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**Evolution and Gene Flow of
Azole Resistance and Other
Virulence Factors in *Aspergillus*
*fumigatus***

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Abstract

Aspergillus fumigatus is a commonly encountered fungus in the environment and has a primary ecological role as a saprotroph in the breakdown and recycling of organic matter. It is also an opportunistic pathogen and the major cause of invasive aspergillosis (IA) in humans, especially in immunocompromised individuals. The main method of treatment of infection is the use of azole antifungals. However, the past twenty years has seen an ever-increasing appearance of resistance to azole antifungals in isolates of *A. fumigatus* from both clinical and environmental settings. Therefore, the overall theme of work in this thesis was to increase our understanding about the genetic basis of both azole resistance and pathogenicity in this fungus.

Previous studies have shown that azole resistance in *A. fumigatus* is frequently linked to mutations in the *cyp51A* gene. However, a number of azole resistant isolates have been reported to exhibit non-*cyp51A* based form of resistance. Therefore, exploring the genetic basis of azole resistant in such isolates, in which the genetic basis of resistance is unknown, is required to develop better diagnostic methods for improved disease treatment. A total of 32 isolates from clinical and environmental sources, with supposed non-*cyp51A* high levels of azole resistance, were obtained from collaborating groups. Screening with E-tests revealed that half of the isolates showed low or unstable levels of resistance. The remaining 16 isolates were screened for known resistant mechanisms. Six isolates showed known *cyp51A*-based resistant mechanisms. However, an apparently novel form of azole resistance was detected in eight clinical isolates based on the presence of a relatively large tandem repeat in the *cyp51A* promotor region. These isolates were assigned for further analysis. In addition, one of the two remaining isolates (C286) that exhibited a non-*cyp51A* based form of azole resistance, with unknown resistance mechanism(s), was chosen as a candidate for later bulk segregant analysis.

Further analysis of the eight clinical isolates showing an expansion in size of the *cyp51A* promoter region revealed the presence of a four-fold tandem repeat in six isolates, a three-fold tandem repeat in one isolate and a two-fold tandem repeat in one isolate of the same 130 bp sequence, here termed TR₁₃₀. The presence of TR₁₃₀ was

found to be correlated with elevated resistance to both itraconazole and voriconazole, with the extent of resistance in proportion to the number of tandem repeats. Sexual crossing also demonstrated a direct correlation between the presence of TR₁₃₀ and azole resistance. Furthermore, gene expression studies revealed a significant increase in *cyp51A* expression in isolates harbouring TR₁₃₀, which was correlated with the number of repeats and the level of azole resistance. Gene manipulation studies were then conducted, with different number of repeats of TR₁₃₀ introduced into a wild-type (WT) sensitive lab strain. Transformants showed greatly increased resistance to itraconazole, in proportion to the number of TR₁₃₀ repeats introduced, and also showed concomitant increases in *cyp51A* expression. Azole sensitivity was restored in a complement strain in which TR₁₃₀ repeat was removed. Thus, results demonstrated that the TR₁₃₀ mutation can lead to azole resistance. Moreover, infection of *Galleria mellonella* was used to evaluate the treatment efficiency of *A. fumigatus* strains harbouring different forms of TR₁₃₀. No significant differences in virulence were observed between TR₁₃₀ and WT isolates. By contrast, itraconazole treatment was less efficient in the control of strains harbouring TR₁₃₀ than WT strains, indicating the likely clinical significance of *A. fumigatus* strains harbouring different forms of the TR₁₃₀. The evolutionary origins of the TR₁₃₀ repeat are discussed based on phylogenetic analysis.

Sexual crossing of the azole resistant isolate C286 with sensitive partners revealed a 1:1 segregation pattern of resistance: sensitivity, consistent with monogenic inheritance. BSA was then undertaken in conjunction with next generation sequencing of parents and progeny pools. Three rounds of backcrossing were performed to increase isogeneity of progeny. DNA was then extracted from the parents and progeny pools of 100 resistant and 100 sensitive progeny. DNA was genome sequenced and a BSA bioinformatic pipeline used to identify variants between the azole resistant parent and progeny, and the sensitive parent and progeny pools. This BSA pipeline had recently been used successfully to identify a single linked region of the genome containing a causal mutation of azole resistance in *A. fumigatus*. A total of 636 SNPs and 57 INDELs were found that varied between the azole resistant and sensitive groups. However, unlike the previous study there was no clear single linked region containing these variants so no definitive answer as to the resistant mechanisms as yet.

In addition, the sexual cycle of *A. fumigatus* was exploited to investigate the genetic basis of a novel form of oxidative stress response observed in the fungus, together with aspects of the population biology of the species. Previous studies had identified clinical isolates with a striking phenotype linked to pathogenicity relating to the ability to grow in a low oxygen environment and/or under oxidative stress produced from reactive oxygen species (ROS) compounds. Crosses were set up between isolates exhibiting and lacking this phenotype, and progeny screened under oxidative stress conditions. Results showed that progeny exhibited a wide variety of morphologies, indicating that the phenotype has a polygenic basis linked to more than one gene. Finally, there is recent evidence of two distinct clades within global populations of *A. fumigatus* with most azole resistant isolates clustered into one group. Crosses were set up between azole resistant isolates and highly fertile isolates from the opposing clade. Results showed successful crossing between a series of isolates under laboratory conditions, indicating no barrier to gene flow of azole resistance between the two groups.

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Chapter- 1 Introduction

1.1 General Overview of the Fungal Kingdom

Biologists currently categorize living organisms into six distinct kingdoms; one of these taxonomic ranks is the fungal kingdom. The fungal kingdom comprises a diverse and large set of organisms, however, only about 150,000 species have been described, with probably a few million awaiting discovery (Rokas, 2022). Well known examples from the fungal kingdom include mushrooms, yeasts, moulds, puffballs, rusts, truffles, smuts, and morels. Most of the constituents of the fungal kingdom affect almost all other forms of life on earth. They play a significant role in the ecosystem through the decay of organic material and by creating symbiotic relations with members of other kingdoms, including plants and animals (Heitman, 2011; Moore *et al.*, 2011).

Fungi are eukaryotic organisms that reproduce by means of spores. These spores are produced when environmental conditions are suitable for reproduction. Dependent on the type of species and conditions, fungi may produce asexual and/or sexual spores. The spores germinate to form hyphae, which appears as microscopic fingertips, and networks of hyphae collectively form a mycelium (Stephenson, 2012). Unlike members of the plant kingdom, which utilise chlorophyll and sunlight energy to make their own food, fungi lack chlorophyll and have to acquire food from other sources. Therefore, they are heterotrophic organisms which must obtain their nutrients from other sources through absorption (Stephenson, 2012). Unlike plants whose cell walls consist of cellulose; most fungi have cell walls made up of chitin and polysaccharide polymers. Most species store their carbohydrates as glycogen (Moore *et al.*, 2011).

Genetic studies have provided key insights into the nature of fungal genes, control of gene expression, and heredity. For many fungal species the fungal nuclei are mainly haploid in the hyphal form, containing only a single set of chromosomes (Dyer *et al.*, 2018). This aspect is valuable in the analysis of fungi mutations that are typically recessive and consequently can be masked in diploid organisms. The analysis and sequencing of fungal genomes over the past decade has provided considerable insights into many diverse aspects of fungal biology. According to Dyer *et al.* (2018), the genome size of most fungi range between 10 – 45 Mb which encode between 5,000 –

15,000 proteins that are spread over 7 – 20 chromosomes. For example, the genome of *Aspergillus fumigatus* (*A. fumigatus*) is approximately 30 Mb in size and comprises eight chromosomes.

1.1.1 The Phylum Ascomycota

The vast majority of identified fungal species belonging to the subkingdom called *Dikarya* (Moore *et al.*, 2011). The subkingdom comprises two phyla, the Basidiomycota and Ascomycota. The Ascomycota represents the largest phylum of the fungal kingdom, comprising around 65% of all identified fungi, with over 64,000 known species in 6,355 genera (Wijayawardene *et al.*, 2017). The distinctive aspect of this phylum is their ascus, which are sac-like structures produced during sexual reproduction that enclose haploid spores known as ascospores. However, certain species of the Ascomycota are asexual, and thus, do not have ascospores. Nevertheless, phylogenetic analysis has demonstrated that such asexual species can clearly be assigned to the Ascomycota (Schoch *et al.*, 2009). Ascomycetes are found in terrestrial, freshwater, and marine habitats. Many members of this phylum have a critical ecological responsibility as agents of decomposition (Moore *et al.*, 2011). Meanwhile, several agriculturally and medicinal significant pathogens, such as certain *Penicillium* and *Aspergillus* species, are ascomycetes. A number of ascomycete species have proved useful as model microorganisms for research in cell biology and genetics (Wijayawardene *et al.*, 2017). Biologists have subdivided the phylum Ascomycotina into three further monophyletic subphyla (Moore *et al.*, 2011) as follows.

- I. Taphrinomycotina: includes, among others, *Pneumocystis jirovecii*. This fungus commonly causes pneumocystosis in persons with weakened immune systems.
- II. Saccharomycotina: includes the yeasts *Saccharomyces cerevisiae* and *Candida albicans*, among others.
- III. Pezizomycotina: they represent the majority (ca. 90%) of species of the Ascomycota and comprise the vast majority of filamentous fungi overall.

Work in the current project is focussed on the occurrence of resistance to antifungal drugs in *A. fumigatus*, a member of the Pezizomycotina. Therefore, additional

background will now be provided about this group of fungi and the mechanisms of resistance to anti-fungal drugs such as azoles.

1.1.2 Filamentous Fungi

Fungi can develop as yeast or filamentous fungal (mold) forms. Molds develop through apical extensions of their filaments (hyphae), whereas yeast divide via budding or fission. Hyphal development can occur with or without septation, which refers to the separation of cellular compartments by specialised pores or cell walls. A mycelium is a large complex network of hyphae that forms as a result of continued development and extensions of the hyphae (Bennett *et al.*, 2010; Powers-Fletcher *et al.*, 2016). Filamentous fungi are almost universally present in terrestrial environments, where they obtain their nutrients from the breakdown of organic matter (Gow and Gadd, 2007; Bennett *et al.*, 2010; Powers-Fletcher *et al.*, 2016). They are typical eukaryotic organisms, having a variety of membrane-enclosed sub-cellular compartments that act as sites for specialised cellular functions. Notably, filamentous fungi have all the key eukaryotic organelles, with the exception of a chloroplast (Gow and Gadd, 2007; Bennett *et al.*, 2010; Moore *et al.*, 2011).

1.1.3 Role of Filamentous Fungi in Nature

Regardless of their minuscule appearance, the filamentous fungi play a critical role in nature. For example, filamentous fungi grow on dead organic matter, decomposing such materials and making their chemical constituents available to succeeding generations of organisms. Through this process, they help in recycling dead organic matters in the environment (Moore *et al.*, 2011). The filamentous fungi also contribute a vital role in plant nutrient uptake. Plant roots have symbiotic fungi, mycorrhiza, which help plants to take up nutrients and distribute them to the other parts of the plant (Powers-Fletcher *et al.*, 2016).

In industry, filamentous fungi are useful in biotechnological applications, including significantly the manufacture of antibiotics such as penicillin, fermentation of cheese, and production of citric acid (Moore *et al.*, 2011). Additionally, filamentous fungi are

helpful in the treatment of contaminated wastewater. Certain fungal species can convert organic components in the wastewater into valuable bio-chemicals and fungal proteins that can be used as animal feeds (Sankaran *et al.*, 2010).

By contrast to their beneficial economic importance, certain filamentous fungi are also disease-causing pathogens. They have the potential to cause infection in plants, animals and humans as well as the capability to trigger allergic reactions in oversensitive hosts (Dickinson, 2004; Heitman, 2011; Powers-Fletcher *et al.*, 2016; Sharma Ghimire and Jin, 2017). In term of plant infection, it has been estimated that up to 20% of the world's crop yield is lost due to fungi infection. One of the top examples is *Magnaporthe oryzae* which might lead to yield losses up to 100%. Moreover, fungi have a significant impact in the spoilage of food such as *Aspergillus niger* infecting fresh product such as fruit (Davies *et al.*, 2021).

In the case of *A. fumigatus*, there is no differential morphology between the parasitic and saprophytic natures of the organism (unlike for example *Coccidioides* spp.). The species *A. fumigatus* is almost unique amongst the genus *Aspergillus* because it is both a primary opportunistic pathogen but can as well be a key allergen linked with acute sinusitis and asthmatic complications. Notably, the fungi can cause ‘aspergillosis’ infection, particularly in humans with a weakened immune system and complicating lung infections (Howard *et al.*, 2009; Verweij *et al.*, 2016a; Chowdhary *et al.*, 2017).

1.2 Medical Mycology

1.2.1 Fungi as Human Pathogens

Fungi are one of the three main eukaryotic kingdoms, along with plants and animals (Rokas, 2022). They are characterized by having a specific cellular wall structure and cytoplasmic membranes composed primarily of sterols, particularly ergosterol (Ivanov *et al.*, 2022). In terms of causing disease, despite the fact that the fungal kingdom has an estimated six million species spread throughout the globe, only around 200 species are known to cause illness in humans (Mancuso *et al.*, 2022). Fungi have received less attention than bacteria and viruses, largely because their causation is opportunistic and have been less well diagnosed. Fungal diseases have become more prevalent in the past

few decades due to factors such as lengthier lifespans, extended treatments that can lead to compromised immunity, more invasive procedures (Rokas, 2022), and climate change (Gnat *et al.*, 2021).

1.2.2 Rising Numbers and Threat of Fungi

On a global scale, infectious diseases are the second leading cause of death worldwide after cardiovascular diseases. Even though exposure to fungi is near-constant in most environments, fungal infections are relatively uncommon diseases in healthy humans compared with bacterial and viral infections (Gnat *et al.*, 2021). The types of fungi that can cause diseases in humans in terms of morphology can be moulds, yeasts, and biphasic (dimorphic) fungi (Ivanov *et al.*, 2022). These fungal types can cause both superficial and invasive infections. Invasive fungal infections occur with a much lower incidence rate, but their mortality rates are deemed unacceptably high, exceeding 50% even with multiple effective antifungal drugs available (Brown *et al.*, 2012).

Humans have been highly resistant to fungal infections due to four factors: a high thermal (body heat) barrier; limited penetration pathways, mainly through air passages; difficulty in digesting and absorbing multiple components of human tissue and defeating the human immune system long enough to establish sustainability (Köhler *et al.*, 2015). With immune-compromised people, the resistance barriers decrease significantly, and fungal infection rates may surge. Another factor increasing the threat level is climate change, causing adaptation in many previously harmless fungal species, as well as expanding environments in which fungi-human interactions can occur (Gnat *et al.*, 2021).

Fungal illnesses kill more than 1.6 million people globally each year, a rate equivalent to tuberculosis (TB) and more than three times that of malaria (Rokas, 2022). But in terms of mortality, species belonging to one of four genera: *Cryptococcus*, *Candida*, *Aspergillus*, and *Pneumocystis*, account for almost 90% of all documented fungal-related mortality (Brown *et al.*, 2012), with the estimation of a killing rate of more than 1 million people yearly (Ivanov *et al.*, 2022). However, nearly every fungal species can be an opportunistic or accidental pathogen in a human host whose immune system is severely weakened (Rokas, 2022).

1.2.3 Immune System Response to Fungal Infection

1.2.3.1 Diagnostic Challenge

The fact that the underlying immune response to fungal infectious agents is not as well understood as that of bacteria and viruses provides a daunting challenge to modern medicine (Drummond *et al.*, 2015). At the same time, the diagnostic precision of dealing with fungal infections is often thwarted by the fact that evaluations based on clinical symptoms are not easy because of nonspecific symptoms, and because individuals might interact with a wide range of fungal spores daily, many of which present no health risks, as opposed to comparatively few that pose significant clinical risk (Mancuso *et al.*, 2022).

A separate-but-related fact is that fungi can be commensal with bacteria and viruses and can cause the immune system to react in many of the same ways. The potential delay in clinical diagnosis and subsequent targeted treatment of fungal infections, particularly in immuno-compromised patients, may be a significant contributing factor to persistently high mortality rates, especially for patients in intensive care (Mancuso *et al.*, 2022).

1.2.3.2 Immune System Response

Like bacteria and viruses, fungi are recognized by the specialized cells of the immune system such as macrophages and dendritic cells, using pattern recognition receptors (PRR) which bind to specific components of fungal cell membranes, walls or other elicitors. This binding triggers the PRR to signal others cells to destroy the bound cell through phagocytosis, which also helps to create adaptive immunity against future infections (Drummond *et al.*, 2015).

The main specialized pattern recognition receptors that respond to fungal infections are Toll-like receptors (TLRs), C-type lectin like receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), all of which are responsible for initial immune responses that eventually trigger adaptive immunity (Mancuso *et al.*, 2022).

By responding to the pathogen-associated molecular patterns (PAMPs) such as glucans, glycolipids, and glycoproteins of fungi, the PRRs can trigger other responses beyond phagocytosis (Salazar *et al.*, 2022). After being activated by specific PAMPs, PRRs trigger signalling pathways that encourage the transcription of a number of genes, such as nuclear factor (NF-) κ B, activator protein-1 (AP-1), and mitogen-activated protein kinase (MAPK); the latter is essential for the production of proinflammatory cytokines like interleukin (IL-) 1 and tumour necrosis factor (TNF-) α see **Figure 1.1** (Mancuso *et al.*, 2022).

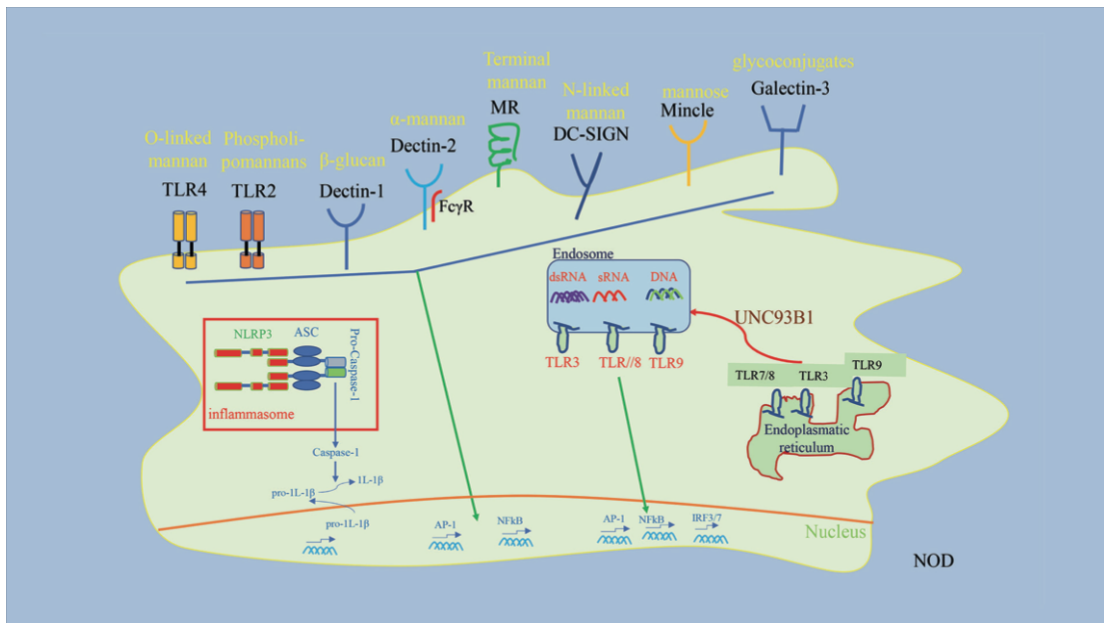


Figure 1-1: Illustration of the role of Pattern Recognition Receptors involved in detecting fungal infection as presented in (Mancuso *et al.*, 2022).

A primary factor in the clinical analysis of fungal infections is that these innate immune responses, particularly phagocytosis and cytokine release, produce inflammation. While this can serve as a proper and highly-effective immune response, the triggering effect of fungi and the often compromised immunity of the patient can lead to the innate response becoming as large a threat, or even larger, than the isolated infection itself, especially in case of fungal coinfection (Salazar *et al.*, 2022). The inflammatory response in patients with normal immune systems tends to maintain near-stasis and self-correct, but in immune-compromised patients, the balancing process can fail. The most common form of excessive response is the cytokine storm, which can

often lead to serious complications, especially in patients who are immuno-compromised and with weakened pulmonary systems (Rokas, 2022).

This over-reaction by the immune system is seen in patients who have COVID-19 (Rokas, 2022), but also in patients who have other bacterial or viral infections in which the lungs become compromised. The coinfection potential rises as the immune system weakens, so it is quite possible that the fungal agent is not the direct cause of the disease or cytokine storm, but serves as an exacerbating factor, an opportunistic threat making the most of the brief window within which it can act (Salazar *et al.*, 2022).

1.2.3.3 Emerging and Increasing Resistance

Four factors play primary roles in the emergence and increasing resistance of antifungal drugs by fungal infectious agents. The first is increasing contact between fungal agents in a time of expanding environments and only minimal knowledge of fungal species (Rokas, 2022). The second is that invasive procedures, such as catheters, allow the fungus an additional layer of potential protection. The third is that prolonged use of antifungal medications in long-term treatment of cancer or immuno-suppression allows for prolonged and expanded chances of mutations that can resist the drugs' effects. And the fourth is that suboptimal use of antifungals, due to incomplete treatment or misdiagnosis, can lead to natural selection of drug-resistant strains (Mancuso *et al.*, 2022). Taken together, these factors indicate that the clinical threat of fungal infections is increasing, possibly well beyond the short-term capacity of medical science to effectively meet the challenge when the immune system is overwhelmed.

1.2.4 Main Opportunistic Fungi and Related Diseases

The opportunistic behaviour of most fungal pathogens means that many of their genetic elements and traits have evolved to allow survival independently of human infection (Rokas, 2022). In terms of epidemiological data, fungal infections are often misdiagnosed, and even correct diagnoses go uncounted as the World Health Organization (WHO) has no tracking program for fungal infections, and the U.S. Centres for Disease Control and prevention (CDC) only track coccidioidomycosis ("valley fever") (Brown *et al.*, 2012).

Human mycosis can be categorized as exogenous (such as dermatomycoses), endogenous (such as candidiasis), and exo-endogenous (such as cryptococcosis). Further categorization can be based on location. Superficial infections are common on the nails, eyes, and ears, at the cutaneous and sub-cutaneous levels. Deep or invasive fungal infections can propagate in deep tissues and organs, ranging from blood and bones to lungs, liver, heart, and brain (Ivanov *et al.*, 2022). As noted by Brown *et al.* (2012), the vast majority of deaths related to fungal infections are due to *Cryptococcus*, *Candida*, *Aspergillus*, and *Pneumocystis*, with particularly high rates related to pulmonary diseases (Mancuso *et al.*, 2022; Salazar *et al.*, 2022). An overview of these opportunistic pathogens will now be provided.

1.2.4.1 Cryptococcus

The genus *Cryptococcus* is composed primarily of anamorphic basidiomycetous yeasts that do not cause infections in humans or animals (Gnat *et al.*, 2021). However, the resulting disease, cryptococcosis, has a high mortality rate, and nearly every case is caused by two particular species, *Cryptococcus neoformans* and the closely related species *Cryptococcus gattii*. Exposure mainly happens through inhalation of airborne spores into the lungs. Local and systemic spread are likely to occur, with the fungus targeting the central nervous system (Brown *et al.*, 2012). Superficial symptoms include nodules, papules, pustules, abscesses, cellulitis, and ulcers, while invasive symptoms include fever, cough, fatigue, meningeal signs, and focal neurological deficits (Gnat *et al.*, 2021).

1.2.4.2 Candida

The genus *Candida* is comprised of over 200 species, and of these 15 have so far been isolated as infection vectors in humans and animals. About 80% of infections are caused by *Candida albicans*, although the incidence of candidiasis due to non-albicans candida (NAC) species is increasing: between 1997 to 2016, infections caused by other more drug-resistant species increased while *C. albicans* infections dropped from 57.4% to 46.4% (Gnat *et al.*, 2021).

Candida species are the most common fungal agent causing life-threatening invasive infections in patients who are severely immunocompromised, have undergone invasive clinical procedures, and/or have experienced major trauma that requires extended stays

in intensive care units (ICUs). *Candida* species are the fourth most common cause of hospital-acquired bloodstream infections (Brown *et al.*, 2012).

Superficial symptoms of *Candida* infections include bright pink-red papules/vesicles, diaper dermatitis, balanitis, onychomycosis, stuck on white papules or plaques, folliculitis-like eruption, subcutaneous nodules, and haemorrhagic bullae, while invasive symptoms include fever, chills, fatigue, and constellations of endocarditis that can cause severe cardiac damage (Gnat *et al.*, 2021).

1.2.4.3 Aspergillus

Of the more than 450 known *Aspergillus* species (Houbraken *et al.*, 2020), only a few are known to cause aspergillosis, for example *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus lentulus*, and *Aspergillus niger*. The mould and its spores are often found in soil, dust, seeds, grains, decomposing vegetation and building materials. The increase of carbon dioxide in the atmosphere, due to climate change, enhances the burden of *Aspergillus*-driven chronic allergic diseases (Gnat *et al.*, 2021). This is especially concerning as *Aspergillus* infections can cause a gradual destructive disease called chronic pulmonary aspergillosis, one that complicates numerous pulmonary disorders including tuberculosis, and the systemic inflammatory disease sarcoidosis especially in individuals with impaired immune function. Chronic pulmonary aspergillosis is estimated to affect more than three million people around the world especially patient with underlying lung conditions such as asthma (Brown *et al.*, 2012). Superficial symptoms of *Aspergillus* infections are part of its invasive course, and they include nodules, ulcers, skin necrosis, vesicles, and indurated erythema, with fully invasive symptoms often manifesting as fever, chest pain, cough, haemoptysis, and dyspnea (Gnat *et al.*, 2021).

1.2.4.4 Pneumocystis

The species *Pneumocystis jirovecii* is an opportunistic fungus that co-evolved with mammals, including humans. This yeast cannot survive outside of a mammalian host and becomes pathogenic in hosts with weakened immune systems (Rokas, 2022). The high prevalence of *P. jirovecii* pneumonia (PJP) in patients with acquired immunodeficiency syndrome (AIDS) makes the disease a marker for AIDS (Brown *et al.*, 2012).

A factor in the difficulty of treating PJP is that there has been no effective method developed for culturing *Pneumocystis* in the laboratory (Brown *et al.*, 2012). It is also a relatively recently defined disease, identified in isolated cases after World War II in European premature and malnourished infants in orphanages (Truong and Ashurst, 2022). Along with people diagnosed with AIDS, other high-risk groups include transplant patients, patients with prolonged immunosuppression treatment, and those with haematological malignancies. Symptoms are often similar to that of bacterial and viral pneumonia, as well as tuberculosis, namely high fever, dyspnoea, tachypnoea, and tachycardia (Truong and Ashurst, 2022).

1.2.5 Invasive Pulmonary Fungal Infections

1.2.5.1 Pneumocystis Pneumonia

Pneumocystis is one of a few potentially serious fungal infections that attack the pulmonary system and is reported most commonly among immunocompromised patients (Gingerich *et al.*, 2021). *Pneumocystis jirovecii pneumonia* (PCP, or PJP) was once a rare but almost always fatal disease that showed an increase in cases with HIV-infected patients after 1980 (Tasaka, 2020). According to Brown *et al.* (2012), the global incidence of PJP is unknown due to diagnostic limitations, but it is estimated to reach 400,000 cases per year, with mortality rates of more than 13% (that is, more than 52,000 deaths per year) and probably as high as 80%.

An interesting clinical aspect of *Pneumocystis pneumonia* is that it manifests differently in patients with HIV and those without, with much higher severity in the latter than in the former (Tasaka, 2020). In non-HIV patients, PCP/PJP has an abrupt onset of respiratory distress and insufficiency, with the course of the pneumonia taking around a week to progress from dry cough and fever to respiratory failure. However, in HIV-infected patients, even those with immune-suppressing treatment, the progression from initial symptoms to full respiratory failure can average between two weeks and two months (Tasaka, 2020).

The data indicates that the mortality rates from PCP/PJP also vary widely, with a low range of 10% to 20% among HIV-infected patients, and a high range of 30% to

60% in those without an HIV infection (Tasaka, 2020). Part of the difference can be ascribed to the body's immune response, where in the HIV-infected patient is found to have fewer neutrophils in the alveoli, unlike the higher counts of non-HIV patients (Gingerich *et al.*, 2021). Although neutrophils do not lead directly to pulmonary damage, the lower count in HIV-infected patients does result in less severe oxygenation impairment, meaning that inflammation (related to neutrophil count) may be a more significant damage factor than the fungus itself (Tasaka, 2020).

Treatment of PCP/PJP relies mainly on trimethoprim-sulfamethoxazole (TMP-SMX), but drug resistance and side effects can be serious negative factors. Negative side effects are common and range from rashes, fever, cytopenia, and hyperkalemia to marrow suppression, nephritis, meningitis, renal failure and pancreatitis (Gingerich *et al.*, 2021).

1.2.5.2 Cryptococcosis

The two main infectious fungal species in this genus are *Cryptococcus neoformans* and *Cryptococcus gattii*. *C. neoformans* is most often the fungal cause of infections in immune-compromised patients (Gnat *et al.*, 2021), while *C. gattii* is more prevalent in healthy patients (Brown *et al.*, 2012). The range for *C. gattii* was primarily sub-tropical and tropical zones, but climate change has apparently allowed the fungal species to thrive in new areas, such as Vancouver, Canada, and the North-western states of Oregon and Washington, with mortality rates of 33%, indicating a possible public health crisis (Brown *et al.*, 2012). The worldwide number of *Cryptococcus neoformans* cases exceeded one million, with a mortality rate ranging from 20% to 70% (Brown *et al.*, 2012). Various subtypes of *C. neoformans* have been recognised and the latest divisions have been proposed as followed: *C. neoformans* var. *grubii* (serotype A) with 3 genotypes (VNI, VNII, VNB); *C. neoformans* var. *neoformans* (serotype D or VNIV); and 5 other cryptic species, *C. gattii*, *C. bacillisporus*, *C. deuterogattii*, *C. tetragattii*, and *C. decagattii* (serotypes B/C or VGI-IV) as stated in (Maziarz and Perfect, 2016).

Cryptococcal infections are initiated primarily by inhalation of the infectious propagules (yeast cells or basidiospores) that become deposited in pulmonary alveoli. The lodged propagules may remain dormant in the host tissue for years, although it can also cause pulmonary infection that is largely asymptomatic or minimally symptomatic

(Maziarz and Perfect, 2016). There may also be migration of the propagules to cause the development of subcutaneous lesions as one of the milder reactions, though the most severe damage done by cryptococcal infections are due to infection and damage to the central nervous system (Gnat *et al.*, 2021). Such a transition, from pulmonary infection to central nervous system, is more likely to occur with *C. neoformans* than with *C. gattii* (Maziarz and Perfect, 2016).

The main antifungal used to treat cryptococcal infections is Amphotericin B deoxycholate (AmBd). Moreover, Flucytosine (5-FC) is used as first-line treatment in cryptococcal meningitis or severe pulmonary cryptococcosis in conjunction with AmBd at a dose of 100 mg/kg/d in split doses (Maziarz and Perfect, 2016; Molloy *et al.*, 2018).

1.2.5.3 Candidiasis

Given the widespread presence of *Candida* species on and around humans, the prevalence of *Candida*-based pulmonary infections is relatively low. The adaptability of the species, capable of switching between yeast, pseudohyphae, and hyphae during infection and disease, means it can adapt to surviving on and inside more tissues and organ systems, thus reducing its need for respiratory system entry and incubation (Ivanov *et al.*, 2022). This makes *Candida* a particularly difficult fungal agent to protect against and treat. For example, a specific antifungal resistance mechanism in certain *Candida* strains involves upregulation of the ATP-binding cassette transporter genes CDR1 and CDR2. This leads to the development of high resistance to azole-based antifungal treatments, a commonly used therapeutic method against fungal infections (Ivanov *et al.*, 2022).

The most common *Candida* species, *C. albicans*, is thought to be present in most humans, but remains relatively inert in terms of infections due to the competition for mucosal and epithelial cell adhesion sites in which bacteria are more aggressive, better adapted, and thus more successful. The end result is that *C. albicans* is rarely the cause of an infection, even in the case on immune-compromised patients, and may actually be a co-relevant factor more so than a direct cause, even in cases of pneumonia where *C. albicans* is readily detected (Pendleton *et al.*, 2017).

1.2.6 *Aspergillus fumigatus* and Aspergillosis

Studies in this thesis are focussed on *A. fumigatus* and therefore a deeper dive into the biology and range of pulmonary fungal infections with which the fungus is associated will be provided. The species may cause a range of lung infections collectively categorized as Aspergillosis. Other aspergilli such as *A. flavus*, *A. terreus*, and *A. niger* may also rarely cause aspergillosis, but globally *A. fumigatus* is the main etiological agent (Gnat *et al.*, 2021). In this closer look, the ecological niche, life cycle, occurrence and other characteristics of *Aspergillus fumigatus* will be described, along with specific reviews of different types of pulmonary infections caused by this fungal species.

1.2.6.1 *Aspergillus fumigatus*

Aspergillus fumigatus is one of the most common and widespread airborne fungi worldwide. It is saprophytic, feeding on organic matter across a wide range of ecological niches. The fungus grows optimally at 37°C, and in soil-based environments with pH levels of 3.7 to 7.6, but has been found at a range of soil temperatures of between 12° and 65°C, and in pH ranges as wide as between 2.1 to 8.8 (Kwon-Chung and Sugui, 2013). This extensive range of viable niches means that most people on the planet will breathe in spores of *A. fumigatus* on a daily basis (Latgé and Chamilos, 2019).

The species reproduces asexually via the production of conidia. Conidia (asexual propagules) are pigmented and ca. 2.5µm in diameter in size. They are generated in chains on independent phialides that emerge from conidiophores borne on conidial heads, with each conidial head capable of producing thousands of conidia (Latgé and Chamilos, 2019). Furthermore, conidia can survive sub-12°C temperatures easily, having been shown to tolerate being submerged in liquid nitrogen for up to 18 years (Kwon-Chung and Sugui, 2013). *A. fumigatus* does not have any elaborate type of release mechanism to launch its conidia into the air, but rather relies on dissemination through disturbances of the environment (physical manipulation of the organic matter, for example) and air currents. Once the conidia are in the air, their small size makes them buoyant, tending to keep them airborne both indoors and outdoors. Even very slight air currents can cause conidia to disperse because of their high level of hydrophobicity, and these airborne conidia are further protected from ultraviolet

irradiation due to the melanin in their cell wall (Kwon-Chung and Sugui, 2013). The survival potential is thus enhanced by the thick outer wall of the conidia, which is also a barrier to the immune system (Latgé and Chamilos, 2019).

Reproduction was long thought to be purely by asexual means, but accumulating evidence implied the presence of a cryptic sexual cycle. For example, various *A. fumigatus* population genetic studies provided evidence of recombination (Dyer and Paoletti, 2005; Paoletti *et al.*, 2005; O’gorman *et al.*, 2008). In addition, bioinformatic analysis of the first sequenced genomes of *A. fumigatus* revealed over 200 annotated genes associated with sexual reproduction (Galagan *et al.*, 2005; Nierman *et al.*, 2005). These included so called mating-type (*MAT*) genes, which act as master regulators of sexual reproduction. Analysis of *MAT* gene distribution then led to the identification of putative sexually compatible MAT1-1 and MAT1-2 isolates in global and regional surveys of isolates (Paoletti *et al.*, 2005; O’gorman *et al.*, 2008). Sexual reproduction was then conclusively first identified by O’gorman *et al.* (2008) who found that crossing MAT1-1 and MAT1-2 isolates could induce a complete sexual cycle under certain laboratory conditions (**Figure 1.2**).

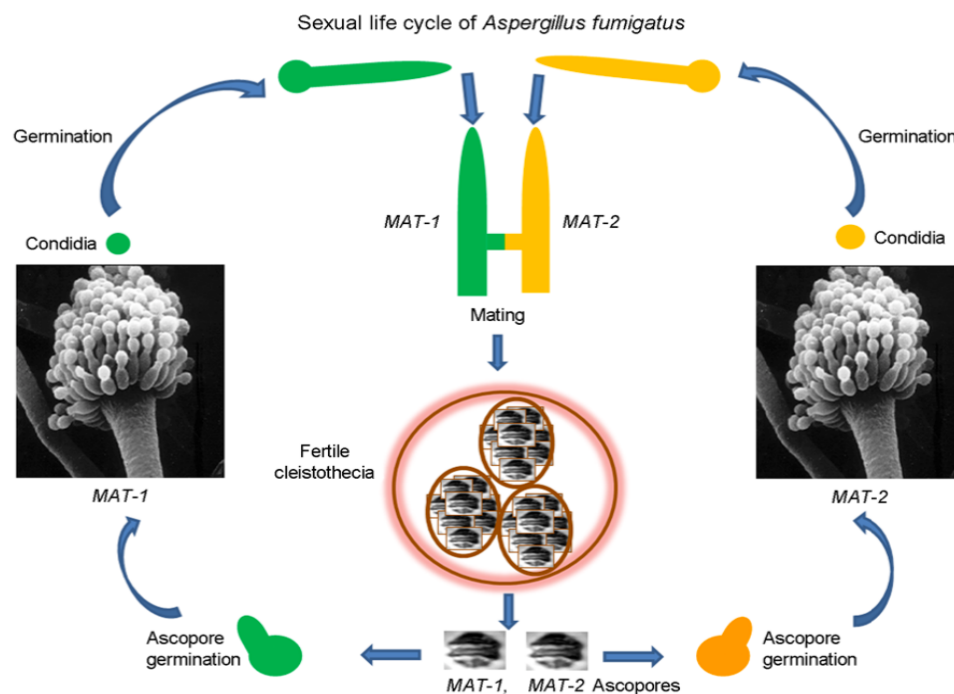


Figure 1-2: Typical life cycle of heterothallic species such as *A. fumigatus*. Adapted from (Kwon-Chung and Sugui, 2011).

The resulting sexual reproductive structures consisted of yellowish cleistothecia (*Neosartorya* morph), typically averaging 150 to 500 μm in diameter, that formed singly or in small clusters, with the clusters primarily arranged along the junction where hyphae of the parental isolates came into contact. The cleistothecia contained asci, which themselves contained eight ascospores, sexual propagules ranging in colour from yellowish-white to greenish-white, lenticular in shape with two equatorial crests, and measuring from 4 - 5 μm in diameter (O’gorman *et al.*, 2008). The ascospores of *A. fumigatus* were found to be heat-resistant, being able survive heat stress of 70°C for several hours and have been shown to require a heat shock for optimal germination (Swilaiman *et al.*, 2020; O’gorman *et al.*, 2008). More recently a study of sexual fertility of global isolates found that ca. 85% of isolates were able to undergo sexual reproduction with laboratory ‘supermater’ strains (Swilaiman *et al.*, 2020). Nevertheless, sexual reproduction appears to be relatively rare in the field, and asexual reproduction is instead thought to be the most common form of propagation (Latgé and Chamilos, 2019).

The factors that make *A. fumigatus* such a potentially dangerous pathogen, especially for immune-compromised patients, include the facts that; (a) it survives and grows in a wide range of environmental conditions; (b) it has very effective dispersal in the air; (c) the physical characteristics of the conidia allow them to bypass the nasal and sinus filters to reach the distal airways; and (d) the *A. fumigatus* genome content provides swift adaptability and defensive reactivity to the host environment (Kwon-Chung and Sugui, 2013). Unlike other fungal species, for whom the human immune system is a nearly impregnable barrier, the genome of *A. fumigatus* contains a series of characteristics which taken together allow infectivity. The genome has 22 secondary metabolism gene clusters and 16 different secondary metabolites, allowing for greater adaptability against immune system cells. The genome also has an abundance of effective enzymes, such as catalases, superoxide dismutases, and glutathione transferases for the detoxification of reactive oxygen species, reducing the negative impact of some of the basic immune system processes (Kwon-Chung and Sugui, 2013).

