCAAATCT
GTCCAGATCATGGTTGACCGGTGCCTGGATCTTCCTATAGAATCATCCT
TATTCGTTGACCTAGCTGATTCTGGAGTGACCCAGAGGGTCATGACTTG
AGCCTAAAATCCGCCGCCTCCACCATTTGTAGAAAAATGTGACGAACTC
GTGAGCTCTGTACAGTGACCGGTGACTCTTTCTGGCATGCGGAGAGAC
GGACGGACGCAGAGAGAAGGGCTGAGTAATAAGCCACTGGCCAGACA
GCTCTGGCGGCTCTGAGGTGCAGTGGATGATTATTAATCCGGGACCGG
CCGCCCCTCCGCCCCGAAGTGGAAAGGCTGGTGTGCCCCTCGTTGAC
CAAGAATCTATTGCATCATCGGAGAATATGGAGCTTCATCGAATCACCG
GCAGTAAGCGAAGGAGAATGTGAAGCCAGGGGTGTATAGCCGTCGGC

GAAATAGCATGCCATTAACCTAGGTACAGAAGTCCAATTGCTTCCGATC
TGGTAAAAGATTCACGAGATAGTACCTTCTCCGAAGTAGGTAGAGCGAG
TACCCGGCGCGTAAGCTCCCTAATTGGCCCATCCGGCATCTGTAGGGC
GTCCAAATATCGTGCCTCTCCTGCTTTGCCCGGTGTATGAAACCGGAAA
GGCCGCTCAGGAGCTGGCCAGCGGCGCAGACCGGGAACACAAGCTGG
CAGTCGACCCATCCGGTGCTCTGCACTCGACCTGCTGAGGTCCCTCAG
TCCCTGGTAGGCAGCTTTGCCCCGTCTGTCCGCCCGGTGTGTCGGCG
GGGTTGACAAGGTCGTTGCGTCAGTCCAACATTTGTTGCCATATTTTCC
TGCTCTCCCCACCAGCTGCTCTTTTCTTTTCTCTTTCTTTTCCCATCTTCA
GTATATTCATCTTCCCATCCAAGAACCTTTATTTCCCCTAAGTAAGTACT
TTGCTACATCCATACTCCATCCTTCCCATCCCTTATTCCTTTGAACCTTT
CAGTTCGAGCTTTCCCACTTCATCGCAGCTTGACTAACAGCTACCCCGC
TTGAGCAGACATCACCATGCCTGAACTCACCGCGACGTCTGTCGAGAA
GTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCG
GAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGA
TATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTT
ATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGC
TTGACATTGGGGAATTCAGCGAGAGCCTGACCTATTGCATCTCCCGCC
GTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCG
CTGTTCTGCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGCC
GATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATC
GGTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCC
ATGTGTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCG
TCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCC
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ACGGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATG
TTCGGGGATTCCCAATACGAGGTCGCCAACATCTTCTTCTGGAGGCCG
TGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCAT
CCGGAGCTTGCAGGATCGCCGCGGCTCCGGGCGTATATGCTCCGCATT
GGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGATG
CAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCC
GGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTG
GACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCC
CAGCACTCGTCCGAGGGCAAAGGAATAGAGTAGATGCCGACCGCGGG
ATCCACTTAACGTTACTGAAATCATCAAACAGCTTGACGAATCTGGATAT
AAGATCGTTGGTGTCGATGTCAGCTCCGGAGTTGAGACAAATGGTGTTC
AGGATCTCGATAAGATACGTTCATTTGTCCAAGCAGCAAAGAGTGCCTT
CTAGTGATTTAATAGCTCCATGTCAACAAGAATAAAACGCGTTTTCGGGT
TTACCTCTTCCAGATACAGCTCATCTGCAATGCATTAATGCATTGACTGC
AACCTAGTAACGCCTTNCAGGCTCCGGCGAAGAGAAGAATAGCTTAGC
AGAGCTATTTTCATTTTCGGGAGACGAGATCAAGCAGATCAACGGTCGT
CAAGAGACCTACGAGACTGAGGAATCCGCTCTTGGCTCCACGCGACTA
TATATTTGTCTCTAATTGTACTTTGACATGCTCCTCTTCTTTACTCTGATA
GCTTGACTATGAAAATTCCGTCACCAGCNCCTGGGTTCGCAAAGATAAT

TGCATGTTTCTTCCTTGAACTCTCAAGCCTACAGGACACACATTCATCGT
AGGTATAAACCTCGAAATCANTTCCTACTAAGATGGTATACAATAGTAAC
CATGCATGGTTGCCTAGTGAATGCTCCGTAACACCCAATACGCCGGCC
GAAACTTTTTTACAACTCTCCTATGAGTCGTTTACCCAGAATGCACAGGT
ACACTTGTTTAGAGGTAATCCTTCTTTCTAGAAGTCCTCGTGTACTGTGT
AAGCGCCCACTCCACATCTCCACTCGACCTGCAGGCATGCAAGCTTGG
CACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTA
CCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAA
TAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCT
GAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGC
GGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATG
CCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCG
CCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTG
ACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCG
AAACGCGCGAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTA
ATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGG
AAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATAT
GTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAA
AAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTT
TTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGA
AAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCG
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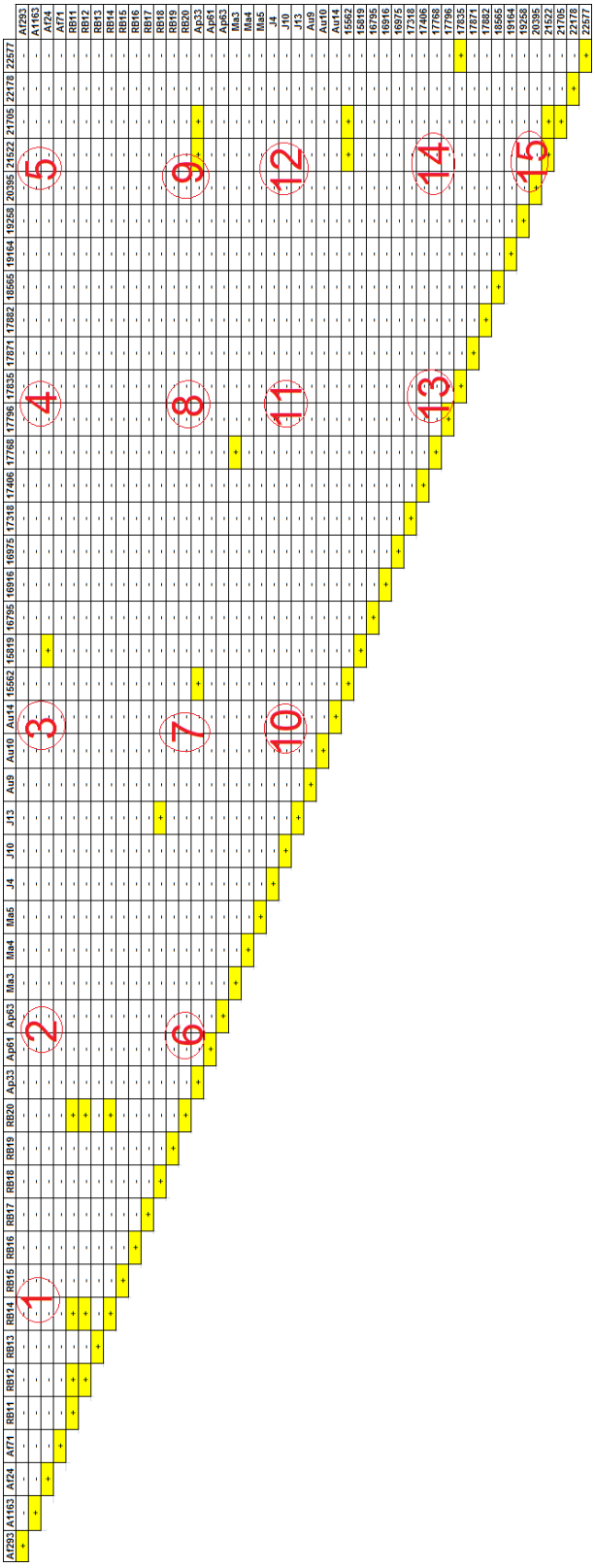
ACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTA
TTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACAC
TATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATC
TTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCAT
GAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACC
GAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGC
CTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAG
CGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTA
TTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACT
GGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTC
CGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGT
CTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTA
TCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAA
ATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACT
GTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTT
TTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCA
AAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGA
AAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCT
GCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGG
ATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGC
GCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCAC
TTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGT

TACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGG
ACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGG
GGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAAC
TGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAG
GGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGA
GAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGT
CCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCT
CGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTT
TTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGC
GTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCT
GATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAG
CGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGC
GTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGA
AAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTA
GGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGA
ATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATT
AC

Appendix 4: Full Table of Pair-Wise Compatibility Grouping of Clinical and Environmental *A. fumigatus* Nit Mutants

Fully constructed image of Table 3.4. Numbered sections are labelled in red.



Appendix 5: Predicted *het* KO Fusion Cassette DNA Sequences

The predicted 5'-3' DNA sequences of the *het* KO fusion cassettes described in Table 2.5 and section 3.3.1.1. Fusion cassettes for *het2* and *het5* are omitted from this appendix as they were not produced.

> *het1* predicted fusion cassette

TCTCATGATAGGTGGGTGTGTTAACATTCCATTAGGTTGTTGTTTACTTA
ACCTAATCATTCACCAAGTTTTGATGTTACTTCTTAAAAGGAGTTCTGCT
AATTTTATGAATTGTATATTAAAAATTATAATAAATTTAGTTACAGTTTGAT
TATAGAGCAGTCTAGAACCAGTAAAAGCAGAGTCTGATTAATCAGAGAA
TTTGCTTTTCCCACACAATGGTGCTTTGTTCATGTAATATAGTAGAAAAT
GTTTAGTTTAGAGTAGTTAGAACCTATTACATTTCAGGTATTATCTCTATA
AGTGGCCTGACCTGCATTCTGATCTCTATAATAAGTAATTACTTAGTTAA
TTAACTAATAAAGATTATGTATTTAACTCCGGTTATCACCCCACTCCTTTA
GTAATAGCCAGTCTATCATGGCACTTACTGCTAAAAGCTAAAAGACTACT
TACATTCCATCAACTTATCAGTGATAATCCTACTTTCTTTTTTTTTATGTAT
GTTGATACTTCGCTATACTACACTGATCTATTTGTTATTACTCATATGAGT
ACAGTTTGAAGCTTAGTGCCACAACTCTTCTATAATATCCTGGCGCAAC
CCTGGTTAATTGACAGTTGGCAGCTCGATACTGACTCGAGTGGAGATGT
GGAGTGGGCGCTTACACAGTACACGAGGACTTCTAGAAAGAAGGATTA
CCTCTAAACAAGTGTACCTGTGCATTCTGGGTAAACGACTCATAGGAGA
GTTGTAAAAAAGTTTCGGCCGGCGTATTGGGTGTTACGGAGCATTCACT
AGGCAACCATGCATGGTTACTATTGTATACCATCTTAGTAGGAANTGATT
TCGAGGTTTATACCTACGATGAATGTGTGTCCTGTAGGCTTGAGAGTTC
AAGGAAGAAACATGCAATTATCTTTGCGAACCCAGGNGCTGGTGACGG
AATTTTCATAGTCAAGCTATCAGAGTAAAGAAGAGGAGCATGTCAAAGT
ACAATTAGAGACAAATATATAGTCGCGTGGAGCCAAGAGCGGATTCCTC
AGTCTCGTAGGTCTCTTGACGACCGTTGATCTGCTTGATCTCGTCTCCC
GAAAATGAAAATAGCTCTGCTAAGCTATTCTTCTCTTCGCCGGAGCCTG
NAAGGCGTTACTAGGTTGCAGTCAATGCATTAATGCATTGCAGATGAGC
TGTATCTGGAAGAGGTAAACCCGAAAACGCGTTTTATTCTTGTTGACAT
GGAGCTATTAAATCACTAGAAGGCACTCTTTGCTGCTTGGACAAATGAA
CGTATCTTATCGAGATCCTGAACACCATTTGTCTCAACTCCGGAGCTGA
CATCGACACCAACGATCTTATATCCAGATTCGTCAAGCTGTTTGATGATT
TCAGTAACGTTAAGTGGATCCCGCGGTCGGCATCTACTCTATTCCTTTG
CCCTCGGACGAGTGCTGGGGCGTCGGTTTCCACTATCGGCGAGTACTT
CTACACAGCCATCGGTCCAGACGGCCGCGCTTCTGCGGGCGATTTGTG
TACGCCCGACAGTCCCGGCTCCGGATCGGACGATTGCGTCGCATCGAC
CCTGCGCCCAAGCTGCATCATCGAAATTGCCGTCAACCAAGCTCTGATA