The relatively small size of the spores of *A. fumigatus* also enables them to penetrate to lower airways where initiation of infection is more successful. Unlike the typical fungal spore that is $\geq 5.0 \mu\text{m}$ in diameter, spores of *A. fumigatus* are 2.0-3.0 μm in

diameter, a size that enables them to reach the alveoli when inhaled (Abad *et al.*, 2010; Kwon-Chung and Sugui, 2013). Moreover, even with increasing humidity, the size of the conidia of *A. fumigatus* does not change significantly due to its hydrophobicity, a factor that ensures it retains the size needed for entry into lower airways (Kwon-Chung and Sugui, 2013; Ortiz *et al.*, 2022). Further, *A. fumigatus* conidia are resistant to reactive oxygen species (ROS) due to the melanin in the cell wall and have exposed sialic acid residues that facilitate adhesion to the host's basal lamina (Sugui *et al.*, 2008). The resistance to ROS will be discussed in more detail in **Chapter 5**. Such ease of entry, protection from host defence systems, and adhesion enable colonization in immunocompromised individuals leading to aspergillosis infections.

1.2.6.2 Aspergillosis Diseases

Invasion of the body by *A. fumigatus* can cause three types of illness: allergic, based on sensitivity to the fungus; chronic, in which the fungus causes long-term negative effects to health, and invasive, affecting deep tissues and organs. Because *A. fumigatus* is so widespread, the potential for all three illness types is higher than with other fungal species (Latgé and Chamilo, 2019). The primary mortality risk is associated with invasive illnesses, with *A. fumigatus* being a primary cause, particularly in pulmonary diseases in immuno-compromised patients (Gnat *et al.*, 2021). The three categories of illness associated with *A. fumigatus* are now reviewed in terms of their symptomology, risk population, mortality rates, and COVID-related impact, among other factors.

1.2.6.2.1 *Allergic Bronchopulmonary Aspergillosis (ABPA)*

Allergic bronchopulmonary aspergillosis (ABPA) is a lung illness that affects individuals who are allergic to the fungus *Aspergillus*. This allergic reaction to *Aspergillus* leads the immune system to overreact, resulting in lung inflammation. The inflammation caused by the allergic reaction can become chronic, leading to impaired lung function, and may eventually be fatal (Suzanne Lareau, 2014). Prevalence of ABPA covers all age groups, with patients at risk being those with already compromised respiratory systems such as patients with asthma, cystic fibrosis, or chronic obstructed pulmonary disease (COPD) (Tracy *et al.*, 2016).

The diagnostic criteria and symptoms for ABPA are: observation of episodic bronchial obstruction, coughing that worsens periodically, coughing up mucus plugs

that are often brown in colour or bloody (haemoptysis), chest pain, shortness of breath, intermittent fever, elevated total serum IgE, and immediate cutaneous reactivity to *A. fumigatus* (Suzanne Lareau, 2014; Tracy *et al.*, 2016). Chest X-rays are often not helpful in diagnosing ABPA as the infection does not tend to leave visible signs until the late stages, but a CT scan can reveal early stages more readily. Spirometry is used to see how severe the lung problem is and to determine the effectiveness of treatment (Suzanne Lareau, 2014).

Early diagnosis is key to effective treatment, using primarily anti-inflammatory medications to control the allergic reaction and anti-fungal drugs to help eliminate the *A. fumigatus* infection. The most effective treatment developed for a majority of ABPA cases is based on using oral prednisone (a glucocorticoid medication) at a dosage of 0.5 mg/kg/day for 7-14 days, on a schedule of alternate days for three months, and then tapering by 5 mg every two weeks for an additional three months (Tracy *et al.*, 2016). This protocol combines anti-inflammatory effect with the body's natural defences to reduce potential side effects (Suzanne Lareau, 2014).

The recent COVID pandemic has placed more immune-compromised and pulmonary-debilitated patients in the at-risk population (Ezeokoli *et al.*, 2021). Acute respiratory distress syndrome (ARDS), caused by coronavirus disease-19 (COVID-19) and characterized by difficulty in breathing, has emerged as an important condition that predisposes patients to subsequent pulmonary aspergillosis, similar to severe influenza, with the increased reported of mortality related to COVID-19 associated pulmonary aspergillosis (CAPA) (Arastehfar *et al.*, 2020).

1.2.6.2.2 Chronic Pulmonary Aspergillosis (CPA)

CPA is a collection of consuming illnesses that are characterised by prolonged and relapsing cough, dyspnea, and weight loss, but also ranging into haemoptysis and bronchial or pulmonary haemorrhage (Schweer *et al.*, 2014). Diagnosing CPA is made more difficult because many of its related symptoms, such as fatigue, chest pain, shortness of breath, and excessive sputum mirror that of other illnesses. The most effective diagnostic method consists of long-term radiology comparisons that can reveal the development of an aspergilloma or other structural changes involving pulmonary

cavities, as well as the presence of detectable *Aspergillus* IgG (Maghrabi and Denning, 2017).

The mortality rate of CPA is considered low because the condition rarely develops on its own, but more often as a co-morbidity condition (Brown *et al.*, 2012). If left untreated CPA can eventually lead to the development of multiple aspergilloma and/or chronic pulmonary haemorrhaging, which can often require surgery for effective treatment (Maghrabi and Denning, 2017).

Patients who present stable and asymptomatic simple aspergillomas over a period of 6 months to 2 years do not require antifungal treatment, relying on the body's immune and healing systems to manage the fungal infection. In symptomatic patients with severe haemoptysis, surgical resection is often required cure. Oral triazoles, which inhibit the conversion lanosterol to ergosterol, are the primary treatment medications for less-severe cases, except for fluconazole, an azole that has been found to have little or no effect on the *Aspergillus* cell membrane (Maghrabi and Denning, 2017).

1.2.6.2.3 Invasive Aspergillosis (IA)

An invasive fungal infection is one in which a species, such as *A. fumigatus*, penetrates beyond subcutaneous tissue to invade the bloodstream, bone marrow, deep tissues, and organs (Ivanov *et al.*, 2022). These are the most dangerous of the fungal infections due to the difficulty of treatment based on having a limited range of effective antifungals (Ezeokoli *et al.*, 2021), the ubiquitous spread of the fungus throughout the tissues (Brown *et al.*, 2012), the increased use of invasive procedures and long-term immune-suppression treatments, and the fact that invasive infections almost always arise in already immune-compromised patients (Rokas, 2022). Add to this the fact that the COVID-19 pandemic has increased the number of people with seriously-compromised pulmonary systems as well as cascading immune system over-reactions (cytokine storms) (Mancuso *et al.*, 2022) and it is easy to understand how these types of infections are raising serious concerns.

The mortality rate for invasive Aspergillosis is well above 50%, and can reach close to 100% if diagnosis is missed or treatment delayed, for patients in the highest risk categories, such as those in prolonged ICU stays, transplant recipients, patients undergoing long-term chemotherapy, and those who have chronic conditions that

weaken the immune system (Brown *et al.*, 2012; Mancuso *et al.*, 2022). Treatment depends on what tissues and/or organs are affected, the co-morbidities present, and the range of effective anti-fungal medications applicable to the case (Ezeokoli *et al.*, 2021). The growing danger of invasive fungal infections risks is also enhanced by climate change, as it is exposing more people to a wider range of fungi, along with the increased level of exposure due to routine and emergency medical treatments (Gnat *et al.*, 2021).

1.2.7 Treatment of Aspergillosis Infections

1.2.7.1 Antifungals in use

It is no easy task to diagnose aspergillosis infection especially in immunosuppressed patients. It is also a difficult condition to treat and is associated with a high mortality rate. Allergic and long-term sinus and pulmonary aspergillosis have been detected in several clinical settings (Howard *et al.*, 2009). Aspergillosis infections is treated using prescriptions of antifungal drugs in order to prevent the propagation of the diseases to other organs. The core therapy for these conditions is treatment with azole antifungals such as itraconazole and voriconazole, as well as a more recent treatment by posaconazole that is active against many fluconazole and voriconazole resistant strains. This is because azoles are a class of drugs which are licensed for treating aspergillosis, and which have fewer side effect for the host compared to the other available classes of antifungal drugs such as the polyenes and the echinocandins. Although echinocandins (caspofungin) and polyenes (amphotericin B) are licensed intravenous agents for invasive aspergillosis, their use for long-term and allergic aspergillosis is restricted due to possible side effects and possible lack of efficacy (see below) (Howard *et al.*, 2009; Wei *et al.*, 2015).

Azoles target and inhibit the enzyme Lanosterol 14- α -demethylase (encoded by the *cyp51A* and *cyp51B* genes in *A. fumigatus*) which is critical for ergosterol biosynthesis illustrated in **Figure 1.3**. Through their azole rings, these antifungal agents create a stoichiometric complex when it encounters the iron atom of the heme group present on the active site of P-450 demethylase in the CYP51A protein. The resultant inhibition of ergosterol synthesis and subsequent malfunction of the fungal membrane, and associated build-up of lanosterol and additional 14-methylated sterols, results in a

severe inhibition of fungal growth (Hof, 2006; Shapiro *et al.*, 2011). Therefore, treatment with azole results in ergosterol exhaustion and accrual of toxic intermediate sterols (Snelders *et al.*, 2011; Zhang *et al.*, 2015).

Apart from azoles, two other categories of antifungal medications exist, namely polyenes and echinocondins (Snelders *et al.*, 2011). The most widely used polyene is amphotericin B which binds to ergosterol in the cell membranes of the fungi cells, forming channels through these membranes and making the cells leaky, leading to cell death. However, this type of drug has limited use because of its side effects of host toxicity, such as renal dysfunction which might be because of the similar structure between ergosterol and cholesterol in mammalian cell membrane (Shapiro *et al.*, 2011; Ivanov *et al.*, 2022). The second type of antifungal drug is echinocandins, which block and inhibit 1,3- β -D glucan synthesis resulting in loss of cell wall integrity (Cappelletty and Eiselstein-McKittrick, 2007; Snelders *et al.*, 2011). Echinocandins such as caspofungin, have a better safety profile and are commonly used as first-line therapy for *Candida* infections; nevertheless, their range of activity against *Aspergillus* is inconsistent, and they do not acquire enough drug levels when infection reach brain tissue (Shaw and Ibrahim, 2020).

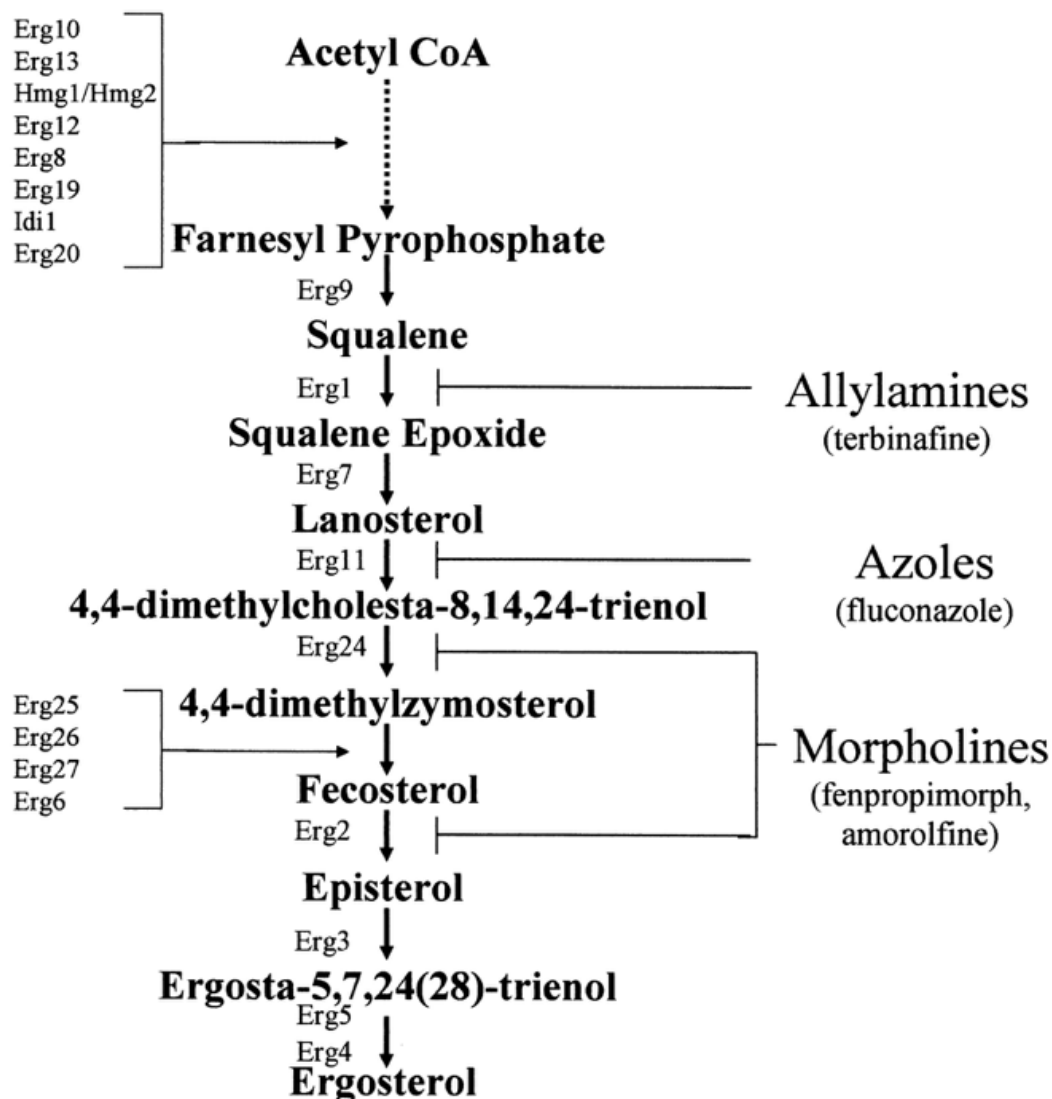


Figure 1-3: A model of ergosterol biosynthesis pathway adapted from *Saccharomyces cerevisiae* which show the commonly used antifungal drugs and genes involves in this process. Adapted from (Onyewu *et al.*, 2003)

1.2.7.2 New Antifungals

With increasing reports of the evolution of novel mechanisms for resistance to antifungal drugs resulting in treatment failure, combined with possible side effects of the currently available antifungals, there is an urgent need for new antifungal medications for the life-threatening invasive fungal infections. Promising studies of new antifungal agent under clinical development have recently been reviewed (Ivanov *et al.*, 2022). These include Fosmanogepix, an N-phosphonooxymethylene prodrug of manogepix (MGX), which is being explored for the treatment of invasive fungal

infections caused by *Candida*, *Aspergillus*, other moulds. Its mode of action is inhibition of GPI-anchored wall transfer protein 1 (Gwt1), which is required for mannoprotein trafficking and attachment to the cell membrane and outer cell wall. Gwt1 suppression by MGX has several physiological effects since these mannoproteins are necessary for cell wall integrity, adhesion, pathogenicity, and evading the host immune system. Reduced cell wall-linked mannoproteins, hyphal development, biofilm formation, cell size and shape malformation, glucan layer exposure, and endoplasmic reticulum stress are some of the side effects (Shaw and Ibrahim, 2020; Ivanov *et al.*, 2022). Fosmanogepix, has been found to be effective against resistant strains such as echinocandin-resistant *Candida* and azole-resistant *Aspergillus* because of its new method of action, and its efficiency has been evaluated in vivo using mouse and rabbit as infection models.

A second promising candidate is Olorofim which is one of the orotomide class of antifungals, which has been tested in clinical trials studies for the treatment of invasive mould infections. Olorofim acts by inhibiting the enzyme dihydroorotate dehydrogenase (DHODH), an oxidoreductase that is involved in the biosynthesis of pyrimidine, which leads to the inhibition of formation of uridine-5'-monophosphate (UMP) and uridine-5'-triphosphate (UTP). These two uridine compounds are required for a variety of biological functions such as the formation of UDP-sugars, which are important for synthesis of the cell wall components 1,3- β -D-glucan and chitin. As a result, Olorofim's reduction of pyrimidine production may disrupt the fungal cell wall, resulting in cell lysis (Wiederhold, 2020; Ivanov *et al.*, 2022). Olorofim's mode of action against pyrimidine biosynthesis is unique, and should be unaffected by processes that generate resistance to currently available antifungals such azoles and amphotericin B. However, its ineffectiveness against yeasts, such as *Candida* and *Cryptococcus* species, as well as members of the *Mucorales* group, is a serious drawback (Wiederhold, 2020; Ivanov *et al.*, 2022). **Figure 1.4** shows the chemical structure of the various antifungals described above.

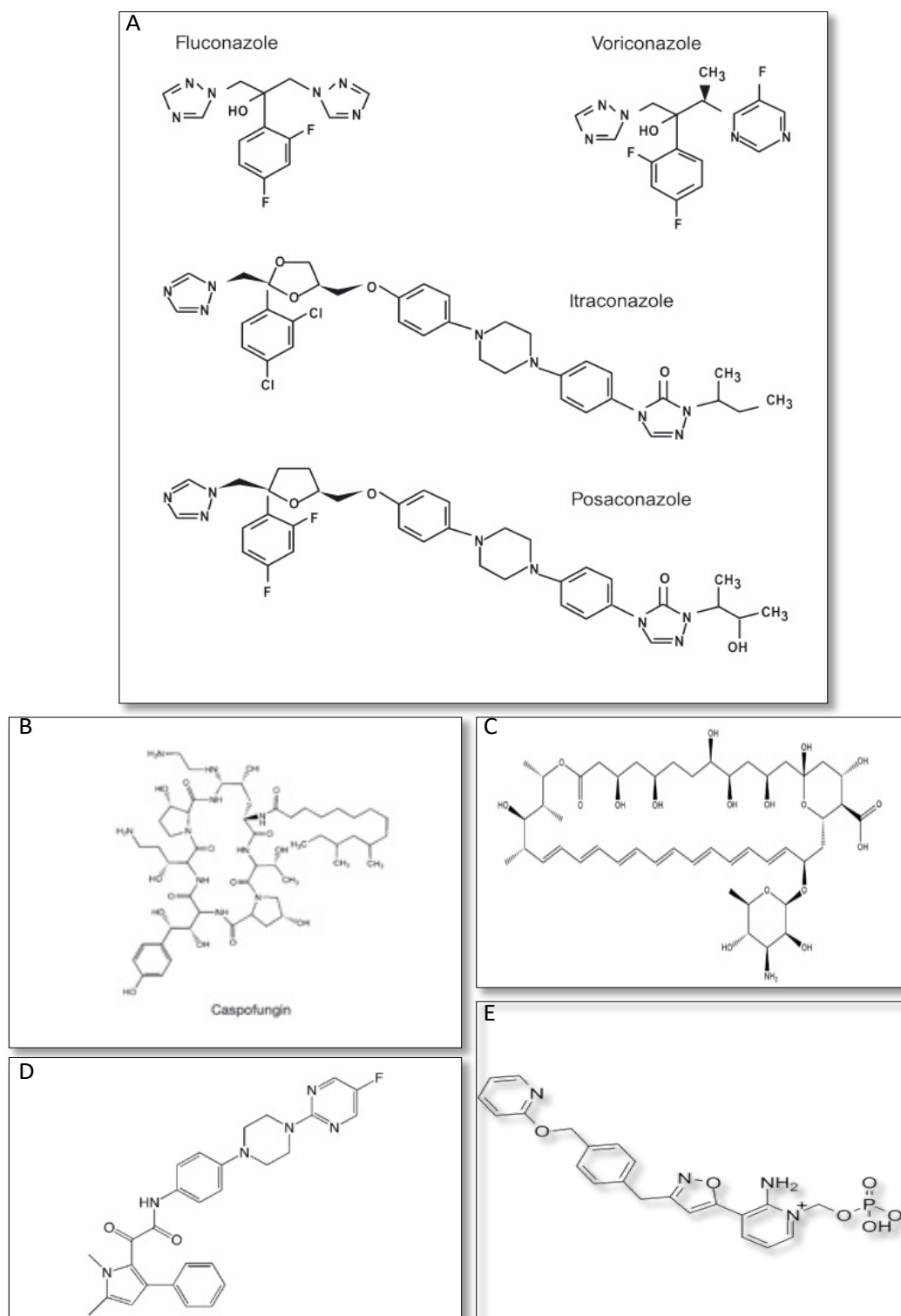


Figure 1-4: Chemical structure of most antifungal in use (A, B, and C) and the new promising identified antifungal (D and E) for treating Aspergillosis infections. (A) various azole antifungals, adapted from (Hof, 2006). (B) Echinocandins such as caspofungin, adapted from (Cappelletty and Eiselstein-McKittrick, 2007). (C) Polyene such as amphotericin B, adapted from (Ivanov *et al.*, 2022). (D) Olorofim, adapted from (Wiederhold, 2020). (E) Fosmanogepix, adapted from (Shaw and Ibrahim, 2020).

1.3 Azole Resistance in *Aspergillus fumigatus* and Treatment Failure

The past twenty years has seen an ever-increasing appearance of resistance to azole antifungal drugs in isolates of *A. fumigatus* both from a clinical and environmental setting. The precise evolutionary mechanism leading to selection for azole resistance in the environment has not, at present, been completely explained. However, there is growing evidence that the use of azole fungicides in agriculture might be an important factor. Azole treatment will not be successful in patients who have an invasive fungal infection due to the presence of an azole-resistant *A. fumigatus* isolate. Since the azoles are the principal agents used for treatment and prophylaxis, azole resistance in *A. fumigatus* isolates impacts the management of IA (Snelders *et al.*, 2011).

The first azole resistance in *A. fumigatus* was detected in the United States in 1997 (Howard *et al.*, 2009; Snelders *et al.*, 2011). Since then, several incidences of azole resistance have been reported in numerous countries. In the last few years, the increased detection of azole-resistant *A. fumigatus* strains poses a risk to public health since there is a lack of effective substitute treatments (Rivero-Menendez *et al.*, 2016).

The development of azole resistance is believed to be arise in two possible ways (**Figure 1.5**). The first source is in patients with chronic aspergillosis, who are under long-term azole medication (Rivero-Menendez *et al.*, 2016; Chowdhary *et al.*, 2017). In this scenario, resistance may develop through exposure and direct selection against azole fungicides. Thus, these patients are primarily infected by a susceptible *A. fumigatus* that later mutates into a resistant phenotype when exposed to azole drugs. Therefore, these resistant mutants are isogenic to the preliminary ones that triggered the infection. The second source of azole resistance is due to the usage of azole fungicides in the environment (Wei *et al.*, 2015; Rivero-Menendez *et al.*, 2016; Chowdhary *et al.*, 2017; Verweij *et al.*, 2020). In this scenario, resistance may again develop through exposure and direct selection against azole fungicides but this time in the general environment. Fungal plant pathogens also pose a threat to the quality of human health owing to the production of mycotoxins which can be either carcinogenic, teratogenic or neurotoxic (Davies *et al.*, 2021). Therefore, fungicides are useful in the control of fungi in both plants and animals with the fact that azole are the most used antifungals for crop

protection in the world as they have proven to be so effective in the eradication of fungi (Becher and Wirsal, 2012). Azole fungicides have been used all around the world and currently used to treat more than half of the farming land in the European Union. By contrast, azole use in the USA is less than 5% of acreage compared to the European Union. Even though the use of azoles in the EU has decreased over the past few decades, they remain widely used in the Netherlands where triazole usage doubled in use between 1995 and 2007 (Verweij *et al.*, 2009). Meanwhile, azoles are also used in the general environment for treatment of building materials prone to fungal decay, and as a protectant against food decay (Verweij *et al.*, 2009).

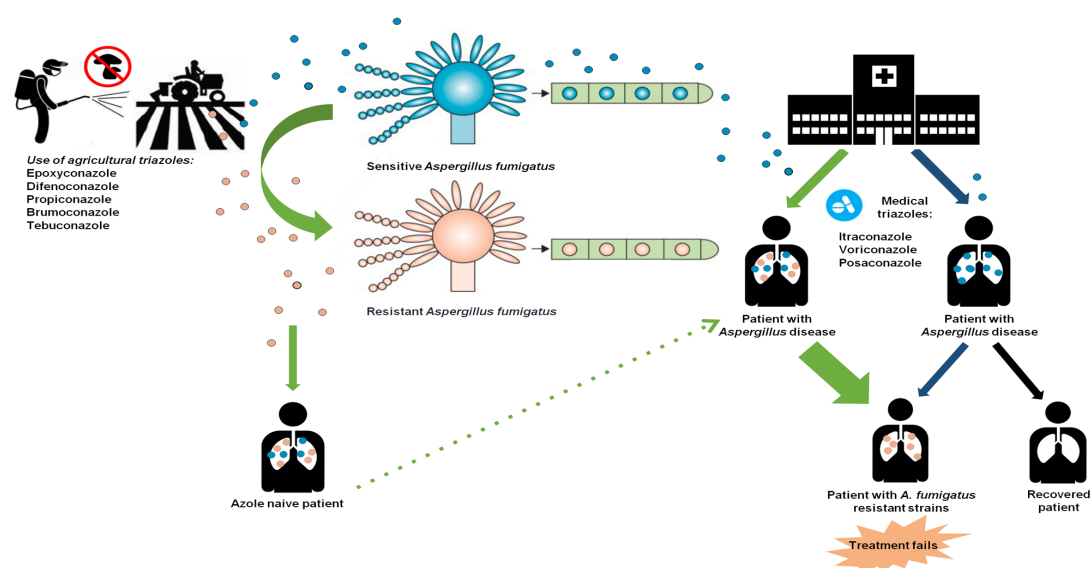


Figure 1-5: Possible proposed routes of azole resistance development in *A. fumigatus*. The extensive utilization of azole agents in the agriculture is believed to be the primary basis of resistance selection in *A. fumigatus*. Azole resistance can also occur when the client is under azole medication for long period. Adapted from (Berger *et al.*, 2017).

Azole antifungals are the only category of pesticides that are used both for agricultural use and in clinical medicine. Resistance to pesticides occurs when the antifungals fail to meet expected outcomes of disease control. Total disease failure has in the past been experienced with benzimidazole fungicides (Ma and Michailides, 2005), and there are growing concerns at the moment to the long-term efficacy and possible disease treatment failure in use of azoles, with reports of lowered susceptibilities to triazoles in fungal pathogens being experienced all over Europe (Verweij *et al.*, 2009). Resistance to azoles can be attributed to various mechanisms. These include amino acid substitution (s) in CYP51A resulting from DNA changes in the coding region of the *cyp51A* gene, as well as transcriptional enhancers which lead

to overexpression of the *cyp51A* gene (Becher and Wirsal, 2012). The most frequently reported mechanism of resistance which has been found in clinical and environmental isolates of *A. fumigatus* has been termed ‘TR₃₄/L98H’. The appearance of the amino acid change (L98H) in the *cyp51A* gene is believed to be a result of a long treatment with azole. However, the effect of utilizing fungicide in controlling plant pathogenic moulds in the general environment was then thought to later lead to the presence of a tandem repeat (TR) in the *cyp51A* promoter region (see **Section 1.3.1** below for more detail). Thus, the use of azoles fungicides has induced cross-resistance in *A. fumigatus* isolates present in the environment (Verweij *et al.*, 2009).

Understanding the emergence of azole resistance in *A. fumigatus* is critical for public health. Zhang *et al.* (2017) hypothesized that asexual sporulation that is plentiful in nature is necessary for the phenotypic emergence of azole resistance mutants in *A. fumigatus*. This concept relies on the fact that whenever a resistance mutation occurs during asexual reproduction inside the multinucleate mycelium, the ensuing resistant nucleus is originally accompanied by hypersensitive nuclei within a heterokaryotic cell. All else being constant, if resistance mutants are recessive, resistance is not wholly translated into the phenotype when the hypersensitive nuclei also exist inside the mycelium. Therefore, the production of uninucleate asexual spores allows resistant nuclei to break out from the heterokaryotic mycelium enabling the resistant phenotype to be fully expressed after germination. Consistent with this hypothesis is the observation that asexual sporulation may be possible from aspergilloma(s) inside the lungs of patients, and these are linked with the possible evolution of azole resistance (Zhang *et al.*, 2015). Evidently, azole resistance is a mounting concern because patients infected with azole-resistant *A. fumigatus* develop a high likelihood of treatment failure and substitute treatment choices are limited.

1.3.1 The Molecular Mechanisms of Azole Resistance in *Aspergillus fumigatus*

Azoles act as antifungals by hampering ergosterol biosynthesis by inhibiting the activity of 14- α -lanosterol demethylase that is encoded by both *cyp51A* and *cyp51B* in *A. fumigatus*. Most studies have associated changes specifically in the *cyp51A* gene in

a majority of clinical *A. fumigatus* azole-resistant isolates (Gsaller *et al.*, 2016; Rivero-Menendez *et al.*, 2016). Several mechanisms of resistance, with varying levels of azole resistance, have been identified such as point mutations in *cyp51A* that affect the drug interaction with the target protein or cause overexpression of *cyp51A* (Camps *et al.*, 2012a; Chowdhary *et al.*, 2014; Wei *et al.*, 2015)

Various point mutations in the *cyp51A* gene cause reduced susceptibility to azole drugs in *A. fumigatus*. The majority of azole-resistant isolates of *A. fumigatus* reported in the existing literature harbour mutations in *cyp51A*, either in the coding or associated promoter region. Point mutations of the *cyp51A* gene can alter the amino acid sequence of the encoded CYP51A protein and the resulting drug-enzyme interaction, leading to azole drug resistance in isolates. Lanosterol demethylase represents one of a large class of mono-oxygenases referred as P450 proteins since they have such a spectral absorbance peak in their cysteine-linked heme molecules at their active sites (Wei *et al.*, 2015). The CYP51A protein possesses two ligand channels that allow the lanosterol substrate and azoles to encounter the protein's active site. However, point mutations of *cyp51A* gene could result in structural changes of the encoded CYP51A protein which may directly limit the docking of the azole, reducing affinity for the target by varying the opening of the structure in each channel, or alter the site of the heme molecules, whilst still retaining enzymatic function (Wei *et al.*, 2015; Liu *et al.*, 2016). Commonly reported sites of the point mutation in *cyp51A* gene, denoted as hot spots, are G54, G138, M220, and G448 (**Figure 1.6.A**)

The first point mutation to have been explored in depth is linked with a change from glycine at position 54 in the protein (G54). Various point alterations of this gene at G54 are associated with cross-resistance to posaconazole (POS) and itraconazole (ITC) (Mellado *et al.*, 2007). A study by Diaz-Guerra *et al.* (2003) described the presence of this single base mutation in six ITC-resistant strains of clinical origin. These single point modifications resulted in alterations in the triplet encoding glycine amino acid at position 54 which is located at a very conserved region of the CYP51A protein, for example change of G-to-T, at position 161 lead to change the amino acid glycine (G) to glutamic acid (E) amino acid.

An amino acid change at methionine 220 constitutes another common form of point mutation associated with *cyp51A* alteration leading to reduced azole resistance

susceptibility. Mellado *et al.* (2004) and Ashton *et al.* (2022) found a methionine to lysine (M220K) change in CYP51A was correlated with greatly increased resistance to itraconazole. Meanwhile, mutations in glycine 448 with substitution of serine (G448S) can cause resistance to voriconazole (VRC), and various alterations in glycine 138 (G138) can result in resistance to VRC and ITC. Additional possible point mutations that require further examination to validate their influence in azole resistance include F219C, P216L, F219I, G434C, G432S, Y431C, and A284T (Rivero-Menendez *et al.*, 2016).

Another major mechanism of azole resistance is the combination of a mutation(s) within the *cyp51A* coding region, together with a tandem repeat (TR) within the promoter region of *cyp51A* (**Figure 1.6.A**). Examples include a TR₃₄ merged with a lysine to histidine switch at position 98 within the protein of *cyp51A* (termed as ‘TR₃₄/L98H’) (Mellado *et al.*, 2007) and TR₄₆ with two amino acid changes, T289A and Y121F, within the coding region of *cyp51A* (termed ‘TR₄₆/T289A, Y121F’) (Van Der Linden *et al.*, 2013). Further molecular-genetic research has revealed the likely mechanistic basis of azole resistance is due to much increased *cyp51A* gene expression seen in the various tandem repeat mutants, hence reducing the impact of azole binding efficacy. More in depth information about the tandem repeats linked to azole resistance in *A. fumigatus* will be presented in **Chapter 3**.

1.3.2 Other Resistance Mechanisms: Non-Cyp51A

The recent rise in azole resistance in populations of *A. fumigatus* has been accredited mostly to mutations in the *cyp51A* gene, with more than 30 different *cyp51A* mutations having been reported in clinical or environmental isolates. However, some researchers have identified certain collections of *A. fumigatus* clinical isolates where up to 50% of resistant isolates have no apparent mutation in *cyp51A* or its promoter region (Rivero-Menendez *et al.*, 2016; Fraczek *et al.*, 2013; Paul *et al.*, 2017; Wei *et al.*, 2017; Hagiwara *et al.*, 2018; Furukawa *et al.*, 2020). This suggests the presence of other resistance mechanisms that are non-*cyp51A* based. This has been suggested to be linked to mechanisms such as point mutation in several genes e.g. (*hapE* gene, *cox10* gene, *Hmg1* gene and *erg6* gene), overexpression of an ABC transporter *cdr1B* gene, overexpression

of efflux pumps and overexpression of *cyp51B*, as will now be described (lower section of Figure 1.6.B).

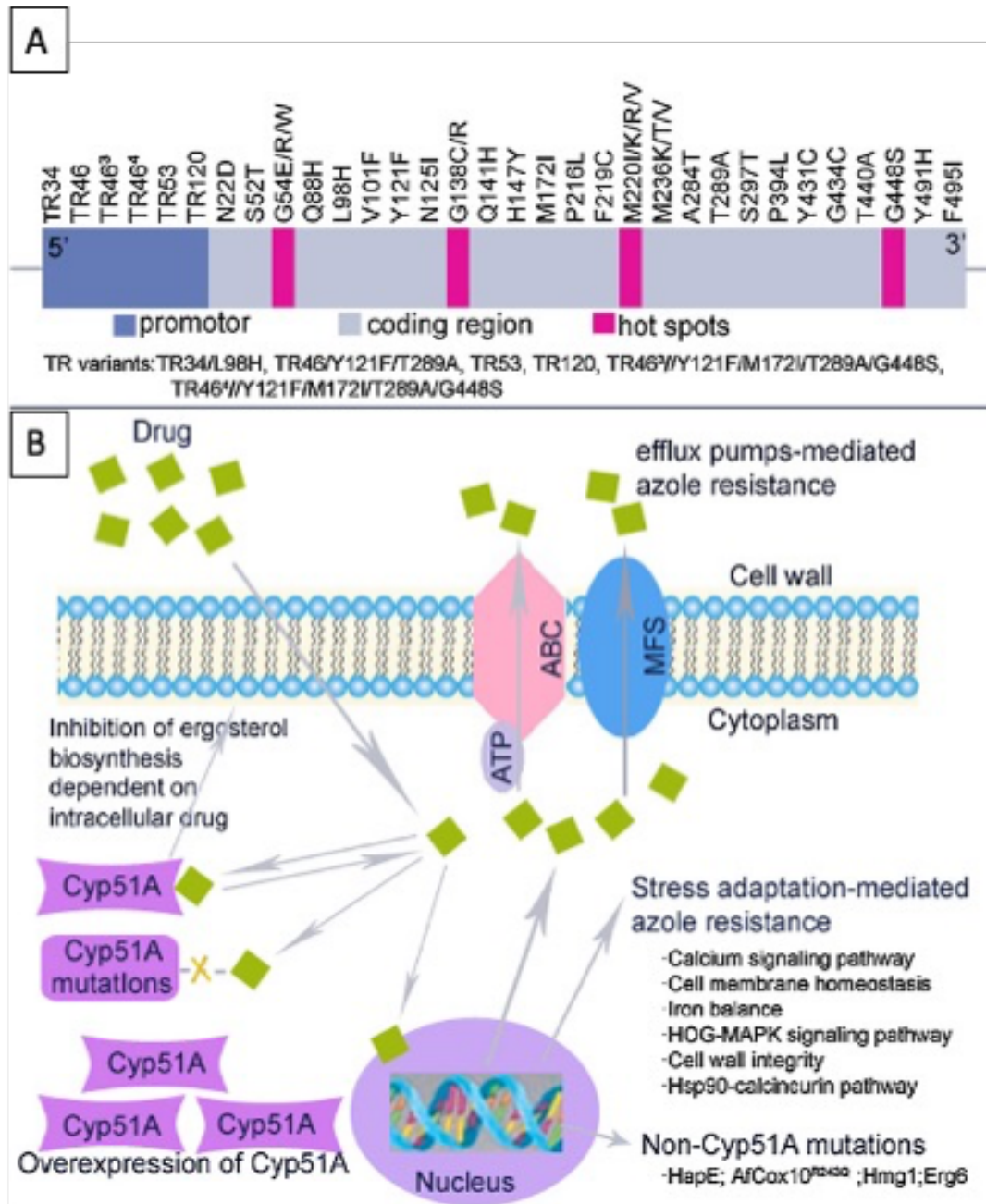


Figure 1-6: Representative diagram showing the frequently reported azole resistance mechanisms in *A. fumigatus*. (A) The most reported mutations in the azole target Cyp51A. (B) Mechanisms of action of *cyp51A* mutations, and other forms of resistant mechanisms. Adapted from (Chen *et al.*, 2020a).

Firstly, point mutations in several genes other than *cyp51A* have been shown to confer azole-resistance phenotypes. Camps *et al.* (2012c) reported a discovery of a new resistance mechanism resulting from a mutation in the “CCAAT-binding-transcription-

factor-complex” (CBC) subunit called *HapE* (AFUA_6G05300). The CBC serves as a negative regulator of ergosterol biosynthesis and its absence leads to higher sterol levels through transcriptional derepressing of 14 α -demethylase. Mutations in the gene can significantly hinder the binding of the CBC to its target site. Notably, amino acid-P88 of HapE plays a key role in fastening the regulatory CCAAT component (Camps *et al.*, 2012c). Thus, the HapE subunit of the CBC serves as a repressor of *cyp51A*; however, the P88L mutation can eliminate this repressor function (Gsaller *et al.*, 2016).

An azole resistance mechanism has recently been linked to the *A. fumigatus* farnesyltransferase *Afcox10* gene (AFUB_065450) (Wei *et al.*, 2017). Sequence analysis showed that a point mutation R243Q in this gene contributed to itraconazole resistance in *A. fumigatus* in an *algA* (a calcium- dependent protein encoding gene) independent manner. Microscopy analysis indicates that the Cox10 protein affects calcium responses, resulting in anomalous activation of calcium signalling. In vitro, *cox10* deletion also resulted in reduced-colony growth and sporulation, and an abnormal cell wall composition (Wei *et al.*, 2017).

Mutation in the *hmg1* and *erg6* genes can provide further non-*cyp51A* mediated azole-resistance in *A. fumigatus*. By comparing the genomic sequences of resistant and sensitive isolates of *A. fumigatus*, which showed no alterations in the *cyp51A* gene, Hagiwara *et al.* (2018) found evidence that mutations in *erg6* (which encodes sterol 24-C- methyltransferase) and *hmg1* [which encodes hydroxymethylglutaryl-CoA (HMG-CoA) reductase] were associated with azole resistance in *A. fumigatus*. Modification of *hmg1* (S269F) was situated at the start of the sterol-sensing domain (SDD), while the alteration of *erg6* (A350T) was located at the sterol_MT_C domain in the C-end (Hagiwara *et al.*, 2018). Since these genes are directly involved in the biosynthesis of ergosterol, the authors presumed that these mutations interrupted this pathway leading to hyperaccumulation of ergosterol in the cells; hence, stimulating azole resistance. The findings affirmed that all isolates displaying multi azole resistance contained mutations in both *erg6* and *hmg1* genes. Another study by Rybak *et al.* (2019) also showed that alteration in the conserved sterol-sensing domain of Hmg1 led to increased triazole resistance in *A. fumigatus*. In their work three different mutations (F262 Δ , I412S and S305P) were introduced into *akuB*^{KU80} susceptible strains which all resulted in significantly increased resistance to triazole.

Secondly, increased expression of the *cdr1B* gene in azole-resistant strains of *A. fumigatus* has been observed which, according to most scholars, is directly associated with efflux transporter activity (Rivero-Menendez *et al.*, 2016). It is noted that mutation of the *cdr1* homolog has also been reported to commonly cause azole resistance in *C. albicans*, hence the naming of the protein (Candida drug resistance 1) (Shukla *et al.*, 2007). Fraczek *et al.* (2013) observed increased expression of the *cdr1B* gene (>5-fold) in eight of ten azole-resistant strains of *A. fumigatus*, three of which displayed >30-fold amplified expression. A knockout of *cdr1B* led to a 4-fold decline in VRC, POS, and ITC minimum inhibitory concentrations (MICs). Hagiwara *et al.* (2017) demonstrated the influence of *AtrR* (a Zn₂-Cys₆ transcription factor) in the expression of *cdr1B*. In particular, *AtrR* appeared to exert transcriptional control on *cdr1B* and *cyp51A* gene expressions through promoter binding (Hagiwara *et al.*, 2017). Recently, a study by Furukawa *et al.* (2020) described in considerable detail the importance of two transcription regulators from the CBF/NF-Y family, AFUB 029870 (NctA) and AFUB 045980 (NctB) (Negative cofactor two A and B), whose loss of activities lead to multi antifungal resistant phenotype. They demonstrate that the NCT complex is a crucial regulator of ergosterol biosynthesis and the azole exporter CDR1B in *A. fumigatus*.

A third possible mechanism of azole resistance concerns overexpression of efflux pumps, which act by pumping the drugs out of the cell resulting in reduced accumulation of drugs in the fungal cell (Wei *et al.*, 2015). This mechanism represents a ubiquitous resistance process that activates the membrane-associated efflux pumps to distinguish diverse chemicals, facilitating multidrug resistance. In fungi, two dissimilar drug efflux systems control azole resistance, namely the ATP-binding cassette (ABC) proteins, and the major facilitator super-family (MFS) pumps. In *A. fumigatus* around 49 ABC family transporters and 278 MFS genes have been identified (Wei *et al.*, 2015). However, there is as yet very little clear demonstration of azole resistance in *A. fumigatus* as a result of altered expression of efflux pumps, the only examples being a clinical strain (strain AF72) that has been found to overexpresses the *atrF* gene (by five times), which encodes an ABC transporter efflux pump. This overexpression only occurs when it is grown in the presence of itraconazole (Slaven *et al.*, 2002).

Finally, in *A. fumigatus* Cyp51 proteins are encoded by two presumed paralogous genes *cyp51A* and *cyp51B*, which share 63% amino acid identity (Mellado *et al.*, 2001).

Most of the reported azole resistance mechanisms have been linked to a change in *cyp51A* or its promoter. However, overexpression of *cyp51B* might also be capable of inducing an azole-resistance phenotype. A study by Buied *et al.* (2012) found high-level constitutive and over-expression of *cyp51B* in certain azole-resistant isolates. Another study by Rybak *et al.* (2019) found that the expression of *cyp51B* among three triazole-resistant clinical isolates was significantly higher than a control sensitive strain.

1.3.3 Azole Resistance in Other *Aspergillus* Species

Azole resistance is becoming a major concern to medics tasked with caring for patients with high risk of invasive mycoses. *A. fumigatus* is the leading cause of IA (85%), followed by species including *Aspergillus flavus* (5% - 10%), *Aspergillus terreus* (2% - 10%) and *Aspergillus niger* (2% - 3%) (Rivero-Menendez *et al.*, 2016). As in *Candida* species, recent attention has also begun to focus on azole-resistant of these *Aspergillus* species which can cause superficial diseases related with vaginal and oral mucosa in addition to disseminated deep-tissue and bloodstream infections (Garber, 2001; Wiederhold, 2017).

Aspergillus flavus represents the second major cause of IA in people with a suppressed immune system and it has been reported to be prevalent in countries with arid climates (Rivero-Menendez *et al.*, 2016). Although the basis of azole resistance has been well researched in *A. fumigatus*, mechanisms that lead to azole resistance in *A. flavus* are not well-understood (Dudakova *et al.*, 2017). However, some mutations have been identified in *cyp51A* of *A. flavus*, including K197N, Y132N, D282E, M288L, and T469S, and in the *cyp51B*, mutations such as, D411N, H399P, T486P, and T454P, all of which have been linked to increased azole resistance in *A. flavus* (Hagiwara *et al.*, 2016). Interestingly, *A. flavus* contains an additional *cyp51C* paralogue, unlike other clinically important aspergilli. Dudakova *et al.* (2017) suggested that mutations of the *cyp51C* gene might be the cause of azole resistance in some *A. flavus* isolates, and it was found that Y319H mutation in this gene was associated with azole-resistance (Hagiwara *et al.*, 2016). Additional studies by Sharma *et al.* (2018) resulted in the discovery of further novel substitutions in the *cyp51C* gene: S196F, N423D, A324P, and V465M, which were possibly linked to azole resistance. Meanwhile, a point

mutation L558W in the *yap 1* gene (which encodes Yap 1, a transcription factor) of *A. flavus* was found to result in augmentation of activity of an ATP transporter *atrF*, which was correlated with low VRC susceptibility (Pérez-Cantero *et al.* (2020). Lastly, according to Sharma *et al.* (2018) the overexpression of several genes such as *cyp51B*, ABC transporters (*mdr1*, *mdr2*, *mdr4*, and *atrF*), and *mfs1* were linked to VRC resistance in *A. flavus*.

Regarding other aspergilli, Pérez-Cantero *et al.* (2020) reported that the presence of H382R and V104I and an amalgamation of I377V/S507I/L511M mutations in the *cyp51A* gene of *A. niger* resulted in a decline in susceptibility to VRC and ITC. In parallel, distinct *cyp51A* gene alterations, including T321A and L21F or the amalgamation of A185G/ T321A/ 327S/ V422I/ L492M/ I503F/ Q504P or a combination of A9V/ L21F/ A140V/ P413S/ D505E mutations led to decreased susceptibility to azole and associated agents in *A. tubingensis*. In *A. terreus*, only a M217 mutation in *cyp51A* has so far been associated with reduced azole vulnerability (Pérez-Cantero *et al.*, 2020).

Other than azole resistance in *Aspergillus* species, azole resistance has also been reported in other fungal species that are opportunistic human pathogens, including *Candida albicans*, *Candida parapsilosis*, and *Candida tropicalis* (Wiederhold, 2017). Azoles constitute the most frequently prescribed antifungals used in the treatment of *C. albicans* infections. However, several studies have reported the ability of *Candida* to develop resistance to azole prescriptions, which can vary according to the type of infection. *C. albicans* isolates collected from patients with candidemia exhibit the least likelihood of azole resistance ranging from 0–5%. By contrast, the likelihood of azole resistance from *C. albicans* isolates collected from oropharyngeal candidiasis patients is high, thought to be due to previous azole treatments and prior infections. Additionally, *C. glabrata* exhibits the highest probability of azole resistance among *Candida* species and demonstrates intrinsic decreased receptiveness to azole antifungal (Whaley *et al.*, 2017). Other clinical fungi in which azole resistance has been reported include *C. parapsilosis* and *C. tropicalis*. The worldwide occurrence of azole resistance in *C. parapsilosis* disseminated diseases varies between 2 and 5%. In the Asia-Pacific region, azole resistance in *C. tropicalis* varies between 0 to 83% (Whaley *et al.*, 2017).

1.3.4 Azole Resistance is a Worldwide Problem

Up to the present time, Europe has a greater reported incidence of azole resistance in *A. fumigatus* than any other continent. Concern about the increase in such cases was expressed by two reports produced in the late 2000s, one in the UK and the other in the Netherlands (Rivero-Menendez *et al.*, 2016). The first report, produced in 2007, concerned some Dutch patients whose IA condition was caused by pan-azole resistant strains. This even included some patients who had not received previous azole treatment. Upon examination of these strains, a new resistance mechanism was discovered; namely, TR₃₄/L98H (Mellado *et al.*, 2007; Verweij *et al.*, 2007). Meanwhile, a second study undertaken in the UK in 2009 revealed a rapid increase in the incidence of azole resistance in *A. fumigatus*, with resistance attributed to a variety of mechanisms (Howard *et al.*, 2009), which was updated the year after to show evidence of the presence of multiple azole resistance isolates with unknown form(s) of resistance mechanisms (Bueid *et al.*, 2010). On the same report the authors demonstrated that the high frequency of azole resistance in clinical isolates of *A. fumigatus* had increased from 14% in 2008 to 20% in 2009, with 43% of these isolates exhibiting unknown forms of azole resistance.

From that time onwards, almost every nation in Europe has reported azole resistant cases in clinical samples. Such nations include: Austria, Belgium, Denmark, France, Germany, Greece, Italy, the Netherlands, Poland, Portugal, Romania, Spain, Sweden, Turkey and the UK (Rivero-Menendez *et al.*, 2016). Although the two-point mutations M220 and G54 have sometimes been reported in Europe (Howard *et al.*, 2009; Camps *et al.*, 2012a), by far the most frequently occurring mutation, in both in the clinical and environmental samples, is the TR₃₄/L98H genotype. Subsequent to its initial 2007 report in Dutch and Spanish isolates, TR₃₄/L98H has been identified throughout Europe (Rivero-Menendez *et al.*, 2016). An additional new TR-based resistance mechanism known as TR₄₆/Y121F/T289A was then detected in the Netherlands in 2009 (Van Der Linden *et al.*, 2013). This has subsequently been reported in many nations from environmental and clinical setting (Rivero-Menendez *et al.*, 2016).

Within Europe, there has been frequent identification of azole resistance in environmental strains. In this setting, TR₃₄/L98H and TR₄₆/Y121F/T289A have been

the most often detected resistance forms and consequently their appearance has been suggested to be associated with the widespread utilisation of agricultural fungicides (Rivero-Menendez *et al.*, 2016). For example, Van Der Linden *et al.* (2013) reported that 14 out of 140 environmental resistant strains had the TR₄₆/Y121F/T289A mechanism, whereas 126 had TR₃₄/L98H. An analysis of 455 environmental isolates in Germany showed that 45 harboured the TR₃₄/L98H mutation and six harboured the TR₄₆/Y121F/T289A. Furthermore, in Italy 16% of environmental isolates of *A. fumigatus* sampled between May 2011 and June 2012 were found to show resistance to the azoles ITC and POS (Prigitano *et al.*, 2014).

Azole-resistant in *A. fumigatus* is also becoming recognized beyond Europe and has now been reported from other countries such as the United States, Colombia, Tanzania, China, India, Iran, Japan, Kuwait, and Pakistan (Rivero-Menendez *et al.*, 2016; Friedman and Schwartz, 2019). The most recent estimation of reported azole resistance isolates of *A. fumigatus* in the US was 1.5% with 30% of those isolates show unknown form of resistance linked to *cyp51A* gene (Friedman and Schwartz, 2019). The worldwide distribution of reported *A. fumigatus* azole resistance by mechanisms and the reported isolates with unknown form of resistance mechanism or non-*cyp51* mutations are shown in **Figure 1.7**.

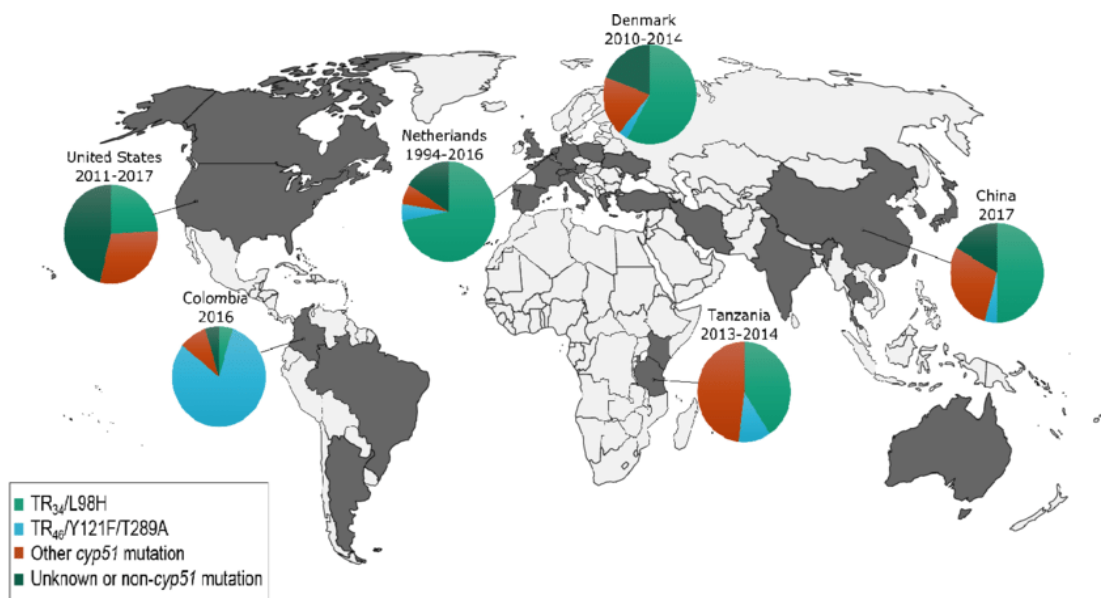


Figure 1-7: Global map showing the worldwide distribution of different forms of azole resistance exhibited by *A. fumigatus* according to resistance mechanism, showing mechanisms related to *cyp51A* and also unknown or non-*cyp51A* mutations. Adapted from (Friedman and Schwartz, 2019).

Triazole resistance in *Aspergillus* species, both from clinical and in environmental settings, is one of the most significant public health hazards spreading through the world (Verweij *et al.*, 2020). Despite the fact that Europe has the greatest rates of triazole resistance, many cases have been documented on every continent, and novel resistance mechanisms are being discovered. Regardless of the fact that *A. fumigatus* is one of the most prevalent *Aspergillus* species, triazole resistance has also been found in a number of cryptic *Aspergillus* species. Therefore, molecular approaches for detecting azole resistance in clinical cultures from infected patients might be extremely valuable in laboratory practise. Moreover, it is critical to look into the origins of environmental samples that are resistant to triazoles in greater depth, because limiting the usage of agricultural azoles might be a key step in lowering resistance rates in the clinical situation (Rivero-Menendez *et al.*, 2016).

1.4 Pathogenicity and Other Virulence Factors of *Aspergillus fumigatus*

While the lack of specific virulence factors in *A. fumigatus* enables the immune system of healthy individuals to prevent colonization by *A. fumigatus*, the coincident adaptation of the fungus to the host environment facilitates infection in immunocompromised individuals. For example, since the optimum temperature for germination and growth of *A. fumigatus* corresponds to the human body temperature (due to its occurrence in rotting vegetation, often at elevated temperatures, in the wild), entry of conidia leads to significant changes within a short period. Within four hours the conidia shed the outer layer and swell, germinating to form short hyphae between six and eight hours (Kwon-Chung and Sugui, 2013). Such quick germination also arises from gene expression that enables *A. fumigatus* to overcome growth-limiting conditions such as low glucose, oxygen, and nitrogen levels.

For a fungal pathogen to cause disease, it must first survive the hostile environment of the host, primarily through evasion of the host's immune system, especially the phagocytic mechanisms. This involves macrophages and neutrophils attacking invading microorganisms, developing reactive oxygen, nitrogen, and chlorine species that alter

or inactivate vital proteins, lipid membranes, and DNA in any foreign organism, and thus increase the efficacy of the host response (Missall *et al.*, 2004). Part of this reaction is the production of ROS to cause severe oxidative stress on the fungal pathogen, where radicals can overwhelm the fungus while the host's immune system balances out the oxides with more abundant resources (Gessler *et al.*, 2007). Many of these mechanisms defence pathways have some equivalent similar mechanisms present in plants as well such as production of reactive oxygen species (ROS) and expressions changes of some genes (Dickinson, 2004).

However, oxidation by the immune system also involves nitrogen, and chlorine species, therefore it is possible that the where one specific oxidation species is largely ineffective, others may be significantly more efficacious in destroying the fungal pathogen (Missall *et al.*, 2004). Further complicating the immune response to a fungal pathogen is the fact that certain fungal species such as *A. fumigatus* are able to use the oxidative reactions to trigger additional defence mechanisms that aid its survival and propagation within the host. Filamentous fungi have a larger number of antioxidant enzymes, secondary metabolites with antioxidant functions, as well as enzymes like the NADPH oxidases, which regulate multicellular development through ROS production and enhance in-host survival potential (Aguirre *et al.*, 2006). More detailed information of oxidative stress and how *A. fumigatus* can overcome this stress will be provided in **Chapter 5**.

1.5 Aims

Aspergillus fumigatus is an ascomycete fungus found worldwide, where it plays an important role in nutrient recycling and can be found in soil and rotting vegetation. It is also an opportunistic pathogen and the major cause of invasive aspergillosis (IA) in humans. The inhalation of its spore is unavoidable, and it is one of the leading causes of IA diseases, especially in immunocompromised individuals. The wide distribution of *A. fumigatus* in nature threatens human life because: (a) this fungus can grow in a wide range of environmental conditions; (b) the size of the spores that this fungus produces are very small, facilitating dispersal in the air; (c) the fungus was found to be able to adapt and survive inside the host by developing certain mechanisms to avoid the host defence system; and (d) many isolates of *A. fumigatus* have developed resistant to antifungal drugs (e.g. azoles), which increases the mortality rate of humans (Kwon-Chung and Sugui, 2013). A major breakthrough in the understanding of the biology of the species has recently been made with the discovery of sexual fertility in global populations of the fungus, offering a valuable tool for laboratory studies (Swilaiman *et al.*, 2020; O’gorman *et al.*, 2008).

The overall aims of the present doctoral project were therefore to exploit the newly identified sexual cycle of *A. fumigatus* to try and identify the genetic basis of so-far ‘unknown’ forms of azole resistance in the species, and to also try to understand the genetic basis of other virulence factors in *A. fumigatus* in order to deepen our medical knowledge in this area. It was anticipated that such studies might help to inform better methods of disease management in a medical setting via, for example, the development of molecular diagnostics to detect the presence of such mechanisms and their possible spread in the environment. In particular, specific aims of this study were as follows:

- 1- To characterize a newly discovered azole resistance mechanism termed “TR₁₃₀” in *A. fumigatus*, using a combination of sexual crossing and complementary laboratory and genomic analyses to analyse this resistance mechanism.
- 2- To explore the genetic basis of azole resistance in certain non-*cyp51A* resistant isolates of *A. fumigatus*, for which the genetic basis of resistance was unknown, using bulk segregant analysis (BSA) which has recently been developed for use with sexual crosses of *A. fumigatus*.

- 3- To exploit the sexual cycle of *A. fumigatus* to provide insights into the genetic basis of a recently identified phenotypic trait linked to the evolution of pathogenicity in this fungus (specifically a hypoxia response), for which the genetic basis is so far unknown.

Chapter- 2 Materials and Methods

Material and methods which are used in multiple chapters of the thesis will now be described. Materials and methods that are specific to only certain chapters will instead be presented in the relevant chapter. Unless otherwise noted, all chemicals were obtained from Sigma-Aldrich (UK) or Fisher Scientific (UK). The compounds were all of analytical or molecular biology grade. Unless otherwise stated, all chemical solutions and media were routinely sterilised by autoclaving at 121°C for 20 minutes before use.

2.1 Materials:

2.1.1 Fungal Strains

Various *Aspergillus fumigatus* strains were used in this thesis. The first set of strains were reference strains available from the University of Nottingham (either the BDUN collection or personal gifts), which included supermater crossing strains (known to be wild-type azole sensitive) (Swilaiman *et al.*, 2020), previously obtained azole resistant strains with the TR34 and TR46 genotypes, and host strains for genetic manipulation (GM) work.

The second group of 32 isolates were supposed to be azole resistant and have non-*cyp51A* forms of resistance to azoles antifungals. These isolates were obtained from different locations (London, Netherlands, and Germany) via collaborations with different groups who had previously tested these isolates for azole susceptibility and examined them for the presence of known *cyp51A*-based mutations (**Table 2.1**). These isolates were used for investigation of unknown forms of azole resistant mechanisms in *A. fumigatus* using a bulk segregant analysis approach. An early screening of these isolates resulted in the apparent identification of a unique tandem repeat in the promoter of the *cyp51A* gene in eight isolates, results of which will be presented in Chapter 3. Studies using other strains from this group will be presented in Chapter 4.

The final group of 34 *A. fumigatus* isolates were obtained from two different locations (London-UK and Dartmouth-USA) via collaboration with different groups

(Table 2.1). These isolates were used in investigations exploiting sexual crossing to give insights into the pathogenicity and the population biology of *A. fumigatus*, the results of which will be presented in Chapter 5.

Table 2-1: List of *A. fumigatus* strains obtained externally during this project.

Number of isolates	Location	Collaboration with	Presented in
9	Imperial College, London, UK	Prof. Matthew Fisher, Dr. Thomas Sewell and Dr. Darius Armstrong-James	Chapter 3
1	Doctoral student at Nottingham (George Ashton)	Former PhD student	Chapter 4
5	Radboud Medical University, Netherlands	Prof. Paul Verweij	
17	Imperial College, London, UK	Prof. Matthew Fisher, Dr. Thomas Sewell and Dr. Darius Armstrong-James	
10	Germany	Dr. Amelia Barber (Leibniz Institute for Natural Product Research, Jena, Germany)	
28	Imperial College, London, UK	Prof. Matthew Fisher, Dr. Thomas Sewell and Dr. Darius Armstrong-James	Chapter 5
6	Dartmouth, USA	Dr. Robert A. Cramer	

2.1.2 Strain Maintenance and Revival

All *A. fumigatus* isolates/ strains used in this project were maintained as stocks in 10% glycerol at -80 °C. When required, appropriate strains were thawed, and a cotton swab (SLS, UK) was used to inoculate conidia on Aspergillus complete media (ACM) agar in 9 cm diameter Petri plates (Thermo Scientific, USA) and ACM slopes (STARLAB, Germany) (Paoletti *et al.*, 2005) for revival before the stocks were refrozen. Longer-term stocks of *A. fumigatus* were also prepared by making spore suspensions in 10% glycerol which were then stored under liquid nitrogen.

2.1.3 Growth Conditions and used Media

Strains of *A. fumigatus* were grown on either 9 cm diameter Petri plates (Thermo Scientific, USA) or on slopes in 30 ml universal containers (STARLAB, Germany) containing ACM solid media (see below). A sterile swab was used to transfer spores

from a pre-existing culture or from minus -80 °C or liquid nitrogen stocks. Unless otherwise stated, fungal cultures were grown under light at 28 °C for 5-7 days.

In preparation for DNA extraction, all isolates were grown in ACM liquid media. The same ingredients as standard ACM were used but without addition of agar. A sterile swab was used to transfer the spores from seven-day-old cultures to 250-ml conical flasks, containing ACM liquid media (50 ml). Flasks were set up overnight on a rotary shaker at 28 °C.

2.1.3.1 Aspergillus Complete Media (ACM)

All isolates were grown in ACM (Paoletti *et al.*, 2005), which consisted of the following ingredients: D-glucose, 10 g (Fisher Scientific, UK); yeast extract, 1 g (Oxoid, UK); peptone, 2 g (Bacto, USA); casamino acids, 1 g (Bacto, USA); Aspergillus vitamin solution, 10 ml (see below); Aspergillus salt solution, 20 ml (see below); adjusted to pH 6.5 and made up to a final volume of 1 L, using distilled water. Agar, 20 g (Sigma, UK) was added for solid ACM or the medium was used direct without agar for liquid ACM.

2.1.3.2 Aspergillus Minimal Medium (AMM)

AMM consist of the following ingredients: D-glucose, 10 g (Fisher Scientific, UK); 1.46 g glutamine (Acros, USA), 20 ml of 50x salt solution (see below) and 1 ml 1000 x trace element solution (see below). Agar, 20 g (Sigma, UK) was added for solid AMM and without agar for liquid AMM. Adjusted to pH 6.5 and made up to a final volume of 1 L, using distilled water.

2.1.3.3 Aspergillus Vitamin Solution

Aspergillus vitamin solution stock was prepared by dissolving the following components: 400 mg p-aminobenzoic acid (Duchefa Biochemie, The Netherlands), 50 mg thiamine HCl (Sigma, UK), 2 mg d-biotin, 100 mg nicotinic acid, 250 mg pyridoxine hydrochloride, 1.4 g choline chloride and 100 mg riboflavin in 1 L distilled water.

2.1.3.4 50x Salt Stock

One litre of the 50x salt solution was made using; 26 g potassium chloride (KCl), 26 g magnesium sulfate heptahydrate (MgSO₄·7H₂O) and 76 g potassium dihydrogen

phosphate (KH_2PO_4) and made up to a final volume of 1 L with distilled water. The salt solution was stored at 4°C after adding 2 ml chloroform.

2.1.3.5 1000x Trace Elements Solution

Trace elements solution was prepared by dissolving the following components: 40 mg sodium borate decahydrate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$), 8 g zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), 800 mg manganese sulphate monohydrate ($\text{MnSO}_2 \cdot 4\text{H}_2\text{O}$), 800 mg iron sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), 400 mg copper II sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), 800 mg sodium molybdate decahydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$), in 1L distilled water. Vacuum filtration was used to sterilise the solution.

2.1.3.6 2x RPMI Media

Roswell Park Memorial Institute (RPMI) media was prepared as previously described (Pfaller *et al.*, 2000; Pfaller *et al.*, 2003) containing RPMI 1640 (Sigma Aldrich, UK), glucose (2%), buffered with 0.165 M morpholinepropanesulfonic acid (MOPS) and adjusted to a final pH of 7.0 with NaOH. Vacuum filtration was used to sterilise the solution. RPMI was mixed with a sterilized 2x water agar (see below) for solid media when required.

2.1.3.7 2x Water Agar

A 7.5 g of agar (Oxoid, U.K.) was dissolved in 250 ml distilled water.

2.1.3.8 Oatmeal Agar (OA)

Odlum's Irish Pinhead Oatmeal was used to prepare the required media for crossing of *A. fumigatus* (O'gorman *et al.*, 2008; Ashton and Dyer, 2019). Eighty grams of oatmeal were added to 2 L of tap water in a 5-L flask. The mixture was boiled, using a hotplate and then cooked by simmering for 45 minutes. The mixture was then filtered, using two layers of cheese cloth and made up a final volume of 2 L with tap water. The total volume was split into four bottles, each containing 10 g of agar, which was then mixed thoroughly for five minutes, using a magnetic flea inside each bottle. The four bottles were then autoclaved for 30 min at 117 °C using a programme with slow cooling to prevent loss of media from bottles (Ashton and Dyer, 2019)

2.1.3.9 Malt Extract Agar (MEA)

MEA was prepared by dissolving 20 g malt extract, 2 g yeast extract and 20 g agar in 1L of distilled water. The medium was then autoclaved in 250 ml aliquots.

2.1.4 Solutions and Buffers

2.1.4.1 Tris Acetate EDTA (TAE) Buffer

One litre of 50X TAE solution was prepared as follows; 242 g of tris (hydroxymethyl) aminomethane, 57.1 ml of glacial acetic acid and 100 ml of 0.5M EDTA were combined and made up to 1 L with distilled water.

2.1.4.2 Tris Borate EDTA (TBE) Buffer

A 10X TBE solution was prepared by dissolving 108 g tris (hydroxymethyl) aminomethane, 55 g boric acid and 9.3 g EDTA in an appropriate volume of distilled water, which was then made up to a final volume of 1 L with distilled water.

2.1.4.3 0.1% Tween 80

One ml Tween 80 was made up to 1 L with distilled water, the solution was aliquoted into 10 ml glass universals prior to autoclaving. 0.05% Tween 80 lab stocks were also used where appropriate,

2.1.4.4 Phosphate Buffered Saline (PBS)

Prepared by adding 1 preformed tablet (Oxoid) to 100 ml distilled water.

2.2 Methods

2.2.1 Spore Suspensions

Isolates were grown on ACM slopes for seven days at 28 °C under lights. Five millilitres of PBS or 0.1% Tween 80 were then added to each slope to assist in harvesting spores. A sterile swap was used to collect the spores by gently rubbing across the surface of the mycelium on the slope. The solution, which contained a considerable number of

spores, was then transferred to a universal container (10 ml). The arising suspension was filtered through a sterile funnel with Miracloth (Merck Chemical Ltd., UK) to remove hyphal debris. An improved Neubauer haemocytometer (depth 0.1mm); was used to count the number of spores. Spores were counted either in small (central) or medium (corner) squares until a count of approximately 200 spores was obtained. Then the average number of spores per square was multiplied by either 4×10^6 or 2.5×10^5 in small (central) or medium squares, respectively, to obtain the number of spores/ml. The spore suspension was then diluted to a final desired concentration with 0.05% Tween 80.

2.2.2 Antifungal Susceptibility Testing

2.2.2.1 E-test

E-test strips were used to routinely test the susceptibility of *A. fumigatus* isolates against the azole antifungal itraconazole as described before by (Espinel-Ingroff and Rezusta, 2002). These consisted of a calibrated plastic strip impregnated with a continuous concentration gradient of drug (itraconazole) (Biomerieux, France). Twenty-five millilitres of the RPMI 1640 medium were transferred to a 9 cm diameter Petri plates and allowed to set. E-test methods were then used to evaluate *A. fumigatus* isolates as described in **Chapters 3 and 4**. Isolates were first grown on slopes, containing ACM at 28 °C for five days. Spore suspensions were then prepared of a concentration of 1×10^6 spores/ml in 0.05% Tween 80. One millilitre of the spore suspension was added and spread all over the surface of the RPMI agar using a sterile spreader. A pipette was used to remove the excess spore suspension, and the plates were left to be completely dry for 15 minutes before placing the E-test strip at the centre of the plate. Finally, the plates were incubated at 35 °C for 48 hours, according to the E-test strip manufacturer instructions (Biomerieux, France).

2.2.2.2 EUCAST

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) method was also used to test the susceptibility of *A. fumigatus* isolates to the azole antifungals itraconazole or voriconazole. This method is considered a reference method used to establish the minimum inhibitory concentration (MIC) of antifungal agents. MIC

is defined as the lowest concentration of the drug, recorded in mg/L, that inhibits fungal growth (EUCAST, 2003; Messer *et al.*, 2007).

2.2.2.2.1 Preparation of Microdilution 96-well Plate

Plates were prepared following precisely the EUCAST recommended method and dilutions. Stock solutions of azole drugs dissolved in dimethyl sulfoxide (DMSO) were made 200 times higher than the highest concentration required for each drug (3200 mg/L and 1600 mg/L for voriconazole and itraconazole, respectively). The recommended growth medium RPMI with 2% glucose was made double-strength as well to allow for 1:1 dilution once the fungal inoculum is added. In order to produce a dilution series of the drugs at 200-fold, the final concentration steps shown in **Table 2.2** were followed.

Ten serial dilutions of each drug were prepared as described (**Table 2.2**) and then 100 µl of each dilution was transferred to one of 10 tubes containing 9.9 ml $2 \times$ RPMI (1:100 dilution). The 10 tubes containing serial dilutions of the drug ($2 \times$ final concentration) were mixed with RPMI medium by inverting the tubes several times. A 100 µl amount from each tube was dispensed into wells 1–10 of the microdilution plate. The $2 \times$ RPMI medium without drug (control) was dispensed into wells 11 and 12. These plates had been prepared in advance and stored at -20°C for no more than one month. Spore suspensions were prepared as described above (**Section 2.2.1**) at a concentration of 5×10^5 , because the final inoculum must be between 1×10^5 cfu/ml and 2.5×10^5 cfu/ml. A 100 µl amount of spore suspension was added to wells 1–11. In well 12, 100 µl of water was used to dilute the spore suspension. Plates were then covered with plastic bags and incubated at 37°C for 48 h. Readings were recorded after 24 h and a final reading taken after 48 h.

Table 2-2: Preparation steps of voriconazole dilution series with a final concentration of 0.0312 – 16 mg/L. The same steps were followed for itraconazole with a final concentration of 0.0156 – 8 mg/L starting with stock concentration of 1600 mg/L.

Step	Concentration (mg/L)	Volume of antifungal (µl)	Volume of solvent (µl)	Intermediate concentration (mg/L)	Concentration (mg/L) after 1:100 dilutions with double-strength RPMI	Final inoculum concentration after adding spore suspension
1	3200	200	0	3200	32	16
2	3200	100	100	1600	16	8
3	3200	50	150	800	8	4
4	3200	50	350	400	4	2
5	400	100	100	200	2	1
6	400	50	150	100	1	0.5
7	400	50	350	50	0.5	0.25
8	50	100	100	25	0.25	0.125
9	50	50	150	12.5	0.125	0.0625
10	50	25	175	6.25	0.625	0.0312

2.2.3 DNA Extraction

Mycelium was collected from overnight cultures grown in ACM broth (**Section 2.1.3.1**) and filtered using a double layered Miracloth. Mycelium was washed using sterilised distilled water to remove any remaining media and then squeezed in blue towel roll to remove the excess water and media. Mycelium was frozen using liquid nitrogen and ground into a fine powder using a mortar and pestle. Genomic DNA was isolated from 40 mg aliquots of ground mycelia using either a Wizard® Genomic DNA Purification Kit (Promega, UK), or DNeasy® Plant Mini Kit (QIAGEN, UK) according to the manufacturer instructions. The concentration of the resulting DNA was measured using a Nanophotometer P330 (Implen, Germany). Purified gDNA was kept at 4 °C for long-term storage.

2.2.4 PCR Amplification and Sequence Analysis of *cyp51A*, *hapE* and *cox10* Genes

DNA sequence analysis was performed to ensure that the provided azole resistant isolates (**Table 2.1**) did not have any known azole resistance mechanisms, such as a point mutation within the coding regions of a azole target gene *cyp51A*. After extracting

the gDNA (**Section 2.2.3**), PCR and a specific set of primers for each gene were designed using (Primer3) function of MacVector (version 18.2.5 for Macintosh; MacVector, UK), and used to amplify the full coding sequence of *cyp51A*, *hapE* and *cox10*. Specific sets of primers were also used to amplify the promoter region of *cyp51A* gene. All primers were designed using the primer 3 design function of MacVector (v18.2.5 for Macintosh). **Tables 2.3, 2.4 and 2.5** list the primers, the PCR reaction mixture and the thermocycling conditions that were used in PCR amplifications. All reactions were performed using a Techne TC-5000 PCR thermal cycler. One per cent (w/v) of agarose gel electrophoresis was used to visualise 5 µl aliquot of each amplified product to assure the correct product size. Following the manufacturer instructions, NucleoSpin®Gel and PCR clean-up kit were used to clean up the remaining PCR products. Afterwards, the sample was sent to be sequenced at the Deep Seq facility at Queen's Medical Centre (QMC), University of Nottingham.

Table 2-3: Specific sets of primers for *cyp51A*, *hapE* and *cox10* genes.

Name of the primer	Sequence
Cyp51A F1	TGTCTCCTCGAAATGGTGCC
Cyp51A R1	CGTTGAAAAGTCGCAGGTGG
Pro Cyp51A F2	GGACTGGCTGATCAAACATATGC
Pro Cyp51A R 2	GTTCTGTTTCGGTTCCAAAGCC
HapE F3	TATTCACAGGTCCCAGCAACC
HapE R3	TCTTGGTCTTGTCCCATGCC
Cox10 F4	TTATCTCCGGTCCTCGTTGC
Cox10 R4	TTATCTCCGGTCCTCGTTGC

Table 2-4: PCR reaction mixture.

PCR reaction per sample	Volume (µl)
Distilled water	35
5x Phusion HF Buffer	10
dNTPs (AB gene, UK)	0.5
10 mM forward primer	2
10 mM reverse primer	2
Phusion DNA polymerase	0.5
50 ng/µl DNA template	1
Final volume	50

Table 2.2-5: Thermocycling conditions.

Step	Temperature (°C)	Time
Initial denaturation	95	5 minutes
30 cycles	98	20 seconds
	65	30 seconds
	72	30 seconds
Final extension	72	5 minutes
Hold	4	∞

2.2.5 Mating-type PCR

The determination of mating type was important for each isolate as this allowed suitable pairings to be set up for sexual crossing. The PCR diagnostic method of Paoletti *et al.* (2005) was used, whereby a *MAT1-1*-specific primer—AFM1 (5'-CCTTGACGCGATGGGGTGG-3'), and a *MAT1-2*-specific primer—AFM2 (5'-CGCTCCTCATCAGAACAACATCG-3'), together with a 'common' primer—AFM3 (5'-CGGAAATCTGATGTCGCCACG-3'), flanking the *MAT* locus, were used in a

multiplex PCR mating-type assay. The PCR cycling condition was performed according to the manufacturer instructions for use of Phusion® High-Fidelity DNA polymerase (NEB, USA) (**Table 2.6**), and all reactions were performed using a Techne TC-5000 PCR thermal cycler. One per cent (w/v) agarose gel electrophoresis was used to visualise the amplicon sizes, whose sizes were estimated by comparison to a 100 bp DNA ladder.

Table 2-6: Mating-type PCR reaction mixture

PCR reaction per sample	Volume (µl)
Distilled water	34
5x Phusion HF Buffer	10
dNTPs (AB gene, UK)	0.5
10 mM AFM1	1
10 mM AFM2	1
10 mM AFM3	1
Phusion DNA polymerase	0.5
50 ng/µl DNA template	1
Final volume	50

2.2.6 Agarose Gel Analysis

In order to visualise DNA, RNA and PCR products, 0.7-2.0% (w/v) of agarose (Lonza, Rockland, Maine, USA) was dissolved in TAE or TBE buffer, and ethidium bromide was added to a final concentration of 0.2 µg/ml. The DNA or RNA was combined with 5X loading dye, and then loaded into the wells alongside with a DNA ladder for size preference. The gel was then exposed to an electric current and was run at 100 -120 V for at least 30 to 40 min to separate products. A ChemiDoc™ XRS system (Bio-Rad, USA) was used to capture gel images, using Quantity One 4.6.9 software.

2.2.7 Crossing Protocol

Methods for crossing isolates of *A. fumigatus* were followed as described by Ashton and Dyer (2019) based on the original protocols of O’Gorman *et al.* (2008). Spore suspensions (**Section 2.2.1**) of the crossing partners were diluted and adjusted to a target concentration of 5×10^5 spores/ml. *A. fumigatus* isolates were then crossed in pairwise *MAT1-1* and *MAT1-2* combinations in triplicate on oatmeal agar plates (**Section 2.1.3.8**). Two x 1 µl aliquots of the spore suspensions from partners of the opposite mating-type were placed about 4 cm apart from each other (O’gorman *et al.*, 2008). This

provided four interaction zones (barrage) for mating between the two isolates of each cross. Each plate was sealed with two layers of parafilm and incubated at 30 °C in dark for 3-4 months.

2.2.7.1 Harvesting Cleistothecia

Each cross was evaluated for the formation of cleistothecia after three to four months of incubation as described by (Ashton and Dyer, 2019). To determine the state of cleistothecia development, sexual crossings were examined under a dissection microscope in a laminar flow hood and inspected for the formation of cleistothecia in particular at the barrage zones between the two mating partners. When required, cleistothecia were isolated using a sterile fine point needle. The cleistothecia were then washed in sterile distilled water to remove any extra conidia by rolling them in the liquid. Cleistothecia were transferred to the 2 ml Eppendorf tube (Sarstedt, Germany), usually one cleistothecia/ Eppendorf tube, and crushed against the side of the tube containing 2 ml 0.05% Tween 80. To guarantee that all ascospores were discharged and asci were disrupted, the ascospore suspension was vortexed for 1 minute (Vortex Genie 2, Scientific Industries, UK). The ascospore suspension was then heat treated for 1 hour at 70 °C in a heated water bath (Grant Instruments, UK) to eliminate any leftover conidia. Following heat treatment, 100 µl aliquots of the ascospore suspension were spread over the surface of 9 cm diameter MEA plates with a sterile spreader and incubated at 28 °C for 36 hours.