GAGTTGGTCAAGACCAATGCGGAGCATATACGCCCGGAGCCGCGGCG
ATCCTGCAAGCTCCGGATGCCTCCGCTCGAAGTAGCGCGTCTGCTGCT
CCATACAAGCCAACCACGGCCTCCAGAAGAAGATGTTGGCGACCTCGT
ATTGGGAATCCCCGAACATCGCCTCGCTCCAGTCAATGACCGCTGTTAT
GCGGCCATTGTCCGTCAGGACATTGTTGGAGCCGAAATCCGCGTGCAC
GAGGTGCCGGACTTCGGGGCAGTCCTCGGCCCAAAGCATCAGCTCATC
GAGAGCCTGCGCGACGGACGCACTGACGGTGTCGTCCATCACAGTTTG
CCAGTGATACACATGGGGATCAGCAATCGCGCATATGAAATCACGCCAT
GTAGTGTATTGACCGATTCCTTGCGGTCCGAATGGGCCGAACCCGCTC
GTCTGGCTAAGATCGGCCGCAGCGATCGCATCCATGGCCTCCGCGACC
GGCTGCAGAACAGCGGGCAGTTCGGTTTCAGGCAGGTCTTGCAACGTG
ACACCCTGTGCACGGCGGGAGATGCAATAGGTCAGGCTCTCGCTGAAT
TCCCCAATGTCAAGCACTTCCGGAATCGGGAGCGCGGCCGATGCAAAG
TGCCGATAAACATAACGATCTTTGTAGAAACCATCGGCGCAGCTATTTA
CCCGCAGGACATATCCACGCCCTCCTACATCGAAGCTGAAAGCACGAG
ATTCTTCGCCCTCCGAGAGCTGCATCAGGTCGGAGACGCTGTCGAACT
TTTCGATCAGAAACTTCTCGACAGACGTCGCGGTGAGTTCAGGCATGGT
GATGTCTGCTCAAGCGGGGTAGCTGTTAGTCAAGCTGCGATGAAGTGG
GAAAGCTCGAACTGAAAGGTTCAAAGGAATAAGGGATGGGAAGGATGG
AGTATGGATGTAGCAAAGTACTTACTTAGGGGAAATAAAGGTTCTTGGA
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GCAGCTGGTGGGGAGAGCAGGAAAATATGGCAACAAATGTTGGACTGA
CGCAACGACCTTGTCAACCCCGCCGACACACCGGGCGGACAGACGGG
GCAAAGCTGCCTACCAGGGACTGAGGGACCTCAGCAGGTCGAGTGCA
GAGCACCGGATGGGTCGACTGCCAGCTTGTGTTCCCGGTCTGCGCCG
CTGGCCAGCTCCTGAGCGGCCTTTCCGGTTTCATACACCGGGCAAAGC
AGGAGAGGCACGATATTTGGACGCCCTACAGATGCCGGATGGGCCAAT
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CATCGGTCCAGACGGCCGCGCTTCTGCGGGCGATTTGTGTACGCCCGA
CAGTCCCGGCTCCGGATCGGACGATTGCGTCGCATCGACCCTGCGCC
CAAGCTGCATCATCGAAATTGCCGTCAACCAAGCTCTGATAGAGTTGGT
CAAGACCAATGCGGAGCATATACGCCCGGAGCCGCGGCGATCCTGCA
AGCTCCGGATGCCTCCGCTCGAAGTAGCGCGTCTGCTGCTCCATACAA
GCCAACCACGGCCTCCAGAAGAAGATGTTGGCGACCTCGTATTGGGAA
TCCCCGAACATCGCCTCGCTCCAGTCAATGACCGCTGTTATGCGGCCA
TTGTCCGTCAGGACATTGTTGGAGCCGAAATCCGCGTGCACGAGGTGC
CGGACTTCGGGGCAGTCCTCGGCCCAAAGCATCAGCTCATCGAGAGCC
TGCGCGACGGACGCACTGACGGTGTCGTCCATCACAGTTTGCCAGTGA
TACACATGGGGATCAGCAATCGCGCATATGAAATCACGCCATGTAGTGT
ATTGACCGATTCCTTGCGGTCCGAATGGGCCGAACCCGCTCGTCTGGC
TAAGATCGGCCGCAGCGATCGCATCCATGGCCTCCGCGACCGGCTGC
AGAACAGCGGGCAGTTCGGTTTCAGGCAGGTCTTGCAACGTGACACCC
TGTGCACGGCGGGAGATGCAATAGGTCAGGCTCTCGCTGAATTCCCCA
ATGTCAAGCACTTCCGGAATCGGGAGCGCGGCCGATGCAAAGTGCCGA
TAAACATAACGATCTTTGTAGAAACCATCGGCGCAGCTATTTACCCGCA

GGACATATCCACGCCCTCCTACATCGAAGCTGAAAGCACGAGATTCTTC
GCCCTCCGAGAGCTGCATCAGGTCGGAGACGCTGTCGAACTTTTCGAT
CAGAAACTTCTCGACAGACGTCGCGGTGAGTTCAGGCATGGTGATGTC
TGCTCAAGCGGGGTAGCTGTTAGTCAAGCTGCGATGAAGTGGGAAAGC
TCGAACTGAAAGGTTCAAAGGAATAAGGGATGGGAAGGATGGAGTATG
GATGTAGCAAAGTACTTACTTAGGGGAAATAAAGGTTCTTGGATGGGAA
GATGAATATACTGAAGATGGGAAAAGAAAGAGAAAAGAAAAGAGCAGCT
GGTGGGGAGAGCAGGAAAATATGGCAACAAATGTTGGACTGACGCAAC
GACCTTGTCAACCCCGCCGACACACCGGGCGGACAGACGGGGCAAAG
CTGCCTACCAGGGACTGAGGGACCTCAGCAGGTCGAGTGCAGAGCAC
CGGATGGGTCGACTGCCAGCTTGTGTTCCCGGTCTGCGCCGCTGGCC
AGCTCCTGAGCGGCCTTTCCGGTTTCATACACCGGGCAAAGCAGGAGA
GGCACGATATTTGGACGCCCTACAGATGCCGGATGGGCCAATTAGGGA
GCTTACGCGCCGGGTACTCGCTCTACCTACTTCGGAGAAGGTACTATCT
CGTGAATCTTTTACCAGATCGGAAGCAATTGGACTTCTGTACCTAGGTT
AATGGCATGCTATTTCGCCGACGGCTATACACCCCTGGCTTCACATTCT
CCTTCGCTTACTGCCGGTGATTCGATGAAGCTCCATATTCTCCGATGAT
GCAATAGATTCTTGGTCAACGAGGGGCACACCAGCCTTTCCACTTCGG
GGCGGAGGGGCGGCCGGTCCCGGATTAATAATCATCCACTGCACCTCA
GAGCCGCCAGAGCTGTCTGGCCAGTGGCTTATTACTCAGCCCTTCTCT
CTGCGTCCGTCCGTCTCTCCGCATGCCAGAAAGAGTCACCGGTCACTG
TACAGAGCTCACGAGTTCGTCACATTTTTCTACAAATGGTGGAGGCGGC
GGATTTTAGGCTCAAGTCATGACCCTCTGGGTCACTCCAGAATCAGCTA
GGTCAACGAATAAGGATGATTCTATAGATCGACATCCAGCATACTCCCA
TTGCTTGAACTCAAAGGCCGAGTCCTAGGGTTTAAGTATACCACGTAGG
CTCCGGGTACTCATGCAGCAGCACATATCTGCCGAGCTCCCGATTCTT
GTATGCCACAGGGTCATGCAAAGTGTGCGTCCTGATATCCCGCCAGAA
CCGGTCCAGTCCCACCTTCAGCGACGTTGCTCGAGCGCCAGTCACCTC
GAATATACCAGACGTGACTCGGAGTCCGACATCCGTAGTGACCACCTT
CACACTGGCGACCCATTCAGCCACCTCCCCTCGTTCCTCCGCTGTCAC
CTTCAACCGATCGCCTGAATGGCGCTCGTACAGCTCCGAGAGTCGATC
GCCGGCTTTGTCGGCCAGTGCTTCCACAGCACGGAGGTGTGCAAAGAA
GTTCCCGTATCGCTCGAGAATGTAGAACTCATCGGTGGCTCTCTCTTTG
TTGTCCCCGCCATAGGGCCAGGCGCGTGTGGACGTGGTGGTGTACTTG
GCTGCGAATTCAAGGGCGCCGATGGCGATGCCGAGGTAGAAATTGCTG
AACACCAGCTGAATCCTAGTATGGTCAGTACGGACTGTGGGGAAGCAG
GAGTCCTATGGCACTTACGTAGGCAGAAACAACGAGGCAAAAGGGATG
GTGAGAATCTCTGCCAGAGGTCGTTTGGTTGTTGCGTCCCATCCCAGT
GCATCCGTCCAAGATGCACGAACATTTTCGATCTTCACGCTTCCGGACT
CGGTCAACCGCAAACCAATATTCTTCCAGTTGTACGCGAATTGAATTCC
TGGTTGGTTGGTTTCCACAACAGCGAATATATGGTCCTGTGTTCCGTCC
AGCACGCCTTCCAGGACCGTGAGATC

> *het8* predicted fusion cassette

TCAAAACTGGAGCCCATCAGTCTTCTTTGTTTGCTACTTTCGCTTAACAT
AGGCGCAGGGGCTGAGGTGGTTAATAGCATATTTGTATCTAGCATATTG
ATATGGTACATATAGGGTTCAAGGATGGTGATTAAAATTATTCCTACCAT
ATAGTGATTATCGAAGATGAATCGATTTATCTATCTATAAATAGGTACGG
AGTACCACTACTTTTATACTAATCCATGACCATAACTGAGATGATTTTCA
CAGTTCTGTTATGTAACTCTAGTTGCACTTAAAAGGATTACTAAGACAAT
AAAGGAGATCTATAGGGAGAGATGAACAGAATCTTACATAGCGGAATTA
CTAGCAATCTACATATAATTATTTCAGTCTTGCCCCAATGGATTACTATG
CCTAATATTCTTGGCAGTGAGATTGATCACTCTTTCCCCGCCAGCCCCG
CCTTTGATAAGCGCCGTCAGACAGACCACCCTTTCACCCTCTTCTTCAT
ATGACTTGTCTTTGGAAGTCTCAATCGGGGCAGCAGACTGATATTGGAC
CAGAACAGAAAAGTTCTATAACGCTCACACACGCTACTTAACCCTGAGA
CTGGGGAGCCACCACCCAAGTCCGTTGGTGAGACTTCGGACATTCAAC
ATTTGGCAGATCTGGTGGGTTGCAACTTTTCCTCATATATGAATGAACTT
CCGAGCCAGTCAAGTTGCCGTAGCCATTCACGCCGCTGCAGTGTCAGC
GGCGCATGCATGAACGAGGCGGGTTCTACAGCGGCCTGACGGAAGTA
CTTGACGGCATTTTGGGCTTCTGGCAGCTGACAGGTAATAGTTCAGAAC
GACTTCGAGTGGAGATGTGGAGTGGGCGCTTACACAGTACACGAGGAC
TTCTAGAAAGAAGGATTACCTCTAAACAAGTGTACCTGTGCATTCTGGG
TAAACGACTCATAGGAGAGTTGTAAAAAAGTTTCGGCCGGCGTATTGGG
TGTTACGGAGCATTCACTAGGCAACCATGCATGGTTACTATTGTATACC
ATCTTAGTAGGAANTGATTTCGAGGTTTATACCTACGATGAATGTGTGTC
CTGTAGGCTTGAGAGTTCAAGGAAGAAACATGCAATTATCTTTGCGAAC
CCAGGNGCTGGTGACGGAATTTTCATAGTCAAGCTATCAGAGTAAAGAA
GAGGAGCATGTCAAAGTACAATTAGAGACAAATATATAGTCGCGTGGAG
CCAAGAGCGGATTCCTCAGTCTCGTAGGTCTCTTGACGACCGTTGATCT
GCTTGATCTCGTCTCCCGAAAATGAAAATAGCTCTGCTAAGCTATTCTTC
TCTTCGCCGGAGCCTGNAAGGCGTTACTAGGTTGCAGTCAATGCATTAA
TGCATTGCAGATGAGCTGTATCTGGAAGAGGTAAACCCGAAAACGCGTT
TTATTCTTGTTGACATGGAGCTATTAAATCACTAGAAGGCACTCTTTGCT
GCTTGGACAAATGAACGTATCTTATCGAGATCCTGAACACCATTTGTCT
CAACTCCGGAGCTGACATCGACACCAACGATCTTATATCCAGATTCGTC
AAGCTGTTTGATGATTTCAGTAACGTTAAGTGGATCCCGCGGTCGGCAT
CTACTCTATTCCTTTGCCCTCGGACGAGTGCTGGGGCGTCGGTTTCCA
CTATCGGCGAGTACTTCTACACAGCCATCGGTCCAGACGGCCGCGCTT
CTGCGGGCGATTTGTGTACGCCCGACAGTCCCGGCTCCGGATCGGAC
GATTGCGTCGCATCGACCCTGCGCCCAAGCTGCATCATCGAAATTGCC
GTCAACCAAGCTCTGATAGAGTTGGTCAAGACCAATGCGGAGCATATAC
GCCCGGAGCCGCGGCGATCCTGCAAGCTCCGGATGCCTCCGCTCGAA
GTAGCGCGTCTGCTGCTCCATACAAGCCAACCACGGCCTCCAGAAGAA
GATGTTGGCGACCTCGTATTGGGAATCCCCGAACATCGCCTCGCTCCA
GTCAATGACCGCTGTTATGCGGCCATTGTCCGTCAGGACATTGTTGGA