2.2.7.2 Ascospore Isolation

Single germinating ascospores were spotted using a dissecting microscope in a laminar flow hood after the ascospore suspension had been cultured for 36 hours. The germinating ascospores were isolated using a sterile fine point needle after identification under the dissection microscope. Each germinating ascospore was inoculated onto a 9 cm diameter Petri plate containing ca. 25 ml MEA and cultured at 28 °C to allow further colony development (Ashton and Dyer, 2019).

2.2.8 Primer Design

Unless otherwise stated, all primers used in this study were designed using the primer design (Primer3) function of MacVector (version 18.2.5 for Macintosh; MacVector, UK).

2.2.9 Statistical Analysis

All statistical analyses were performed using the graphing and statistical software package GraphPad Prism (Graph Pad v 9.3.1 for Macintosh; Graphpad Software, San Diego, California USA, www.graphpad.com).

Chapter- 3 Identification of a Novel *cyp51A* Azole Resistance Mechanism in *Aspergillus fumigatus*

3.1 Introduction

3.1.1 Tandem Repeats as a Mechanism of Azole Resistance in *Aspergillus fumigatus*

Aspergillus fumigatus and associated species can cause invasive aspergillosis, with associated high rates of morbidity and mortality in immunocompromised individuals. Triazole antifungal drugs are one of the recommended first line medications for treatment (Ullmann *et al.* (2018). These drugs are usually effective against *A. fumigatus* infection, where they act by inhibiting the activity of CYP51A resulting in decreased levels of ergosterol in the plasma membrane of the fungus and the accretion of noxious sterol intermediates (Dhingra and Cramer, 2017). However, over recent years, the effectiveness of this class of drugs has been declining, thought to be due to reduced efficacy in treating infections where azole resistance *A. fumigatus* strains are present (Fisher *et al.*, 2018). One of the major mechanisms of triazole resistance in *A. fumigatus* that has been reported both clinically and environmentally is the combination of mutations within the *cyp51A* coding region, together with a tandem repeat such as, TR₃₄, TR₄₆, in the promoter region of *cyp51A* gene which leads to overexpression of the azole target gene *cyp51A* and hence production of more copies of the CYP51A protein. The presence of increased levels of the target protein, together with possible altered drug binding affinity, therefore, leads to decreased efficacy of the azole antifungal.

TR₃₄ was the first identified form of tandem repeat mutation and has now become very well known, being manifested by the duplication of a single 34 bp sequence within the promoter region of *cyp51A* (**Figure 3.1**), merged with a lysine to histidine switch at position 98 within the protein (Mellado *et al.*, 2007). Hence this is often referred to as ‘TR₃₄L98H’ and results in resistance to itraconazole and partial resistance to other azoles such as voriconazole. Azole tolerance increases slightly when the L98H

modification is introduced into a wild-type (WT) isolate of *A. fumigatus*. Furthermore, the introduction of variant TR₃₄ into *cyp51A* promoter leads to a doubling of expression of the *cyp51A* gene and an associated increase in azole tolerance (Mellado *et al.*, 2007). However, it is only when the L98H alterations occur together with the appearance of TR₃₄ that isolates attain tolerance levels that surpass clinically pertinent breakpoints (Mellado *et al.*, 2007; Gsaller *et al.*, 2016). According to Gsaller *et al.* (2016) the particular 34 bp sequence provides a binding site for the *SrbA* transcription factor, which plays an essential role in the control of sterol biosynthesis. Hence, overexpression of *cyp51A* in strains harboring two tandem repeated copies of this 34 bp sequence (i.e. TR₃₄) is due to amplified *SrbA* activity due to the duplication of its consensus attachment sites SRE1 and SRE2 within the 34 bp sequence (Gsaller *et al.*, 2016). Consistent with this hypothesis, *A. fumigatus* strains that did not have the *SrbA* display showed a decline in resistance to azoles (Gsaller *et al.*, 2016).

TR₄₆ was the second widespread form of tandem repeat to be identified, being found to be present in a variety of environmental isolates. Strains of *A. fumigatus* carrying this alternative 46 bp tandem repeat have all found to be highly voriconazole-resistant (Van Der Linden *et al.*, 2013). This tandem repeat is situated in the same region of the *cyp51A* gene promoter section as that occupied by other tandem repeats such as TR₃₄ above (**Figure 3.1**). The TR₄₆ mutation has been reported to occur simultaneously with two amino acid changes, Y121F and T289A, within the coding region of *cyp51A* in several countries across Europe (Rivero-Menendez *et al.*, 2016). The mechanism of resistance is common in geographically distant countries such as Colombia, the Netherlands, the UK, India, Tanzania, and several others (Meis *et al.*, 2016). This tandem repeat also affords resistance to additional azoles such as isavuconazole, ITC, and POS (Meis *et al.*, 2016). This form of tandem repeat was first described by Van Der Linden *et al.* (2013) who reported TR₄₆/ Y121F/T289A as a new *cyp51A* resistance mechanism which they observed in 21 azole-resistant isolates from 15 clients in six hospitals. In all these 21 isolates, the activity of POS and ITC was diminished (Van Der Linden *et al.*, 2013). Indeed, among the 15 patients from whom these isolates were obtained, eight were diagnosed with azole-resistant invasive aspergillosis, confirming the influence of this mutation as a mechanism of resistance (Van Der Linden *et al.*, 2013). Four years later, Zhang *et al.* (2017) identified a related novel resistance alteration, TR₄₆³/Y121F/

M172I/ T289A/ G448S. However, little is known concerning the migration rate and evolution of this mechanism although this new mutation, just like TR₄₆/Y121F/T289A, was speculated to have arisen through both sexual and asexual reproduction (Zhang *et al.* 2017). The TR₄₆³/Y121F/M172I/T289A/G448S form was shown to exhibit a faster mycelial growth rate (MGR) on a medium containing POS compared with the previously identified TR₄₆/Y121F/T289A and TR₃₄/L98H forms (Zhang *et al.*, 2017). The TR₄₆³/Y121F/M172I/T289A/G448S form also displayed the highest azole resistance in MIC tests, revealing a pan-triazole-resistance phenotype to ITC, POS, and VRC (Zhang *et al.*, 2017).

Meanwhile, there have also been reports of other forms of tandem repeats within the *cyp51A* promoter region leading to azole resistance in *A. fumigatus* strains, although this time without any linked mutation in the coding region of the gene. In all cases these additional tandem repeat forms are located in the same region of the promoter of *cyp51A* as the previous TR₃₄ and TR₄₆ forms, albeit with some extended sequence that differs between the different forms of the TR (**Figure 3.1**). Thus it can be hypothesised that altered binding of the *SrbA* transcription factor again leads to amplified *SrbA* activity due to the duplication of its consensus attachment sites (Gsaller *et al.*, 2016). Examples include TR₅₃ and TR₁₂₀, which will now be described.

TR₅₃ has been shown to be a possible cause of azole resistance in *A. fumigatus*. This tandem repeat is found in the promoter area without any linked amino acid change from the wild-type form of CYP51A. Hodiamont *et al.* (2009) first described the presence of TR₅₃ in the promoter region of *cyp51A* in isolates from a patient with chronic granulomatous disorder and confirmed that the *A. fumigatus* isolates displayed resistance to VRC and ITC, and lowered sensitivity to POS (Hodiamont *et al.*, 2009). Further analysis of this isolate confirmed the presence of TR₅₃, which was found to amplify the expression of *cyp51A* by five-fold (Hodiamont *et al.*, 2009). The tandem repeat is located in the same location of the *cyp51A* promoter region as other previously reported TR₃₄ and TR₄₆ forms, which are indeed contained within the TR₅₃ sequence (**Figure 3.1**). Even though a TR₅₃ isolate has been discovered in the environment in Colombia (Chowdhary *et al.*, 2017), there is presently no further confirmation for migration of this particular resistance genotype.

TR₁₂₀ is an extended tandem repeat that has been reported fairly recently in *A. fumigatus*, and which has been correlated with azole resistance (Hare *et al.* 2017). This tandem repeat is located in the same location of the *cyp51A* promoter region as all the other previously reported tandem repeat forms, and indeed all of these are encompassed within the TR₁₂₀ sequence (**Figure 3.1**). Isolates bearing this genotype were obtained from a patient with COPD undergoing long-term azole treatment. According to Hare *et al.* (2017) TR₁₂₀ represents a new form of azole-resistance machinery in this fungus and the *in-vivo* selection of TR in the promoter location of *cyp51A* is exceptional. Whereas most promoter tandem repeat forms have primarily been linked to environmental selection pressure (Snelders *et al.*, 2009; Snelders *et al.*, 2011; Hare *et al.*, 2017), in this case the two isolates with the presence of TR₁₂₀ arose from *in vivo* selection (Hare *et al.*, 2017). Hence, Hare *et al.* (2017) suggested that their results might contradict the hypothesis that TR azole-resistance mechanisms arise purely by environmental selection pressures. It is noted that the TR₁₂₀ isolates showed elevated azole resistance but no gene expression or GM work was not undertaken to confirm that this was the causal mechanism.

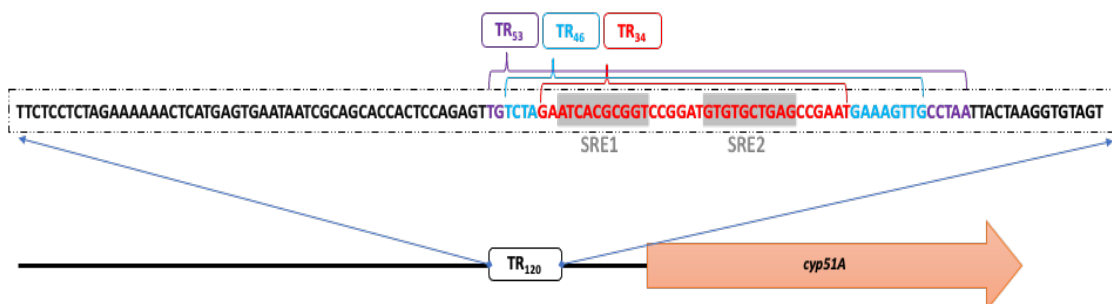


Figure 3-1 Diagram showing the position of different forms of tandem repeat in the promoter of *cyp51A* of *A. fumigatus* and the overlapping sequences between them. Sequence of the different form of the TR adapted from (Gsaller *et al.*, 2016; Hare *et al.*, 2017).

3.1.2 Chapter Aims

This chapter aimed to characterise a novel tandem repeat mutation (TR₁₃₀) in the promoter *cyp51A* gene of *A. fumigatus* that was found in certain clinical isolates during initial experimental work of the present thesis studies. This included evaluation of different forms of the repeated sequence (2 repeats '2R', 3 repeats '3R', and 4 repeats '4R') that were found in different clinical isolates. Specific aims of work were as follows.

1. To confirm the presence of the novel TR₁₃₀ tandem repeat in the promoter region of certain clinical isolates exhibiting increased azole resistance, and to assess possible variation in number of repeats.
2. To use classical genetic analysis via sexual crossing to assess if the resistance phenotype was a monogenic trait i.e. to determine if resistance was linked to mutation in a single gene or other region.
3. To introduce the different forms of the TR₁₃₀ (x2TR, x3TR, or x 4TR) into an azole-sensitive parental background strain, involving replacement of the wild-type *cyp51A* promoter of an $\DeltaakuB^{KU80}$ *A. fumigatus* strain with a *cyp51A* promoter harbouring different numbers of repeats of the TR₁₃₀ mutation.
4. To compare the minimum inhibitory concentration (MIC) values of the newly developed lab *A. fumigatus* strains harbouring the different forms of the TR₁₃₀ with those of the parental control and also the original patient strains harbouring the same pattern of mutation in the *cyp51A* promoter. This would ideally include testing all strains by using two different antifungal susceptibility tests i.e. E-test and EUCAST.
5. To evaluate the expression level of *cyp51A* in the lab strains harbouring different forms of the TR₁₃₀ and compare result(s) with: (a) the *cyp51A* expression level of original patient strains; and (b) the *cyp51A* expression level of previously reported forms of the TR shown to be linked to a resistant phenotype.
6. To test the effect(s) of deleting the ATP-binding cassette transporter (*cdr1B*) gene on the azole resistance level of strains harbouring the TR₁₃₀ X 4R, given

that it has been argued that *cdr1B* (synonym *abcG1*) is required for azole resistance even in a tandem repeat background (Paul *et al.*, 2017).

7. To evaluate treatment efficiency against *A. fumigatus* strains harbouring different forms of the TR₁₃₀ mutation using *Galleria mellonella* as a model of *in-vivo* infection.

3.2 Material and Methods:

3.2.1 Materials:

3.2.1.1 Fungal Strains:

Several putative azole resistant strains of *A. fumigatus* were obtained from Imperial College London (through a collaboration with Profs. Matthew Fisher and Darius Armstrong and Dr. Thomas Sewell). These isolates were from clinical sources from two different cystic fibrosis patients from London (**Table 3.1**). In addition, a *ΔakuB* strain of *A. fumigatus* (Resendiz-Sharpe *et al.*, 2022) obtained from Dr Matthias Brock (University of Nottingham) was used for genetic manipulation (GM) work (see **Section 3.2.2.5.2**), and supermater strains for sexual crosses were obtained from the the University of Nottingham BDUN collection. Strains were stored as described in **Section 2.2**.

Table 3-1: List of clinical isolates used in the present study, including original code and subsequent Nottingham BDUN assignment, London patient identification and date of isolation.

Isolate original code	Date of isolation	Patient ID	Nottingham BDUN code
C128	16/11/2015	0800346	47- 386
158	03/03/2015	D48387	47- 454
C136	02/10/2015	D48387	47- 388
C137	29/01/2016	D48387	47- 389
82	28/06/2016	D48387	47- 455
80	04/07/2016	D48387	47- 456
19	14/03/2017	D48387	47- 457
32	07/04/2017	D48387	47- 458
169	25/10/2018	D48387	47- 459

3.2.1.2 Media, Buffers, and Solutions

3.2.1.2.1 *Glucose Glutamine 10 (GG10)*

GG10 (Geib *et al.*, 2016; Geib and Brock, 2017) was prepared by dissolving the following components in 900 ml distilled water: 9.91 g D-glucose, 1.46 g glutamine (Acros, USA), 50 ml 20x salt stock (see below), 1 ml 1000x Hutner's trace element solution (see below), and 218.6 g sorbitol. When all components had dissolved, the pH was adjusted to 6.5 with NaOH, and the solution was made up to a final volume of 1 L

with distilled water. For solid media 2% agar was added. The medium was autoclaved at 117 °C for 30 min.

3.2.1.2.2 20x Salt Stock

The 2x salt solution was made using; 10.4 g potassium chloride (KCl), 10.4 g magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and 30.4 g potassium dihydrogen phosphate (KH_2PO_4), which were dissolved and made up to 1 L with distilled water. Vacuum filtration was used to sterilise the solution.

3.2.1.2.3 1000x Hutner's Trace Elements Solution

Hutner's trace elements solution was prepared by dissolving the following components: 10 g ethylenediaminetetraacetic acid (EDTA), 4.4 g zinc sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), 2.2 g boric acid (H_3BO_3), 1 g manganese chloride tetrahydrate ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$), 1 g iron sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), 0.32 g cobaltous chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), 0.32 g copper sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), and 0.22 g ammonium molybdate tetrahydrate $[(\text{NH}_4)_6 \text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}]$ in 100 ml distilled water.

3.2.1.2.4 Luria Bertani Broth (LB-B):

Ten grams tryptone, 5 g yeast extract and 10 g sodium chloride (NaCl) were added to 800 ml distilled water, adjusted to pH 7 with sodium hydroxide (NaOH), then the volume made up to 1 L before autoclaving at 121°C for 15 minutes. For Luria Bertani agar LB-A media the same components were used but with the addition of 15 g agar per litre prior to autoclaving.

3.2.1.2.5 Yeast Extract Peptone Dextrose (YEPD)

This medium was prepared using; 20.0 g peptone and 10.0 g yeast extract added to 900 ml distilled water.

3.2.1.2.6 Citrate Phosphate Buffer

A final concentration of 90mM citrate phosphate buffer was prepared by dissolving 16 g of di-sodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) and 17.2 g citric acid monohydrate in 1 L distilled water and adjusting the pH to 7.3 with NaOH. Vacuum filtration was used to sterilise the solution.

3.2.1.2.7 1M DTT Stock Solution

A 1.54 g of dithiothreitol (DTT) was dissolved in 10 ml of sterile distilled water and sterilised through 0.2 µm syringe filter. Aliquots of this solution were stored in 2 ml Eppendorf tubes and kept at -20 °C for up to 1 year.

3.2.1.2.8 Osmotic Solution

One litre of the osmotic solution was made using; 44.73 g potassium chloride (KCl) and 1.6 g sodium phosphate (Na₃PO₄) adjusted to pH 5.7 and made up to 1 L distilled water. Vacuum filtration was used to sterilise the solution.

3.2.1.2.9 Solution A:

One litre of the osmotic solution was made using; 44.73 g potassium chloride (KCl), 5.55 g calcium chloride (CaCl₂) and 1.21 g Tris/HCL, adjusted to pH 7.5 and made up to 1 L distilled water. Vacuum filtration was used to sterilise the solution.

3.2.1.2.10 Washing Solution

Washing buffer solution was prepared by dissolving 44.73 g KCl and 12.14 g Tris/HCL in 1 L distilled water. The solution was adjusted to pH 7.0 and vacuum filtrated for sterilising.

3.2.1.2.11 25 % PEG 8000 Solution

This solution was prepared by dissolving 25 g PEG 8000 (Fisher, UK), 0.12 g Tris/HCL base, and 0.55 g calcium chloride (CaCl₂) in 100 ml distilled water. The solution was adjusted to pH 7.5 with HCl and was filtered through a 0.22 µm filter (Sarstedt, UK) to sterilise the solution.

3.2.1.2.12 10% Sodium Dodecyl Sulphate (SDS)

The 10% SDS (Melford, UK) solution was prepared by dissolving 10 g SDS in 100 ml distilled water.

3.2.1.2.13 10X SSC Buffer

The 10X SSC buffer was prepared by dissolving 44.12 g Na₃-Citrate (Sigma, UK) and 87.66 g sodium chloride (NaCl) in 1 L distilled water.

3.2.1.2.14 Depurination Solution

The depurination solution was prepared by diluting 40 ml of 10 M HCl in 1 L distilled water.

3.2.1.2.15 Denaturation Solution

The denaturation solution was prepared by dissolving 20 g sodium hydroxide (NaOH) and 87.66 g sodium chloride (NaCl) in 1 L distilled water.

3.2.1.2.16 Neutralization Solution

The neutralization solution was prepared by dissolving 87.66 g sodium chloride (NaCl) and 60.57 g Tris/HCl in 1 L distilled water. The solution was adjusted to pH 7.5 with HCL.

3.2.1.2.17 Washing Solution I

The washing solution I was prepared by diluting 200 ml of 10X SSC solution and 100 ml of 10% SDS in 1 L distilled water.

3.2.1.2.18 Washing Solution II

The washing solution II was prepared by diluting 100 ml of 10X SSC solution and 100 ml of 10% SDS in 1 L distilled water.

3.2.1.2.19 Solution A

The solution was prepared by dissolving 5.8 g maleic acid (AppliChem, Germany), 4.38 g sodium chloride (NaCl), and 1.5 ml Tween 20 in 500 ml distilled water. The solution was adjusted to pH 7.5 with sodium hydroxide (NaOH).

3.2.1.2.20 Maleic Acid Buffer

The maleic acid buffer was prepared by dissolving 11.67 g maleic acid and 8.77 g NaCl. The solution was adjusted to pH 7.5 with sodium hydroxide (NaOH).

3.2.1.2.21 Blocking Solution

The solution was prepared by mixing 36 ml maleic acid buffer and 4 ml Western blocking reagent (Roche, Germany).

3.2.1.2.22 Detection Buffer

The detection buffer was prepared by dissolving 12.11 g Tris and 4 g sodium chloride (NaCl) in 1 L distilled water adjusting to pH 9.5.

3.2.1.2.23 DNA Extraction Buffer

A 300 ml of DNA extraction buffer was made using 100 mM Tris/HCL; pH 8.0 (3.63 g Tris adjusted to pH 8.0), 50mM EDTA; pH 8.0 (5.58 g EDTA adjusted to pH 8.0) and 8.77 g NaCl made up to 300 ml distilled water. The solution was adjusted to pH 8.0 and autoclaved at 121 °C for 15 mins. After autoclaving, β -Mercaptoethanol was added to a final concentration of 10 mM in the sterilized solution.

3.2.1.2.24 5 M Potassium Acetate Solution (Kac)

The 5 M potassium acetate (AppliChem, Germany) solution was prepared by dissolving 73.6 g KAc in 150 ml distilled water.

3.2.1.2.25 3 M Sodium Acetate Solution (NaAC)

The solution was prepared by dissolving 36.9 g NaAC in 150 ml distilled water.

3.2.1.2.26 Tris-EDTA (TE) Buffer

The TE buffer was prepared by dissolving 6.05 g Tris and 0.2 mL 0.5 M EDTA in 100 ml distilled water. The solution was adjusted to pH 8.0 using HCL and autoclaved at 121 °C for 15 mins.

3.2.1.2.27 Colony PCR- DNA Extraction Buffer

The Buffer was prepared by dissolving 1 mM NaCl, 10 mM EDTA and 10 mM Tris HCl pH 7.5 in 100 ml distilled water.

3.2.2 Methods:

3.2.2.1 Antifungal Susceptibility Testing (see Section 2.2.2)

3.2.2.2 DNA Extraction, PCR Amplification, Gel electrophoresis and Sequence Analysis (see Sections 2.2.3, 2.2.4 and 2.2.6)

3.2.2.3 Mating- Type Analysis and Sexual Crossing (see Sections 2.2.5 and 2.2.7)

3.2.2.4 Generation of Lab *A. fumigatus* Strains Harboursing Different Forms of the TR₁₃₀ 4-repeat (4R), 3-repeat (3R) and 2-repeat (2R)

Experimental gene manipulation (GM) work was undertaken to generate lab strains for *A. fumigatus* harbouring different numbers of the TR₁₃₀ repeat ranging from two to four repeats as follows.

3.2.2.4.1 *Plasmid construction, isolation, digestion, and purification*

In order to prepare the 4R plasmid, a set of primers was designed to encompass 300 bp upstream of the 4R sequence (F1) and 300 bp downstream of the 4R sequence (R1). These positions were chosen based on the nearest restriction site (CCC GGG) of the *Sma*I enzyme used in the process of preparing the plasmid (**Figure 3.2**).

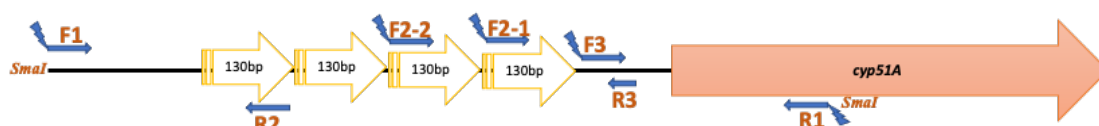


Figure 3-2: Diagram showing the position of PCR primers used in order to clone a specific part of the *cyp51A* and its promoter in which the product can be used for building the 2R, 3R and 4R constructs.

All PCR reactions for generating the individual constructs were performed using Phusion® High Fidelity DNA polymerase (NEB, UK) and oligonucleotides described in (**Table 3.2**). These primers were used to amplify the 4R x TR₁₃₀ sequence by using gDNA from one of the TR₁₃₀ isolates (**Table 3.1**), following the PCR reaction mixture and the thermocycling conditions listed in (**Tables 3.3 and 3.4**). The 3R the 2R cassettes

were built using two different fragments. The F2 set of primers were designed with a 15 bp extension sequence homologous to R2 primer to help during the ligation between fragment of the 2R fragments and the 3R fragments (**Figure 3.2**). The 3R construct was prepared from two fragments, F1/R2 containing one copy of the TR₁₃₀ and F2-2/R1 including two copy of the TR₁₃₀ sequences. The 2R plasmid was prepared from two fragments, F1/R2 containing one copy of the TR₁₃₀ and F2-1/R1 containing one copy of the TR₁₃₀ (**Figure 3.2**). One percent (w/v) agarose gel electrophoresis (**Section 2.2.6**) was used to visualise the PCR products.

Bands with the correct size were excised from agarose gels and cleaned with a NucleoSpin® Gel and a PCR clean-up kit according to the manufacturer's instructions. The PCR products were then cloned into the pJet1.2/blunt vector (Thermo Scientific™). The cloning reactions were undertaken following the manufacturer's instructions and incubated at RT for 5 min. These reactions were then transformed into *Escherichia coli* DH5α and cells spread on plates containing LB-A supplemented with ampicillin. The plates were incubated overnight at 37 °C. The next day, *E. coli* colonies were picked and used for colony PCR using F1/R3 primers (**Table 3.2**) using the same PCR reaction mixture and thermocycling conditions listed in (**Tables 3.3 and 3.4**). Agarose gel electrophoresis (1 % w/v) was used to visualise the PCR product. Positive colonies containing the correct plasmid size were incubated in liquid LB-B supplemented with ampicillin overnight at 37° C in order to get a high concentration yield of the plasmid. The next day, all plasmids were isolated using a NucleoSpin® plasmid kit following the manufacturer's instructions (Macherey-Nagel kit, Germany). The plasmids were digested using the *SmaI* restriction enzyme to free the 4R, 3R, 2R DNA cassette from the pJet1.2 vector, and the digested reactions were resolved by agarose gel electrophoresis (1% w/v) to separate the 4R, 3R and 2R product from the pJet1.2 vector. Bands of the correct size were excised from the gel and cleaned with a NucleoSpin®Gel and PCR clean-up kit, following the manufacturer's instructions. These DNA cassettes contained the different form of the TR₁₃₀ to be used for later protoplast transformation.

Table 3-2: Sequences of various oligonucleotide primers used for PCR of TR₁₃₀ repeats and transformation studies

Primer name	Sequence
F1	GGGAATAGACGCCGTTTACC
R1	CGGGTCGTCAATGGACTATAGAC
F2-1/ F2-2	ctcactactacggtaggtaga TCTACCTACCGTAGTATGAGTTAG
R2	GGTGCTGCGATCTACCTACC
F3	ctcactactacggtaggtagaTCTACTTACCTATGAACCTATATTGG
R3	GGGATTATTTTCATATACTGGATTCC

Table 3-3: PCR reaction mixture for generating TR₁₃₀ repeat transformation fragments

PCR reaction per sample	Volume (μl)
Distilled water	14.3
5 × Phusion HF Buffer	4
dNTPs (AB gene, UK)	0.5
MgCl ₂	0.5
100 mM forward primer	0.2
100 mM reverse primer	0.2
Phusion DNA polymerase	0.3
200 ng/μl DNA template	1
Final volume	20

Table 3-4: Thermocycling condition for generating TR₁₃₀ repeat transformation fragments

Step	Temperature (°C)	Time
Initial denaturation	95	5min
Denaturation	98	30 s
Annealing	60	30 s
Extension	72	60 s
Final extension	72	2 min
Hold	4	∞

3.2.2.4.2 Preparation of protoplasts for transformation

Plasmids containing the 4R, 3R and 2R repeats were planned to be used in GM work in which they would be transformed into a sensitive isolate of *A. fumigatus* in order to see the effect of introducing different numbers of the TR₁₃₀ repeats in relation to azole resistance. A laboratory strain of *A. fumigatus* (Af_Δ*akuB*::*luc*_{OPT_red_ptrA} No. 5) here termed Δ*akuB*^{KU80} was used as an azole sensitive host isolate for the transformations.

This isolate was developed in our lab by Dr Matthias Brock and his group through gene manipulation of *A. fumigatus* wild-type strain CBS144.89 and has recently been used in similar work as the parental strain for transformation (Resendiz-Sharpe *et al.*, 2022). The isolate has a deletion of the *A. fumigatus* *KU80* gene which has an essential role in the homologous recombination process (da Silva Ferreira *et al.*, 2006). Thus, the use of the $\DeltaakuB^{KU80}$ strain was predicted to result in improved gene targeting in transformation experiments when generating the lab TR₁₃₀ strains.

Protoplast-mediated gene transformation was performed as previously described (Fleck and Brock, 2010; Brandenburger *et al.*, 2017). The $\DeltaakuB^{KU80}$ parental strain was grown overnight for 24hr in YEPD medium supplied with 2% glucose. Mycelium was filtered through a sterile funnel covered with Miracloth and washed using citrate phosphate buffer. 300 ml citrate phosphate buffer (**Section 3.2.1.2.6**) containing 1M DDT (**Section 3.2.1.2.7**) was added to an empty sterile 250 ml flask. One spoonful of mycelium was transferred into this solution and incubated at 28 °C for 1 h. During the incubation time, 1.3 g VinoTaste + 100 mg lysing enzyme was dissolved in 10 ml osmotic solution (**Section 3.2.1.2.8**) and filter sterilised by passing through a 0.2 µm syringe filter into an empty sterile 250 ml flask. After incubation, the mycelium was filtered again using the same funnel covered with Miracloth and washed using osmotic solution. A half-spoon of the mycelium was transferred to the enzyme solution and incubated for 1.5–2.5 h at 50 x g at 28 °C. After 1.5 h, protoplasts were checked under the microscope to ensure the formation of protoplasts. After 2.5 h, protoplasts were harvested by filtering, using a new sterile funnel covered with Miracloth, into a sterile 50 ml Falcon tube and centrifuged at 4 °C at 2800 x g for 8 min. All subsequent protoplasting steps were completed on ice. The protoplast pellet formed was re-suspended by pipetting gently in a 20 ml wash solution, and centrifuged at 4 °C, 2800 x g, for 8 min. The supernatant was discarded, and the pellet was re-suspended gently in 10 ml of solution A; 10 µl of the protoplasts were transferred to a haemocytometer counting chamber to determine the concentration. The protoplast solution was then centrifuged again at 4 °C at 2800 x g for 8 min. and the resulting pellet was resuspended in an appropriate volume of solution A to yield a final concentration of 5×10^7 to 2×10^8 protoplasts per ml. One hundred µl aliquots of the protoplasts were transferred into 2 ml tubes containing 5–10 µl of the transforming DNA (i.e. 4R, 3R or 2R plasmids)

with a total amount of 1–20 µg (in small volume of 1–5 µl) used for transformation. For positive and negative controls, 10 µl elution buffer (EB) was used instead of the DNA. Twenty-five µl of 25% PEG8000 solution (**Section 3.2.1.2.11**) was added to each tube and mixed gently. The mixtures were incubated on ice for 25 min. After the incubation time, 500 µl of 25% PEG8000 solution was added to the mixture and mixed gently.

Regeneration plates with the bottom agar were prepared in advance, using 25 ml GG10 medium incorporating 4 µg/ml itraconazole as the selectable marker, except for positive control plates which lacked azole supplementation. For the top agar, 12 ml of the same medium supplied with 4 µg/ml itraconazole was poured into a series of tubes and stored at 50 °C, except for one tube without itraconazole for the positive control.

Six hundred µl of the protoplasts with EB was added to the top agar tube without itraconazole for the positive control and mixed by inverting the tube several times and then pouring in the top agar. The same process was repeated for the negative control with itraconazole. For the other DNA tubes, 400 µl, 500 µl or 600 µl of the transformed cells was added to separate tubes containing the top agar with itraconazole, inverted several times and poured on the bottom agar plates. Plates were then incubated at 37° C for 4–5 days.

3.2.2.4.3 Selection of positive colonies

The first sets of colonies that were able to grow in the presence of itraconazole in the transformation plates were transferred to new GG10 agar plates supplemented with 4 µg/ml itraconazole. The gDNA from those colonies was extracted as described in **Section 2.2.3**. Then, PCR using primers F1/R3 (**Table 3.2**) and the same PCR reaction mixture and thermocycling conditions listed in **Tables 3.3 and 3.4**, was used to select positive colonies with the 4R, 3R and 2R repeat inserts.

3.2.2.5 Southern Blotting

The selected positive transformants harbouring the different forms of *cyp51A*/TR₁₃₀ were tested for single-copy integration of the different forms of the TR₁₃₀ locus in each strain. In this context, Southern blotting was used through a digoxigenin-UTP (DIG-UTP) labelled probe against the prenatal *ΔakuB*^{KU80} strain.

3.2.2.5.1 gDNA Extraction

To obtain a high-quality gDNA for Southern blotting, a specific gDNA method was used as recommended by Dr Matthias Brock (University of Nottingham). A 1 ml volume of 1×10^8 spores/ml of *A. fumigatus* suspension was used to inoculate 50mL ACM/B media placed in a sterilized flask which was then incubated at 28 °C for 24 hours. Mycelial mats, formed overnight on the surface of incubated liquid cultures, were collected and filtered using a double-layer of Miracloth. Mycelium was washed under tap water to remove the remaining medium and squeezed to remove excess water. Mycelium was then transferred to a 15 ml centrifuge tube and frozen with liquid nitrogen. Frozen mycelia were then ground into a fine powder using a mortar and pestle. Genomic DNA was isolated from about 30-40 mg aliquots in a 2 ml Eppendorf tube of ground mycelia using the stated washing steps. A master mix of 500 µl extraction buffer (**Section 3.2.1.2.23**) along with 60 µl 10% SDS and 2 µl RNase per sample was prepared. 562µl was added to each tube containing the ground mycelia and vortexed till a homogenous solution was obtained. Tubes were then incubated at 65 °C for 15 min, with periodic vortexing during incubation. Tubes were then transferred to ice and a 160 µl 5 M Kac was added to each sample, vortexed, and kept on ice for 5 min to help precipitate protein and polysaccharides. Samples were then centrifuged at 4 °C for 10 min at 13,000 x g. Approximately, 700 µl clear supernatant was transferred into a new 2 ml Eppendorf. 700µl isopropanol was added to each tube and inverted gently 30 times to mix before placing tubes at -20 °C for 20 minutes. Afterwards, the samples were centrifuged at 4°C for 10 minutes at 13000 x g. The supernatant was removed from each tube without touching the DNA pellet at the bottom of the tubes. This pellet was then resuspended in 400µl TE buffer and incubated at 60 °C until the pellet dissolved completely. Subsequently, 55 µl of 3 M NaAc was added to the sample. The mixture was then centrifuged at 4 °C for 5 minutes at 13000 x g. The supernatant was transferred to a new 2ml Eppendorf tube. A 500 µl isopropanol was added to each tube and mixed by gently inverting the tube 30 times. Tubes were then placed at -20 °C for 10 minutes. Samples were then centrifuged at 4 °C for 10 minutes at 13000 x g. After discarding the supernatant, the DNA pellet was washed with 400 µl 70% EtOH. Samples were then centrifuged at 4 °C for 10 minutes at 13000 x g. The supernatant was discarded again and the DNA pellet was dried for 5-10 minutes. Finally, the gDNA pellet was dissolved

in 60 µl of TE buffer. The gDNA samples were stored at -20 °C until used in Southern blotting.

3.2.2.5.2 Generation of Southern Blot Probes

The same set of primers (F3/R1; **Table 3.2**) that was used to amplify the TR₁₃₀ upstream fragment for generating the 3R and 2R mutants were used to generate the Southern blot probe by replacing partial proportions of the dTTPs with DIG-UTPs. To create the (DIG-UTP) labelled probe, a 10mM mixed dNTP set with a ratio of (A: C: G: T - 1:1:1:0.7) was used in PCR as shown in (**Tables 3.5 and 3.6**) with an appropriate plasmid with the 4R, 3R, or 2R construct used as a template for PCR. PCR products were gel-purified using a NucleoSpin® Gel and PCR clean-up kit according to the manufacturer's instructions and stored at 4°C.

Table 3-5: PCR reaction mixture for generating Southern blot probe

Reagent	Volume per sample (µl)
Thermos polymerase buffer	5
Prepared 10mM dNTP mix	1
10 mM F4	1
10 mM R3	1
30 ng constructed plasmid	1
Taq polymerase	0.5
DIG- UTP	0.5
dH ₂ O	40
Final volume	50

Table 3-6: Thermocycling conditions for generating Southern blot probe

Step	Temperature (°C)	Time
Initial denaturation	95	30 s
Denaturation	95	30 s
Annealing	55	30 s
Extension	72	60 s
Final extension	72	5 min
Hold	4	∞

3.2.2.5.3 Fragmentation

The gDNA of putative positive transformants along with the parental strain *ΔakuB*^{KU80} with WT *cyp51A* (of *A. fumigatus* wild-type strain CBS144.89) were digested

overnight at 37 °C with a specific restriction enzyme that created different-sized fragments in the WT strain as compared to transformant strains. Digested gDNA samples were then run on a 0.8% agarose gel along with 1kb ladder for around 3h at 100V and then visualised and pictured on the GelDoc system (Bio-Rad, UK).

3.2.2.5.4 Denaturing the DNA

Resulting gels were then denatured by washing several times with three different solutions. The solution was poured off after each wash and rinsed with water before switching to the next solution. Each step was repeated two times with each solution. The gel was placed in a tray (larger than the gel) during the washing steps and moderate shaking was used. Gels were initially immersed in 0.25 M HCl for 7.5 minutes to induce depurination of large fragments. The gel was then denatured by complete immersion in 1.5 M NaCl and 0.5 M NaOH for 15-30 minutes. Finally, the previous solution was replaced with 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.5) and the gel was left in this solution for another 15 minutes.

3.2.2.5.5 Transference to Membrane using the Vacuum Blotting System

During the last washing steps, a nylon membrane (Cytiva Amersham™ Hybond™ -XL Membranes) of a suitable size for the gel and a plastic bag for the transfer vacuum was prepared and wetted with distilled water. The gel was then placed above the exposed nylon membrane of the vacuum (VacuGene XL Vacuum Blotting System) to start transference of the DNA from the gel to the membrane. The vacuum was switched on and 10x SSC buffer was added immediately to a point till the gel was covered completely. The vacuum was run for 1 hour.

3.2.2.5.6 Fixing DNA to the Transfer Membrane

After the sample was transferred to the membrane, it was essential to bind the DNA to the membrane. The membrane was marked with a pencil in the left-hand bottom corner to save the orientation of the DNA samples. The membrane was then lifted from the gel and placed on Whatman filter paper (with the DNA side upwards). A few drops of 10x SSC were added to wet the membrane which was then placed in the Bio-Rad Cross Linker (Bio-Rad, UK) to cross-link the DNA molecules to the nylon membrane using 700mj/cm³ of UV light.

3.2.2.5.7 Hybridisation

Washing the nylon membrane with the pre-hybridisation solution or blocking solution was required to overcome any non-specific DNA interaction and background noise.

3.2.2.5.7.1 Pre-hybridization of the Membrane

The hybridisation oven was turned on and the hybridisation solution was warmed up to 65 °C beforehand. The cross-linked membrane was then rolled and placed at the bottom of a hybridisation tube by ensuring that the membrane folded inside the tube completely. Next, 20 ml of pre-warmed hybridisation solution was added to the hybridisation tube containing the membrane. The membrane was pre-hybridised for at least 1 hour at 65 °C. Afterwards, 10 µl of the labelled probe was added to the hybridisation tube and the mixture incubated overnight at 65 °C.

3.2.2.5.7.2 Post-hybridization Washes

During the previous step, the probe molecules should have been hybridised with DNA fragments on the membrane that contain the complementary sequence. However, to counter the risk that the probe had hybridised to similar DNA sequences (despite the use of a 65 °C temperature), a salt solution wash was used. Both wash solution I and wash solution II (**Section 3.2.1.2.18 and 3.2.1.2.18**) were pre-warmed to 65 °C. Then, the membrane was washed with 20 µl solution I for 5 minutes twice at 65 °C and then 20 µl solution II for 10 minutes twice at 65 °C.