GCCGAAATCCGCGTGCACGAGGTGCCGGACTTCGGGGCAGTCCTCGG
CCCAAAGCATCAGCTCATCGAGAGCCTGCGCGACGGACGCACTGACG
GTGTCGTCCATCACAGTTTGCCAGTGATACACATGGGGATCAGCAATCG
CGCATATGAAATCACGCCATGTAGTGTATTGACCGATTCCTTGCGGTCC
GAATGGGCCGAACCCGCTCGTCTGGCTAAGATCGGCCGCAGCGATCG
CATCCATGGCCTCCGCGACCGGCTGCAGAACAGCGGGCAGTTCGGTTT
CAGGCAGGTCTTGCAACGTGACACCCTGTGCACGGCGGGAGATGCAAT
AGGTCAGGCTCTCGCTGAATTCCCCAATGTCAAGCACTTCCGGAATCG
GGAGCGCGGCCGATGCAAAGTGCCGATAAACATAACGATCTTTGTAGA
AACCATCGGCGCAGCTATTTACCCGCAGGACATATCCACGCCCTCCTA
CATCGAAGCTGAAAGCACGAGATTCTTCGCCCTCCGAGAGCTGCATCA
GGTCGGAGACGCTGTCGAACTTTTCGATCAGAAACTTCTCGACAGACGT
CGCGGTGAGTTCAGGCATGGTGATGTCTGCTCAAGCGGGGTAGCTGTT
AGTCAAGCTGCGATGAAGTGGGAAAGCTCGAACTGAAAGGTTCAAAGG
AATAAGGGATGGGAAGGATGGAGTATGGATGTAGCAAAGTACTTACTTA
GGGGAAATAAAGGTTCTTGGATGGGAAGATGAATATACTGAAGATGGG
AAAAGAAAGAGAAAAGAAAAGAGCAGCTGGTGGGGAGAGCAGGAAAAT
ATGGCAACAAATGTTGGACTGACGCAACGACCTTGTCAACCCCGCCGA
CACACCGGGCGGACAGACGGGGCAAAGCTGCCTACCAGGGACTGAGG
GACCTCAGCAGGTCGAGTGCAGAGCACCGGATGGGTCGACTGCCAGC
TTGTGTTCCCGGTCTGCGCCGCTGGCCAGCTCCTGAGCGGCCTTTCCG
GTTTCATACACCGGGCAAAGCAGGAGAGGCACGATATTTGGACGCCCT
ACAGATGCCGGATGGGCCAATTAGGGAGCTTACGCGCCGGGTACTCG
CTCTACCTACTTCGGAGAAGGTACTATCTCGTGAATCTTTTACCAGATC
GGAAGCAATTGGACTTCTGTACCTAGGTTAATGGCATGCTATTTCGCCG
ACGGCTATACACCCCTGGCTTCACATTCTCCTTCGCTTACTGCCGGTGA
TTCGATGAAGCTCCATATTCTCCGATGATGCAATAGATTCTTGGTCAAC
GAGGGGCACACCAGCCTTTCCACTTCGGGGCGGAGGGGCGGCCGGTC
CCGGATTAATAATCATCCACTGCACCTCAGAGCCGCCAGAGCTGTCTG
GCCAGTGGCTTATTACTCAGCCCTTCTCTCTGCGTCCGTCCGTCTCTCC
GCATGCCAGAAAGAGTCACCGGTCACTGTACAGAGCTCACGAGTTCGT
CACATTTTTCTACAAATGGTGGAGGCGGCGGATTTTAGGCTCAAGTCAT
GACCCTCTGGGTCACTCCAGAATCAGCTAGGTCAACGAATAAGGATGAT
TCTATAGTGCTAAGAATGTAAGGAAGCAGATATTCTGCACCAGAAGAGT
ATTTCTTTATGATTCTGAGAGGAGAGCCTATACTTCTCTGGCTTAGATGG
AAGAGGAGACAAAAACGCATGATTTTTCACCAGTATGCCTATCTCCCAG
ATCTAGATGCAATTCGCAGTCTTTCTGGTGTTTATTTTCAGAGTTCACTG
ACTACTCTGCTAGGATGCGTAATGTAGTAGTAGCTAGAATTTGATTTAAA
CAGACAGTGAAGAAGACAAGGACTGATAATGAGGGAGGGGAAACCTGT
CATAGTGCAGGTGATATATAAGGACAACAGGGAATAGCACCTACAAGGT
CCTAATTAGTGACTTACCTGAGTTCAGACTGAATGCAACTCTGGTAGCT
AGATGCTCTGAAAGCCTACTGCCGGGTTACATGGCCGATGGCCCTACT
TGGCATGAAATTAGTGACTTGTGTTGGTTTTTCGTCCCCACCATTTCGG
ATATTCCGGACTCTCCAGATGCGGGTTATCTGGGGTCGGCAGAACAAC

CACCGAAATGTTCTATAACCCCCGCGAAGCTTGTCCTGTCTCCACATCG
GGTCCGCTAAAGTGATCATTGCTTTGAGCTTCAGGATAAAAGCTCTTTT
CCATCCGGTTAGCATATGTATTTACACCAATTTATCTTCATCTTTCCTGC
ACGCAATTACGGAGGAATATCGTGTTCGACTCATCTCCCACAATGTCTC
TCGCCTTCACGAATCAACCGCCCGAGGAGAGGGTCCCCTACGCCATCC
CTCAATTGGAAGGTGAACGCATTACTATCCCCGGTAGCAAGGGAACCTT
TCGCATTCTTGCAT

Appendix 6: Bidirectional fusion cassette DNA sequences

het KO fusion cassettes were sequenced in both directions using the appropriate FP1N (forward) and FP6N (reverse) primers as described in section 3.3.1.1. Recovered DNA sequences were approximately 1 kb in length. The sequences were aligned with the predicted sequences (Appendix 5) to ensure the sequences matched around the recognition sites of FP2, FP3, FP4 and FP5 as shown in Figure 3.13.

> *het1* fusion cassette forward

ANNNNNNNNNGGTNNNNGNNTCTTAACCTAATCATTCACCAAGTTTTG
ATGTTACTTCTTAAAAGGAGTTCTGCTAATTTTATGAATTGTATATTAAAA
ATTATAATAAATTTAGTTACAGTTTGATTATAGAGCAGTCTAGAACCAGT
AAAAGCAGAGTCTGATTAATCAGAGAATTTGCTTTTCCCACACAATGGT
GCTTTGTTCATGTAATATAGTAGAAAATGTTTAGTTTAGAGTAGTTAGAA
CCTATTACATTTCAGGTATTATCTCTATAAGTGGTCTGACCTGCATTCTG
ATCTCTATAATAAGTAATTACTTAGTTAATTAACTAATAAAGATTATGTATT
TAACTCCGGTTATCACCCCACTCCTTTAGTAATAGCCAGTCTATCATGG
CACTTACTGCTAAAAGCTAAAAGACTACTTACATTCCATCAACTTATCAG
TGATAATCCTACTTTCTTTTTTTTTATGTATGTTGATACTTCGCTATACTA
CACTGATCTATTTGTTATTACTCATATGAGTACAGTTTGAAGCCTAGTGC
CACAACTCTTCTATAATATCCTGGCGCAACCCTGGTTAAATGACAGTTG
GCAGCTCGATACTGACTCGAGTGGAGATGTGGAGTGGGCGCTTACACA
GTACACGAGGACTTCTAGAAAGAAGGATTACCTCTAAACAAGTGTACCT
GTGCATTCTGGGTAAACGACTCATAGGAGAGTTGTAAAAAAGTTTCGGC
CGGCGTATTGGGTGTTACGGAGCATTCACTAGGCAACCATGCATGGTT
ACTATTGTATACCCATCTTAGTAGGAATGATTTTCGAGGTTTATACCTAC
GATGAATGTGTGTCCTGTAGGCTTGAGAGTTCAAGGAAGAAACAGTGCA
ATTATCTTTGCGAACCCAGGGCTGGTGACGGAATTTTCTTAGTCAAGCT
ATCANAGTAAAGAAGAAGGAGCATGTCCAAGTACAATTAGAGACAAATA
TATAGTCGCGTGANCCAGAAGCGGAATTCCTCAGNCTCCGTAAGTCTCT
TGACGACCGT

> *het1* fusion cassette reverse

NNNNNNNNNNNNNNTTGNGTTANANTACTGCAACTAATCTAATTCAGA
GACAATGCTCCATTGAATAGAACCACTTATATCATTCTCTCAAGTACAGA

TACAACAACTTATAGACAGTTCTGACACTGTACATCATATCTCAGGATGT
AATTATGTATTATAGAGCAGTGTAATAATAGAAACAGTCACTTTGCTTATT
GTTGCTTAATGCAACTTCCTCCCTCAGGTTATTCTGCTAAAGAAGCATCC
GTGAAAAAAATCTTTATGGGGATAAGCATGTATTGCTGTTGCATCTCCAC
GTCGTTCAGGCGTATTCACTGCACTAGCCCACAGCCTAGTTGCCGACA
CCTCACTGGCTAGAAACCCCCTTAGGCTGATTACCTCTGATTGCCTTAC
ACATTATTATCATTCTCAGCAAATAGTGATACAATCTCCTATCCCATGCG
GGCGATCAAACACTGAGAGCTTAAGCAAACCTATAGGACAAGTCGGCG
ACTGCTCTATAGAATCATCCTTATTCGTTGACCTAGCTGATTCTGGAGTG
ACCCAGAGGGTCATGACTTGAGCCTAAAATCCGCCGCCTCCACCATTT
GTAGAAAAATGTGACGAACTCGTGAGCTCTGTACAGTGACCGGTGACT
CTTTCTGGCATGCGGAGAGACGGACGGACGCAGAGAGAAGGGCTGAG
TAATAAGCGCCACTGCGCCAGACAGCTCTGGCGGCTCTGAGGTGCAGT
GGATGATTATTAATCCGGGACCGGCCGCCCCTCCGCCCCGAAGTGGAA
AGGCTGGTGTGCCCCTCGTTGACCAAGAATCTATTGCATCATCGGAGAA
TATGGAGCTTCATCGAATCACCGGCAGTAAGCGAAGGAGAATGTGAAG
CCAGGGGTGTATAGCCGTCGGCGAAATAGCATGCCATTAACCTAGGTA
CAGAAGTCCAATGCTTCCGATCTGGTAAAAGATTCACGAGATAGTACCT
TCCTCGAAGTAGGTAGAGCGAGTACCCNNCGCCGTAAGCTCCCTATTG
GGCCCATTCGGGCATCTGTAAGGG

> *het3* fusion cassette forward

NNNNNNNCCNCCNNNNNTCTCCGCGGCCACGGGGGCGATGTTCATCT
CGATCTCGAACGCGTCGTCGGCGGTCGGGAAGGTGGTCATCGGGCAT
GTGGCGGACCGCGCGGGGCGCATCAATGTCTTCCTGCTGACGACGCT
GATGAGCGCGATCGCGACGCTGGGCCTGTGGCTGCCGTCCACGGTAT
CGGGGGGGAAGGCCCTGTTCGTCGCGTTTGCGATGTTCTATGGCGTGT
TCGCTGGGGCGTATGTGAGTTTGTTTCCGGCGACGCTGGTGGAGTTGT
TCGGGGTGGAGCACTTTGCCAGTGTCAATGGGTTTCTGTACATGGTGC
GCGGGTTTGCCGCGCTGGTCGGGACGCCGGTGGCTGGGGCGCTGATC
CGGAGTGCGGGCTCGCATGCTGATGGAAAAGTCATGAGCCAGCTGTTC
TTCCATACGAGTATCTTGGTGGGTGCGTTGCTGGTCGGTGCGACGGTG
GGTGTCGTGTGGGTGAGGATCGAGGCTGCGGTCAGTCAGGCTGGCTT
CAATGGGGGGTCTACTGGAGGGTCTGCTAGAGGGTCTACTGGAGGGT
CCAATAGAAGGTCTAACGGAGGATTCAAATGGAGGATTTGATCTATTCC
ATGTCTCTGTATATAGAAACACTCTCGGCAATCAAACACCCCTCACTTCA
CCATCCCAAACTCTCGGCATCGTATAACCCATCAGCCTCGCACCGGAC
ACTAGGAACGAATACGAGCTTAGTTCGACTAGCCACGGACTACATATAT
AAGCCCATCATCCCCCGCAACACGATCATTTCTCTGGGATTCCTCCGTG
CCCCCAAGCGAAGCAAATCGAGTGGAGATGTGGAGTGGGCGCTTACAC
AGTACACGAGGACTTCTAGAAAGAAAGATTACCTCTAAACAAGTGTACC
TGTGCATTCTGGGTAAACGACTCATAGGAGAGTTGTAAAAAAGTTTCGG