3.2.2.5.8 Single Generation and Detection of Specific DNA

The DNA probe was labelled with DIG-UTP molecules and could therefore be bound by Anti DIG-AP Fab to generate a coloured product. The membrane was washed with 20 µl solution A for 5 minutes at room temperature, followed by rinsing with 20 µl malic acid buffer (**Section 3.2.1.2.20**). Next, the membrane was incubated in 20 µl blocking solution (**Section 3.2.1.2.21**) for 30 minutes. Later, a new 20 µl aliquot of blocking solution with 0.8 µl of Anti DIG AP Fab fragment was added and incubated for 30 minutes. Afterwards, the membrane was rinsed with solution A for 10 minutes twice, with malic acid buffer for 5 minutes once, and with detection buffer (**Section 3.2.1.2.22**) for 1 minute once consecutively.

3.2.2.5.9 Staining

A special plastic wallet with only one side remain stuck was prepared and the membrane was placed inside the plastic wallet. A few drops of CDP^{star} reagent were splashed onto the membrane and spread over the whole membrane surface using a 10 ml glass pipette. The plastic wallet containing the membrane was then sealed using a heat sealer and visualized with a GelDoc system using the chemiluminescence setting.

Finally, after ensuring the correct binding of the labelled probe to the target DNA from the first staining, a second staining was performed. The membrane was removed from the plastic bag and washed using malic acid buffer and then allowed to rest in a small amount of detection buffer. The membrane was then stained using NBT/BCIP and left in the dark for 2-3 hours or overnight till the best results could be seen/observed. To stop the staining, the membrane was placed in water with a few drops of HCl.

3.2.2.6 Generation of complementary $\Delta cyp51A$ TR₁₃₀ X 4R isolate.

3.2.2.6.1 *Complementation Plasmid*

To confirm that the observed resistance phenotype in the positive transformants was linked to the introduction of the TR₁₃₀ in the promoter of *cyp51A*, these mutations were replaced with the WT-*cyp51A* gene and its promoter. This was achieved by using a complementation plasmid constructed by Dr Matthias Brock and his group for similar work (Resendiz-Sharpe *et al.*, 2022). This plasmid consisted of the following parts; a 986 bp fragment of the promoter region, a 1,619 bp fragment of the entire CDS, and a 228bp fragment of the terminator region of the *cyp51A* gene which were all generated from wild-type *cyp51A* gene of (CBS144.89). Another fragment of (792bp) of the downstream region was amplified separately. The two fragments were assembled by in vitro recombination in a *HindIII*-digested pUC19 plasmid, and the construct was amplified in *E. coli* DH5 α . In addition, this complementation plasmid contained a hygromycin B (2,800bp) resistance marker placed between the two fragments. For the constructed plasmid for replacing the altered azole-resistant *A. fumigatus* strain (TR₁₃₀ X 4R) to the wild-type *cyp51A* gene including the promoter region of this gene, see the plasmid map adapted from (Resendiz-Sharpe *et al.*, 2022) (**Figure3.3**).

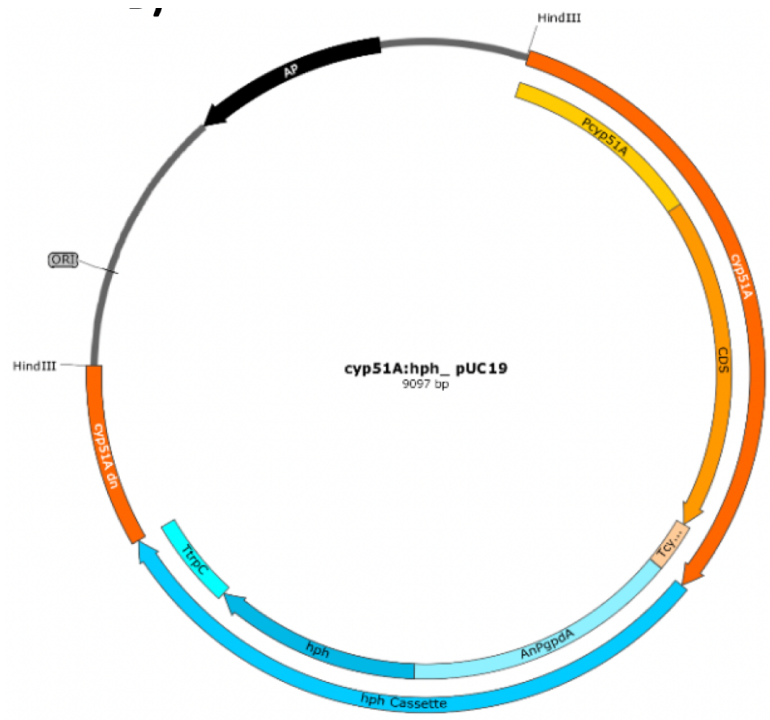


Figure 3-3: Plasmid map of the *cyp51A* and hygromycin complementation cassette, adapted from (Resendiz-Sharpe *et al.*, 2022).

3.2.2.6.2 Digestion of the Plasmid

The complementation plasmid was digested using the *HindIII* restriction enzyme allowing the construct ($\approx 6,425$ bp) to be released from the pUC19 vector backbone.

3.2.2.6.3 Transformation Procedure

The complementation was done using the PEG-mediated protoplast transformation (as described in **Section 3.2.2.4**) except that hygromycin B ($180\mu\text{g/ml}$) was added to the GG10 media as a selection marker. Two control plates were included; positive control without hygromycin B and with protoplasts without DNA, and negative control with hygromycin B and protoplasts without DNA. The positive control was included to confirm that protoplast formation and regeneration had occurred. The negative control was included to verify that no growth would be observed if the protoplast does not carry the hygromycin selectable marker.

3.2.2.6.4 Confirmation of Successful Transformation

Positive transformants that were able to grow in the presence of hygromycin were then selected in a second media containing $8\mu\text{g/ml}$ itraconazole. Afterwards, colonies

that were unable to grow in the presence of itraconazole were selected for further screening using standard PCR.

3.2.2.7 Antifungal Susceptibility Testing of Positive Transformants

After selection of the positive transformants harbouring the different forms of TR₁₃₀ as well as the complementary $\Delta cyp51A$ TR₁₃₀ X 4R transformants, E- test (**Section 2.2.2.1**) and EUCAST (**Section 2.2.2.2**) methods were used to determine the MIC values of the transformants in comparison to the parental strains.

3.2.2.8 Growth Susceptibility Assay of Positive Transformants

Tests were conducted as previously described by Valiante *et al.* (2008), where they use this assay to evaluate the impact of different cell wall stress agents on the growth of *A. fumigatus*. Here this assay was used order to evaluate possible differences in growth between the transformation parental strain and the 4R, 3R, 2R and the complementary $\Delta cyp51A$ TR₁₃₀ X 4R transformants in the present of itraconazole. Plates were prepared containing AMM agar (**Section 2.1.3.2**) supplemented with itraconazole ranging in concentration from 1 µg/ml to 16 µg/ml. Spore suspensions of all four strains were prepared as previously described (**Section 2.2.1**), whereby serial 10-fold dilutions were used to obtain a concentration range between 1 x 10⁶ and 1 x 10²-spores/ml. Five agar plate containing different itraconazole concentrations were spotted using five different spore suspension concentrations for each strain.

3.2.2.9 Quantification of TR₁₃₀ Strain Growth in the Presence of Itraconazole

The resistance levels of the TR₁₃₀ lab and patient strains were measured using different methods, including E- test, Tablet, and EUCAST. However, the difference between the level of resistance of the lab strains and patient strains harbouring 3R and 4R of the TR₁₃₀ required more validation. Therefore, the quantification method that described by (Meletiadis *et al.*, 2003) was used in combination with a microdilution EUCAST method, with some modifications in the way data was presented to fit the purpose of our use. Firstly, EUCAST plates were prepared as described in (**Section 2.2.2.2**). After the incubation period of 48 h at 37 °C, plates were then transferred to an

Epoch microplate spectrophotometer (BioTek Instruments, USA). Later, the optical density (OD) of each well was monitored at 405nm (OD_{405nm}). The data was then exported as an Excel sheet and corrected by subtracting the background ODs (ODs of wells contain the media only). Afterwards, the percentage of growth at five different concentrations of itraconazole (8, 4, 2, 1, and 0.5 mg/L) was calculated using the following equation:

$$\% \text{ growth} = (\text{OD}_{405\text{nm}} \text{ of wells containing the drug} / \text{OD}_{405\text{nm}} \text{ of the drug-free well}) \times 100$$

Data were then exported to GraphPad Prism (Graph Pad v 9.3.1 for Macintosh) for further analysis.

3.2.2.10 Growth Curve of the TR₁₃₀ Strains

To confirm that the EUCAST growth percentage results were not subject to strain-specific growth behaviour in the RPMI media, a comparison of growth between patient and lab TR₁₃₀ strains, including WT strains, was performed as previously described (Gomez-Lopez *et al.*, 2014; Meletiadiis *et al.*, 2001; Meletiadiis *et al.*, 2003), whereby parallel growth measurements were made using 96-well flat-bottomed plates (Cellstar®; Greiner Bio-one, Austria) incubated in an Epoch microplate spectrophotometer (HORATIO; BioTek Instruments, USA). First, spore suspensions for each strain were adjusted to a specific concentration (1 x 10⁶ conidia/ml) by counting the conidia in the haemocytometer chamber. Then, 100 µl of either RPMI or AMM was dispensed into each well. Plates were then inoculated with 100 µl of spore suspension (1 x10⁵ spore/ml). Plates were then sealed with a single layer of breathable film and finally incubated in the plate reader at 37 °C without shaking. The optical density (OD) of each well was monitored at 405 nm (OD_{405nm}) every 30 minutes over 48 h. For each time point, six replicate wells per strain were used to build the growth curve. The read values of the OD over time were then exported as an Excel sheet, with further calculations performed before plotting the curves. Calculations included removing the background ODs (i.e ODs of wells contain the media without inoculation with spores) from all reads. Another calculation was done by adding the value of 0.01 to all ODs read

to avoid complications with the logarithmic plot of value 0. Data were then exported to GraphPad Prism (Graph Pad v 9.3.1 for Macintosh).

3.2.2.11 RNA Extraction and cDNA Synthesis

3.2.2.11.1 *RNA Extraction*

Mycelial mats, formed after 72 h of incubation on ACM (**Section 2.1.3.1**) were collected and filtered using Miracloth. Mycelium was washed with tap water to remove the remaining medium and squeezed to remove excess water and medium. Mycelium was then frozen with liquid nitrogen and ground into a fine powder using a mortar and pestle. The ground mycelium was transferred to a 2 ml tube (containing 1 ml TRIzol™ Reagent) and left at room temperature for 10 minutes. A 200 µl chloroform was added to the tube and vortexed. The tube was then left for 2-3 minutes at room temperature and re-vortexed to ensure a homogenous solution was obtained. The tube was then centrifuged at 17000 x g for 10 minutes. The top aqueous layer was transferred to a new 1.5ml tube. A similar amount of isopropanol was added to the solution to help precipitate the RNA. The mixture was inverted several times and left at -20°C for 20 minutes. The tube was centrifuged at 17000 x g for 10 minutes; thereby causing a pellet to form. The pellet was washed with 700µl of 75% ethanol and 25% DEPC water. It was then centrifuged at 17000 x g for 10 minutes. Ethanol was removed and the tube was left to dry for 10-15 minutes. The pellet was resuspended in 100 µl DEPC water. Afterwards, a NucleoSpin® RNA clean-up kit (Macherey Nagel) was used to remove DNA, following the on-column DNase digestion protocol and the manufacturer's instructions.

After the isolation of total RNA, the concentration was measured using a Nanodrop ND-1000 spectrophotometer (Labtech International). RNA purity and integrity were checked by gel electrophoresis. Two µl of the samples mixed with 2 µl RNA was run alongside a 100bp DNA ladder in 1.5% TBE agarose gel at 100V for 40 minutes. Next, the agarose gel containing the RNA samples was visualised under UV light using the ChemiDoc™ XRS system (Bio-Rad, UK) with Quantity one 4.6.6 Software for capturing gel images (Bio-Rad, Hemel Hempstead, Herts, UK). The gel image was then checked for the appearance of both RNA bands - 28S and 18S ribosomal subunits. RNA samples were then stored at -80°C for long-term storage.

3.2.2.11.2 *cDNA Synthesis*

To convert RNA into first-strand cDNA, a GoScript™ reverse transcript kit (Promega, UK) was used following the manufacturer's instructions. A mixture of all components, including nuclease-free water (4 µl), reaction buffer that contains oligo dTs (4 µl), and enzyme mix (2 µl), was prepared separately to generate 10 µl of this mixture for each cDNA reaction. The mixtures were then mixed gently through pipetting and kept on ice until needed. Afterwards, 5 µg of total RNA was prepared in a separate tube to a final volume of 10 µl with nuclease-free water. The 10 µl reverse transcription mix and 10 µl RNA were combined for each sample and mixed by pipetting. The mixtures were then incubated at 65°C for 60 minutes. Finally, the reaction was inactivated by incubating the mixtures at 70°C for 15 minutes. The cDNA samples were stored at -20°C for long-term storage to be later used as a template for either qRT-PCR or end-point PCR.

3.2.2.12 Expression Level of *cyp51A* using Semi-quantitative End-point PCR and Real-time PCR (qRT-PCR)

3.2.2.12.1 *End-point PCR*

Two different methods, end-point RT-PCR and qRT-PCR, were used to evaluate *cyp51A* transcription levels in the various TR₁₃₀ azole-resistant and control sensitive strains. A specific set of primers for the *cyp51A* gene and two different housekeeping genes - β -tubulin (*tub1*) and glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) – were used as a reference for gene expression (**Table 3.7**). It is noted that these two housekeeping genes were chosen as they had been used in similar previous *cyp51A* expression studies (Gsaller *et al.*, 2016; Mellado *et al.*, 2007). Primer pairs were designed to either include an intron within the gDNA amplicon, or spanned an intron region, thereby allowing assessment of whether bands produced by RT-PCR arose correctly from cDNA or were from gDNA contamination (as shown by the size of resulting amplicons). Primer pairs were used with different numbers of PCR cycles (26, 28, and 30 cycles) with the PCR reaction mixture and thermocycling conditions as shown in **Tables 3.8 and 3.9** for the end-point PCR, with cDNA used as a template for all samples and gDNA used as a negative control template.

Table 3-7 :Primer list for end-point PCR and qRT-PCR studies to evaluate *cyp51A* gene expression.
*EXON/INTRON junction

Primer name	sequence
RTcyp51A-f	GTGCAGAGAAAAG*TATGGCG
RTcyp51A-r	TCCGCATTGACATCCTTGAG
RTgpdA-FW	GAGCTCAAAA*ACATCCTCGGC
RTgpdA-RV	CGAAGTTGGGGTTGAGGGAG
tub5-f	TGACCCAGCAGATGTT
tub6-r	GTTGTTGGGAATCCACTC

Table 3-8: End -point PCR reaction mixture for studies to evaluate *cyp51A* gene expression.

Reagent	Volume per sample (µl)
nuclease-free water	16
5 × Phusion HF Buffer	5
dNTPs (AB gene, UK)	0.5
10 mM forward primer	1
10 mM reverse primer	1
Phusion DNA polymerase	0.5
cDNA	1
Final volume	25

Table 3-9: Thermocycling condition for end-point PCR

Step	Temperature (° C)	Time
Initial denaturation	95	30 s
Denaturation	95	30 s
Annealing	26 or 28 or 55	30 s
Extension	72	60 s
Final extension	72	5 min
Hold	4	∞

3.2.2.12.2 *qRT-PCR*

The same sets of primers used for endpoint PCR (**Section 3.2.2.13.1**) were also used for qRT-PCR, which was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems) using the Fast SYBR Green Kit. The transcript level of *cyp51A* was measured using cDNA as a template from different azole-resistant strains and compared to the values obtained from WT control strains. The target gene (*cyp51A*) along with the reference genes (*gpdA*, *tub1*) and negative control (no template) were analysed in one

plate. A standard curve method was employed for quantification against known concentrations of purified PCR products. Three biological replicates were utilised for each measurement, each of these using triplicate technical repeats. Ten microliter of PCR reaction mixture containing 0.5 µl of cDNA, 0.5 µl of gene-specific primers (10 mM), 5 µl of FAST SYBR-Green Master Mix (Applied Biosystems), and 3.5µl of nuclease-free water was used. The PCR programming was denaturation at 95°C for 15 seconds, up to 40 cycles of hybridization at 67 °C for 30 seconds, and a final extension at 60 °C for 60 seconds.

3.2.2.12.3 Statistical Analysis

The relative quantitation method was used to normalise gene expression data. This method relies on calculating the difference(s) in cycle thresholded C_T values, known as delta C_T (ΔC_T), between the target gene and the housekeeping gene. C_T values were obtained with the appropriate rise of the fluorescence above the background fluorescence, which was automatically determined by the PCR machine.

To investigate the change in *cyp51A* expression between the TR mutant strains and wild type, the comparative C_T or ($\Delta\Delta C_T$) method was used, assuming 95-100% PCR efficiency. Calculations were conducted in Microsoft Excel version 16.58. According to the Delta- C_T method (Livak and Schmittgen, 2001), relative expression was calculated according to the expression of reference genes and was defined as the relative expression of a target gene in different samples by using the following equation:

$$\Delta\Delta C_T = |(\Delta C_T \text{ Mutants}) - (\Delta C_T \text{ WT})|$$

$$\text{Ratio} = 2^{-|\Delta\Delta C_T|}$$

All statistical analyses were performed using the graphing and statistical software package GraphPad Prism (Graph Pad v 9.3.1 for Macintosh). The data of the three biological replicates and the three technical repeats of the different groups sharing were analysed using one/ tow-way ANOVA.

3.2.2.13 Deletion of ABC transporter *cdr1B* gene from TR₁₃₀ strains

A fusion PCR method was used to generate the *cdr1B* knockout cassette according to (Szewczyk *et al.*, 2006). This method involves producing a linear fragment that

contains a selectable marker flanked by ca. 1 kb fragments upstream and downstream of the target gene. This targeted construct could be used for transformation direct without requiring any cloning procedure. To delete *cdr1B*, the hygromycin B gene (*hph*) was chosen as a selection marker for fungal transformation. The four following steps were performed.

3.2.2.13.1 *Designing Primers*

Using the primer 3 design function of MacVector (Version v 18.2.5 for Macintosh), eight primers were designed. The first set of primers - P1 (forward primer) and P2 (reverse primer) - were designed to amplify the upstream 5' flank fragment of the *cdr1B*. The second set of primers – the P3 (forward primer) and P4 (reverse primer) - were designed to amplify the downstream 3' flank fragment of the *cdr1B*. The third set of primers - the P7 (forward primer) and the P8 (reverse primer) - were designed to amplify the *hph* fragment (**Figure 3.4**).

To increase the probability of correct linkage between the three fragments, generic linker sequences were used as followed: A tag tail with a complementary sequence to P7 primer was attached to the 5' end of the P2 primer. Similarly, another tag tail with a complementary sequence to P8 primer was attached to the 5' end of P3 primer. The last pair of primers - P5 and P6 (nested primers) - were used to aid the amplification and to increase the specificity of the fusion PCR, whereby all the three fragments were joined together as a final product of the *cdr1B* knockout cassette. **Figure 3.4** shows the position of each primer and the fusion PCR designed knockout cassette. Primers sequences are listed in (**Table 3.10**).

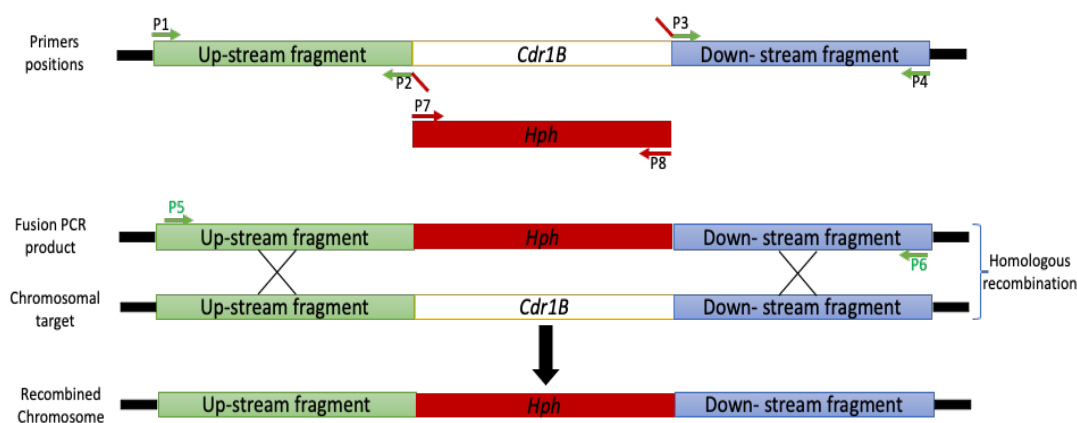


Figure 3-4: Diagrammatic illustration of gene replacement using the fusion PCR method. The *cdr1B* gene was planned to be replaced by the selectable marker hygromycin B (*hph*), using fragments amplified with various sets of primers namely P1, P2, P3, P4, P5, P6 and P7. Complementary sequence to primer P7 and P8 attached to primer P2 and P3 shown in red.

Table 3-10: PCR oligonucleotides sequences used in fusion PCR amplifications

Primer's name	Sequences
P1	CCAGGACCTTCTTGAGTTGC
P2	gttgtgtgacttttggttacgcATGGCCGATTGAGTGAGACA
P3	gggagacgagatcaagcagaCCTGACCTCCTTCCCTTCTAG
P4	GACCAGATCCTTCGCCTCC
P5 ¹	CATACGGCTTGCGTCTTCTC
P5 ²	TTAAGTGCCTCAGGACCAGG
P6 ¹	GCTCTCCTTGACGGACTGT
P6 ²	ACCTGGAGCCATCTGATACG
P7 ¹	GCGTAACCAAAAGTCACACAAC
P7 ²	ACGGCGTAACCAAAAGTCAC
P8 ¹	TCTGCTTGATCTCGTCTCCC
P8 ²	TCTTGACGACCGTTGATCTG

3.2.2.13.2 Amplifying Fragments

The designed primers (P1/P2 and P3/P4; **Table 3.10**) were used to amplify the up-stream and the down-stream regions of *cdr1B*, respectively, using *A. fumigatus* gDNA as a template. The selectable marker *hph* was amplified from an existing plasmid (**Section 3.2.2.6**) by using two different pairs of primers P7¹/P8¹ and P7²/P8². To amplify all fragments, the same PCR cycle conditions and reaction components as shown in **Tables 3.3 and 3.4** were applied using the proof-reading Phusion® High Fidelity DNA polymerase (NEB, UK). PCR products were visualised using gel

electrophoresis and UV light as described in **Section 2.2.6**. Amplicons were purified using a NucleoSpin® Gel and PCR Clean-up kit according to the manufacturer's instructions.

3.2.2.13.3 Fusion PCR

To generate the linear DNA for transformation, the template for the fusion PCR consisted of the two amplified flanking fragments of *cdr1B* and the selectable *hph* marker fragment. Nested primers (P5 and P6) were used to increase the specificity of the final PCR product. Fusion PCR was performed using previously described PCR conditions and components (**Tables 3.11 and 3.12**) according to (Szewczyk *et al.*, 2006).

Table 3-11: Fusion PCR thermocycling conditions

Number of cycles		Temperatures	Duration	Ramp
1 cycle	Initial denaturation	94 °C	2 min	
10 cycles	Denaturation	94 °C	20 sec	
	Extension	70 °C	1 sec	
	Annealing	55 °C	30 sec	Ramp from 70 °C to 55 °C at 0.1°C/sec
	Extension	68 °C	5 min	Ramp from 55 °C to 68 °C at 0.2°C/sec
5 cycles	Denaturation	94 °C	20 sec	
	Extension	70 °C	1 sec	
	Annealing	55 °C	30 sec	Ramp from 70 °C to 55 °C at 0.1°C/sec
	Extension	68 °C	5 min + 5 sec for each subsequent cycle	Ramp from 55 °C to 68 °C at 0.2°C/sec
5 cycles	Denaturation	94 °C	20 sec	
	Extension	70 °C	1 sec	
	Annealing	55 °C	30 sec	Ramp from 70 °C to 55 °C at 0.1°C/sec
	Extension	68 °C	5 min + 20 sec for each subsequent cycle	Ramp from 55 °C to 68 °C at 0.2°C/sec
1 cycle		68 °C	10 min	

Table 3-12: Fusion PCR reaction mixture

Reagent	Volume per sample (µl)
Up-stream fragment	2
Down-stream fragment	2
<i>Hph</i> fragment	2
10 mM forward primer P5	2.5
10 mM reverse primer P6	2.5
Phusion DNA polymerase	1
dNTPs (AB gene, UK)	1
5 × Phusion HF Buffer	10
dH ₂ O	27
Final volume	50

3.2.2.13.4 Transformation Procedure

PEG-mediated protoplast transformation (described in **Section 3.2.2.4**) was used to delete *cdr1B* using the linear cassette DNA with *hph* as a selectable marker. The only difference from the procedure described previously was that the transformation GG10 media was supplemented with 180 µg/ml hygromycin as selectable media for the transformants. Two azole-resistant *A. fumigatus* strains harbouring TR₁₃₀ X 4R were used (10.17 as a lab strain and C136 as a patient strain) for *cdr1* deletion studies. Two control plates were included i.e. (i) a positive control without hygromycin B and with protoplasts without DNA; and (ii) negative control with hygromycin B and protoplasts without DNA. The positive control was included to confirm that protoplast formation and regeneration had occurred. On the other hand, the negative control was included to prove that no growth could be observed if the protoplasts did not carry the hygromycin selectable marker.

3.2.2.13.5 Confirmation of Successful Transformation

A colony PCR method was used for quick screening of putative positive transformants. Mycelium from colonies able to grow in the presence of the hygromycin were picked off the media using toothpicks. The picked mycelia were then transferred to 1.5 ml tubes containing 2 µl of 1M NaOH and 98 µl of colony PCR solution (**Section 3.2.1.2.27**). Toothpicks with mycelia were then incubated at 100°C for 10 minutes. 2 µl of the resulting solution was later used as a colony PCR template to check for positive colony harbouring the deletion cassette using P5/P6 primers (**Table 3.10**)

Colonies that produced a positive PCR product were then transferred to ACM slopes and allowed to grow for 7 days in preparation of gDNA extraction (as described in **Section 2.2.3**). PCR was performed to ensure deletion of the *cdr1B* in those selected transformants. Two sets of primer - P1/P8 – were used to check whether those colonies contained the up-stream of *cdr1B*+ *hph*. Similarly, P4/P7 was used to check the down-stream of *cdr1B*+ *hph*.

3.2.2.13.6 Antifungal Susceptibility Test

E-tests were used to evaluate the effect of deleting *cdr1B* on the azole resistance level of the various TR₁₃₀ and control strains as described in (**Section 2.2.2.1**).

3.2.2.14 Virulence of TR₁₃₀ *A. fumigatus* strains and Itraconazole Efficacy in an *In-vivo* Model of Infection (*Galleria mellonella*)

In order to examine the virulence of azole-resistant *A. fumigatus* strains with different forms of the TR₁₃₀ (2R, 3R, 4R), as well as azole-susceptible control strains, both patient-derived and lab GM strains were tested by infecting larvae of *Galleria mellonella* *in vivo*. Eight strains in total were tested; lab strains included one strain with WT *cyp51A* (the $\DeltaakuB^{KU80}$ strain), one GM strain with two repeats of the TR₁₃₀ sequence (12.8/ 2R), one GM strain with three repeats of the TR₁₃₀ (2.4/ 3R), and one GM strain with four repeats of the TR₁₃₀ (10.17/4R). Patient-derived strains included one strain with a WT *cyp51A* (32 strain), one strain with two repeats of the TR₁₃₀ sequence (158/ 2R), one strain with three repeats of the TR₁₃₀ (19/ 3R), and one strain with four repeats of the TR₁₃₀ (169/4R).

Galleria mellonella wax moth larvae-killing assays and treatment efficacy tests were performed as previously described (Garcia-Rubio *et al.*, 2018; Maurer *et al.*, 2019). Fungal spore suspensions (1×10^8 spores per ml) were prepared by harvesting conidia from 7-day old cultures using sterile phosphate-buffered saline (PBS). Ten healthy-appearing wax moth larvae per strain were selected, 0.2-0.3 g in weight and 2 cm in length as supplied from wax moth *Galleria mellonella* (Waxworms Ltd - Sheffield, UK). They were then injected in the last proleg using a Hamilton syringe (Fisher Scientific) to reach the hemocoel, with 10 μ l (i.e. 1×10^6 spores) injected per larvae. After each use the Hamilton syringe was flushed using PBS and was then sterilized by rinsing with 70% ethanol after finishing each group to prevent syringe blockage and cross-

contamination. Each group of 10 larvae were placed in 9 cm diameter Petri dishes and then incubated at 37 °C for 7 days. Two control groups were included: untouched larvae and larvae that were injected with PBS only (10 larvae for each group).

To evaluate the possible impact of itraconazole treatment on outcomes, parallel sets of the same groups of larvae were treated with itraconazole 3h after infection with *A. fumigatus*. Itraconazole concentrations were calculated considering the therapeutic dose (10mg/kg of body weight) and the toxicity of the solvent dimethyl sulfoxide (DMSO). It was concluded that 3 µg/larvae was equivalent to a standard therapeutic dose, therefore 50µl containing this dose in DMSO and PBS was injected in a different proleg to prevent injury to the larvae. For this set, another control group was included i.e. 10 larvae were injected with itraconazole solution only. Mortality was recorded twice a day by monitoring the larvae's colour and movement. Larvae were considered dead when no response to touch was observed and they became melanized. Each experiment was performed in triplicate, and mean survival values were used to generate Kaplan-Meier curves [which could be analysed by long-rank (Mantel-Cox) test] as described in (Jemel *et al.*, 2020).

3.2.2.15 Phylogenetic Analysis

A phylogenetic analysis was performed to identify the relationship between the various TR₁₃₀ isolates derived from the two different source patients (**Table 3.1**). This analysis and the production of the phylogenetic tree was undertaken by Renad Aljohani and Dr. Joanna Rhodes through a collaboration with the Imperial College (London) Group. The analysis is based on using a whole-genome sequence of 100 *A. fumigatus* strains and observed single nucleotide polymorphism (SNPs) on those isolates. The data was analysed by comparing the presence/absence of SNPs in those isolates along with the *A. fumigatus* reference isolate AF293 (Genbank accession NC_007194.1). Maximum-likelihood phylogenies were constructed to assess sequence similarity between isolates using rapid bootstrap analysis over 1,000 replicates by employing the BINCAT Model of Rate Heterogeneity in RAxML v8.2.9. Phylogenies were visualised in FigTree v1.4.2 (Rhodes *et al.*, 2022).

3.3 Results:

3.3.1 Antifungal susceptibility test

A series of supposedly azole-resistant *A. fumigatus* clinical isolates were supplied through a collaboration with Imperial College (London) which did not exhibit any known *cyp51A* forms of azole resistance (**Table 3.1**). To confirm that isolates were indeed azole resistant, the minimum inhibitory concentration (MIC) point was determined using EUCAST methodology for susceptibility testing of molds (version 9.3.2) with two different azole antifungals, itraconazole and voriconazole. The MIC values were read after 48 h incubation of the isolates with different drug concentrations in 96-well plates. The MIC value was defined as corresponding to the concentration of the drug in the well at which no growth was visible by the unaided eye i.e. the well in which fungal growth stopped was considered the minimum azole concentration that inhibits fungal growth, and thus the MIC. EUCAST was used to test all the clinical isolates in **Table 3.1**. The results showed that six of the isolates, namely C128, C136, C137, 82, 80 and 169, were highly resistant to itraconazole MICs values were $\geq 8 \mu\text{g/ml}$ which is higher than the recommended resistance breakpoint for itraconazole $>1 \mu\text{g/ml}$. However, all isolates showed lower MIC values for voriconazole with MICs values of $2 \mu\text{g/ml}$, although this remains above the recommended resistance breakpoint for voriconazole of $>1 \mu\text{g/ml}$. Isolate NO.19 and NO.158 were also resistant to itraconazole with MIC of $8 \mu\text{g/ml}$ and $2 \mu\text{g/ml}$ but were sensitive to voriconazole. By contrast, isolate 32 was totally sensitive to both drugs (**Table 3.13**).

Table 3-13: The minimum inhibitory concentration (MIC) values of various clinical isolates supplied by Imperial College (London) as determined by EUCAST testing with itraconazole (ITZ) and voriconazole (VOR)

Isolate	ITZ MICs value ($\mu\text{g/ml}$)	VOR MICs value ($\mu\text{g/ml}$)
C128	≥ 8	2
158	2	0.25
C136	≥ 8	2
C137	≥ 8	2
82	≥ 8	2
80	≥ 8	2
19	8	1
32	0.5	0.125
169	≥ 8	2

3.3.2 PCR amplification and sequence analysis of *Cyp51A*, *HapE* and *Cox10* genes

To double check that the supplied isolates did not contain any previously identified form of resistance to azole antifungals, PCR was used to amplify candidate *cyp51A*, *hapE* and *cox10* genes using specific sets of primers for each gene. In addition, a *de novo* specific set of primers was designed to amplify around 655 bp of the promoter region of the *cyp51A* gene directly upstream of the ATG start codon to check for the presence of tandem repeats in the promoter region. Five µl of the PCR products was run in 1% agarose gel electrophoresis to check the fragment size for each gene, which were estimated in comparison to 1kb of DNA ladder.

Very interestingly, the PCR products of the *cyp51A* promoter for eight of the clinical isolates were larger than the expected size of 655 bp for isolates lacking any tandem repeat. The fragment size for six of these isolates (C128, C136, C137, 80, 82 and 169) was instead ca. 1,045 bp (**Figure 3.5**). Moreover, a further two isolates showed larger fragment sizes of ca. 915 bp and 785 bp (19 and 158, respectively) that also differed from the expected size of 655 bp, which was seen only in one of the strains (32) examined (**Figure 3.5**). Regarding the source of these isolates, all of the isolates except one had been obtained from a single patient during the period March 2015 till October 2018, with the exception being C128 from a different patient in November 2015 (**Table 3.1**). The change in the predicted size of the PCR products of these strains indicated that those isolates might have duplication of a specific sequence within the promoter region. In order to confirm this hypothesis all PCR products were sent for sequencing at the Deep Seq unit (University of Nottingham).

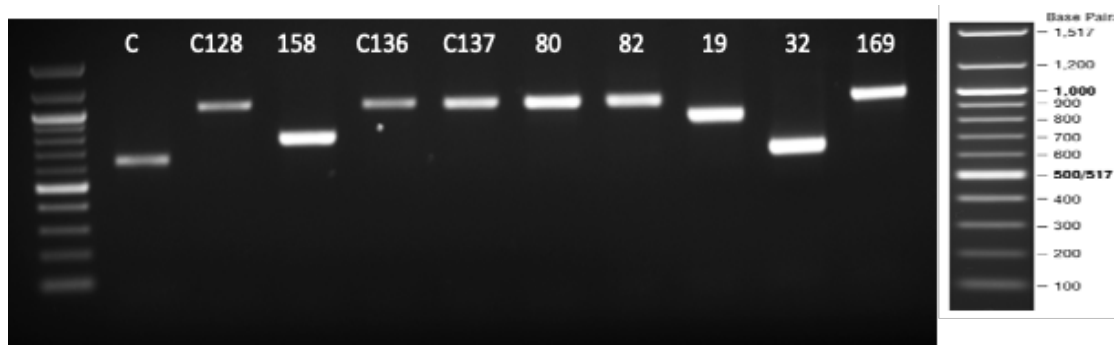


Figure 3-5 Gel electrophoresis of PCR products of the promoter of *cyp51A* using gDNA from different azole-resistant *A. fumigatus* isolates. Bands of the expected size 655bp was observed in the (C) sample using gDNA of a control sensitive strain as well as sample number 32. Six isolates (C128, C136, C137, 80, 82 and 169) produced amplicons with larger fragment size (1,045 bp) than the expected (655 bp) observed in the control sample of the *cyp51A* promoter. PCR product of the two strains 158 and 19 were 785bp and 915bp, respectively, which were larger than the expected size of the control sample. Products were run alongside 100bp DNA ladder (M) in 1% agarose gel.

Arising sequence data of *cyp51A* and its promoter region were analysed using (MacVector v 18.2.5) software. Six of the clinical isolates, (C128, C136, C137, 80, 82 and 169) appeared to have a totally novel fourfold tandem duplication of a 130 bp sequence in the *cyp51A* promoter. One isolate number (19) appeared to have three tandem copies of the same sequence and one isolate number (158) appeared to have two tandem copies of the same sequence as shown below:

**(5'TCGCAGCACCACTTCAGAGTTGTCTAGAATCACGCGGTCCGGAT
GTGTGCTGAGCCGAATGAAAGTTGCCTAATTACTAAGGTGTAGTTCCA
GCATACCATACACCCTAACTCATACTACGGTAGGTTAGA-3')**

This 130 bp sequence is located between positions 224 and 354 upstream from the *cyp51A* start codon in the WT sequence and overlaps fully with previously described tandem repeat regions from *A. fumigatus* (**Figure 3.6**). The other isolate (32) also had the same 130-bp sequence within its promoter region, but in this case the sequence was not duplicated.

Sequence results of *cyp51A* for all the resistant isolates (C128, C136, C137, 80, 82, 19, 158 and 169) showed single nucleotide polymorphisms genotype (SNPs) at several protein codons (46, 127, 248, 255, 427) compared to the reference *A. fumigatus* (Af293) azole susceptible control strain (GenBank accession number AF338659). However, when the sequences were aligned against the azole-susceptible reference genome sequence of *A. fumigatus* A1163 the sequences of almost all *cyp51A* from these strains were identical to *cyp51A* from A1163 (GenBank accession number AF222068) (data not shown). This indicated these SNPs were not correlated with azole resistance, consistent with the findings of (Howard *et al.*, 2009), although it is cautioned that little is known about their effect as presented in combination with the newly identified TR₁₃₀ mutation. However, the C128 clinical isolate had a unique point mutation A1362G in the *cyp51A* gene, that differed from both azole susceptible genome reference isolates Af293 and A1163. This change results in a substitution of the amino acid lysine for glutamine at position 351 in the CYP51A protein (Q351K) and might conceivably contribute to azole resistance.

Meanwhile, sequence alignment of *HapE* and *Cox10* genes from all the isolates against the sensitive control strain Af293, revealed that all appeared to have identical DNA sequence and there were no changes within the codon region that might explain the occurrence of azole resistance in those isolates.