CCGGCGTATTGGGTGTTACGGAGCATTCACTAGCAACCATGCATGGTTA
CTATTGTATACCCATCTTAGTAGAATGATTTTCGAGGTTTA

> *het3* fusion cassette reverse

NNNNNNNNNNNNCCGGNCACCCATCATCGACGTAAAGCAGGGAATACA
CCTCAACGGGGTCGGAGACGACGGACACGAGCCTCTCTGTTTGGACC
GACACGGCAACGCCGGATTTTCGTTCATCAAGACGGCTCGGAAGCCAT
ATAACGAGGTGGTTGCGTGTATCTTGCTCCGGGCGGCTGTGCTGGCTC
CGACCTGTGTTTGCTTGAGGTGAGGATGGCAGATCATGGTCGGTTGGA
GTAAGCTGACGGCGCTGACAGCTCGGATGGTGATTGGGAGCATGATTG
GTCTGCGCCTCGCCAGCTGTCTAGGGATTTGTGGGGTGTGGATGTGGA
GTGTCCTTGGAGTGAGACGGAGGTTGCGGATGATTAGTGTTGCCGGGG
CCTCTCGACCGATGTCTTAGGTTGTGTAGAGGGTATTAGCACGTTTTGC
TTGCTGTTGCGAGAGATCGGTTTGACTGATAACGGTGGACTTTGTAGAA
GGTCGGCAGCTAAGGCGCCTGTATTCTTCTCTATCTAATCCTATGACAC
ACCGGTCCTTATACGTCTACTCGTATCATCTAAGCGAAGTCGACCTTGT
CTCTGTTGCTGGCAGCACCCTTGGCAAGAATGTAGATACATCTAAGTCG
CGCTCCTTGCGAGGCTCTCTTAAAGCTCGCCGGCTATATGCGCTGGCG
TCTTTCGTAATATGTAGAGCGAACTGATCGACACTGTTCTGTCCTTGACA
CGGTCTCACGAAGTTAACCTTCAGGACGGTCAATTTCAGAGAACCGGT
GTCTGGGATGAATCGTTTCCGATTCTTTCATGCAGTAACCGCAGAGATC
AAAGTCTGCGCAGTCATTGCATCTATAGAATCATCCTTATTCGTTGACCT
AGCTGATTCTGGAGTGACCCAGAGGGTCATGACTTGAGCCTAAAATCC
GCCGCCTCCACCATTTGTAGAAAAATGTGACGAACTCGTGAGCTCTGTA
CAGTGACCGGTGACTCTTTCTGGCATGCGGAGAGACGGACGGACGCA
GAGAGAAGGGCTGAGTAATAAGCGCCACTGCGC

> *het4* fusion cassette forward

NNNNNNNNNNNNNNGGNAGGTGATCCNGANAGGGCTTTAAGGTTTTCT
TGTTGTTTTTGTTTCCATTTCTGGATGGTCTGTGGATCGGAGAACCAGC
CGCAGATTGTACGTTGTCTTTGCAGCTCAGCGAATTGATCAGTGTTGTG
TAGATTGACAGGTTCGAGGAGGCATTTAGCAGCAATGGATGAGGATTG
CATTTGTGATGCGATATAGATTCGAGTTGAAAGGATTCTGCGTCGCTGA
AGGTTTGACTGGTAAATGTCGCACCCATCCGCTTATGAGTCACGCTGGC
TTGGATCGGGAGTCTTGGTGGCAGCCATCAAACCATGTCTAGCCGACC
GTTTCTAGCATGCCTCTATACAGATTTTTAGAAATCCAGGATCAAATAGG
TGCATATTCTAGATCCTCAATGAGTTGATACTGCTGTAGCCATGCTGCC
ATACTTGATAGTTCACTAATTTCTACAGATTAATTGTCCTTTTTTCTTCAT
CTATATAATCCAAATACATAACGTTTTTAAGTAGTATCGCTACTAATAAAG
GACCGATTTAATCTCACTTCATACGAGATTTACTACTAGCAGAAGATATA
AATACTAACCGACTTCAAGACCTACAGAGGTTTATGGGTTTCTCCCAAT

GGCTTGCCGTACCAAGCGAGATGCGTCGCTGTGATCGTGAACCTCGTA
TTCTGTCAGCCTTCAGACCTCGGTCTCTTTCCCCCTGTCACACATTTGG
AGTCGGCAAAGATAGTAGCCTTCCAGTCAGAGAGCAATTCAAGATTGAG
AGCCATCATCACACACGCAATAGGTCGAGTGGAGATGTGGAGCGGGCG
CTTACACAGTACACGAGGACTTCTAGAAAGAAAGGATTACCTCTAAACA
AGTGTACCTGTGCATTCTGGGTAAACGACTCATAGGAGAGTTGTAAAAA
AGTTTCGGCCGGCGTATTGGGTGTTACGGAGCATTCACTAGCAACCAT
GCATGGTTACTATTGTATACCCATCTTAGTAGGATGATTTTCGAGGTTTA
TACCTACGATGATGTGNGTC

> *het4* fusion cassette reverse

NNNNNNNNNNTNNCTCTCGCTTTATCACTCCGATCTTCGTGGGCTTTGA
CATTGTGGCCCTCCTGCTGCAGCTCGGCGGTGCAGTCTTGATCACATC
AGCCGATGGTACTAGCAGTGATGCGAAGGACAAGTTCGACCGTGGTCG
CAATATTGCATTGATTGGCGTCATAGTCCAGATGGTCGCCTTTGGGCTA
TTCTCCCTTGCTGCGTTTCGGTTCAACTTCACATCGAAACGGTTTGCGA
AGCCTGTCGACGAGCAATTCGAAATGCTTGCGAGCAATGATGCAGGAC
CTGGTGGCCGGGAAAAAAGTGCCAACTGGAATGCGCTGCTGAGAGTG
GTGAACTTTTCTACTCTGATGATTCTTGTAAGTGTTTCATACAGATCGTC
TCCCGTGTTAGATCATCGCTGAATCCTGCCTACAGATACGATCGGTCTA
CCGTCTGGTTGAATTCACCGAAGGCAAGAACGGATATATCAATTTACAT
GAGTGGTGCCTGTATGTCTTTGACGCATTGCCAATCTTCCCATGTGCGG
CATTGTTCGTCTATTGGCATCCGGCCAAATACCTCCCATATCTGGGATT
TCGGCTGCCAAAGCATGCACGGTAGATATTGCGTGGGGGCTTTATCAC
GGTAGCACCGACCTACCCTTCCATTGAGTTCGTGAGAAGGTCTGGAAC
ATGGCCAACTTGCATTTGGGATCTTTGTGTGGAATATATCCAATCTTGCT
ATAGAATCATCCTTATTCGTTGACAATGGCTGATTCTGGAGTGACCCAG
AGGGTCATGACTTGAGCCTAAAATCCGCCGCCTCCACCATTTGTAGAAA
AATGTGACGAACTCGTGAGCTCTGTACAGTGACCGGTGACTCTTTCTGG
CATGCGGAGAGACGGACGGACGCAGAGAGAAGGGCTGAGTAATAAGC
GCCACTGCGCCAGACAGCTCTGGCGGCTCTGANTGCAGTGGATGATTA
TTATCCGGGACCGGGCCGCCCTCCGCCCGAAGTGAAAGGCTGTGTGC
CCTCGTTGACCAGATCTATNCATCATCGGAN

> *het6* fusion cassette forward

NNNNNNNTNNNCTCTCGACTGCGGATTGGCGAGATTGAGGACGCGCTG
GCGGGAGATGTTCAAGAATGTTTTCTCAGCCCCTCAATAAGAGGAATGC
CGATAATGAGACCTTTCCGGGATCAGTCGAGGACCGTTTTCACATGAAA
TGCCCCAGTACATGTCAAATCACAGTGCCACTCAGCGTCCTTGCTCCTC
CTGCACCGAATGAAGCGTTCCGTCTGGCAACGCCAACGGGGGAGAATC
TGCCCATTACTCTGGTGGTCCGGATATGTGCCCCTTGTGGTTGCAGAA

GGTCGTAAGCTCGTGGGCCTCCCTCGAGCAAGTCAGAATACCAGGAAG
AGCATGCCTCCAGCCTCGCCAAGCAGAGTATTAAGTTTAAGGCAAACAG
CTAAGAGGTCTTTCTCTCAAGAGATCATCCTACTACTTCTACTCCGTAGA
CACTTTGATACTAGATTATTGTCTAATTAGAGTAGCTATAGGCTTCTAGG
ATGGAAGGAATGCGGGGGATCCGGAGATCGAGCAGCAGTGGGGTCTG
GTATAGGCTACGTGCTATGATGCTACGCTTGCCAAGGATAATGAAATAT
ATTTACAACGATATACTTGAATGCTGTATTACTTGTCGAGGCATAGGCTT
CTCTCAGCCATAAAACCACTAAGCTAAAAAGGCGTCTAGACCTCCGTTT
CTAGTATAGTAGGAATTATAAGGCATCTTCTGGTTGACAGCTTCATTAGC
CTTCGAGCCACATGGTCAAAGCGCTCGGACGGCCAACACTACTTAAAT
CAGTCTATCTCCACCTCGAATTTGCATCCTCACACTTCACCAGATCGTA
CGTAGACCTCCATATTAGCGATCCTGTCTGCCCGATCGAGTGGAGATGT
GGAGTGGGCGCTTACACAGTACACGAGGACTTCTAGAAAGAAAGATTA
CCTCTAAACAAGTGTACCTGTGCATTCTGGGTAAACGACTCATAGGAGA
GTTGTAAAAAAGTTTCGGCCGGCGTATTNNTGTTACGGAGCATTCACTA
NCAACCATGCATGGTTACTATGTAT

> *het6* fusion cassette reverse

NNNNNNNNNNNNNTCTGNTTAATACTCCTTTCCTGACAAAGACCACTAC
GGTAGAAAGGTAGCTCCGGGTTTACCAATCTTGATCCGCCAAGGACAA
CTCAACCGGTGACTTACAAAGGTCATTTCGCTAGGGACTTGTCTCGAGG
AGATCCTGATGGGCTGTGGAGGCGCTGTGGTAATACTTCGGAAGTTATT
TATGGGTTAATGAGATGGTCAAAAGGCGTCTGATGAATTTAATCCTTAAC
CTGCCGGAGTCCTCAAAGAACAATTGAACATATCAATCGATGTCTCGGA
GAGCACTGAAGTATAGCATCCAGACAGCAGCAGGAAAGGATGCTTAGG
AGGGATATCATATCATTGACAAACCACTGCCATATATGATCAGCAAAACT
CACCATTGCTCCTGTCCAAAGGGAGCTGGTATGCCAACTCCCTGCTGC
TTTTCCCAAGAAGGAACAGCAGGCTGATAAATCTATCTCCTACCGGTCT
TGCGCCAGGGGTTATAGACTGAAATAGATATTAATTTAGATGCCTGTATT
GATACGCCACTTCTTCAACGCAAACTTTATGGGGCTAAAGAGAGGTAAG
CTGTCTATGCTGTTGTAGCATTGTTGGCTGAACAGAAGAGGGTGCTATG
GAGAGGTAGCAAAGTTTCACAGGAATACAGGCCTGTCGTTCCTGGACA
TGGAGCCAGGGCATCTGTTTCCGCGCTACGTGGCCTGGACCTGGTATA
AACGTTTATATTGATGGTTCAACGCATAGTCATTTACTGTCATCACAGGC
TACAACCATTACTACTATAGAATCATCCTTATTCGTTGACCTAGCTGATT
CTGGAGTGACCCAGAGGGTCATGACTTGAGCCTAAAATCCGCCGCCTC
CACCATTTGTAGAAAAATGTGACGAACTCGTGAGCTCTGTACAGTGACC
GGTGACTCTTTCTGGCATGCGGAGAGACGGACGGACGCAGAGAGAAG
GCTGAGTAATAAGCGCCACTGCGCCAGACAGCTCTGGCGGCTCTGAGT
GCAGTGGATGATTATTAATCCCGGN

> *het7* fusion cassette forward

NNNNNNGNTNNGGCAGATTCAGTCTTCAGGGGACTCCGGCAATACAGC
CCTGCTTGGCACCTCCAACAGACCCTTGGCATGACTCGCTTGGGGGCC
AAGGGTCTTCCATTCGCGGAGAAGAGGAGGCTACAACTGACTAGCAAA
GGCTAGCCGAGGCGCTTAGACGGGGAGAGACATATCGTTGGTCACGG
CGGGTGCATTTATCCACATTGACCGATGTTAAGTGTGGTTTAGTGGTAA
ATAGTCCCTGGAAGAGAGATGACCAGAGTTTTATGTTCACCAGAGACAT
GCAGGCAAGAGATTGCATAAATTAATGCTATTCGGACGCTGTTTCTATC
CATTCGCCGCTAACACATATACTACATTCAATGAAATCTTCAACCGAATC
GCATCCCGAGAGCAAGTGGAAGCAAGCATTACAATTCGAATCAAGGCC
AGTTTGATCTGGCCTCAGGAATAGGCAGTTAGGGTCTACAATAAACAAG
TATGAAACTATGTATATGAGCTGAGCCGAAACAATGTATCTCGCTGCTA
ACCATTGATCAGATCTAGTCCGTTTTGATGTCTCCGCGACAGCGCCTGA
TTGCTCTCCAGAACCAACTTGTATCAGGGCATTTCAACCTTTCTCCTGTT
TTCTTTCGCTGTCGCTATGAATCCTGCTACTTGTCTTCTTCGACCAATAG
GCAAAGCACCGTGCTGTGATGGCCGGGCGATCTCGTATTGAATCAGCC
GATCAATCTTAGCCTCCACAGCCTCAACCCCACATTCTCTCTGACAAAC
TTTTGATTTCTTCCTTTTTCACTCTGGAGGGCATTCTTATTTTGACCGCA
AGTCGAGTGGAGATGTGGAGTGGGCGCTTACACAGTACACGAGGACTT
CTAGAAAGAAGGATTACCTCTAAACAAGTGTACCTGTGCATTCTGGGTA
AACGACTCATAGGAGAGTTGTAAAAAAGTTTCGGCCGGCGTATTGGGT
GTTACGGAGCATTCACTANCAACCATGCATGGTTACTATTGTATACCCAT
CTTAGTAGATGATTTTCGAGGTTTA

> *het7* fusion cassette reverse

NNNNNNNNNNNNGACATATATTCGCTGTTGTGGAACCAACCAACCAGG
AATTCAATTCGCGTACAACTGGAAGAATATTGGTTTGCGGTTGACCGAG
TCCGGAAGCGTGAAGATCGAAAATGTTCGTGCATCTTGGACGGATGCA
CTGGGATGGGACGCAACAACCAAACGACCTCTGGCAGAGATTCTCACC
ATCCCTTTTGCCTCGTTGTTTCTGCCTACGTAAGTGCCATAGGACTCCT
GCTTCCCCACAGTCCGTACTGACCATACTAGGATTCAGCTGGTGTTCAG
CAATTTCTACCTCGGCATCGCCATCGGCGCCCTTGAATTCGCAGCCAA
GTACACCACCACGTCCACACGCGCCTGGCCCTATGGCGGGGACAACAA
AGAGAGAGCCACCGATGAGTTCTACATTCTCGAGCGATACGGGAACTT
CTTTGCACACCTCCGTGCTGTGGAAGCACTGGCCGACAAAGCCGGCGA
TCGACTCTCGGAGCTGTACGAGCGCCATTCAGGCGATCGGTTGAAGGT
GACAGCGGAGGAACGAGGGGAGGTGGCTGAATGGGTCGCCAGTGTGA
AGGTGGTCACTACGGATGTCGGACTCCGAGTCACGTCTGGTATATTCG
AGGTGACTGGCGCTCGAGCAACGTCGCTGAAGGTGGGACTGGACCGG
TTCTGGCGGGATATCAGGACGCACACTTTGCATGACCCTGTGGCATAC
AAGAATCGGGAGCTCGGCAGATATGTGCTGCTGCATGAGTACCCGGAG

CCTACGTGGTATACTTAAACCCTAGGACTCGGCCTTTGAGTTCAAGCAA
TGGGAGTATGCTGGATGTCGATCTATAGAATCATCCTTATTCGTTGACC
TAGCTGATTCTGGAGTGACCCAGAGGGTCATGACTTGAGCCTAAAATCC
GCCGCCTCCACCATTTGTAGAAAAATGTGACGAACTCGTGAGCTCTGTA
CAGTGACCGGTGACTCTTTCTGGCATGCGGAGAGACGGACGGACGCA
GAGAGAAGGGCTGAGTAATAGCGCCACTGCGCCAGACA

> *het8* fusion cassette forward

NNNNNNNNNNNNNNTTCGCTTACATAGGCGCAGGGGCTGAGGTGGTTA
ATAGCATATTTGTATCTAGCATATTGATATGGTACATATAGGGTTCAAGG
ATGGTGATTAAAATTATTCCTACCATATAGTGATTATCGAAGATGAATCG
ATTTATCTATCTATAAATAGGTACGGAGTACCACTACTTTTATACTAATCC
ATGACCATAACTGAGATGATTTTCACAGTTCTGTTATGTAACTCTAGTTG
CACTTAAAAGGATTACTAAGACAATAAAGGAGATCTATAGGGAGAGATG
AACAGAATCTTACATAGCGGAATTACTAGCAATCTACATATAATTATTTC
AGTCTTGCCCCAATGGATTACTATGCCTAATATTCTTGGCAGTGAGATT
GATCACTCTTTCCCCGCCAGCCCCGCCTTTGATAAGCGCCGTCAGACA
GACCACCCTTTCACCCTCTTCTTCATATGACTTGTCTTTGGAAGTCTCAA
TCGGGGCAGCAGACTGATATTGGACCAGAACAGAAAAGTTCTATAACG
CTCACACACGCTACTTAACCCTGAGACTGGGGAGCCACCACCCAAGTC
CGTTGGTGAGACTTCGGACATTCAACATTTGGCAGATCTGGTGGGTTGC
AACTTTTCCTCATATATGAATGAACTTCCGAGCCAGTCAAGTTGCCGTA
GCCATTCACGCCGCTGCAGTGTCAGCGGCGCATGCATGAACGAGGCG
GGTTCTACAGCGGCCTGACGGAAGTACTTGACGGCATTTTGGGCTTCT
GGCAGTTGACAGGTAATAGTTCAGAACGACTTCGAGTGGAGATGTGGA
GTGGGCGCTTACACAGTACACGAGGACTTCTAGAAAGAAAGGATTACCT
CTAAACAAGTGTACCTGTGCATTCTGGGTAAACGACTCATAGGAGAGTT
GTAAAAAAGTTTCGGCCGGCGTATTGGGTGTTACGGAGCATTCACTAG
GCAACCATGCATGGGTTACTATTGTATACCCATCTTAGTAGGAATGATTT
TCGAGGTTTATACCTACGATGA

> *het8* fusion cassette reverse

CNNNNNNNNNNNGNNNNNAATGCGTTCACCTTCCAATTGAGGGATGGC
GTAGGGGACCCTCTCCTCGGGCGGTTGATTCGTGAAGGCGAGAGACAT
GGTGGGAGATGAGTCGAACACGATATTCCTCCGTAATTGCGTGCAGGA
AAGATGAAGATAAATTGGTGTAAATACATATGCTAACCGGATGGAAAAG
AGCTTTTATCCTGAAGCTCAAAGCAATGATCACTTTAGCGGACCCGATG
TGGAGACAGGACAAGCTTCGCGGGGGTTATAGAACATTTCGGTGGTTG
TTCTGCCGACCCCAGATAACCCGCATCTGGAGAGTCCGGAATATCCGA
AATGGTGGGGACGAAAAACCAACACAAGTCACTAATTTCATGCCAAGTA
GGGCCATCGGCCATGTAACCCGGCAGTAGGCTTTCAGAGCATCTAGCT

ACCAGAGTTGCATTCAGTCTGAACTCAGGTAAGTCACTAATTAGGACCT
TGTAGGTGCTATTCCCTGTTGTCCTCATATATCACCTGCACTATGACAG
GTTTCCCCTCCCTCATTATCAGTCCTTGTCTTCTTCACTGTCTGTTTAAA
TCAAATTCTAGCTACTACTACATTACGCATCCTAGCAGAGTAGTCAGTGA
ACTCTGAAAATAAACACCAGAAAGACTGCGAATTGCATCTAGATCTGGG
AGATAGGCATACTGGTGAAAAATCATGCGTTTTTGTCTCCTCTTCCATCT
AAGCCAGAGAAGTATAGGCTCTCCTCTCAGAATCATAAAGAAATACTCT
TCTGGTGCAGAATATCTGCTTCCTTACATTCTTAGCACTATAGAATCATC
CTTATTCGTTGACCTAGCTGATTCTGGAGTGACCCAGAGGGTCATGACT
TGAGCCTAAAATCCGCCGCCTCCACCATTTGTAGAAAAATGTGACGAAC
TCGTGAGCTCTGTACAGTGACCGGTGACTCTTTCTGGCATGCGGAGAG
ACGGACGGACGCAGAGAGAAGGGCTGAGTAATAAGCGCCACTGCGCC
AGACAGCTCTGGCGGCTCTGAGGTGC

Appendix 7: PCR Screening DNA Sequences of *het* Gene KO *A. fumigatus* Mutant Strains Using Primers SF and SR

PCR products of gDNA from transformants amplified using primers FP1N and FP6N were used as template material for PCR reactions using primers SF and SR, and these products were then sequenced using primers SF and SR. SR primers targeted between the FP1N and FP2 sites, and SF primers targeted between FPN6 and FP5 sites (Figure 2.5). The primers provided sequence data that included the 50 bp fusion primer target sites (FP2, FP3, FP4 and FP5), and provided a stronger DNA sequencing signal downstream of these sites.

> *het3* KO 3-1 (SF-3)

NNNNNNNNNNCTTCCCNNNNNACACGATCATTTCTCTGGGANTCCTC
CGTGCCCCCAAGCGAAGCAAATCGAGTGGAGATGTGGAGTGGGCGCT
TACACAGTACACGAGGACTTCTAGAAAGAAGGATTACCTCTAAACAAGT
GTACCTGTGCATTCTGGGTAAACGACTCATAGGAGAGTTGTAAAAAAGT
TTCGGCCGGCGTATTGGGTGTTACGGAGCATTCACTAGGCAACCATGC
ATGGTTACTATTGTATACCCATCTTAGTAGGAATGATTTTCGAGGTTTAT
ACCTACGATGAATGTGTGTCCTGTAGGCTTGAGAGTTCAAGGAAGAAAC
AGTGCAATTATCTTTGCGAACCCAGGGGCTGGTGACGGAATTTTCATAG
TCAAGCTATCAGAGTAAAGAAGAGGAGCATGTCAAAGTACAATTAGAGA
CAAATATATAGTCGCGTGGAGCCAAGAGCGGATTCCTCAGTCNCGTAG
GTCTCTTGACGACCGTTGATCTGCTTGATCTCGTCTCCCGAAAATGAAA
ATAGACTCTGCTAAGCTATTCTTCTGCTTCGCCGGAGCCTGAAGGGCGT
ACTAGGGTTGCGAGGTCCAATGCATTAATGCATTGCAGATGAGCTGTAT
CTGGAAGAGGTAAACCCGAAACGCGTTTTATTCTTGTTGACATGGAGCT
ATTAAATCACTAAAAGGCACTCTTTTGCTGCTTGGACAAATGAACGTATC
TTATCGAGATCCTTGAACACCATTTGTCTCAACTCCGGAGCTGACATCG
ACACCCAACGATTCTTATATCCAGATTCGTCAAGCTGTTTGATGATTTCA
GTAACGTTAAGTGGATTCCCGGTCGGCATCTACTCTATTCCTTTGCCCT
CGGACGAGTGCTGGGGGCGGTCGGNTTTCCACTATCGGTCGAGTACTT
CTCTCAACAGCCATCGGTTCAAGACGGCCGNCGCTTTCTGCGGGGCGA
ATTTGTGTACNCCCGACATTCCCGGGCTCCTGAACTGGACGAANTGCG
TTCCCATCGACCCTGCCGCCCAANCN

> *het3* KO 3-1 (SR-3)

NNNNNNNNNNGNNNNNNNNNNTTTNNNAGNNCNGGTGTCTGGGATG
AATCGTTTCCGATTCTTTCATGCAGTAACCGCAGAGATCAAAGTCTGCG
CAGTCATTGCATCTATAGAATCATCCTTATTCGTTGACCTAGCTGATTCT
GGAGTGACCCAGAGGGTCATGACTTGAGCCTAAAATCCGCCGCCTCCA
CCATTTGTAGAAAAATGTGACGAACTCGTGAGCTCTGTACAGTGACCGG
TGACTCTTTCTGGCATGCGGAGAGACGGACGGACGCAGAGAGAAGGG
CTGAGTAATAAGCGCCACTGCGCCAGACAGCTCTGGCGGCTCTGAGGT
GCAGTGGATGATTATTAATCCGGGACCGGCCGCCCCTCCGCCCCGAAG
TGGAAAGGCTGGTGTGCCCCTCGTTGACCAAGAATCTATTGCATCATCG
GAGAATATGGAGCTTCATCGAATCACCGGCAGTAAGCGAAGGAGAATG
TGAAGCCAGGGGTGTATAGCCGTCGGCGAAATAGCATGCCATTAACCT
AGGTACAGAAGTCCAATTGCTTCCGATCTGGTAAAAGATTCACGAGATA
GTACCTTCTCCGAAGTAGGTAGAGCGAGTACCCGGCGCGTAAGCTCCC
TAATTGGCCCATCCGGCATCTGTAGGGCGTCCAAATATCGTGCCTCTCC
TGCTTTGCCCGGTGTATGAAACCGGAAAGGCCGCTCAGGAGCTGGCCA
GCGGCGCAGACCGGGAACACAAGCTGGCAGTCGACCCATCCGGTGCT
CTGCACTCGACCTGCTGAGGTCCCTCAGTCCCTGGTAGGCAGCTTTGC
CCCGTCTGTCCGCCCGGTGTGTCGGCGGGGTTGACAAGGTCGTTGCG
TCAGTCCAACATTTGTTGCCATATTTTCCTGCTTCTCCCCAACCAGCTGC
TCTTTNNTTTCTCTTNCTTTCCCATCTTCAGTATNNTTCATCTCCCATCCA
AGAACCTTTATTTCCCTTAGTAAGTTACTTTGGCTACATCCATACNCCAT
CCTTCCCATCCCTTANNCTTTGGAACTTTCAG