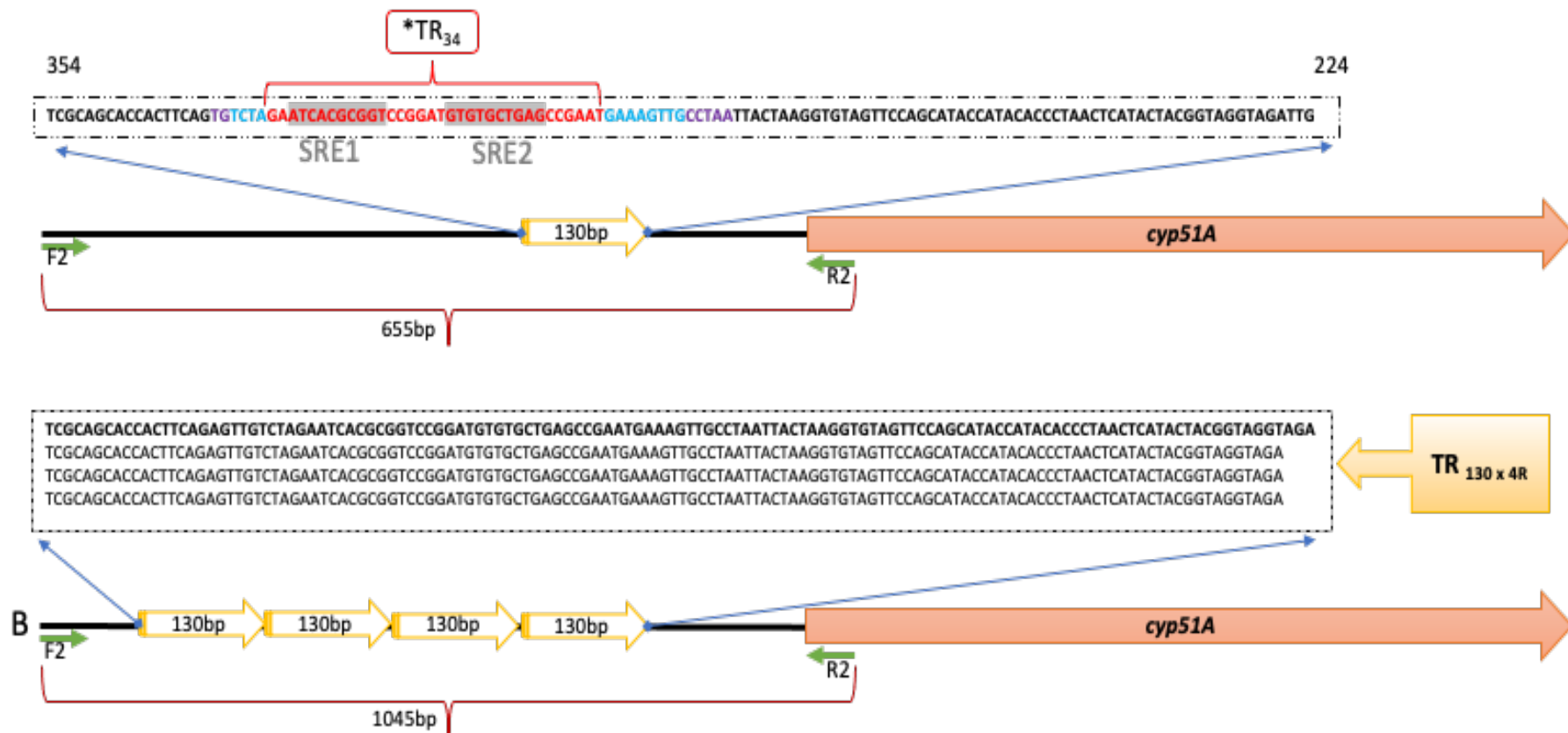


Figure 3-6: Diagram of *Aspergillus fumigatus cyp51A* gene and its promoter region showing the position of the 130 bp sequence in wild-type isolate Af293 (A) and the duplication of TR₁₃₀ in the resistance clinical isolates C128, C136, C137, 80, 82 and 169 (B). The green arrows show the position of the primers used for amplification of the promoter region. The other two isolates number 19 and 158 share the same sequence of the TR₁₃₀ but with different number of repeats TR₁₃₀ X 3R AND TR₁₃₀ X 2R, respectively (not shown in the figure for simplicity). * Note TR₃₄ region in red, the extended part of TR₄₆ in blue and fully-extended TR₅₃ in purple indicating that these sequences with the different form of the previously described TR are presented in each repeat of the TR₁₃₀.

3.3.3 Classical genetic analysis via sexual crossing

Testing with the multiplex mating-type diagnostic of Paoletti *et al.* (2005) revealed that isolates C128, C136 and C137 (harbouring the TR₁₃₀ X 4R tandem repeat) were all of the same *MAT1-2* genotype. These strains were therefore crossed with the azole-susceptible supermaternal strain 47-51 of the opposite *MAT1-1* genotype (Swilaiman *et al.*, 2020). This resulted in a successful crossing between C136 and 47-51, with mature cleistothecia observed after 3 months of incubation. Other crosses did not produce cleistothecia (data not shown). The resulting cleistothecia were collected and around 40 representative progeny were collected following germination of ascospores. Antifungal susceptibility test was performed for all these progeny using the EUCAST method. Twenty of the offspring (50%) were found to be ITZ resistant, with an MIC ≥ 16 μ g/ml, and twenty (50%) were sensitive, with an MIC = 0.5 μ g/ml. This indicated that a 1:1 segregation had occurred, and that the resistance phenotype is due to a single mutation (monogenic trait). To ensure that the resistant progeny harboured the TR₁₃₀, gDNA of 20 progeny was extracted and screened for the presence of TR₁₃₀. All the resistance progeny had the TR₁₃₀, providing very strong evidence that the TR₁₃₀ is responsible for the resistant phenotype in those progeny and that resistance is linked to the single *cyp51A* gene (Figure 3.7).

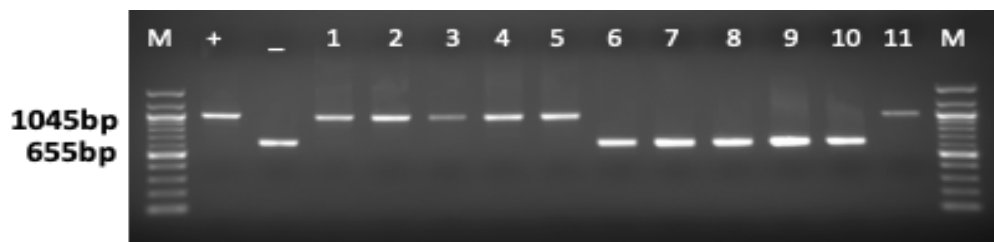


Figure 3-7: Gel electrophoresis showing representative PCR products of the *cyp51A* promoter region obtained from progeny of *A. fumigatus* cross 47-51 $\times$ C136, illustrating that some of the progeny (lanes 1, 2, 3, 4, 5 and 11) have a similar product size (1,045bp) to the azole resistant parent strain C136 (+), indicating that these progenies have four repeats (4R) of TR₁₃₀ while the other progeny are showing the WT PCR product size (655bp) of the azole sensitive 47-51 parent (-) indicating that those progenies do not harbour the TR₁₃₀ in the promoter of *cyp51A*. Products were run alongside a 100bp DNA ladder (M) in a 1% (w/v) agarose gel.

In parallel, the mating-type genotype was determined for the same 20 progeny to confirm Mendelian 1:1 segregation (Figure 3.8). Ten of the progeny were found to be of the *MAT1-1* genotype and 10 of the *MAT1-2* genotype, and there was no relation

between the resistant phenotype and the mating type i.e. results demonstrated independent assortment of these characters as would be expected for non-linked genes.

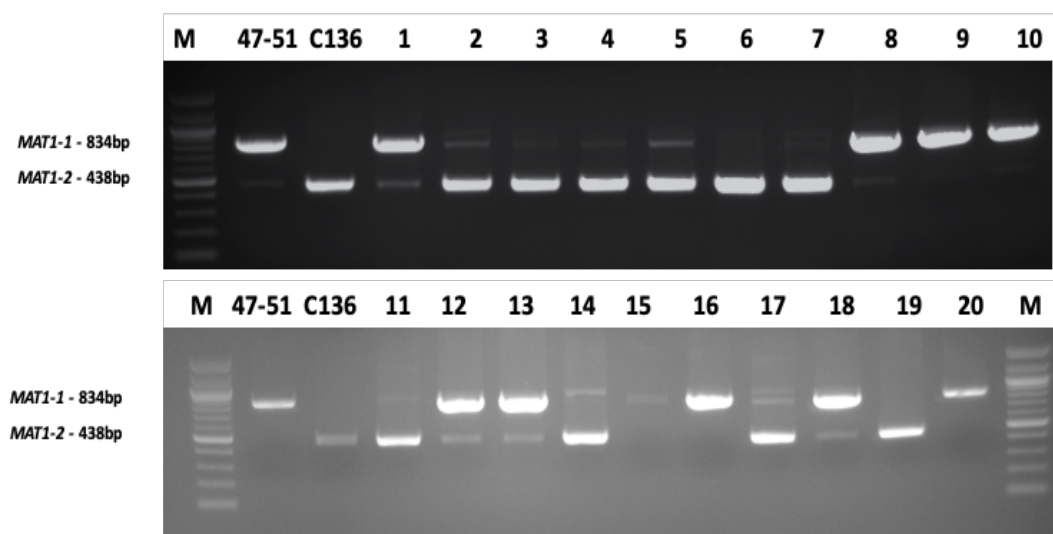


Figure 3-8 Results of the multiplex PCR assay to determine mating type of progeny from *A. fumigatus* cross 47-51 × C136, showing 1:1 segregation of the mating-type genes. Parental strain 47-51 and 10 progenies (1, 8, 9, 10, 12, 13, 15, 16, 18, 20) show band at size of 834bp indicating the present of MAT1-1 gene in those strains. Parental strain C136 and 10 progenies (2, 3, 4, 5, 6, 7, 11, 14, 17, 19) show band at size of 438 bp indicating the present of MAT1-2 gene in those strains. Products were run alongside 100bpDNA ladder (M) in 1% agarose gel.

3.3.4 Generation of lab *A. fumigatus* strains harbouring different forms of the TR₁₃₀ tandem repeat: 4-repeat (4R), 3-repeat (3R) or 2-repeat (2R)

3.3.4.1 Plasmids isolation, digestion and purification

Fragments of the *cyp51A* promoter region were successfully amplified by PCR from various strains exhibiting tandem repeats of the TR₁₃₀ sequence (F1/R1 ~1,500bp in size for 4R, F1/R2 fragment ~578bp in size + F2/R1 top band ~896bp in size for 3R and F1/R2 fragment ~578bp in size + F2/R1 lower band ~766bp in size for 2R) (**Figure 3.9**). Fragments were cloned into *E. coli* and colony PCR with F1/R3 primers used to confirm the presence of positive colonies with either four tandem repeats (4R) (1089 bp), three tandem repeats (3R) (959 bp) or two tandem repeats (2R) (829 bp) (**Figure 3.10**).

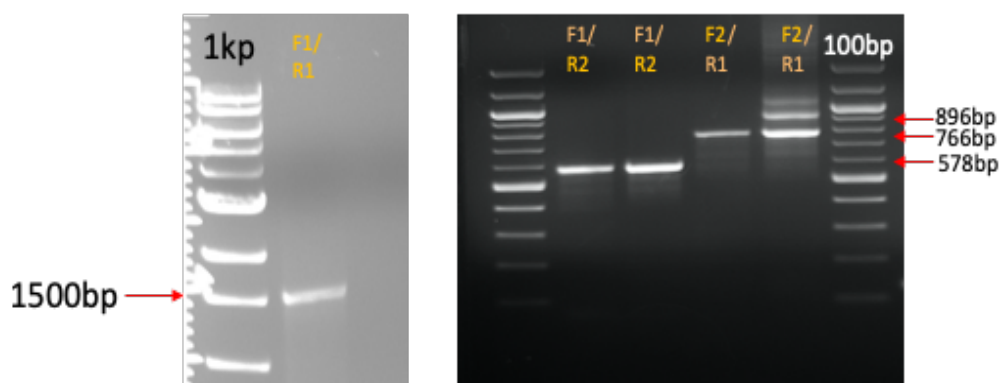


Figure 3-9: Gel electrophoresis of the PCR products using specific sets of primers for cloning the 4R, 3R and 2R TR₁₃₀ tandem repeats. F1/R1 primers generated a fragment (~1,500bp) consistent in size for 4R, F1/R2 primers generated a downstream fragment (~578bp) in size consistent with one copy of 130bp, F2/R1 generated upstream fragments with two different band size (~896bp) consistent with two copies of the 130 bp sequence and (~766bp) that has one copy of the 130bp sequence. Products were run alongside 100bp/or 1kb DNA ladder in 1% agarose gel.

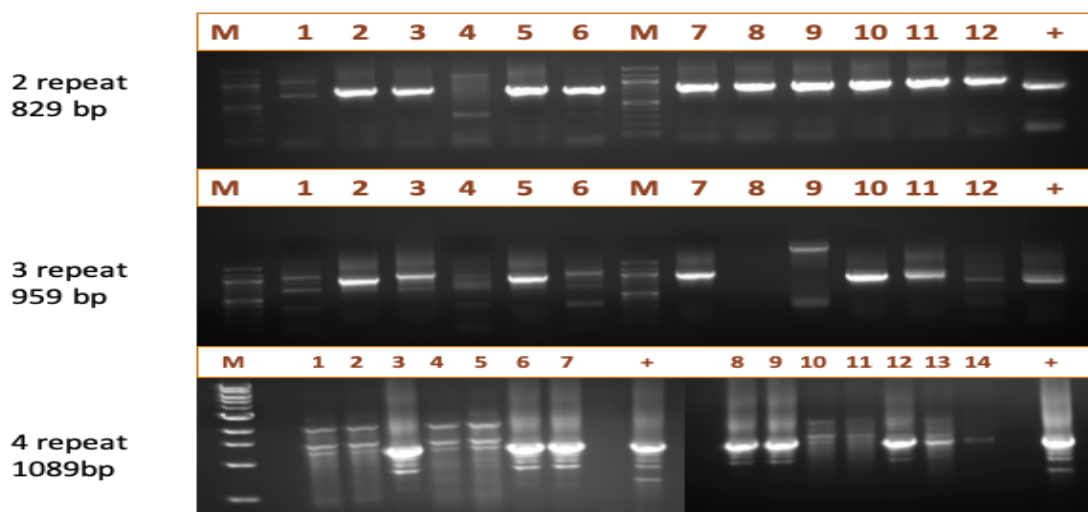


Figure 3-10: Gel electrophoresis of the colony PCR to test for presence of TR₁₃₀ repeat sequences, showing positive 2R colonies in lanes 2, 3, 5, 6, 7, 8, 9, 10, 11 and 12; positive 3R colonies in lanes 2, 5, 7, 10 and 11; and positive 4R colonies in lanes 3, 6, 7, 8, 9, 12 and 13 indicated with brighter bands. Products were run alongside 100bp the top two or 1kb the third image DNA ladder (M) in 1% agarose gel.

After selection of the positive colony with the correct product size from colony PCR, two plasmids from each repeat were isolated and digested using *Sma*I and run on 1% (w/v) of agarose gel electrophoresis to separate the 4R, 3R and 2R product from the pJet1.2 vector (**Figure 3.11**). Bands with the correct size were excised from the gel and used for protoplast transformation.

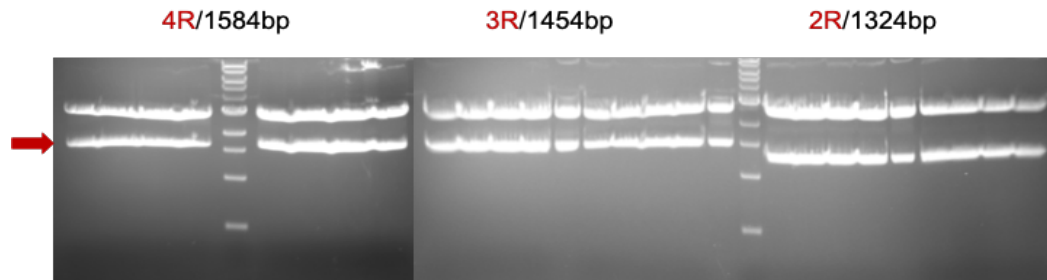


Figure 3-11: Gel electrophoresis of the digested 4R, 3R and 2R plasmids using *Sma*I restriction enzyme. The lower bands show the DNA fragments released from the pJet1.2 vector. Products were run alongside 1kb DNA ladder (M) in 1% agarose gel.

3.3.4.2 Selection of positive transformants

After transformation, colonies were selected on AMM agar plates supplemented with 4 µg/ml itraconazole. Single transformants were confirmed to exhibit the 4R and 2R genotypes, while three positive transformants were recovered with 3R (**Figure 3.12**). The *cyp51A* promotor region was amplified from all putative positive transformant and sent for sequencing. As expected, all positive transformants exhibited the correct number of repeats and correct repeat sequence (data not shown). Transformants 10.17 (which contained four repeats of TR₁₃₀), 2.4 (which contained three repeats of TR₁₃₀) and 12.8 (which contained two repeats of the TR₁₃₀) were selected for further analysis.

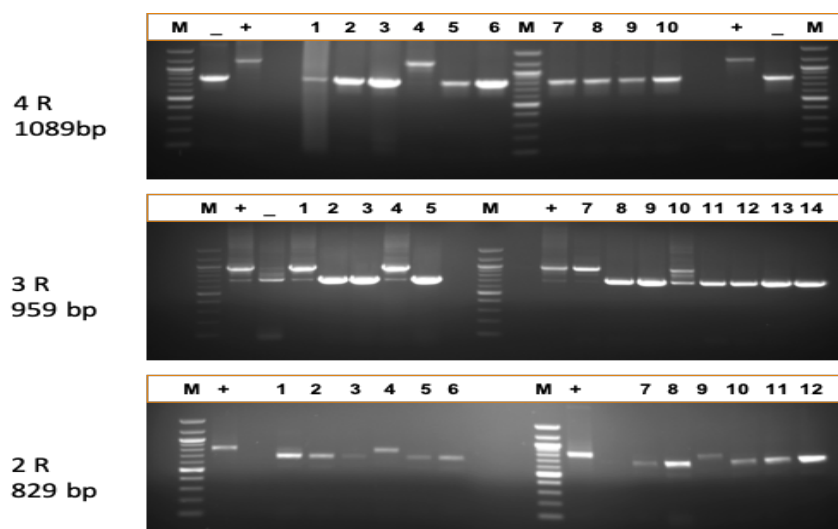


Figure 3-12: Gel electrophoresis of the PCR products of *A. fumigatus* transformants with putative integration of the 4R, 3R and 2R tandem repeats. Bands with the expected size of 4R (1,089 bp) were observed in lane 4. Three bands with the expected size of 3R (959 bp) were observed in lanes 1, 4 and 7. One positive transformant with the expected size (829 bp) was observed in lanes 4 and 9 from the same colony. Products were run alongside 100bp DNA ladder (M) in 1% agarose gel.

3.3.5 Southern Blotting

3.3.5.1 Generating Southern Blot Probe

A specific set of primers were used to amplify a region upstream of the position of TR₁₃₀, containing around 224bp of the promoter region and around 406bp of the start sequence of *cyp51A* gene. Two reactions were set up; one containing the DIG label and one without DIG labelling to ensure that PCR/primers were working correctly. Amplicons with the correct size (≈ 630 bp) were observed in both reactions with no effect of DIG addition (**Figure 3.13**). Fragments with DIG labelling were then excised from the gel and purified to be used as a probe for Southern blotting.

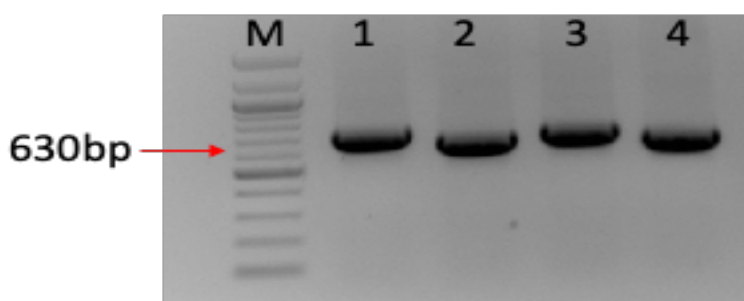


Figure 3-13: Gel electrophoresis of the PCR products for generating Southern blot probe using gDNA of *A. fumigatus* WT isolate. Fragments with an expected size ≈ 630 bp were observed with or without addition of DIG. Lane 1 and 3 contained PCR product + DIG labelling and lane 2 and 4 contained PCR products without DIG labelling. Products were run alongside 100bp DNA ladder (M) in 1% agarose gel.

3.3.5.2 DNA Quality Check

The purity and integrity of gDNA of the putative 2R, 3R and 4R transformants were checked by the gel electrophoresis. Aliquots of 2 μ l of the samples were mixed with 2 μ l 5x loading dye and run alongside a 1kb DNA ladder in 1% TAE Agarose gel at 100V for 40 minutes. Pure intact high molecular weight single-bands were observed in all four samples, showing sufficient gDNA quality for Southern blotting (**Figure 3.14.A**).

3.3.5.3 Digestion of gDNA

The enzyme *NdeI* was used to digest gDNA of all three putative positive transformants (2R, 3R, 4R) along with gDNA of the parental control strain *ΔakuB*^{KU80}.

Fully-digested gDNA could be seen on a gel (**Figure 3.14.B**), which appeared as a smear with a range of fragments of digested DNA. The predicted size for the digested tandem repeat fragments was 3033bp, 3162bp, 3292bp, and 3422bp for the $\DeltaakuB^{KU80}$, 12.8/2R, 2.4/3R, and 17.10/4R, respectively.

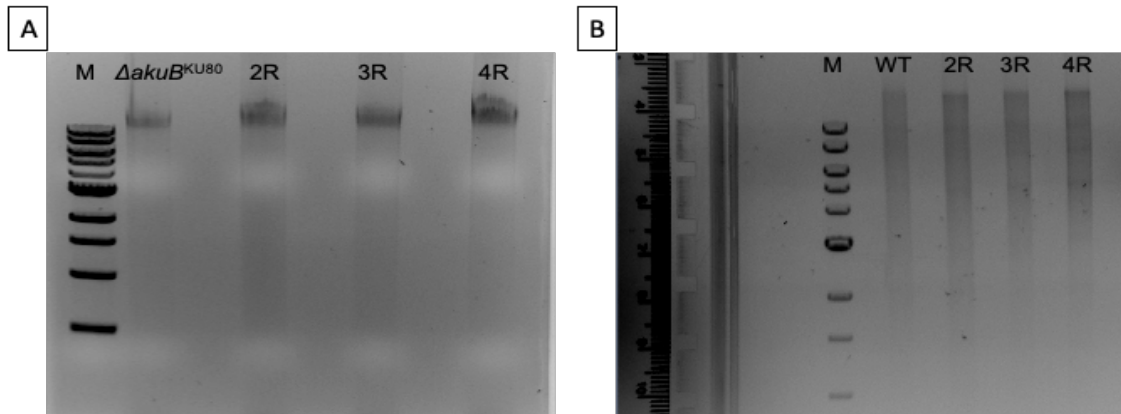


Figure 3-14: Gel electrophoresis showing; (A) The quality of gDNA used for Southern blotting of the positive transformants 12.8/2R, 2.4/3R and 10.17/4R as well as the parental strain for transformation $\DeltaakuB^{KU80}$. (B) The fully-digested gDNA of the putative 2R, 3R and 4R transformants together with the WT parental strain following digestion with the *NdeI* restriction enzyme.

3.3.5.4 Staining

DNA samples were transferred to a nylon membrane through the processes of denaturing, blotting, hybridisation, blocking, and were then probed with a specific sequence labelled probe. The position of the digestion fragment and the binding probe site is presented in (**Figure 3.15.A**). The two staining steps provided proof that a single-copy integration of the *cyp51A* promoter into the *cyp51A* locus had been achieved for each tandem repeat transformant (**Figure 3.15.B**).

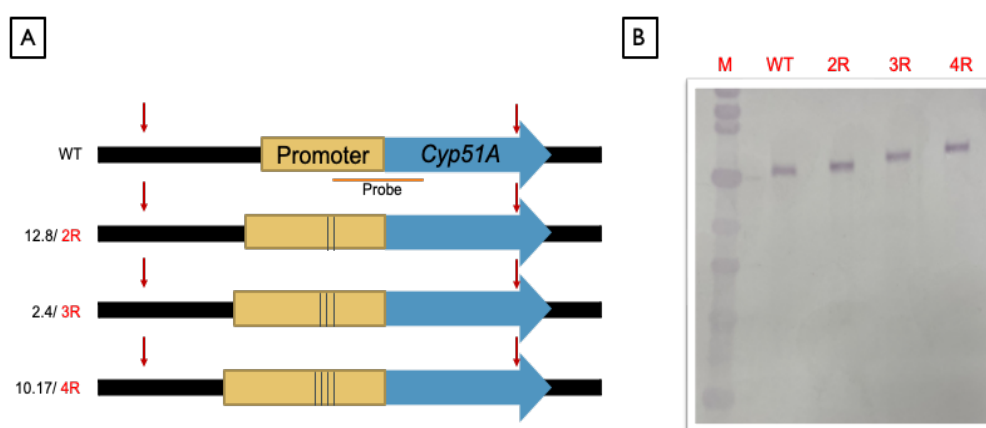


Figure 3-15: Southern blot analysis of generated lab TR₁₃₀ *A. fumigatus* transformants. (A) Schematic illustration of the *cyp51A* gene and its promoter for the WT and different forms of the TR₁₃₀ transformants. *NdeI* restriction site are indicated by red arrows and position of probe binding site is indicated in orange line. (B) Nylon membrane after staining using NBT/BCIP showing fragments of gDNA for the positive transformant as well as the parental strain for transformation $\DeltaakuB^{KU80}$ after digestion using *NdeI*. Only a single copy of the *cyp51A* promoter is detected showing fragment with expected size (3033bp) for WT, compared to larger size (3162bp) for 12.8/2R transformant, (3229bp) for 2.4/3R transformant and (3422bp) for 10.17/4R transformant indicating single integration occurrence during the transformation procedure.

3.3.6 Generation of complementary $\Delta cyp51A$ TR₁₃₀ X 4R isolate

3.3.6.1 Digestion of the Plasmid

The complementary construct was released from the pUC19 vector using the *HindIII* restriction enzyme which resulted in two fragments that were run in gel electrophoresis and visualised using the UV light to check the size. Two fragments with the expected size were achieved after digestion (**Figure 3.16**). The top band was around 6,400bp in size which was the predicted size for the complementary fragment. The bottom band was around 2,600bp which was the predicted size for the pUC19 vector. The top band was excised and purified and stored at -20°C for subsequent transformation work.

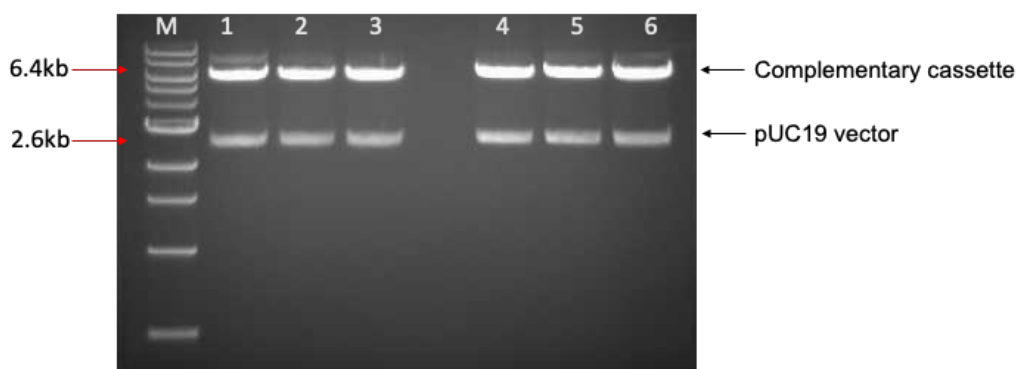


Figure 3-16: Gel electrophoresis of the digested *cyp51A* and hygromycin complementary plasmid. The top bands show the DNA complementary cassette with the expected size of (6.4kb) released from the pUC19 vector (2.6kb) using *HindIII*. The first three lane (1, 2, and 3) are replicates of one plasmid, and the other three (4, 5, and 6) are replicates of second plasmid. Both plasmids and the replicates showed the same digestion pattern. Products were run alongside 1kb DNA ladder (M) in 1% agarose gel.

3.3.6.2 Selecting Positive Transformant

During transformation, the linear fragment with the *hphA* selective marker was predicted to integrate by homologous recombination into the genome and replace the mutated *cyp51A* with the WT *cyp51A*. Colonies that were able to grow in the presence of hygromycin B were therefore selected in a second selection media supplemented with 8µg/ml itraconazole. Around four colonies were unable to grow in the presence of itraconazole indicating that these colonies are positive complementary transformant as they had lost the azole resistance phenotype. On the other hand, twelve colonies were able to grow in itraconazole media indicating that those colonies were still resistant and further analysis was needed (**Figure 3.17.A**). Seven colonies were then selected randomly for further screening using standard PCR after extracting gDNA through primers to screen the promoter of *cyp51A*. Colonies that showed WT *cyp51A* promoter (NO.20, 21, 22 and 27) were selected as positive complementary transformants as the rest showing multiple bands indicating mixed samples (**Figure 3.17.B**). This result provides strong evidence that by deleting the TR₁₃₀ X 4R from the promoter of *cyp51A*, the resulted transformants were restored back to the original sensitive phenotype. This result clearly demonstrated the link between the resistant phenotype and the novel azole resistant mechanism TR₁₃₀ in *A. fumigatus*.

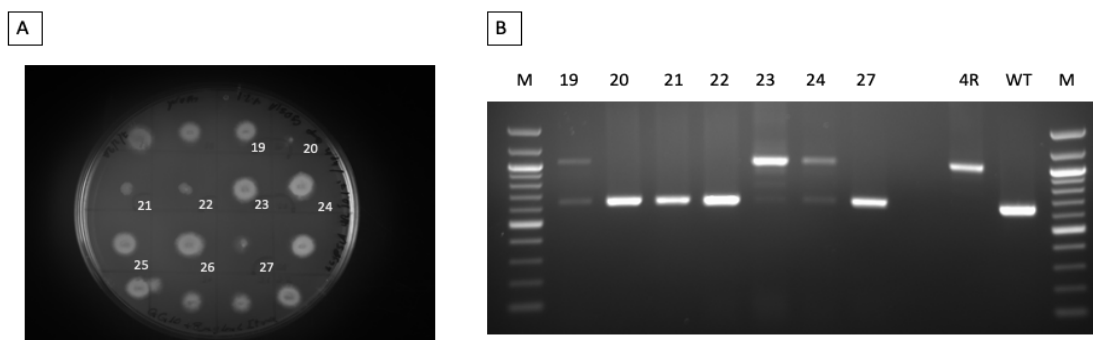


Figure 3-17: Representative result of the complementary transformation work in which the *cyp51A* gene with TR130x4R had been replaced with the WT *cyp51A* hygromycin cassette with just one 130 bp sequence. (A) Selection of transformant colonies on itraconazole selection media showing around 12 colonies that were able to grow in the present of itraconazole and four colonies that showed much reduced growth in the present of itraconazole. (B) Gel electrophoresis of PCR product using primers for screening the promoter region of *cyp51A* of seven selected transformants from the itraconazole plate. PCR product of transformants in lane 20, 21, 22, 27 showing WT promoter size of 655bp indicating that those are positive complementary transformants as they were sensitive to itraconazole, and they had WT promoter of *cyp51A*. Products were run alongside 100bp DNA ladder (M) in 1% agarose gel.

3.3.7 Antifungal susceptibility testing of the lab strains

The MIC values of 4R, 3R and 2R transformants were determined for itraconazole using the E-test and EUCAST methods and compared to an azole-resistant strain with TR₁₃₀ found in the clinical samples, and the azole sensitive $\DeltaakuB^{KU80}$ parent strain used for GM transformation. In addition, four positives complementary transformants, where the *cyp51A* TR₁₃₀/ 4R of the positive transformant (10.17/4R) was replaced with WT *cyp51A*: hygromycin B cassette, were also tested for MIC using EUCAST.

Results showed that the 10.17/4R transformant could grow in the presence of a high concentration ($\geq 8 \mu\text{g/ml}$) of itraconazole unlike the host $\DeltaakuB^{KU80}$ parental strain with an MIC value of only $0.5 \mu\text{g/ml}$ (Table 3.14). Therefore, the insertion of 4R of the TR₁₃₀ into the sensitive $\DeltaakuB^{KU80}$ strain converted this strain into an azole-resistant strain. This result confirmed the hypothesis that the four-fold repeat (4R) of the TR₁₃₀ is responsible for the resistance phenotype. Moreover, the 3R and 2R were also found to be resistant to itraconazole, and although the MIC values were not as high as those of the 4R, they were still considered azole resistant as their level of resistant is higher than the recommended breakpoint of itraconazole for EUCAST testing (Table 3.14). By contrast, the complementary transformants all showed a return to the sensitive phenotype after removal of the TR₁₃₀/4R: *cyp51A* mutation again indicating that the

TR₁₃₀ mutation is the mechanism responsible for the azole resistant phenotype in strains harbouring the TR₁₃₀ mutation. A comparison of the resistance level between lab genetically modified (GM) strains and clinical strains harbouring the different form of TR₁₃₀ will be presented in **Section 3.3.9**.

Table 3-14: MIC values (using EUCAST methodology) of the original UK clinical isolate C136 and $\DeltaakuB^{KU80}$ GM parental control strain as compared to various lab GM strains of *A. fumigatus* with different numbers of the TR₁₃₀ tandem repeat

Isolates	Itraconazole MICs (µg/ml)
$\DeltaakuB^{KU80}$	0.5
TR ₁₃₀ /C136	≥ 8
+VE/4R (10.17)	≥ 8
+VE/3R (2.4)	4
+VE/2R (12.8)	2
+VE/ comp (20)	0.5
+VE/ comp (21)	0.5
+VE/ comp (22)	0.5
+VE/ comp (27)	0.5

3.3.8 Growth susceptibility assay of positive transformants

The growth of the positive transformants on media supplemented with itraconazole was conducted to evaluate possible differences in growth between the parental sensitive strain for transformation $\DeltaakuB^{KU80}$ and the 4R, 3R and 2R transformants. Results showed that 4R, 3R and 2R transformants were able to grow in the presence of itraconazole up to 4 µg/ml, even with inoculation of spore suspensions of relatively low concentrations. The 4R and 3R transformants were also able to grow in the present of higher concentrations of itraconazole up to 8-16 µg/ml, even with lower spore suspension concentrations, unlike the 2R transformant which was largely inhibited above 8 µg/ml ITZ. Growth of the parental transformation strain $\DeltaakuB^{KU80}$ was inhibited at 1 µg/ml and above (**Figure 3.18**).

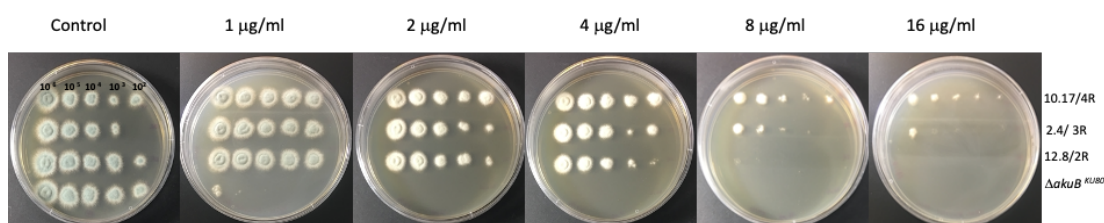


Figure 3-18: Effects of varying itraconazole concentrations on the growth of *A. fumigatus* 4R, 3R and 2R GM transformants. The top row in each plate shows transformants 10.17/4R with different spore suspension concentrations followed by positive transformant with 3R in the second row, then the 2R positive transformant. The bottom row in each plate shows the azole-sensitive $\Delta akuB^{KU80}$ strain with different spore suspension concentrations used as an inoculum. The positive transformants harbouring 4 repeats and 3 repeats of the TR₁₃₀ were able to grow in the presence of high concentration 8-16µg/ml even with inoculation of lower spore suspension concentrations.

In further tests, the same method was used to evaluate azole resistance of one of the positive GM transformants (10.17/4R) in comparison to the one of the original clinical C136/4R isolates, together with a complementary control transformant. Plates were again prepared using AMM agar supplemented with varying itraconazole concentrations up to 8 µg/ml. Results showed that the 10.17 transformant harbouring 4R of the TR₁₃₀ as well as the C136 clinical isolate (also harbouring 4R of the TR₁₃₀) were able to grow in the presence of itraconazole up to 8 µg/ml, even when inoculating with spore suspensions of a relatively low concentration (**Figure 3.19**). By contrast, the complementary transformant was hypersensitive and was not able to grow in the presence of itraconazole, indicating that removal of the TR₁₃₀ mutation resulted in loss of the resistance phenotype.

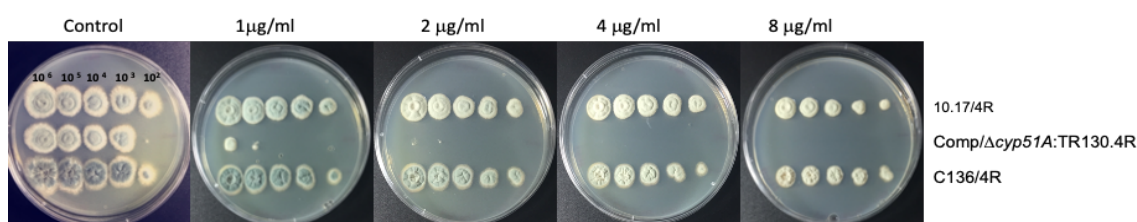


Figure 3-19: Effects of varying itraconazole concentrations on the growth of various *A. fumigatus* strains, namely the GM strain 10.17/4R harbouring the TR₁₃₀ X 4R, the clinical C136 strain also harbouring TR₁₃₀ X 4R, and a GM complementary strain in which the TR₁₃₀ X 4R has been replaced with the WT 1 x TR130 sequence. The top row in each plate shows the parental strain for transformation 10.17/4R inoculated with different suspension concentrations. The middle row in each plate shows the complementary transformant inoculated with different spore suspension concentrations. The bottom row in each plate shows clinical azole resistant C136 /4R TR₁₃₀ isolate inoculated with different spore suspension concentrations.

3.3.9 Comparison between the resistant level of TR₁₃₀ GM lab and clinical strains using E- test

In parallel tests, the E-test method was used to determine azole resistance of the GM transformants showing different numbers of tandem repeats (10.17/ 4R, 2.4/ 3R, 12.8/2R) in comparison to the original clinical patient-derived isolates (C136/4R, 19/3R, 158/2R, C136/4R), together with the GM parental ($\DeltaakuB^{KU80}$) and a WT control strain (32) from the original patient. The minimum inhibitory concentration (MIC) point was read after 48 hours of incubation with the itraconazole strip. The point at which fungal growth stopped was considered as the minimum azole concentration that inhibits fungal growth i.e. the MIC.

Results showed that the 32/WT strain and $\DeltaakuB^{KU80}$ strain were hypersensitive to itraconazole with MICs values of 0.5 $\mu\text{g/ml}$. Strains harbouring 4R of the TR₁₃₀ mutation were highly resistant, demonstrating high MICs value between 24-32 $\mu\text{g/ml}$ against itraconazole. However, there was some inhibition zone around the highest concentration in the top of the E- test strip on testing the 10.17/4R GM strain. By contrast, the clinical C136/4R isolate showed no similar effect and instead showed growth even at the highest levels of itraconazole (**Figure 3.20**). Similarly, the MIC value for the clinical strain harbouring the TR₁₃₀ X 3R mutation was higher (ca. 12 $\mu\text{g/ml}$) than the MIC value for the GM lab strain harbouring the same mutation (ca. 6 $\mu\text{g/ml}$) i.e. a 2-fold difference in MIC value between the ‘same’ TR₁₃₀ genotype in the clinical versus GM strain. Although the MIC values for the lab GM strain 12.8/ 2R and clinical strain 158/2R clinical strain were both very similar at ca. 2 $\mu\text{g/ml}$, there was nevertheless a clear difference in the size of the inhibition zone around the E- test strip of the GM lab strain in comparison to the clinical strain (**Figure 3.20**). Taken as a whole these results indicated that MIC was not determined by the presence of the TR₁₃₀ repeat alone, and that other background genetic factors also contributed. Therefore, further work was undertaken to quantify differences in the resistant level between lab and clinical strains harbouring the same mutation in the promoter of *cyp51A* using a different methodology.

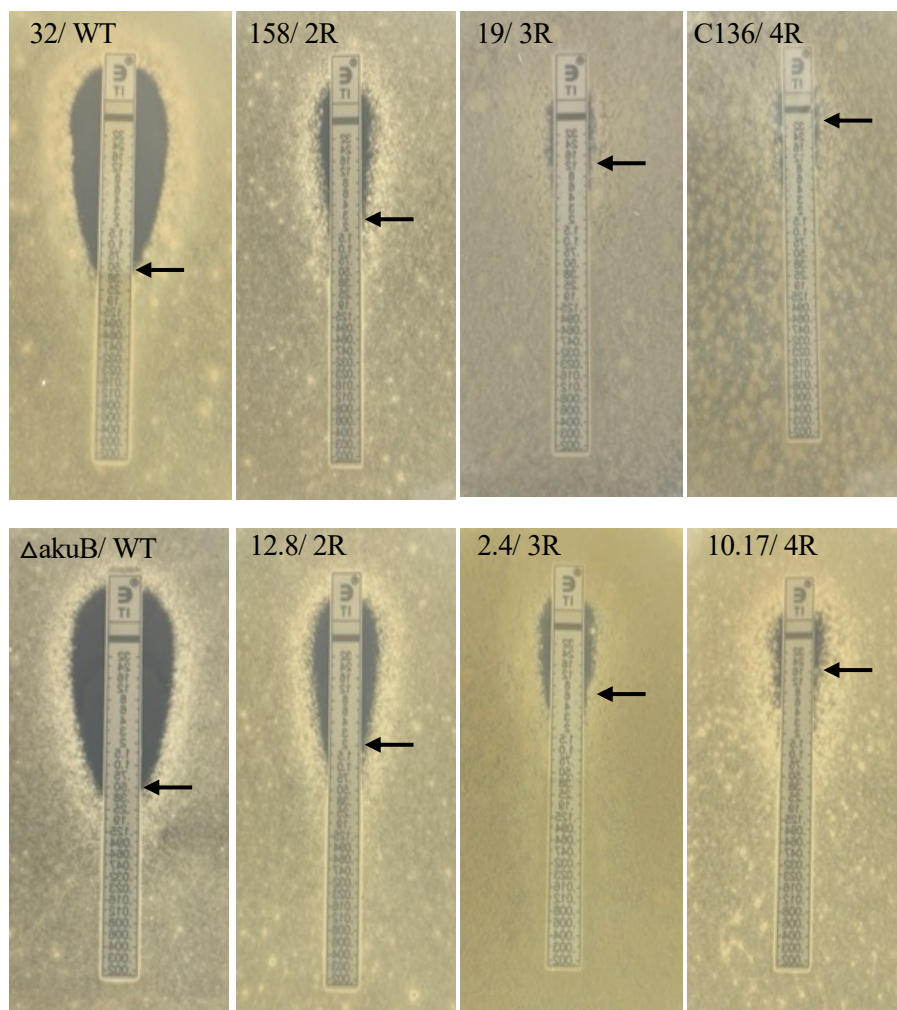


Figure 3-20: A comparison of the resistance level of TR130 clinical and lab strains of *A. fumigatus* using the E-test method. Arrows indicate the MICs value for each strain. MICs value for clinical strains (32/ WT, 158/ 2R, 19/ 3R, C136/ 4R) were as follow. (0.5µg/ml, 2µg/ml, 12µg/ml, ≥32µg/ml). MICs value for lab strains ($\DeltaakuB^{KU80}$ / WT, 12.8/ 2R, 2.4/ 3R, 10.17/ 4R) were as follows: (0.5µg/ml, 2µg/ml, 6µg/ml, ≥24µg/ml). MICs were recorded after 48 hours of incubation at 37 °C.

3.3.10 Quantification of TR₁₃₀ Strains Growth in the Presence of Itraconazole

An OD-based quantification method described by (Meletiadiis *et al.*, 2003) was used in combination with a microdilution EUCAST method (with some modifications) to

further evaluate differences in levels of azole resistance between the GM lab strains and patient-derived strains harbouring 3R and 4R of the TR₁₃₀ sequence,

Usually, the EUCAST result is read visually by recording visible growth (or not) in each well of different antifungal concentrations after a defined period of static growth (**Figure 3.21**). However, to validate the EUCAST result and further quantify any difference in the resistance level of the lab (**Table 3.14**) and patient TR₁₃₀ strains (**Table 3.13**), the growth percentage of strains in the presence of different concentrations of itraconazole (relative to an unamended media control) was analysed using ODs measured spectrophotometrically i.e. a combination of OD-based growth testing together with the EUCAST style use of a range of concentrations of the antifungal, The data was then analysed from grouped OD reads of 8 replicates per well for each strain at different itraconazole concentrations.

Figures 3.22.A and 3.22.B show the results of growth percentage of all GM lab and patient-derived strains at a range of different itraconazole concentrations. The figure shows that both patient and lab strains harbouring different forms of TR₁₃₀ exhibited more or less similar resistance levels, with MICs values of around 8 mg/l, 4 mg/l, and 2 mg/l for the 4R, 3R, and 2R strains, respectively. However, confirmation of this result necessitated two-way ANOVA analysis (**Figures 3.22.C and 3.22.D**). As expected, the 10.17/4R GM strain showed a significantly higher resistance level than the 2.4/3R GM strain at the two highest ITZ concentrations of 8 mg/l and 4 mg/l ($P=0.0009$ and $P=0.0169$, respectively) (**Figure 3.22.C**). However, there was no similar significant difference between the MIC values for the patient-derived C136/4R and 19/3R strains (**Figure 3.22.D**). Further comparison to quantify possible differences in growth percentage between the 4R and 3R lab and patient strains was done (**Figure 3.22.E and 3.22.F**). Interestingly, results showed a significant difference in growth percentage between the 3R strains, namely the 2.4 lab strain and 19 patient strain, whereby the patient-derived strain showed a significantly higher resistance level than the lab GM strain at the highest two itraconazole concentrations of 8 mg/l and 4 mg/l ($P < 0.0001$) and ($P= 0.0058$), respectively (**Figure 3.22.E**). Moreover, the 4R strains also showed a significant difference in the MICs values between the 10.17 lab strain and C136 patient strain, whereby the patient strain showed a higher resistance level compared to the lab strain at the highest 8 mg/l concentration of itraconazole ($P= 0.0119$) (**Figure 3.22.F**).

The evidence presented so far supports the idea that the patient strains might have an additional resistance mechanism rather than TR₁₃₀ alone within the *cyp51A* gene. This was despite previous findings from the classical genetic analysis which indicated that the resistant phenotype was linked only to single gene (Section 3.3.3).

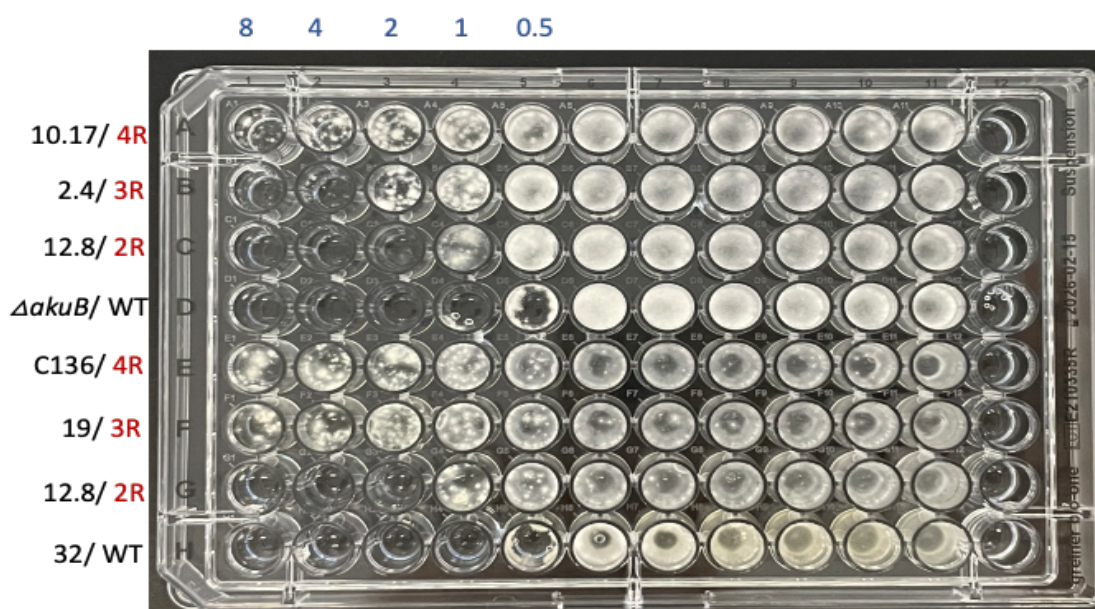


Figure 3-21: Microwell plate showing the EUCAST method, illustrating growth of *A. fumigatus* strains harbouring the different form of the TR₁₃₀ as well as WT strains in the present of varying concentration of itraconazole. Blue numbers at the top indicate the concentration (mg/L) of each well. Each row contains different strains indicated with the labelling. Result of EUCAST are usually recoded by visible growth in each well of different concentrations after 48 h of incubation at 37°C. MICs value of was recoded visually as follow 8mg/l, 4mg/l, 2mg/l and 0.5mg/l for strains 4R, 3R, 2R and WT, respectively.

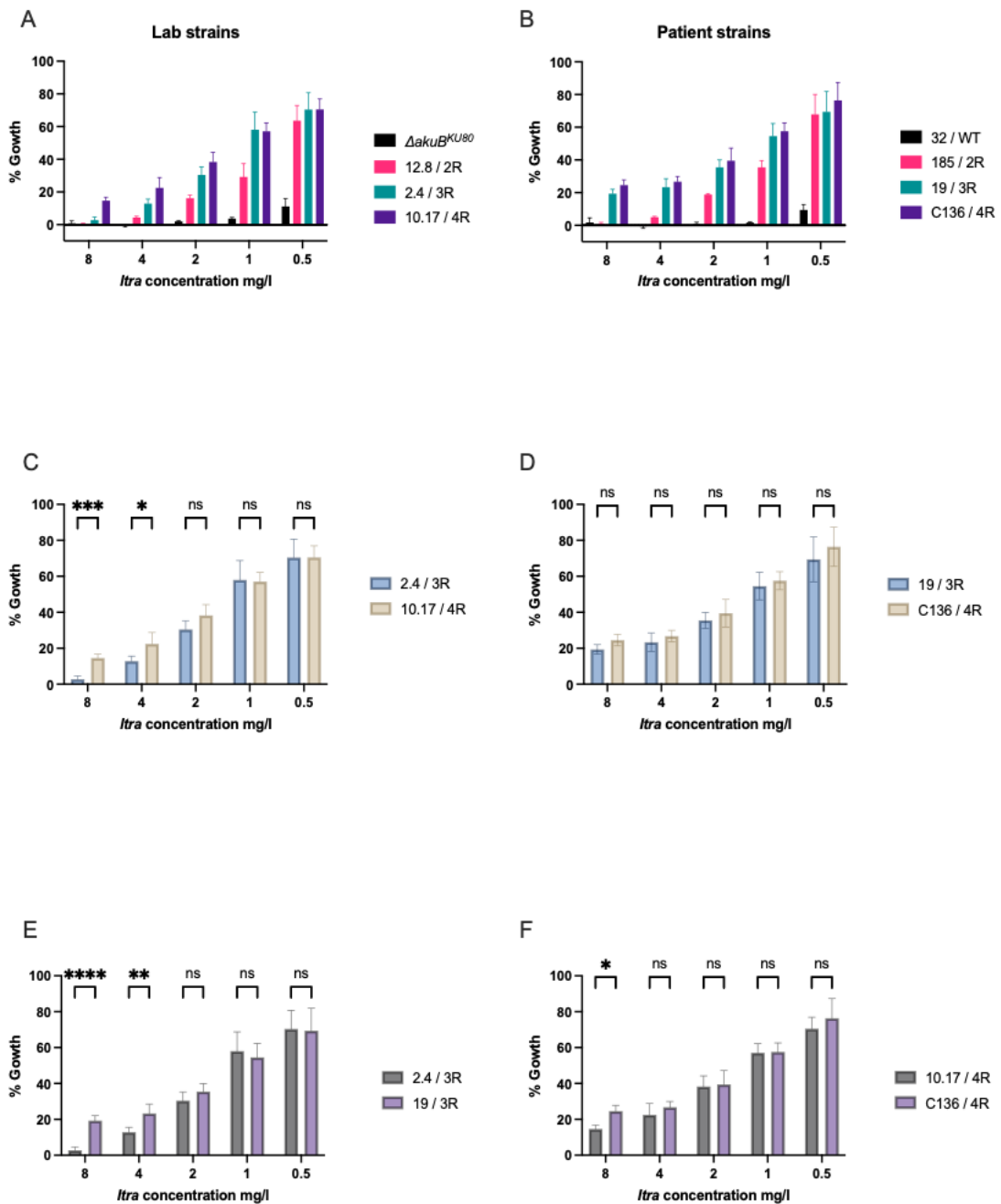


Figure 3-22: Representative data of the growth percentage of *A. fumigatus* strains in the presence of different concentrations of itraconazole (relative to unamended media controls) using a combination of EUCAST method and OD measurements. **(A)** % growth of lab strains. **(B)** % growth of patient strains. **(C)** Comparison between lab strains 4R and the 3R *** $p=0.0009$, * $p=0.0169$. **(D)** Comparison between patient strains 4R and the 3R. **(E)** Comparison between the 3R lab and patient strains **** $p<0.0001$, ** $p=0.0058$. **(F)** Comparison between the 4R lab and patient strains * $=0.0119$. Error bars indicate the mean of the analysed OD reads of 8 replicates. Data of different groups were analysed using two-way ANOVA using GraphPad Prism (Graph Pad v 9.3.1 for Macintosh).

To confirm that the EUCAST growth percentage results were not subject to strain-specific growth behaviour in the RPMI media, a comparison of growth between patient and lab TR₁₃₀ strains, including WT strains, was performed. From the growth curves of all strains in the two different media, there were no notable difference in the growth between RPMI and AMM growth media (**Figure 3.23**). All patient and lab strains seem to behave similarly especially in the RPMI media where all the three phases (lag, exponential and stationery) of the curves looked almost identical, indicating that there was no effect on strains growth as a result of the EUCAST % growth methodology.

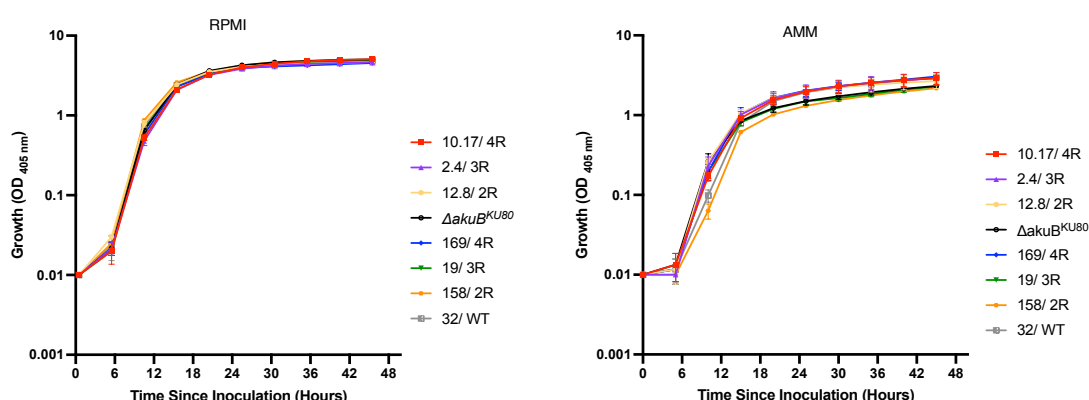


Figure 3-23: Growth of TR₁₃₀ *A. fumigatus* strains in two different media (RPMI and AMM). Error bars indicate the mean of the analysed OD reads of 8 replicates. Curves were generated using GraphPad Prism (Graph Pad v 9.3.1 for Macintosh).

3.3.11 Characterisation of *cyp51A* expression Level using Semi-quantitative End-point PCR and Real-time PCR (qRT-PCR)

3.3.11.1 Comparison between TR₁₃₀, TR₃₄, and TR₄₆

3.3.11.1.1 *End-point PCR*

Expression of the *cyp51A* gene in the azole-resistant clinical strains (C128, C136 and C137; each harbouring a TR₁₃₀ X 4R mutation) was compared to the azole-susceptible WT strain 32 (as a control) initially by semi-quantitative end-point PCR (**Section 3.2.2.13.1**). In addition, expression levels in the azole -resistant isolates C86 and C87, known to harbour the TR₃₄ and TR₄₆ mutations respectively, were also analysed using end-point PCR. The C86 and C87 isolates were chosen for additional

comparison as previous studies had revealed that representative isolates with TR₃₄ and TR₄₆ mutations exhibited an approximately 8-fold increase in *cyp51A* expression, compared to the WT (Gsaller *et al.*, 2016; Mellado *et al.*, 2007). The quality and purity of RNA samples used in this work were checked using gel electrophoresis before being converted to cDNA to be used as a template for PCR (**Figure 3.24**).

The end-point PCR demonstrated far stronger (i.e. intensively staining) PCR product bands were obtained using cDNA of the three clinical isolates harbouring the TR₁₃₀, compared to all of the WT control and TR₃₄ and TR₄₆ isolates, over all of the different 26, 28 and 30 cycles of PCR (**Figure 3.25.A**). The three clinical TR₁₃₀ isolates showed similar levels of expression to each other throughout, although isolate C128 (from a different patient than the other isolates) produced a slightly less strong product than C136 and C137 at the lowest 26 cycles of amplification. Surprisingly, the TR₃₄ strain C86 only showed a very slight increase in band intensity compared to the WT control (e.g. at 28 cycles). By contrast, the TR₄₆ strain C87 showed production of brighter bands at 26 and 28 cycles as compared to the WT and TR₃₄ strains (**Figure 3.25.A**). Meanwhile, the housekeeping β -tubulin gene showed similar brightness of PCR bands for all isolates across all cycle lengths, thereby signifying that the expression level of those reference genes was consistent across all isolates (**Figure 3.25.A**).

3.3.11.1.2 *qRT-PCR*

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR; **Section 3.2.2.12.2**) was also used to evaluate expression of *cyp51A* in the various azole resistant and sensitive strains. Primary results were normalized to the level of transcription of the reference *gpdA* or β -tubulin genes for each strain included in the assay, which show all most similar induction of result. Therefore, data on this thesis will rely on data normalised against β -tubulin gene only for consistency. Results showed a ca.14 to 20-fold increase in *cyp51A* expression in strains harbouring TR₁₃₀ compared to the WT strain (**Figure 3.25.B**), indicating that this change in expression may be the reason for the occurrence of azole resistance in the three isolates C128, C136, and C137. Although the *cyp51A* expression level of C128 was slightly lower than the other TR₁₃₀ isolates (C136 and C137 isolated from a different patient) all three isolates showed similar high levels of *cyp51A* expression with no significant differences. High levels of gene expression were also seen in the TR₄₆ strain, with no significant differences present

when compared to the TR₁₃₀ strains (**Figure 3.25.B**). By contrast, *cyp51A* expression in isolates harbouring the TR₁₃₀ was significantly higher compared to both the WT and the TR₃₄ strains (one-way ANOVA; $P < 0.001$). Surprisingly, it was not possible to show the previously described 2-8-fold increase in *cyp51A* expression in the TR₃₄ strain compared to the WT due to unknown reasons. This was even despite testing gene expression in 7 additional strains harbouring the TR₃₄ mutation, which all showed an almost consistent result.

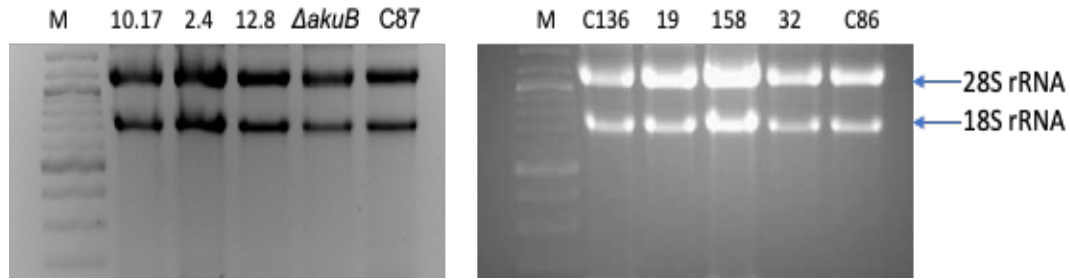


Figure 3-24: Visualization of extracted total RNA from *A. fumigatus* strains used in expression analysis, samples (indicated at top of lanes) run on 1.5% (w/v) agarose gel along with 100 bp marker. RNA integrity was indicated by the presence of intact sharp 18S and 28S bands.

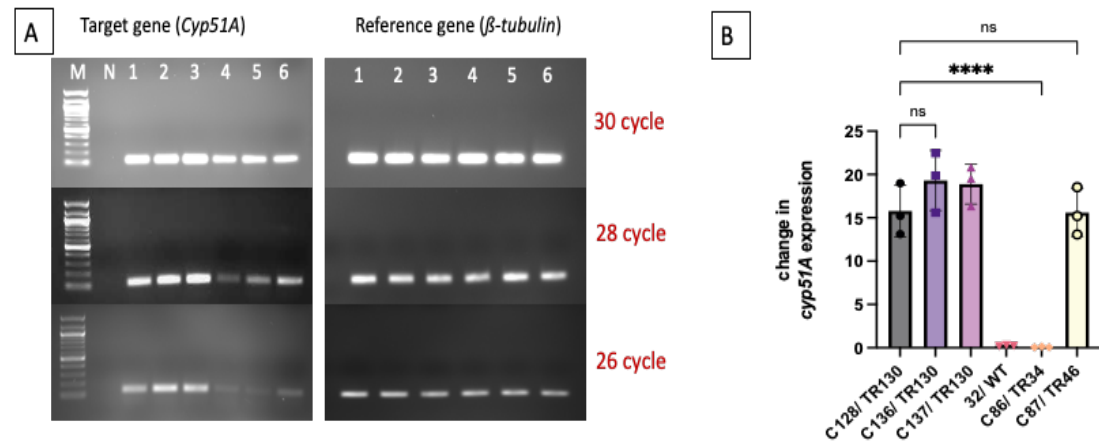


Figure 3-25: Expression analysis of *cyp51A* compared to a reference gene (β -tubulin). **(A)** Gel electrophoresis of *cyp51A* and β -tubulin PCR products using cDNA from the three TR₁₃₀ isolates, C128, C136 and C137 (lanes 1, 2 and 3), control WT isolates 32 (lane 4), control isolate C86 harbouring TR₃₄/L98H mutation (lane 5), and control isolate C87 harbouring TR₄₆ mutation (lane 6). Products were run alongside 100bp DNA ladder (M) in 1% agarose gel. **(B)** Representative results of qPCR showing around 18 to 20-fold change in gene expression of *cyp51A* in the three isolates harbouring TR₁₃₀ (C128, C136 and C137) and in isolate C87 harbouring TR₄₆, compared to WT (32) and TR₃₄ isolate C86. This result was normalized to the level of transcription of the β -tubulin housekeeping gene. Error bars indicate the mean of the three biological replicates. Data of different groups were analysed using ordinary one-way ANOVA. (**** $p < 0.001$) (ns. = not significant).

3.3.11.2 Change in *cyp51A* Expression in the Presence of Sub-lethal Concentration of Itraconazole

Expression *cyp51A* was next examined by qRT-PCR after extracting RNA from *A. fumigatus* strains (TR₁₃₀, TR₃₄, and WT) that were grown in ACM supplemented with a sub-lethal level of 25 µg/ml itraconazole. The *cyp51A* expression level in the TR₁₃₀ strain was markedly higher (one-way ANOVA; $p < 0.0001$) in the presence of itraconazole, with around a 10-fold change compared to the expression of the same strain without exposure to itraconazole (**Figure 3.26**). This indicated that an increase in *cyp51A* expression had occurred as a result of itraconazole challenge. By contrast, the C86 strain harbouring TR₃₄ showed only a slight 2-4-fold increase in *cyp51A* expression between the different conditions with/without itraconazole and this change was not significant. No change in expression was seen in the WT control (**Figure 3.26**).

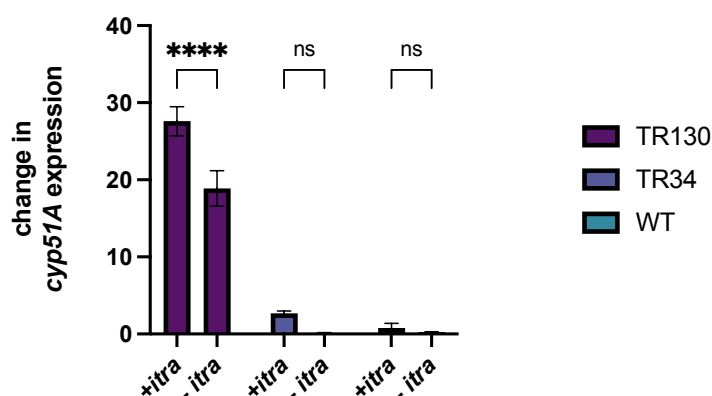


Figure 3-26: Representative results of qPCR showing the difference in *cyp51A* expression using extracted RNA from culture that were exposed to sub-lethal itraconazole concentration. Result showed around 10-fold change in expression of *cyp51A* in isolate harbouring TR₁₃₀ after exposed to the itraconazole challenge (**** $p < 0.0001$). The change in *cyp51A* expression level in isolate harbouring TR₃₄ and WT isolate was similar in the different conditions with/without itraconazole. This result was normalized to the level of transcription of the β -tubulin housekeeping gene. Error bars indicate the mean of the three biological replicates. Data of different groups were analysed using ordinary two-way ANOVA.

3.3.11.3 Comparison between Different Forms of the TR₁₃₀ (4R, 3R, 2R) using End-point PCR and qRT-PCR

3.3.11.3.1 *End-point PCR*

End-point PCR was used to assess *cyp51A* transcript levels present in the different TR₁₃₀ lab strains produced by genetic manipulation (containing between two to four repeats of the TR₁₃₀ sequence) versus the parental control and a patient-derived TR₁₃₀ X 4R strain. RNA of all TR₁₃₀ GM strains along with the transformation parental strain *ΔakuB*^{KU80} was extracted followed by cDNA synthesis and end-point PCR (**Sections 3.2.2.11.2 and 3.2.2.12.1; Table 3.7**). Under all cycle conditions (26, 28, 30) there was a consistent pattern with highest expression in the isolates containing the TR₁₃₀ X 4R and then with a steady decrease in band brightness according to decrease in number of the TR₁₃₀ repeats (**Figure 3.27.A**). Lowest expression was seen in the TR₁₃₀ X 2R strain, but this still showed slightly elevated expression compared to the *ΔakuB*^{KU80} WT control. All isolates showed similar brightness of the bands for the β-tubulin housekeeping gene control, thereby indicating the expression stability of these genes across all isolates (**Figure 3.27.A**).

3.3.11.3.2 *qRT-PCR:*

Expression levels of *cyp51A* in the different TR₁₃₀ lab GM strains (containing between two to four repeats of the TR₁₃₀ sequence) versus the parental control and a patient-derived TR₁₃₀ X 4R strain was also quantified by qRT-PCR. Results showed a clear relationship between number of TR₁₃₀ repeats and levels of *cyp51A* expression (**Figure 3.27.B**). The lab 10.17 X 4R and 2.4 X 3R strains showed 10-15-fold and 7-10-fold, respectively, increased expression relative to the *ΔakuB*^{KU80} parental control strain although there were no statistically significant differences between the 4R and 3R expression levels (one-way ANOVA; $p < 0.0807$). However, there were significant differences in the level of *cyp51A* expression between the lab 4R and the lab 2R strain and the *ΔakuB*^{KU80} control (one way ANOVA; $p < p < 0.0006$ and < 0.0001 , respectively) (**Figure 3.27.B**). Differences in expression between the clincial TR₁₃₀ X 4R and the GM TR₁₃₀ X 4R strain were also observed, which led to subsequent studies presented in the following **Section 3.3.11.4**.

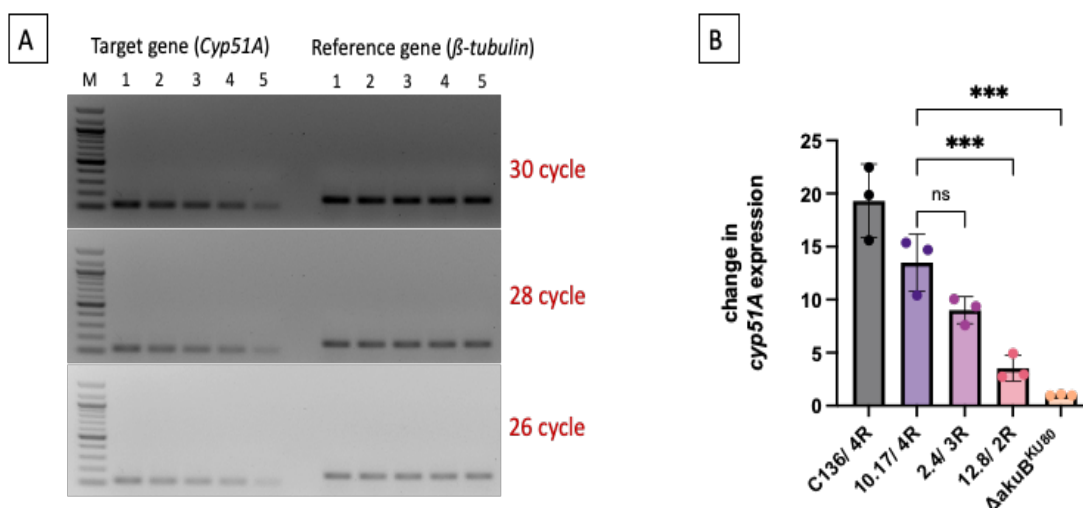


Figure 3-27: Expression analysis of *cyp51A* compared to a reference gene (β -tubulin). (A) Gel electrophoresis of *cyp51A* and β -tubulin PCR products using cDNA from patient isolate C136 harbouring TR₁₃₀ X 4R (lane 1), Lab GM strain 10.17 harbouring TR₁₃₀ X 4R (lane 2), lab GM strain 2.4 harbouring TR₁₃₀ X 3R (lane 3), lab GM strain 12.8 harbouring TR₁₃₀ X 2R (lane 4), and control WT strain Δ akuB^{KU80} (lane 5). Products were run alongside a 100 bp DNA ladder (M) in 1% agarose gel (B) Representative results of qPCR showing changes in *cyp51A* gene expression of *cyp51A* according to number of TR₁₃₀ repeats strains c136/4R, 10.17/4R, 2.4/3R, and 12.8/2R. Data was normalized to the level of transcription of the β -tubulin housekeeping gene. Error bars indicate the mean of the three biological replicates. Data of different groups were analysed using ordinary one-way ANOVA. (***) $p=0.001$ (ns. = not significant)

3.3.11.4 Comparison of *cyp51A* Expression between Patient TR₁₃₀ Strains and Lab TR₁₃₀ Strains:

Ongoing results suggested that there was a difference in *cyp51A* expression between the C136 patient-derived strain and the 10.17 GM-derived strain despite both strains harbouring the same 4R of the TR₁₃₀. Therefore, qRT-PCR was used to investigate possible differences in *cyp51A* expression between patient- and GM-derived strains with different TR₁₃₀ repeat numbers.

Results revealed that for both the X 4R and 3R forms of TR₁₃₀ that the patient-derived strains showed higher expression of *cyp51A* than the GM-derived strains with the same number of repeats (**Figure 3.28**). For example, *cyp51A* expression was 14-20-fold higher in the patient TR₁₃₀ X 4R isolate compared to the WT control, whilst was 10-15-fold higher in the GM TR₁₃₀ X 4R strain, with a statistically significant difference in *cyp51A* expression level between the two strains (two- way ANOVA; $p=0.0051$). A similar result was observed with the 3R patient and GM strains (two- way ANOVA; p

=0.0058). However, there were no significant differences between the 2R patient and GM strains (**Figure 3.28**). Of particular interest was the observation that the patient-derived 3R X TR₁₃₀ showed similar levels of *cyp51A* expression as the 4R X TR₁₃₀ GM strain despite the lower TR copy number. This indicated that the patient-derived strains might have an additional mechanism(s) in addition to the tandem repeats that might lead to an increase in *cyp51A* expression.

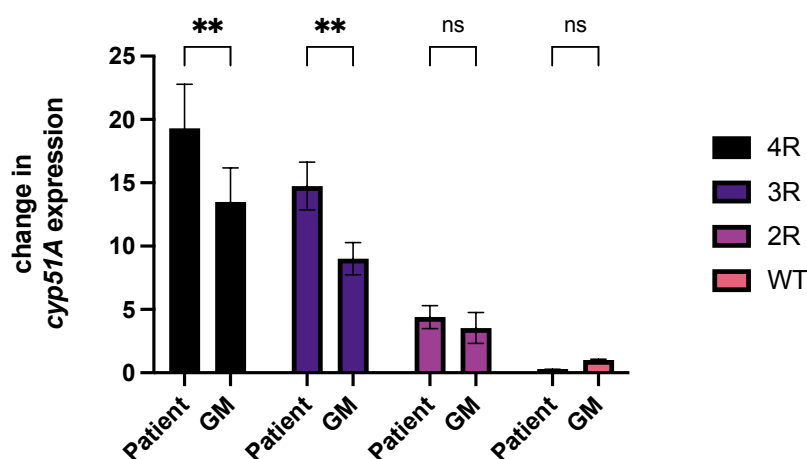


Figure 3-28: Representative results of qPCR showing differences in the levels of *cyp51A* expression between lab and patient strains harbouring different numbers of TR₁₃₀ repeats relative to the WT control strains. Data was normalized to the level of transcription of the β -tubulin housekeeping gene. ** indicates significant differences in expression levels. Error bars indicate the mean of the three biological replicates. Data of different groups were analysed using ordinary two-way ANOVA. (**p=0.0051) for the 4R and (**p=0.0058) for the 3R, (ns. = not significant)

3.3.12 Deletion of ABC transporter *cdr1B* gene from TR₁₃₀ strains

3.3.12.1 Fragment Amplification

To generate the *cdr1B* knockout cassette, the up/down-stream *cdr1B* gene flanking regions and the *hph* fragment were amplified in preparation for fusion PCR. Genomic DNA from three WT *A. fumigatus* strains (47-51,47-55, 47-267 from Nottingham collections (Swilaiman *et al.*, 2020) were used to amplify the up/down-stream fragments to ensure that there was no issue with the PCR templates and primers. In all cases single bands were obtained for the up/down-stream fragments with the expected size of around 1kb for both fragments (**Figure 3.29**). The *hph* fragment was amplified from an existing plasmid (**Section 3.3.6**) using two pairs of primers to ensure sufficient

amplification from the plasmid. Single PCR products with the expected size around 2.8kb were observed using the two different set of primers (**Figure 3.29**).

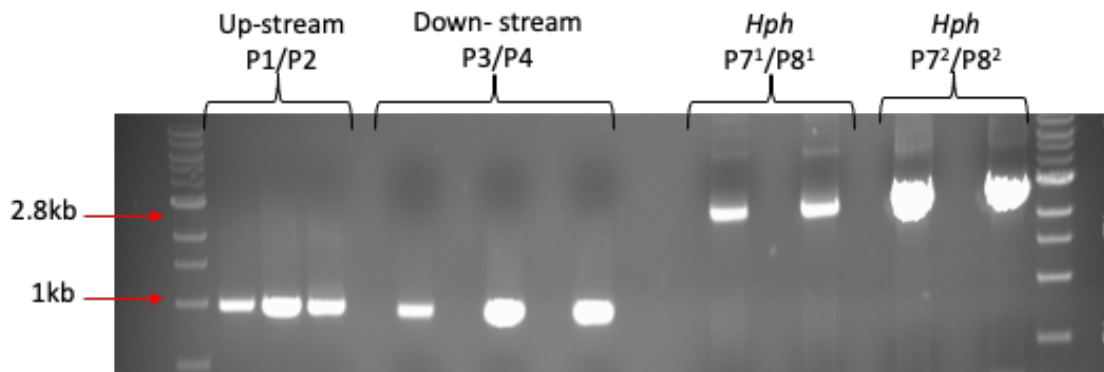


Figure 3-29: Gel electrophoresis of the PCR product to be used for generating the *cdr1B* deletion cassette. PCR products with the expected size of 1 kb was observed for the up/down stream fragment using primer pairs P1/P2 for the up- stream region of *cdr1B* and P3/P4 for the down- stream region of *cdr1B*. *hph* fragments were amplified using two pairs of primers P7¹/P8¹ and P7²/P8² and PCR products with expected size 2.8 kb was achieved. Products were run alongside 1kb DNA ladder (lane 1) in 1% agarose gel. All upstream and downstream fragments were amplified from gDNA of WT *A. fumigatus* strains (47-51,47-55, 47-267, respectively. from Nottingham collections by (Swilaiman *et al.*, 2020). *Hph* fragments were all amplified using plasmid used in this study and full sequence available at (Resendiz-Sharpe *et al.*, 2022).

PCR products were purified and used for the subsequent fusion PCR. The three fragments were fused together using nested primers (two pairs), where P5¹/P6¹ primer pairs generated fragment with around 5 kb in size and P5²/P6² primer pairs generated fragment with 4.7 kb in size. PCR products were then loaded in 1% TEA agarose gel and visualised using UV light, whereby a single product with the expected size of the whole cassette, including the two flanking sequences of *cdr1B* and the *hph* fragment, was observed (**Figure 3.30**). Bands were excised, purified, and finally used for transformation.

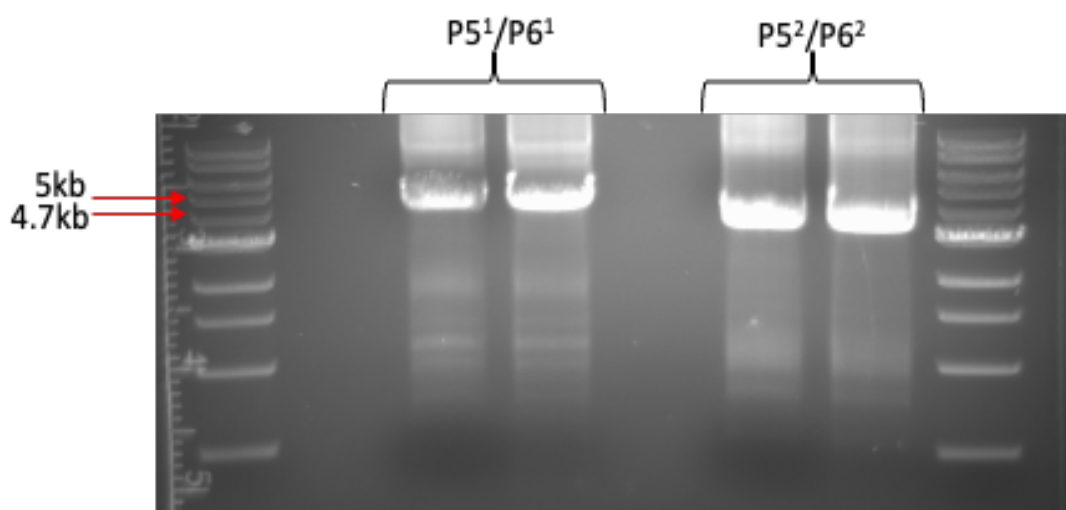


Figure 3-30: Gel electrophoresis of fusion PCR products of the two flanking sequences of *cdr1B* and the *hph* fragment using two pair of primers. P5¹/P6¹ product size around 5kb and P5²/P6² product size around 4.7kb. Products were run alongside 1kb DNA ladder in 1% agarose gel.

3.3.12.2 Selecting Positive Transformants

Attempts were made to delete the *cdr1* gene in two different *A. fumigatus* strains carrying the TR₁₃₀ x 4R resistance genotype, namely the 10.17 GM lab strain and the C136 patient-derived strain. A series of putative positive transformants was obtained from both strains, albeit higher numbers were obtained from transformation of the 10.17 (data not shown). Twelve colonies in total (eight transformants from 10.17 and four from C136) were screened by PCR for the presence of either the *cdr1B* upstream + *hph* fragments or *cdr1B* downstream + *hph* fragments. All transformants of the GM lab strain (10.17) amplified the predicted PCR bands for correct integration of the *cdr1* deletion cassette, although band intensity varied and transformant Number 4 showed multiple bands using either P4/P7 (top gel) or P1/P8 primers (bottom gel) (**Figure 3.31**). By contrast, only two of the four transformants (namely 3 and 4) from the patient strain (C136) showed the predicted PCR bands for correct integration of the *cdr1* deletion cassette with multiple bands or missing bands evident in the other two transformants (2 and 1, respectively) (**Figure 3.31**).