> *het3* KO 3-2 (SF-3)

NNNNNNNNCNNNNNNCCCCCNNNAANNNNGATTCAATTTNNTCTGGGA
TTCCTCCGTGCCCCCAAGCGAAGCAAATCGAGTGGAGATGTGGAGTGG
GCGCTTACACAGTACACGAGGACTTCTAGAAAGAAGGATTACCTCTAAA
CAAGTGTACCTGTGCATTCTGGGTAAACGACTCATAGGAGAGTTGTAAA
AAAGTTTCGGCCGGCGTATTGGGTGTTACGGAGCATTCACTAGGCAAC
CATGCATGGTTACTATTGTATACCCATCTTAGTAGGAATGATTTTCGAGG
TTTATACCTACGATGAATGTGTGTCCTGTAGGCTTGAGAGTTCAAGGAA
GAAACAGTGCAATTATCTTTGCGAACCCAGGGGCTGGTGACGGAATTTT
CATAGTCAAGCTATCAGAGTAAAGAAGAGGAGCATGTCAAAGTACAATT
AGAGACAAATATATAGTCGCGTGGAGCCAAGAGCGGATTCCTCAGTCT
CGTAGGTCTCTTGACGACCGTTGATCTGCTTGATCTCGTCTCCCGAAAA
TGAAAATAGACTCTGCTAAGCTATTCTTCTGCTTCGCCGGAGCCTGAAG
GGCGTACTAGGGTTGCGAGGTCCAATGCATTAATGCATTTGCAGANGA
GCTGTATCTGGAAGAGGTAAACCCGAAACGCGTTTTATTCTTGTTGACA
TGGAGCTATTAAATCACTAGAAGGCACTCTTTGCTGCTTGGACAAATGA
ACGTATCTTTATCGAGATCCTTGAACACCATTTGTCTCAACTCCGGGAG

CTGACATCGACACCAAACGATCTTTATATCCAGATTCGTTCAAGCTGTTT
GATGATTTTCAGTAACCGTTAAGTGGATCCCGGGTCGGCATCTACTCTA
TTCCTTTTGCCCTTCGGACGAGTTGCTGGGACGTCGGTTTCCACTATCG
GCGAGTACTTCTACCACAGCCATCGGTCCAGACGGGCCGCCGNTNTG

> *het3* KO 3-2 (SR-3)

CNNNNNNNNGNNANNGNNNAATTNNNNAGAACCGGTGTCTGGGATGAA
TCGGTTTCCGATTCTTTCATGCAGTAACCGCAGAGATCAAAGTCTGCGC
AGTCATTGCATCTATAGAATCATCCTTATTCGTTGACCTAGCTGATTCTG
GAGTGACCCAGAGGGTCATGACTTGAGCCTAAAATCCGCCGCCTCCAC
CATTTGTAGAAAAATGTGACGAACTCGTGAGCTCTGTACAGTGACCGGT
GACTCTTTCTGGCATGCGGAGAGACGGACGGACGCAGAGAGAAGGGC
TGAGTAATAAGCGCCACTGCGCCAGACAGCTCTGGCGGCTCTGAGGTG
CAGTGGATGATTATTAATCCGGGACCGGCCGCCCCTCCGCCCCGAAGT
GGAAAGGCTGGTGTGCCCCTCGTTGACCAAGAATCTATTGCATCATCG
GAGAATATGGAGCTTCATCGAATCACCGGCAGTAAGCGAAGGAGAATG
TGAAGCCAGGGGTGTATAGCCGTCGGCGAAATAGCATGCCATTAACCT
AGGTACAGAAGTCCAATTGCTTCCGATCTGGTAAAAGATTCACGAGATA
GTACCTTCTCCGAAGTAGGTAGAGCGAGTACCCGGCGCGTAAGCTCCC
TAATTGGCCCATCCGGCATCTGTAGGGCGTCCAAATATCGTGCCTCTCC
TGCTTTGCCCGGTGTATGAAACCGGAAAGGCCGCTCAGGAGCTGGCCA
GCGGCGCAGACCGGGAACACAAGCTGGCAGTCGACCCATCCGGTGCT
CTGCACTCGACCTGCTGAGGTCCCTCAGTCCCTGGGTAGGCAGCTTTG
CCCCGTCTGTCCGCCCGGTGTGTCGGCGGGGTTGACAAGGTCGTTGC
GTCAGTCCAACATTTGTTGCCATTATTTTTCTTGCCTCTCCCCCACCAGC
CTGCTCTTTTTCTTTTTCNNTTTTCTTTTTCCCATCTTCAGTTATATTCCAT
CTTCCCAATCCAAGNAACCTTTTATTTCCCCTAAGTAAGTACTTTGCTAC
ATTCAATACNNCCATCCTTTCCCANTCCCNN

> *het3* KO 3-4 (SF-3)

NNNNNNNNNNNNNNNNNNNNNNCCCCNAGCACGATCATTTCTCTGGGA
TTCCTCCGTGCCCCAAGCGAAGCCGCAAAATCGAGTGGAGATGTGGAG
GGGGCGCTTACACAGTACACGAGGACTTCTAGAAAGAAGGATTACCTC
TAAACAAGTGTACCTGTGCNTCTGGGTAAACGACTCATAGGAGAGTTGT
AAAAAGTTTCGGCCGGCGTATTGGGTGTTACGGAGCATTCACTAGGCA
ACCATGCATGGTTACTATTGTATACCATCTTAGTAGGAANTGATTTCGAG
GTTTATACCTACGATGAATGTGTATCCTGTAGGCTTGAGACGNCAAGGA
AGAAACATGCAATTATCTTTGCGAACCCAGGNGCTGGTGACGGAATTTT
CCATAGTCAAGCTATCAGAGTAAAGAAGAGGAGCNTGTCAAAAGTACAA
TTAGAGACAAATATATAGTCGCGTGGAGCCAAAAGCGGATTCCTCAGTC
TCTGTAAGTCTTNNGACGACCGTTGATTN

> *het3* KO 3-4 (SR-3)

ANNNNNNNNNNNNNNNNCNNNTTTNAAAGAACCGGTGTCTGGGATGA
ATCGTTTCCGATCCTTTCATGCGTAACCGCAGAGATCAAAGTCCGCGCA
GTCATTGCATCTATAGAATCATCCTTATTCGTTGACCTAGCTGATTCTGG
AGTGACCCAGAGGGTCATGACTTGAGCCAAAATCCGCCGCCTCCACCA
TTTGTAAAAAAATGTGACGAACTCGTGAGCTCTGTACAGTGACCGGTGA
CTCTTTCTGGCATGCGGAGAGACGGACNGACGCAGAGAGAAGGGTGA
GTAATAAGCCACTGGCCAGACAGCTCTGGCGGCTCTGAGGTGCAGTGG
ATGATTATTAATCCGGGACCGGCCGCCCCTCCGCCCCGAAGTGGAAAG
GCTGGTGTGCCCCTCGTTGACGAAGAATCTATTGCATCATCGGAGAAAT
ATGGAGCTTCATCGAATCACCGGCAGTAAGCGAAGGAGAATGTGAAGC
CAGGGGTGTATAGCCGTCGGCGAAATAGCATGCCATTAACCTAGGTAC
AGAAGTCCAATTGCTTCCGATCTGGTAAAAATTTCACGAGATAGTACCTT
CTCCGAAGTGGGTAGAGCGAGTACCCGGCGCGTAAGCTCCCTAATTGG
CCCATCCGGCATCTGTTAGGGCGTCCAAATATCGTNGCCTNTCCTGCTT
TGCCCATTGTATGAAACCGGAAAGGCCGCTCAGGAGCTGGCCAGCGG
CGCAGACCGGGAACACAAGCTGGCAGTCGACCCATCCGGTGCTCTGA
CTCGACCTGCNTGAGGTCCCTCAGTCCCTGGTAAGCAGCTTTGTCCCG
TCTGTCCGCCCGGTGTGTCAGCGGGGTTGACAGNGTCGTTGCGTCTGT
CCANACATTTGTTGCCNTATTTTTCCTGCTCTCCCGCCAGCCGCTTTTTT
TTTTCTCTTTCTTTTCCCATNCNTCCTATATAT

> *het3* KO 3-6 (SF-3)

NNNNNNNNNNNNNNNNNCNCNCNNGGCACGACATTTTCTCTAGGGAT
TCCTCCGTGCCCCAAAGCGAAGCCGCAAACCGAGTGGAGATGTGGAGT
GGGCGCTTTACAGTACACGGGGACTTCTAGAAAGAAGGATTACCTCTAA
ACAAGTGTACCTGTGCATTCTGGGTGAAACGACTCATAGGAGAGTTGTA
AAAAAGTTTCGGCCGGCGTATTGGGTGTTACGGAGCATTCACTAGGCA
ACCATGCATGGTTACTATTGTATACCATCTTAGTAGGAANTGATTTCGAG
GTTTATACCTACGATGAATGTGTGTCCTGTAGGCTTGAGAGTTCAAGGA
ANAAAACATGCAATTATCTTTGCGAACCCAGGNGCTGGTGACGGAATTT
TCATAGTTGTGCTATCAGAGTAAAGAAGAGGAGCATGTCAAAGTACAAT
TAGAGACAAATATATAGTCGCGTGGAGCCAAGAGCGGATTCCTCAGTCT
CGTAGGTCTCTTGACGACCGTTGATCTGCTTGATCTCGTCTCCCGAAAA
TGAAAATAGCTCTGCTAAGCTATTCTTCTCTTCGCCGGAGCCTGNAAGG
CGTTACTAGGTTGCAGTCAATGCATTAATGCATTGCAGATGAGCTGTAT
CTGGAAGAGGTAAACCCGAAAAACGCGTTTATTCTTGTTGACATGGAGC
TATTAAATCACTAGAAGGCATCTTTGGCTGCTTTGGACAAATGAACGTAT
CTTATCGAGATCCTGAACATCCANTTTGTCTCAACTCCGGAGNNGACAT
CGACACCAACGATCTTTATATCCAGAAA

> *het3* KO 3-6 (SR-3)

NNNNNNNNNNNNAGNNNATNNANGAGAACCCGGTGTCTGGGATGAATC
GTTTCCGATTCTTTCATGTGTAACCGCAGAGATCAAAGTCTGCGCAGTC
ATTGCATCTATAGAAAGCATCCTTTTTCGTTGACCNAGCTGATTCTGGAG
TGACCTAGAAGGTCATGACTTGAGCCTAAAATCCGCCGCCTCCACCATC
TGTAAAAAAATGTGACGAACTCGTGAGCTCTGTACAGTGACCGGTGACT
CTTTCTGGCATGCGGAGAGACGGACGGACGCAGAGAGAAAGGGCTGA
GTAATAAGCCACTGGCCAGACAGCTCTGGCATCTCTGAGGTGCAGTGG
ATGATTTTTAATCCGGGACCGGCCGCCCCCTCCGCCCCGAAGTGGAAA
GGCTGGTGTGCCCCTCGTTGACCAAGAATCTATTGCATCATCGGGAGA
ANATGGAGCTTCACGAATCACCGGCAGTAAGCGAAGAAAAATGTGAAG
CCAGGGGGTGTATAGCCGTCGGCGAAAATAGCATGCCGTTTAACCTAG
GGTCAGNGGTCTAATCGNNTC

> *het4* KO 4-3 (SF-4)

CNNNNNNNNNNTCANANNNNNNNGNNNTCCAGTCAAGAGCAATTCAA
GATTGAGACCATCATTACCACACNCAATAGGTCGAGTGGAGATGTGGA
GTGGGCGCTTACACAGTACACGAGGACTTCTAGAAAGAAGGATTACCT
CTAAACAGTGTACCTGTGCATTCTGGGTAAACGACTCATAGGAGAGTTG
TAACAAAGTTTCGGCCGGCGTATTGGGTGTTACGGAGCATTCACTAGG
CAACCATGCATGGTTACTATTGTATACCATCTTANGTAGGAANTGATTTC
GAGGTTTATACCTACGATGAATGTGTGTCCTGTAGGCTTGAGAGTTCAA
GGAAGAAACATGCAATTATCTTTGCGAACCCAGGNGCTGGTGACGGAA
TTTTCATAGTCAAGCTATCAGAGTAAAGAAGAGGAGCATGTCAAAGTAC
AATTAGAGACAAATATATAGTCGCGTGGAGCCAAGAGCGNGATTCCTCA
GTCCGTAGGTCTCTTGACGACCGTTGATCTACTTGATCTCGTCTCCCGA
AAATGAAAANTAGCTCTGCTAAGCTATCCTTCTCTTCACTGGANCCTGN
AAGGCGTTACTAGGNTTGTCAGTCGATGTATTAATGCATTGCAGATGAG
CTGTATCTGGAAGAGGTAAACCCGAAAACGCGTTTTATTCTTGTTGACA
TGGAGCTATTAAACACTAGAAGGCACTCTTTGCTACTTGGACAAAGAAC
GTATCTTATCGAGATCCTGAATACCATTNTGCTCAATTCCGGAGCTGAC
ATCGACANCAACGATCTTATATCCAGATTCGTCAAGCTGTTTGATGANTT
CAGTAACGCNAAGTGGATCNCGCGGTCGGCATCTGCTCTTTCCTTGCC
CGCGGACGAGNT

> *het4* KO 4-3 (SR-4)

ANNNNNNNNNNTANCANNNNNNGNNTNGTGNGAAGGTCTGGAACATGG
CCAACTTGCATTTAGGATCTTNGTGTGGAATATATCCAATCTTGCTATAG
AATCATCCTTATTCGTTGACCTAGCTGATCCTGGAGTGACCCAGAGGGT
CATGACTTGAGCCTANAATCCGCTCGCCTCCACCATTTGTAGAAAAATG

TGACGAACTCGTGAGCTCTGTACAGTGACCGGTGACTCTTTCTGGCATG
CGGAGAGACGGACGGGCGCAGAGAGAAGGGCTGAGTAATAAGCCACT
GGCCAGACAGCTCTGGCGGCTCTGAGGTGCAGTGGATGATTATTAGTC
CGGGACCGGCCGCCCCTCCGCCTCGAAGTGGAAAGGCTGTGTGCCCC
TCGTTGACCAAGAATCTATTGCATCATCGGAGAATATGGAGCTTCATCG
AATCACCGGCAGTAAGCGAAGGAGAATGTGAAGCCAGGGGTGTATGGC
CGTCGGCGAAATAGCATGCCATTAACCTAGGTACAGAAGTCCAATTGCC
TCCGATCCAGTAAAGGATTCACGAGATAGTACCTTCTCCGAAGTAGGTA
GAACGAGTACCTNGCGCGTAAGCTCCCTAATTGGCCCATCCGGCATCT
GTAGGGCGTCCAAATATCGTGTCCTCTCCTGCTTTGCCCGGTGTATGAA
ACCGGAAAGGCCGCTCAGGTGCTGGCCAGCGGCGACAGACCCGGGAA
CACAAGCTNGCAGTCGACCCATTCGGNTATCTGCACTCGACCTGCTGA
CGGCCTTCGCACACTGGTAGCNAG

> *het4* KO 4-4 (SF-4)

NNNNNNNNNNNNNTNNNTTGNANNNTTAGNNGCCTTCCAGTCAGACAG
CANTTAAGATTGAGAGCCATCANCACTACACGCAATAGGTCNAGTGGAG
ATGTGGAGTGGGCGCTTACACAGTACACGAGGACTTCTAGAAAGAAGG
ATTACCTCTAAACAAGTGTACCTGCGCATTCTGGGTAAACGACTCATAG
GAGAGTTGTAAAAAAGTTTCGGCCGGCGTNATTGGGTGTTACGGAGCA
TTCACTAGGCAACCATGCATGGTTACTATTGTTACCATCTTAGTAGGAAN
TGATTTCGAGGTTTATACCNTACGATGAATGTGTGTCCTGTAGGCTTGA
GAGTTCAAGGAAGAAACATGCAATTATCTTTGCGAACYCAGGNGCTGGT
GACGGAATTTTCATAGTCAAGCTATCAGAGTAGAGAAGAGGAGCATGTC
AAAGTACAATTAGAGACAAATATATGTCGCGTGGAGCCAAGAGCGAATC
CCTCAGTCTCGTAGGTCTCTTGATNCCGTTGGATCTGCTTGATCTCGTC
TCCCGAAAATGAAAATAGCTCTGCTAAGCTATTCTTCTCTTCGCCGGAG
CCTGNAAGGCGTTACTAAGTTGCAGTCAATGCATTAAATGCATTGCAGG
TGAGCTGTATTGGAAGAGGTAAACCCGAAAACGCGTCTTATTCTTGTTG
ACATGGAGCTATTAAATCACCAGAAGGCACTCTTTGCTGCTTGGACAAA
TGAACGTATCTTATCGAGATCCTGAACACCATTTGTATTNCATCTCCGGA
GCTGACATCGGACACCAACGATCTTATATCCAGATTCGTCAGCTGTTTG
ATGATGACAGTAACGTTAAGTGGATCCGCGGTCGGCATCTACTCNATTC
CTTTGCCACTCGGACGATGCTGGNGCGTC

> *het4* KO 4-4 (SR-4)

NNNNNNNNNNNNTTCCNNNNNNNACGCGAGAAGGTCTGGAACATCGC
CAACTTGCATTTGGGATCTTTGTGTGGAATATATCAATCTTGCTAATAGA
ATCATCCTTATTCGTTGACCTAGCTGATTCTGGAGTGATACAGAGGGTC
ATGATTTAAGCCTAAAATCCGCCGCCTCCACCATTTGTAGAAAGATGTG
ACGAACTCGTGAGCTCTGTACAGTGACCGGTGACTCTTTTCTGGCATGC

GGAGAGACGGACGGACGCAGAGAGAAGGGCTGAGTAATAAGCCACTG
GCCAGACAGCTCTGGNGGCTCTGAGGTGCAGTGGATNAATTATTAATC
CGGGACCGGCCGCCCCTCCGCCCCCGAAGTGGAAAGGCTGGTGTGCC
CCTCGTTGACCAAGAATCTATTGCATCATCGGAGAATATGGAGCTTCAT
CGAATCCCGGCAGTAAGCGAAGGAGANATGTGAAGCCAGGGGTGTATA
GCCGTCGGCGAAAATAGCATGCCATTAACCTAGGTACAGAAGTCCAATT
GCTTCCGATCTGGTAAAAGATTCACGAGATAGTACCTTTCTCCGAAGTA
GGTAGAGCGAGTGCCGGGCGCGTAAGCTCCCTAATTGGCCCATCCGG
CATCTGTAGGGCGTCCAAATATCGTGCCTTCTCCTGCTTTGCCCGGTGT
ATGAACCGGAAAGGCCGCTCAGGAGTNAGCCAGCGGCGCAGACCGGG
AACACAAGCTGGCAGTCGACCCATCCGGTGCTCTGCACTCGACCTGCT
GAACTCCNTCAGTCCCTGTNGGGCAGCTTTGCCCCCANTCTGTCCGCC
CGGCNGNTCGGCGG

> *het6* KO 6-2 (SF-6)

NNNNNNNNNNANNNNACTTTNNNNCAGTCTATCTCCACCTCGAATTTGC
ATCCTCACACTTCACCAGATCGTACGTAGACCTCCATATTAGCGATCCT
GTCTGCCCGATCGAGTGGAGATGTGGAGTGGGCGCTTACACAGTACAC
GAGGACTTCTAGAAAGAAGGATTACCTCTAAATAAGTGTACCTGTGCAT
TCTGGGTTTTTANTTTTAC

> *het6* KO 6-2 (SR-6)

AANNNGNNNNNNNNGNNCTGGTANAACGTTTATATTGATGGTTCAACGC
ATAGTCATTTACTGTCATCACAGGCTACAACCATTACTACTATAGAATCA
TCCTTATTCGTTGACCTAGCTGATTCTGGAGTGACCCAGAGGGTCATGA
CTTGAGCCTAAAATCCGCCGCCTCCACCATTTGTAGAAAAATGTGACGA
ACTCGTGAGCTCTGTACAGTGACCGGTGACTCTTTCTGGCATGCGGAG
AGACGGACGGACGCAGAGAGAAGGGCTGGNTAATAAGCCCCTGCGCC
ATACAGCTCTGGCGGCTCTGAGGTGCATAGTGGATGATTATTAATCCGG
GACCGGCCCGCCCCTCCGCCCCGAAGTGGAAAGGCTGGTGTGCCCCT
CGTTGACCAAGAATCTATTGCATCATCGGAGAATATGGAGCTTCATCGA
ATCACCGGCAGTAAGCGAAGAGAATGTGAAGCCAGGGGGTGTATAGCC
GTCGGCGAAAATAGCATGCCAATAGCCTAGGTACAGAAGTCCAATTGCT
TCCGATCTGGTAAAAGATTCACGAGATAGTACCTTCTCCGAAGTAGGTA
GAGCGAGTTACCCGCGCGTAAGCTCCCTAATTGNNCCATCCTGGCATC
TGTACCTANT

> *het7* KO 7-1 (SF-7)

NNNNNNNNNNNNNNGNNNNNNNNNNNATTGAACAGCCGATCAATCTTA
AGCCCTCCACAGCCTCAACCCCACATTCTCTCTGACAAACTTTTGATTTC
TTCCTTTTTCACTCTGGAGGGCATTCTTATTTTGACCGCAAGTCGAGTG
GAGATGTGGAGTGGGCGCTTACACAGTACACGAGGACTTCTAGAAAGN

> *het7* KO 7-1 (SR-7)

NNNNNNNNNNNNNNNNCCNNGNNNTACGTGGTATACTTAAACCCTAGA
CTCGGCCTTTGAGTTCAAGCAATGGGAGTATGCTNGNANNCNATCTATA
GAATCATCCTTATTCGTTGACCTAGCTGATTCTGGAGTGACTCAGAGGG
TCATGACTTGAGCCTAAAATCCGCCGCCTCCACCATTTGTAGAAAAATG
TGACGAACTCGTGAGCTCTGTACAGTGACCGGTGACTCTTTCTGGCATG
CGGAGAGACGGACGGACGCAGAGAGAAGGGCTGAGTAATAAGCCACT
GGCCAGACAGCTCTGGCGGCTCTGAGGTGCAGTGGATGATTATTAATC
CGGGACCGGCCGCCCCCCCTGCCCCGAAGTGGAAAGGCTGGTGTGCC
CCTCGTTGACCAAGAATCTATTGCATCATCGGAGAATATGGAGCTTCAT
CGAATCACCGGCAGTAAGCGAAGNAGAATGTGAAGCCAGGGGTGTATA
GCCGTCGGCGAAAATAGCATGCCATTAACCTAGGTACAGAAGTCCAATT
GCTCCGATCTGGTAAAAGGTTCACGAGATAGTACCTTCTCCGAAGTAGG
TAGAGCGAGTACCTGGCGCGTAAGCTCCCTAGTTGGCCCATCCGGCAT
CTGTAGGGCNCCAAATATCGTGCCTCTCCTGCTTTGCCCGGTGTATGAA
ACCGGAAAAAGCCGCTCAGGAGCTGGCCAGCGGGCGCAGACCGGAAA
CACAAGCTGGCAGTCGANCCATCCGGCGCTTCTGCNTCAGCT

> *het8* KO 8-2 (SF-8)

TNNNNNNNNNNGGGGNNATGCNTNAACGAGGCGGGTTCTACAGCGG
CCTGACGGAAGTACTTGACGGCATTTTGGGCTTCTGGCAGCTGACAGG
TAATAGTTCAGAACGACTTCAAGTGGAGATGTGGAGTGGGCGCTTACAC
AGTACACGAGGACTTCTAGAAAGAAGGATTACCTCTAAACAAGTGTACC
TGTGCATTCTGGGTAAACGACTCATAGGAGAGTTGTAAAAAGTTTCGGC
CGGGGTATCGGGTGTTACGGAGCATTCACTAGGCAACCATGCATGGTT
ACTATTGTATACCATCTTAGTAGGAANTGATTTCGAGNCTTATACCTACG
ATGAATGTGTGTCCTGTAGGCTTGAGAGTTCAAGGAAGAAACATGCAAT
TATCTTTGCGAACCCAGGNGCTGGTGACGGAATTTTCATAGTCAAGCTA
TCAGAGTAAAGAAGAGGAGCATGTCAAAGTACAATTAGAGACAAATATA
TAGTCGCGTGGAGCCNCGAGCGGTTC

> *het8* KO 8-2 (SR-8)

NNNNNCNNNAAANNNNGCTCTNNTCTCAGAATCATAAAGAAATACTCTT
CTGGTTGCAGAATATCTGCTTCCTTACATTCTTAGCACTATAGAATCATC
CTTATTCGTTGACCTAGCTGATTCTGGAGTGACCCAGAGGGTCATGACT
TGACCCTAAAATCCGCCGCCTCCACCATTTGTAAAAAAACGTGACGAAC
TCGTGAGCTCTGTACAGTGACCGGTGACTCTTTCTGGCATGCGGAGAG
ACGGACGGACGCAGAGAGAAGGGCTGAGTAATAAGCCACTGGCCAGA
CAGCTCTGGCGGCTCTGAGGTGCAGTGGATGATTATTAATCCGGGACC
GGCCGCCCCTCCGCCCGAAGTGGAAAGGCTGGTGTGCCCCTCGTTGA
CCAAGAATCTATTGCATCATCGGAGAATATGGAGCTTCATCGAATCACC
GGCAGTAAGCGAAGGAGAATGTGAAGCCAGGGGTGTATAGCCGTCGG
CGAAATAGCATGCCATTAACCTAGGTACAGAAGTCCAATTGCTTCCATC
TGGTCAAGATTCACGAGATAGTACCTTCTCCGAAGTAGGTAGAGCGAGT
ANCCCGGCGCGTAAGCTCCCTAATTGGCCCATCCGGCATCTGTAGGGC
GTCCAAATATCGTGCCTCTCCTGCTTTGCCCGGTGTATGAAACCGGAAG
GGCCGCTCAGGAGNTGGGCCAGCGGCGCAGACCGGGNTCACAAGCTG
GCCAGTCGACCCATCCGGTGCTCTGCACTCGACCTACTGGGGTCCCTC
AGTCCCTGGTAGGCAGCTTTAGCCCGTCTGNTCCGCCCANTGTGTTGC
GGGGNNA

Appendix 8: DNA Sequence of *A. fumigatus* Gene AFUB_022100

The DNA sequence of the A1163 gene AFUB_022100 has 99.9% homology to the Af293 gene AFUA_2G05070. Both sequences are 1521 bp in length however, there is a substitution of the T nucleotide in position 1517 to a C in AFUA_2G05070.

> AFUB_022100

ATGGACGAGACTATTGATTCAGAATCCGTCCTCGCTCCCGTGAGGGCC
TCGTCGCCGTCCCCCTCCATCCCAGCAACGCCTGCGATCTCCTCGTGT
CCATCCCCTGACCGTACCTTCTCCACCATTTCATCCCTCTCAACCTCGT
CCGCCACATCCGCCGATGCCAGGTCATCAATTTCGGTCTCTTCGAAAC
GACATGGGTACATCCGGCCACAGGGAGCCGAATTCGCCGAGTCGGCT
AAGAATCGCGAGAGTGTGATGAGTCTGGGGAGCATTGCCCACCTACAG
TACTATTTTGCGAGAACCGGTCTGCTAGACGGAAAGGGTGGGCATGCG
CGCGAGTGGAAGAAGAAGAAAAAGCCCGAGGAGGAGCCTCGGCTCCT
GTTGACACCTAATGCTCGGTTCATCGATGATTTGACGGAGAGCCCGAC
GGAGGAATATAGTTCAGATCTTGGTGAGGAGGACCCGGAAGACGAGAT
GATGCTCCCGCCGACGGTGAGCACGTACAGCGTGAAGACGCATCATAT
TCCGCCACCGCCGGATGTACTGGCGCTGCGGAGGGATTTGCTCGATG
CGGTCGACAAGGCCGAGAAGAACATCAAGGACCTGGACTCGCAGAAG
GAGCCTCCGCCGGAGGTGATGCCACCGCGGATCAGTGTATCTCGAGAA
GATACAGAAGTGATTTCGCGGCCAGGCACGGCCAATGGTCCGCCAGGA
TGGCATGAGATTCAAGGCATGCGCATTCTGGACGCGGTTACTCTGGCC
ATCCGAGCGGCCAAGGTTTACTATACAGCGCATGAGCGCCCCGAACGG
TTAGCTTCAATCAAGCCCGAGCGGGAAATCCGGCAGGAATTGTTTCATG
TCCTGGAGGTGCTGAAGCGATGGGCCGCTCGCAATTTTGCGGGGGGC
CTCCGCGAGGACGAGCGATCACCCATGATGGATTGGGTGTCAAATGTA
CGTCAGATGCTTGCCAGAGAGGAGAGCCTAGAGGCTTTGGAAGCGAAA
GAGCGCGAAGGATGGGCCTGGGCGAAGGGCGACTGGAGCGGACGAG
AACGGGAGCGCGAGGAATCGTTCCTGCGCAGCTTGCTAGAATCCGATA
CACCCTTGCCAACATGGACGTCCACCGATGGCGGGACATTGCCGACAC
CGATTCTTGAACGACTGCGGGACGGCCGAGACCTCGTCCGCATCCACA
ACCAGGCGGTCAAGAAATCCAAGCGACCGTTCGGCGAGATCAAGTCGT
ACCATCAAGACGTTGCCAAGCCGTATCGACGTGCGGAAAATCTTCGCTT
CTGGCTGAAGGCGGCAGAGATCCGATGGGAGACGAAGCTGGAAATGG
ACGTGATGGGCGTTGTCCATGGCACCAGCGACGCGGCCTGGAGGCAA
TTCGACACGGCTCTTTTGTCATGGTGCCAGGCAGTCCGAGAAGAATTGA

TACGAGACTGGCGCGAGACACCGACGGCACCGGTCGCTGCATATACC
CCCACTACTGACAGTCAGGGAGGTGGCGACCTTGTATGA

Appendix 9: *impala160* Rescue DNA Sequences

The PCR rescue products of three D1-4ET *impala160* transposon mutagenesis strains matched sections of the *A. fumigatus* gene AFUA_2G05070. ST-PCR step 2 products of recovered transposon mutagenesis isolates were sequenced, and the sequences of strains 1, 21 and 31 matched the Af293 gene (section 3.3.2.2). The homologous segments of the DNA sequences are highlighted in green.

> *impala160* mutagenesis strain 1

CGNNNNNNNNNNNNNNGNTCCNNTTGACCTGAGGCTCGATTGTCGNA
GATTTCCCCTGAACGACCGACCGGAGGAAGTTGTTGAAATATTGGATTT
GAGGACCCGTAAACCGCGGTGAGGTTCGCCCCAAGCGCGACTTGCAAT
GCCTGAATTCCGCATCTGATTCCGCCACCGCCGGATGTTCCCCCCCTG
CGTATGGATTTGTTTCATGCGGTCGACTCCGTACTCCGTATGCTAGGTC
TTGGGGTCTCATATGGACCCGGCAATTCATGTGAGACCTCCTTATAACT
ATGAATCTGATTAAAACTTTGCTTCCCCCCCTTTTTTTCCTTGGCCGTAC
GGGAGTATAACATCCGGCTAGTAAAGGCATGTGCATTTGGGACACGCT
TTTTCTTTACATCAAAGCGACCACGGTTTAAAATAAACCAAGCGGGTGTA
TTTAACGCGATCTTCAAACCCATTCGAGCGGGAATCCGGCTTGAATTGT
TGTATGGCCTGGAATGCGGATGGAATTTTATTCTTGCAGTTTAGCCGCC
GGCCTGCACAAGGGTGAGGATTACCCATGAGGGATCCAGTGTCAAGTG
AACGTCAAATGGTTGTCCAAGTATAAGCTTATACGCTTTTATAATTTAAA
GAACGAGTTGAAACGCCCCGAACATAGGGCACCTGTGNCTAACCAGAA
CCGAATCGTGAGAAATCCTCACTCCACCCCTTGCTGAGTATCTAATGCA
CTGTTTACCAACCNGACACCCATCTATTTGCTACACCTCGACGACTCCC
TCTCTTTAATGGACCCCTGTACCGCTCCNATTTCACCAGGATCCTCAAT
AATGCTGCCGCCAGATCTGTTATCCAGTTTCTCGTACTGCAAGTATTTCT
ACTATGACATTGCAAGGCTGCATCTCCCTGACTGATGCCTACGCTTGAT
GTCTGACGCCCCGGATATCACCAGGGTGTAGAATCCTTGTCATGTTCAT
TATGCTCGTATGCTTGGTTCCNNANAGAGACTGGATGCANATTCACTTC
GCGCCNTTNNTGGTGCATGGACN

> *impala160* mutagenesis strain 21

NNNNNNNNNNNGNGNGCNNTNNNGACCCTGAGGCGCGAATTGTAGG
GGTTTCCACTGAACGACGACGGGAGGAGGTTGTTGAAATAGTGGATTT

GAGGACCTTAAAGACCCGGTCAGGTCCGCGCCAAGGGCGAGTTGCATT
GCCTGAATTCCGCATCTGACGACGCCACCGCCGGANGTTCGCCCCCTG
GGGATGGATTTGTTCCACGCGGGCGACGAGNCCCTCCGTATTGCTAGG
TGTTGGAATCTCATTTTGACCCGGGAACTCATGTGAGGCCTCCTTATAT
CTATGAATCTGATTATTACTTTGCTGGATTTNTTTTTTTCNTTGTGAGTAN
GCGGCCATAACATCCGGATAGTAGAGGCATGTGCATTTGGTACCCGCC
ATCTCTTTACGTCACAGCACCCACCGATTACTATAAACAAAGTGAGCGC
CCTTAACGGCGGACAGAGAACCCNCTCGANCGGGAAACCCGGCTCGA
ATTGTTGCGTGGCCTGCAAAGGTGGATGCAATTTTACTCTCCCAGTTTT
GCCTTCGGCGTTGTACAAGGAGTGAGGGATTACCCATGAGNGATGCCA
GTGTCATGTTGAACGTCACATGCTTGGCACATACAGAAAGCCTATACGC
ATATATGCTTAACGAACGAGCTGGATCTCCGCAGAACAAAGGGCACCC
GTGCCTGACGAGAAATGAAATTGCNAAGTATCTCACATCCACAACTAGC
GGGAATCCTAATACGATGATATGACAAATGAGAACTCATTATTGTGGTA
CACTTTGACCACTCATACTATCTCATGACTGTGTGCCGCTCCCNTTCAA
CTCAGGAATGTCAACGATGCTGCATCNNTACCAGTATACAGANCCTGTA
CTGCCAGTCAATTCTACANGACATACGAGGTCGTATCTCCTGGACNGTA
GCTATGCGGCTGANTGANGCGCCGGAGACCTCAGTGTNTCTATCCTGT
ACACGTACATNATCACNNATNGCCGNTCNTGACNNTGATTGNNGCNNAT
TGATGCNNNTCTTTGTGCTCGGNNCNTNNNN

> *impala160* mutagenesis strain 31

NNNNNNNNNNNNNNGNNCNTTNGACCTGAGGCTCGGTTGTAGGGGT
TTCCCCTGAAAGACCGATGGGAGGAGATTGTGGAAATAGTGGATTTGA
GTCCCCGGAGACGCGGTCAGGTCCGCCCCAAGCTCGAGCTGGAATGC
CGGAATTCCGCGGCTGACGACGCCCCCGCCGGATTGATTGGCCTTACG
GAGGGATTTGTTTGCAACGGCCGAGTGGCCTAGTAGATTGCTAGGTGT
TGGACTCTAATTATGACCCGGGGATTGATGTGAGGCATCCGTAGTGTAT
GTATCTCATTAATACTTTGCTTCCCCCCCTTTTTTTCCTCGGCCCATACT
CGAGTATAANACCCAAATAGTAGAGGCATGTGCATTTGGGACACGCTAT
TTCTTTACGTCAAAGCGACCACGGTTTACTATAAACAGAGTGAGCGTCT
TTAGGTCGGNCTTAGATTCAGCCCGACCGGGAACCCTATTGAATTGTTG
TATGGTCTGNGATGTGGAGCAATTTTCCCTCCTTTTTTGCCGGCGCCCT
CAACAAGGCTAGGGATCACCCATGAGGGAGCCGTCTAAATTGAACGTG
CATGGCTGGCCCAGTAAAGAGCCCATACGCATAGATACTTAAGAAAATA
TGAATCGNTAGAACATAGGGCATCTGTGCCTGACTAGAATGGAATTGTG
GAACTCTCACTCCACTCTAGCTGGAAATCCATATGATGGATANAAATGA
GATCTCATTATGTNGCAACTCTTACCGACTCCCTCCCTCGCTTGGCCGC
TGTACGCCTCCACTCTCTACTCGTATCGTTCATCACGCTGCNCGAGATN
TNGTATCCGCATTCTCTACTGCAGTATTCCTAACATGACGTAGCAGGCC
GCAGCTTCCCTGACGTAGTATCTTATTCTGNTGANTGATCCTCCCAGAT
ACTCGCAGTGTCTCTACTTCTTGGCTACTNATCATTACTGCACGTACCT

GCNCGGCACCCTGACNNACGAATGATTGGTCATCTAACTCGTTGTCTTC
TGTCTATTGNNGNCTTGGNCATTCTNNN