From these screenings, two Δ *cdr1B* transformants were chosen from each parental strain on the basis of production of the correct single bands using different sets of screening primers. These were transformants number 2 and 3 of 10.17/4R, here termed

10.17/4R- *hph.Δcdr1B* (genotype $\Delta cdr1B.hph$) and transformants 3 and 4 of C136, here termed C136-*hph.Δcdr1B* (genotype $\Delta cdr1B.hph$).

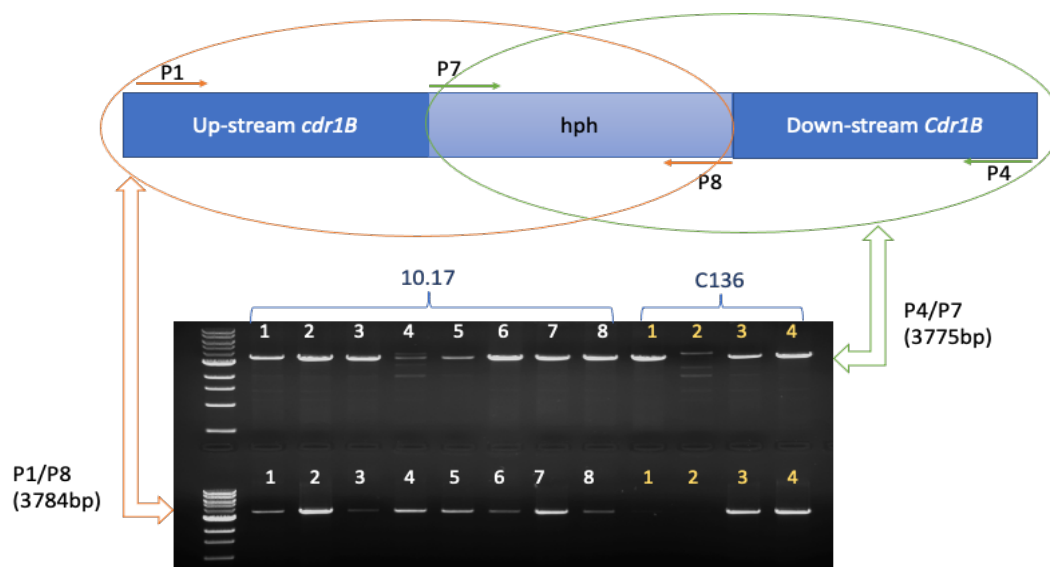


Figure 3-31: Screening for positive transformants of the deletion of *cdr1B* and replacement with *hph* cassette using two set of primers. P1/P8 primers amplified upstream of *cdr1B* + *hph* generating a predicted fragment of 3,784 bp in size. P4/P7 primers amplified downstream of *cdr1B* + *hph* generating a predicted fragment of 3,775 bp in size. All transformants showed a product of the expected size except transformant number 4, the parental strain 10.17, and transformant numbers 1 and 2 of the C136 parental strain.

3.3.12.3 Antifungal Susceptibility Test

E-test strips were used to test the MIC of the four *hph:Δcdr1B* mutants (**Figure 3.32**). Significantly, results showed at least a 2-fold decrease in the MIC values of both the 10.17 GM and C136 patient-derived $\Delta cdr1B$ transformants relative to the parental strains despite the presence of the TR130 4R repeat sequence. The largest decrease in MIC value was seen for the two selected transformants 2 and 3 of 10.17/4R- *hph.Δcdr1B* which showed a 4-6-fold reduction in the MIC value (a change from 24 $\mu\text{g/ml}$ to 2-4 $\mu\text{g/ml}$). By contrast, the two selected transformants 3 and 4 of C136- *hph.Δcdr1B* showed a lower 2-fold reduction in the MIC value (change from 32 $\mu\text{g/ml}$ to 16 $\mu\text{g/ml}$). In both cases though, the MIC values remained higher than the itraconazole 2 $\mu\text{g/ml}$ clinical breakpoint for resistance. The difference in extent of decrease in MIC values between the $\Delta cdr1B$ strains of 10.17 and C136 again suggested that the patient C136 strains might have additional mechanism(s) linked to the resistant phenotype.

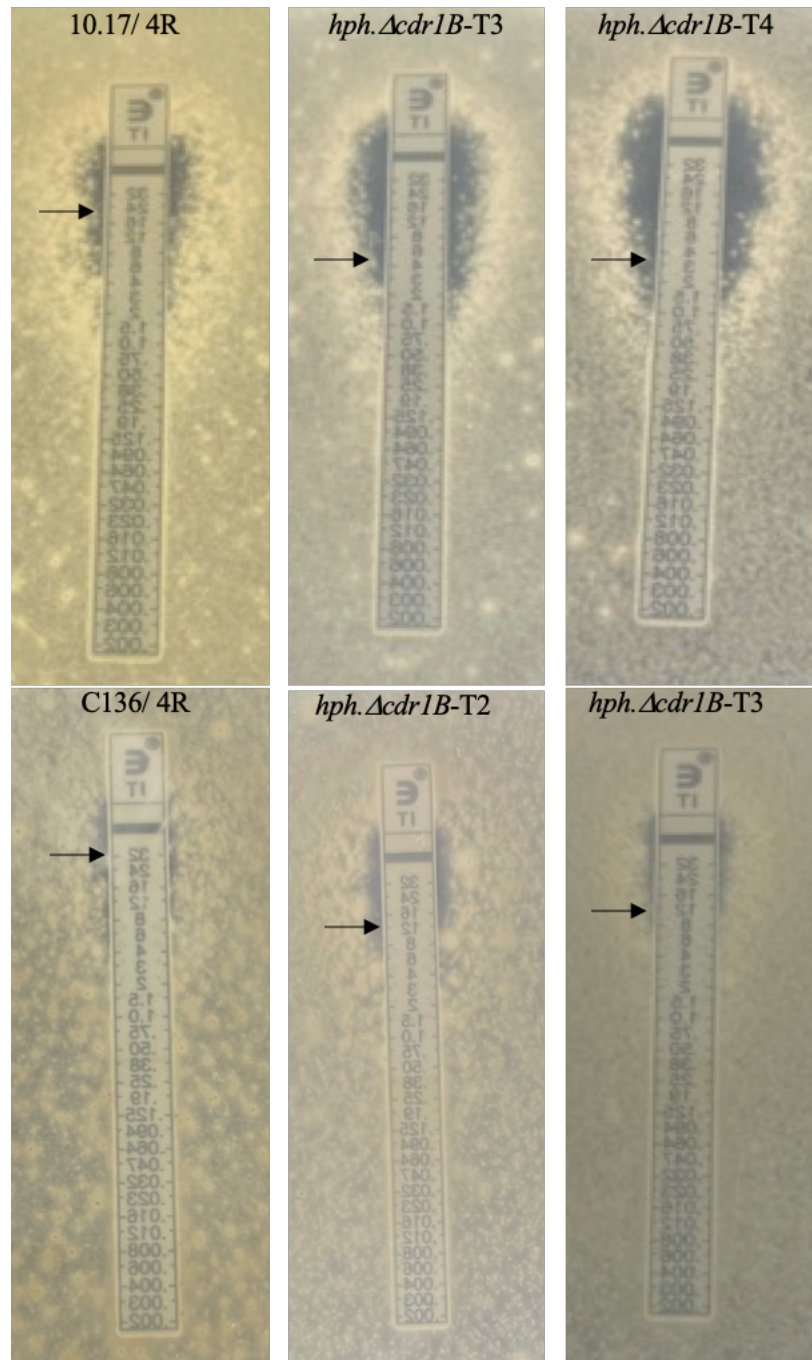


Figure 3-32: Antifungal susceptibility testing using the E-test method. Arrows indicate the MIC values of parental strains and *cdr1B* deletion transformants. MICs were recorded after 48 hours of incubation at 37 °C.

3.3.13 Virulence of TR₁₃₀ *A. fumigatus* and Itraconazole Efficiency in an In-vivo Model of Infection (*Galleria mellonella*)

An established *in vivo* model of infection, *Galleria mellonella*, was used to investigate whether there were any differences in pathogenicity between the lab GM and patient-derived clinical strains harbouring different forms of TR₁₃₀ (2R, 3R, and 4R) and two azole- susceptible *A. fumigatus* control strains (the $\DeltaakuB^{KU80}$ lab strain and WT 32 patient-derived strain). Tests were also conducted to determine whether the presence of strains harbouring different forms of TR₁₃₀ impacted on the efficacy of azole antifungal treatment.

3.3.13.1 Strain Virulence

The survival analysis graphs revealed a slight difference in killing rate, especially between WT *cyp51A* strains and those with TR₁₃₀ X 3R and TR₁₃₀ X 4R forms. Larvae infected with *cyp51A* TR₁₃₀ 3R and 4R strains (both GM and patient-derived) showed 100% mortality after the 4th day of infection as compared to those infected with WT strains that showed 100% mortality only after the 6th or 7th day of infection. By contrast, there were no noticeable differences in the mortality rate between larvae infected with WT and TR₁₃₀ X 2R strains (either GM or patient-derived). However, statistical analysis of the data was unable to detect any significant differences in virulence among all tested strains (long-rank (Mantel-Cox) test; $P=0.0674$ and $P=0.02270$ for patient and GM respectively) (Figures 3.33.A and 3.34.A).

3.3.13.2 Itraconazole Response

Larvae infected with Azole-susceptible *A. fumigatus* WT *cyp51A* strains and the Azole-resistant TR₁₃₀ *cyp51A* were tested for treatment efficiency using the recommended therapeutic dose of itraconazole (10mg/kg of body weight).

3.3.13.2.1 *Lab GM Strains*

Results showed that larvae infected with the azole-susceptible *A. fumigatus* parental strain $\DeltaakuB^{KU80}$ had significantly improved survival rates following treatment with itraconazole (as compared to the untreated larvae infected with the same strain) (long-

rank (Mantel-Cox) test; $P = 0.0095$), with an increased 20% larval survival rate after the 7th day of incubation (**Figure 3.33.B**). By contrast, there was no significant improvement in survival rates of larvae infected with the azole-resistant *cyp51A* 4R (10.17), 3R (2.4) and x 2R (12.8) forms following treatment with itraconazole, although a slight survival improvement and delay in killing was evident.

Survival rates of azole treatment groups of the azole-resistant *cyp51A* TR₁₃₀ strains were compared to those of the WT*cyp51A* ($\DeltaakuB^{KU80}$) treatment group in more detail. Results revealed a remarkable decrease in survival of larvae infected with the 12.8, 2.4, and 10.17 GM strains as compared to the $\DeltaakuB^{KU80}$ parental control (long-rank (Mantel-Cox) test; $P = 0.0288$, $P = 0.0020$, and $P = 0.0002$, respectively) in the presence of itraconazole (**Figures 3.33.B, C, D**). Interestingly, there was a difference in the extent of the drug response of the groups, with a link evident between the number of TR₁₃₀ tandem repeats present and the level of resistance of each strain. Although a significant decrease in survival rates was observed for all the GM strains when the drug response of strains was compared to the WT strain, the extent of this difference was not the same for all groups. TR₁₃₀ X 4R showed the highest significance value, followed by the X3R form and the X2R form with them lowest significance value (**Figures 3.33.B, C, D**). Thus, results overall demonstrated a significantly reduced effectiveness of itraconazole treatment in the presence of azole-resistant strains harbouring the TR₁₃₀ mutation compared to the presence of the azole-susceptible *A. fumigatus* $\DeltaakuB^{KU80}$ strain.

3.3.13.2.2 Patient Strains

Similarly, the efficacy of itraconazole treatment was tested in more detail for all groups of larvae infected either with the *A. fumigatus* WT *cyp51A* susceptible patient strains or with TR₁₃₀ *cyp51A* resistant patient-derived strains. Unfortunately, all groups, including the one infected with the WT strain, showed no significant improvement in survival when treated with itraconazole (**Figures 3.34.B, C, D**). It is noted that some infected groups did show a slight improvement and a delay in the killing rate following azole treatment but results were not significant. For example, the group infected with the WT strain 32 showed some improvement with a 25% larval survival rate after the 7th day of incubation compared to the untreated group that showed 100% mortality on the same day, but this difference was not statistically significant (**Figure 3.34.B**). Therefore results indicated that the 10mg/kg itraconazole dose used was not sufficient

to treat infection with the patient-derived strains, even the 32 WT strain lacking any tandem repeat (**Figures 3.34.B, C, D**). This differed from results of azole treatment of the *cyp51A* WT $\DeltaakuB^{KU80}$ isolate used above where the same azole treatment did demonstrate efficiency, and again illustrated possible differences in the genetic backgrounds of the 32 WT and the *cyp51A* WT $\DeltaakuB^{KU80}$ strains.

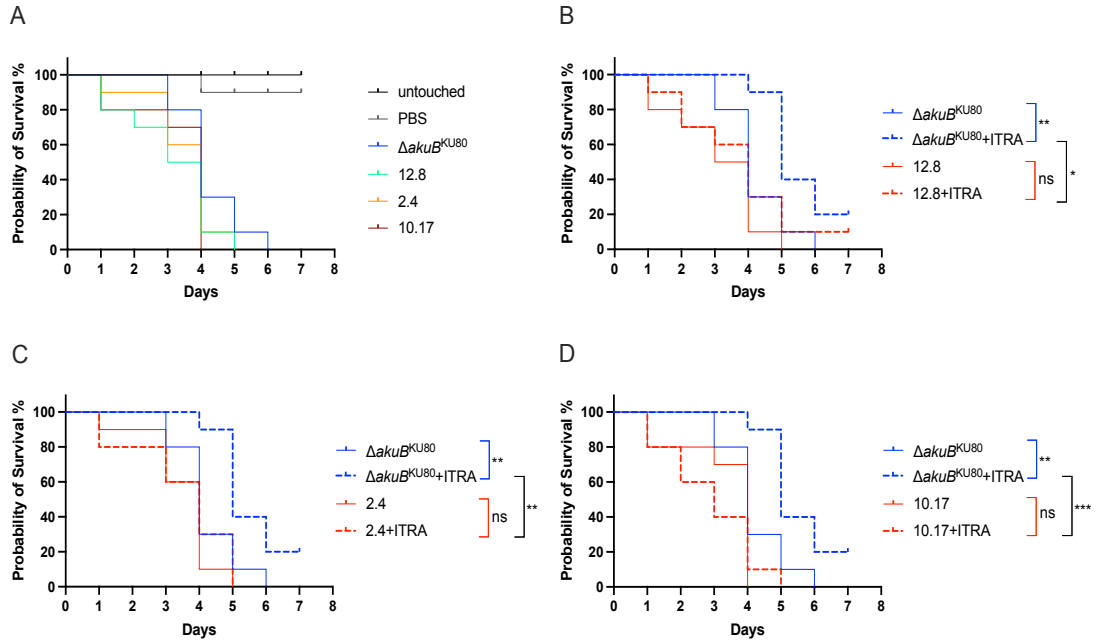


Figure 3-33: Comparison of killing rates and itraconazole (ITRA) responses of *G. mellonella* larvae infected with *A. fumigatus* GM “lab strains” (10.17 4R; 2.4 3R; 12.8 2R) harbouring different forms of the TR₁₃₀ mutation in the promoter *cyp51A* gene as compared to control parental strain ($\DeltaakuB^{KU80}$) or PBS or untouched controls. Kaplan-Meier curves represent the average survival expressed as a %. (A) Survival rates of *G. mellonella* infected with mutant lab strains in comparison with WT $\DeltaakuB^{KU80}$ strain. (B) Survival rates of *G. mellonella* larvae infected with the *cyp51A* WT ($\DeltaakuB^{KU80}$) and GM strain 12.8 with *cyp51A*:TR₁₃₀ X 2R) with or without ITRA treatment. (C) Survival rates of *G. mellonella* larvae infected with the *cyp51A* WT ($\DeltaakuB^{KU80}$) and the GM strain 2.4 with *cyp51A*:TR₁₃₀ X 3R with or without ITRA treatment. (D) Survival rates of *G. mellonella* larvae infected with the *cyp51A* WT ($\DeltaakuB^{KU80}$) and the GM strain 10.17 with *cyp51A*:TR₁₃₀ X 4R with or without ITRA treatment. Statistical analysis was performed using survival curve comparison using long-rank (Mantel-Cox) test (ns =non-significant; **P = 0.0095; *P = 0.0288; ***P = 0.0020; ****P = 0.0002).

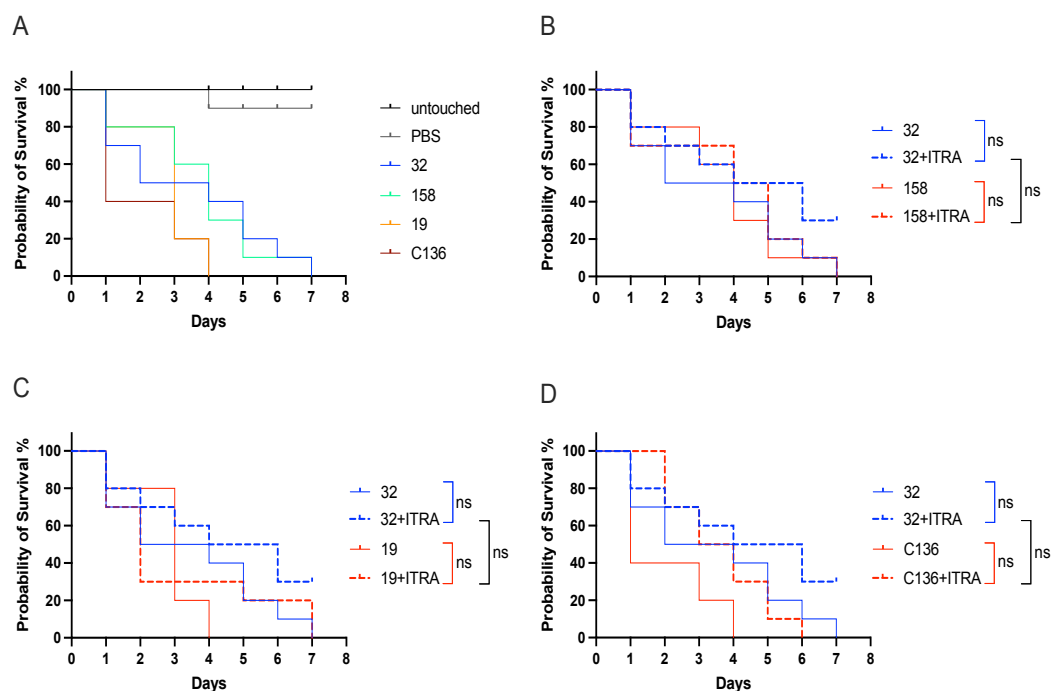


Figure 3-34: Comparison of killing rates and itraconazole (ITRA) responses of *G. mellonella* larvae infected with *A. fumigatus* clinical patient-derived strains harbouring different forms of the TR₁₃₀ mutation in the promoter *cyp51A* gene (158, 19, C136) as compared to control WT strain (32) and PBS or untouched controls. Kaplan-Meier curves represent the average survival expressed as a%. (A) Survival rates of *G. mellonella* infected with clinical mutant strains in comparison with the WT (32) strain. (B) Survival rates of *G. mellonella* larvae infected with the *cyp51A* WT (32 strain) and clinical strain 158 with *cyp51A*:TR₁₃₀ X 2R, with or without ITRA treatment. (C) Survival rates of *G. mellonella* larvae infected with the *cyp51A* WT (32 strain) and clinical strain 19 with *cyp51A*:TR₁₃₀ X 3R, with or without ITRA treatment. (D) Survival rates of *G. mellonella* larvae infected with the *cyp51A* WT (32) and the clinical strain C136 with *cyp51A*: TR₁₃₀ X 4R, with or without ITRA treatment. Statistical analysis was performed using survival curve comparison using long-rank (Mantel-Cox) test (ns =nonsignificant).

3.3.14 Phylogenetic Analysis

Phylogenetic analysis showed that all the TR₁₃₀ isolates coming from both patients, including the different forms of the TR₁₃₀ with different numbers of copies (158/2R; 19/3R; and C128, C136, C137, 80, 82 and 169/4R) were clustered together on one branch (**Figure 3.35**). This indicates an extremely close relationship between all those strains. By contrast, isolate 32 (from patient D48387) that had been identified as being of the itraconazole sensitive WT *cyp51A* genotype was separated from the other isolates. This strongly indicated that it could be considered as a separate infection by *A. fumigatus* of the same patient since it was isolated a month later from isolate 19 harbouring 3R of the TR₁₃₀.

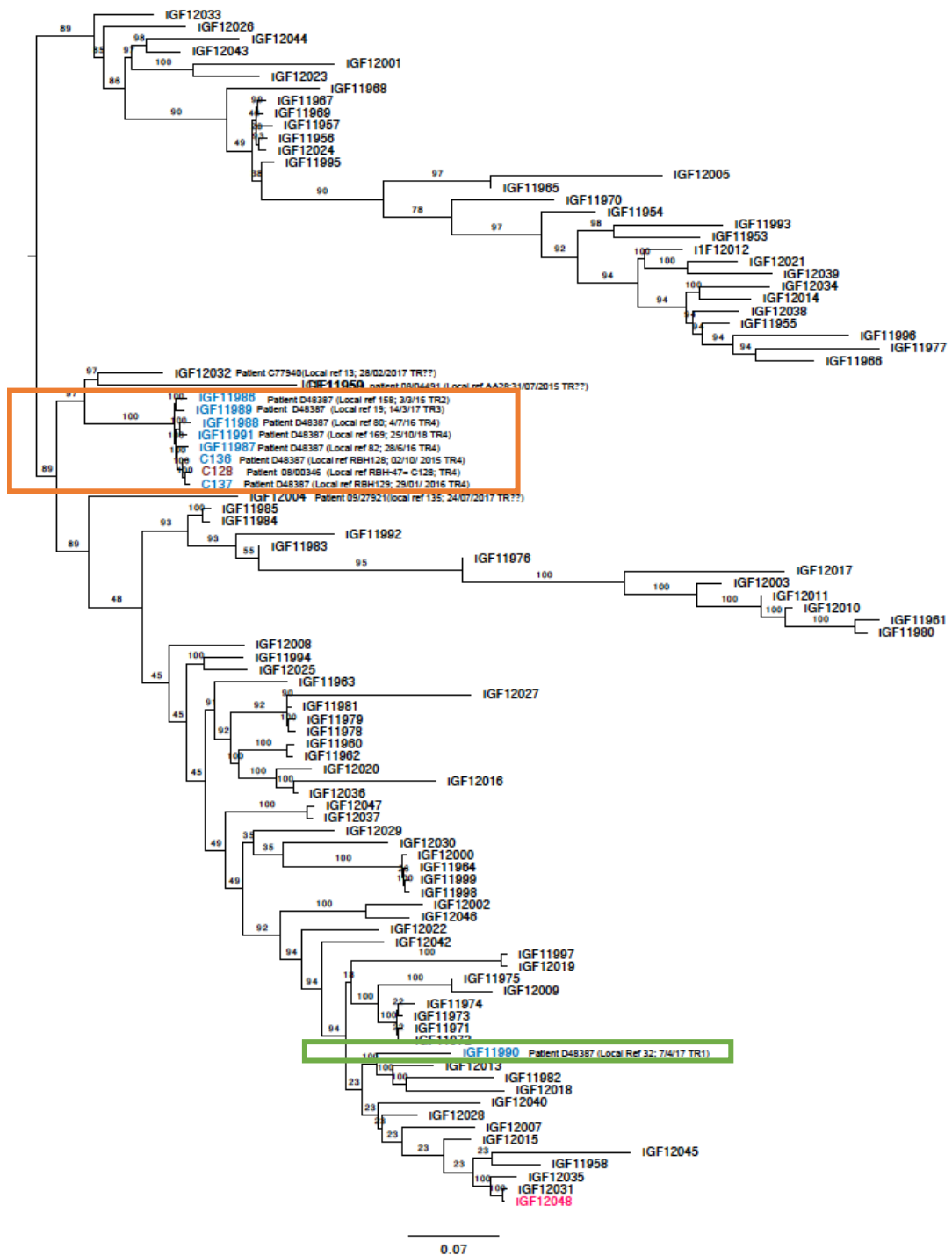


Figure 3-35: Phylogenetic analysis showing the genetic relationship between isolates obtained from two different patients between 2015-2018 relative to a series of other clinical and environmental isolates of *A. fumigatus* for which genome sequence data was available. All eight isolates with TR₁₃₀ mutations clustered together (labelled with orange box). One WT isolate from one of the patients was separated from the rest of the isolates (labelled with green box).

3.4 Discussion

The appearance of what we believe to be a novel tandem repeat mutation in the promoter region of *cyp51A* (TR₁₃₀) has not been previously reported in azole-resistant isolates of *A. fumigatus*. Six clinical isolates (C128, C136, C137, 80, 82, 169; each with supposed azole resistance) isolated from two different patients (D48387 and 0800346) from a London clinic during 2015-2018 were found to produce a larger fragment size (1,045 bp) in PCR amplifications compared to the WT isolate, indicating that they had an additional 390 bp of DNA in this region compared to the expected size (655 bp) in the promoter of *cyp51A* using the pair of primers designed to screen the promoter of *cyp51A* in this study.

Sequence analysis of these isolates showed that they all harbour four copies of a tandem repeated sequence of 130 bp in the promoter of *cyp51A*. In addition, two further isolates of interest were obtained from patients D48387. Isolate 19 contained three copies of the same 130 bp sequence and isolate 158 was found to harbour two copies of the same sequence. All eight isolates exhibiting this novel TR₁₃₀ sequence showed an otherwise identical gene sequence to the azole sensitive reference A1163 *A. fumigatus* genome sequence, although, several point mutations were evident in the *cyp51A* open reading frame for certain isolates at various codons (46, 127, 248, 255, 427) relative to the azole sensitive reference isolate Af293. However, it has been reported that these SNPs are not associated with the azole resistance, and they have been found in other susceptible strains i.e. these SNPs appear to be neutral mutations that occur due to genetic variation within the sensitive population. Although these SNPs might contribute to slightly elevated MIC values, they do not normally reach the azole resistance clinical breakpoint (Rivero-Menendez *et al.*, 2016; Chowdhary *et al.*, 2017).

The sequence analyses therefore suggested that the presence of TR₁₃₀ alone might be the cause of azole resistance in these isolates and might act via an impact on the azole target gene expression as previously described (Gsaller *et al.*, 2016). This was consistent with previous evidence that alterations in *cyp51A* and its promoter region have been reported as a leading cause of azole resistance in *A. fumigatus*, in both clinical and environmental isolates (Chowdhary *et al.*, 2017). Previously reported changes include point mutations in the coding region of *cyp51A* in combination with a tandem repeat in

its promoter, such as TR₃₄/L98H (Mellado *et al.*, 2007) and TR₄₆/Y121F/T289A (Van Der Linden *et al.*, 2013; Zhang *et al.*, 2017), or just a tandem repeat without other substitution, such as TR₅₃ (Hodiamont *et al.*, 2009) and TR₁₂₀ (Hare *et al.*, 2017). Alterations in *cyp51A* and its promoter region have also been linked more widely with resistance to azole antifungals in other fungal animal and plant pathogens (Favre *et al.*, 1999; Marichal *et al.*, 1999; Stergiopoulos *et al.*, 2003; Ma *et al.*, 2006).

Various pieces of evidence were then provided from experimental work in this chapter to confirm that the presence of increased numbers of tandem repeats of the 130 bp sequence was indeed a causal mechanism for azole resistance in these isolates as will now be discussed.

3.4.1 Level of azole resistance is linked to the number of copies of the TR₁₃₀ mutation

The Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) have developed standard methods for testing antifungal susceptibility, facilitating the detection of the MIC breakpoints for azole resistance in *A. fumigatus* (Rivero-Menendez *et al.*, 2016). Based on EUCAST and CLSI data, an MIC breakpoint value for ITC of >2 µg/ml has been proposed for *A. fumigatus* resistant isolates and <2 µg/ml for *A. fumigatus* susceptible isolates. A combination of E-test and EUCAST methodologies were used in the present study to determine the MIC of itraconazole (ITC) for clinical and environmental isolates under examination. The E-test method is very effective and efficient, and yields results comparable to reference broth microdilution assays (Espinel-Ingroff and Rezusta, 2002; Kontoyiannis *et al.*, 2000; Messer *et al.*, 2007; Wei *et al.*, 2015). Moreover, according to Pfaller *et al.* (2000), the overall agreement between ITC E-test MIC values and the reference broth microdilution methods is 100%, when using RPMI media for *Aspergillus* spp. Another study has reported that the agreement between ITC MIC values for the two methods is 95.8% (Pfaller *et al.*, 2003).

Evidence presented in this chapter showed a clear correlation between the presence of a certain form of the TR₁₃₀ and the level of itraconazole resistance in a given isolate.

A gradual increase in level of azole resistance seen as the number of tandem repeats of the 130 bp sequence increased. This was shown using both E-test and EUCAST methods for testing the resistance level of isolates. For example, isolates with four repeats of the 130 bp sequence (TR₁₃₀ 4R) were consistently the most highly azole-resistant with MICs up to 32mg/l, but this resistant level decreased in accordance with decrease in the number of repeats, and isolates with only 2R exhibited much lower MIC values (≥ 2 µg/ml) whilst still remaining above the MIC clinical breakpoint.

3.4.2 Azole resistant phenotype is monogenic in a TR₁₃₀ isolate

Results of the crossing between one of the TR₁₃₀ isolates and a sensitive parent 47-51 clearly showed that 50% of the resulting offspring were itraconazole (ITZ) resistant and 50% were sensitive. This result provided strong evidence that the resistance trait is linked to a single genetic change (e.g. a monogenic trait) (Ashton and Dyer, 2016). In addition, it was demonstrated that all resistant progeny had an amplicon size of 1,045 bp for the *cyp51A* promoter region, indicating that they all harboured TR₁₃₀, whereas the shorter WT form of *cyp51A* (with just a single 130 base pair sequence without any repeat) was found in all ITZ sensitive progeny. This exact correlation between the presence of TR₁₃₀ and ITZ resistance provided clear evidence TR₁₃₀ is linked to the resistance phenotype. Furthermore, an equal distribution of the *MAT1-1* and *MAT1-2* genotypes was found among the offspring and there was no relation between the resistance phenotype and the mating-type genotype, consistent with Mendelian segregation of the traits.

3.4.3 3.4.2 Replacement of wild-type *cyp51A* promoter with different copies of TR130 (4R, 3R and 2R)

The genetic manipulation (GM) work undertaken in the present chapter provided further clear evidence that the presence of tandem repeats of the 130 bp sequence resulted in elevated levels of resistance to azole antifungals. Various repeat numbers of the 130 bp sequence were introduced into an azole-susceptible *A. fumigatus* parental strain (*akuB^{KU80}*). Transformants with either two, three or four 130 bp repeats (TR₁₃₀ X

2R, 3R and 4R, respectively) were successfully generated and selected for on media containing 4 µg/ml itraconazole. The transformants were then shown to gain resistance to itraconazole with MICs of 8 µg/ml, 4 µg/ml and 2 µg/ml for 4R, 3R and 2R, respectively. Moreover, a complementation experiment where an ITZ resistant *cyp51A* transformant with the TR₁₃₀ X 4R was restored back to WT *cyp51A* form resulted in a reversion back to ITZ sensitivity. This result confirms the hypothesis that the newly discovered 130 bp repeat sequences have a significant role in conferring azole resistance, with a gradual decrease in resistance phenotype seen with decrease in number of tandem repeat units. In parallel work Mellado *et al.* (2007); Snelders *et al.* (2015); Resendiz-Sharpe *et al.* (2022) had previously shown via GM approaches that the TR₃₄ and L98H resistance forms were a causal reason of azole resistance in *A. fumigatus*, with deletion of a TR₃₄ resulting in azole sensitivity and introduction of TR₃₄L98H into an azole sensitive isolate resulting in an azole resistance phenotype.

3.4.4 Expression analysis

Gene expression studies revealed a significant positive correlation between the presence of TR₁₃₀ and increased expression of the *cyp51A* gene, whose protein product CYP51A is the target of azole drug binding. As seen from the semi-quantitative endpoint PCR and qRT-PCR results, a significant increase (around 12 to 20-fold) was evident in the basal expression of *cyp51A* in isolates harbouring TR₁₃₀ 4R as compared to a representative WT isolate, with increased *cyp51A* expression also seen in the 3R and 2R TR₁₃₀ forms, although not to the same extent.

There is a great deal of evidence in the scientific literature showing that changes in the expression of the *cyp51* gene can be linked with increased resistance to azole antifungals (Mellado *et al.*, 2007; Van Der Linden *et al.*, 2013; Snelders *et al.*, 2015; Zhang *et al.*, 2017). Molecular genetic research has revealed the likely mechanistic basis of the increased *cyp51A* gene expression seen in the various tandem repeat forms (e.g., TR₃₄, TR₄₆ and TR₅₃), which is thought to be due to the presence of multiple copies of two binding sites for the positive transcription factor regulator *SrbA* that has a fundamental role in regulating sterol biosynthetic genes (Gsaller *et al.*, 2016). It was discovered that *SrbA* interacts directly with a transcription enhancer sequence within

the 34 bp region of the *cyp51A* promoter region (Gsaller *et al.*, 2016). Furthermore, the activating role of *SrbA* is enabled by the action of a CCAAT-binding complex (CBC), which is made up of three protein subunits HapB, HapC and HapE. The subunits are known to interact with a CCAAT motif within the 34 bp sequence of the *cyp51A* promoter. Thus, the 34 bp sequence is indispensable for activation of *cyp51A* gene expression, and the duplication of this region in the TR₃₄ tandem repeat mutants is thought to allow increased *SrbA* binding and, consequently, increased azole resistance (Gsaller *et al.*, 2016). Linked to these findings, it was very significant to note that the TR₁₃₀ region detected in the present study encompasses the same region of the *cyp51A* promoter as that occupied by TR₃₄ and TR₄₆ (**Figure 3.5**). Therefore, a similar impact on *SrbA* binding would be predicted. Indeed, the present results showed a clear differential impact of the number of repeat (2R, 3R, 4R) on the basal level of *cyp51A* expression with a gradual increase in *cyp51A* expression as the number of repeats increased. Therefore, it seems likely that the highest expression levels seen in the 4R X TR₁₃₀ forms can be attributed to the presence of a four-fold increased number of *SrbA* binding sites in these isolates. Indeed, the fact that this represents a doubling in the number of *SrbA* binding sites relative to the previously reported TR₃₄ and TR₄₆ forms (in which only two tandem repeats are present) might explain why there was no need for concomitant mutations in the gene coding region as seen in the TR₃₄L98H and TR₄₆/Y121F/T289A forms i.e. the high increase in *cyp51A* expression in 4R X TR₁₃₀ meant that additional decreased azole binding efficiency to CYP51A was not required for azole resistance. Consistent with this hypothesis it is noted that *cyp51A* gene expression was higher in all the 4R X TR₁₃₀ forms compared to previous TR₃₄ forms, although a similar level of expression was seen in the TR₄₆ form (**Figure 3.24.B**). In parallel, Paul *et al.* (2017) reported that introducing multiple copies of TR₃₄ into *A. fumigatus* resulted in increased *cyp51A* gene expression. Meanwhile, Zhang *et al.* (2017) found that the triple repeat of the TR₄₆ mutation (TR₄₆³/Y121F/M172I/T289A/G448S) resulted in ca. 9-fold increase in *cyp51A* expression compared to WT controls, but only a slight increase compared to regular TR₃₄ and TR₄₆ strains.

The expression of *cyp51A* in isolates exhibiting the TR₁₃₀, TR₃₄, and WT forms was also investigated in the presence of sub-lethal concentration of itraconazole. It was

found that the TR₁₃₀ X 4R strain showed markedly higher *cyp51A* expression in the presence of itraconazole, with around a 10-fold increase in expression compared to basal growth without itraconazole exposure. By contrast, the WT strain did not show any increase in the expression, and strain harbouring the TR₃₄ mutation showed only a slight 2-4-fold increase in *cyp51A* expression following itraconazole exposure. This was again consistent with the hypothesis that the TR₁₃₀ had an important contribution to azole resistance.

One extra interesting observation was that there were differences in both *cyp51A* basal gene expression and levels of azole resistance between the clinical patient-derived isolates and the lab GM strains despite isolates harbouring the same form of the TR₁₃₀ mutation. For example, the clinical 4R and 3R isolates showed higher MIC values of the comparable GM isolates (**Section 3.3.9**), and the clinical 4R and 3R isolates also showed consistently higher *cyp51A* gene expression than the comparable GM isolates (**Section 3.3.11.4**). This indicated that the *A. fumigatus* clinical isolates had a different genetic background than the *akuB*^{KU80} strain used for GM work, and that the clinical isolates might contain some additional resistance mechanism(s). For example, differences might be linked to the presence of SNPs at various codons (46, 127, 248, 255, 427) in *cyp51A* of the clinical strains, which might impact on *cyp51A* expression alongside the TR₁₃₀. Although several studies have shown evidence that these mutations are not associated with the azole resistance phenotype (Escribano *et al.*, 2011; Bueid *et al.*, 2010; Howard *et al.*, 2009), it is not known how these may interact with the novel TR₁₃₀ identified in the present study. Alternatively, there might be extra minor genes (or alternative allelic forms) in the clinical strains conferring elevated azole resistance over the lab GM-strains, despite the findings from sexual crossing that the resistant phenotype is linked to single gene (**Section 3.3.3**).

In parallel work there have been several reports of polymorphisms in the *erg11* gene (homologous to *cyp51A*) from clinical *C. albicans* isolates which have been linked to fluconazole resistance (Favre *et al.*, 1999; Marichal *et al.*, 1999). Resistance in plant pathogenic fungi to various demethylase inhibitors (DMIs) utilised in agriculture has also been generally linked to either point mutations in the *cyp51* azole target gene or changes in gene expression (Mellado *et al.*, 2007). For example, it has been suggested that a potential mechanism for DMI resistance in the plant pathogen *Mycosphaerella*

graminicola is the greater degree of *cyp51* expression (Stergiopoulos *et al.*, 2003). Likewise, a DMI resistance mechanism has been reported for another pathogenic plant mould, *Venturia inaequalis*, in which a highly elevated expression of *cyp51* was correlated with the presence of a 553 bp insertion in the gene promoter region (Schnabel and Jones, 2001). Furthermore, the analysis of 59 DMI-resistant isolates of *Bumeriella jaapii* revealed a 5- to 12-fold increased level of *cyp51* expression associated with different forms of a shortened non-long terminal direct repeat and long interspersed nuclear retrotransposon element in the promoter region (Ma *et al.*, 2006). In *Penicillium digitatum*, a mechanism similar to the aforementioned has been reported for strains having a 126-bp sequence tandemly duplicated DNA sequence in the *cyp51* promoter. The presence of the tandem repeats had a direct association with the extent of resistance to various DMIs, with the number of tandem replications being vital for the creation of a high degree of DMI resistance (Hamamoto *et al.*, 2000).

3.4.5 TR₁₃₀ isolates maintain azole resistance even after deletion of an ABC transporter gene

Azole resistance in *Candida* species such as *C. albicans* and *C. glabrata* and other yeasts such as *Saccharomyces cerevisiae* is in most cases linked to overexpression of ATP-binding cassette (ABC) transporter- encoding genes such as *CDR1*, *CDR2* and *MDR1* (Slaven *et al.*, 2002; Fraczek *et al.*, 2013; Paul *et al.*, 2017). In 2013, Fraczek *et al.* identified an efflux ABC transporter gene linked to azole resistance in *A. fumigatus* which they named *cdr1B* (AFUA-1G14330; although also termed *abcC*, *ABC11* and *abcG1* by other authors). They also identified a gene paralogue which was named *cdr1A* (AFUA-2G15130; although also termed *cdr4* by other authors). Both *cdr1A* and *cdr1B* are orthologues of *C. albicans* *CDR1*. Fraczek *et al.* (2013) found that deletion of *cdr1B* could lead to a four-fold decrease in resistance to itraconazole, and that the expression of *cdr1B* in azole resistant strains of *A. fumigatus* (i.e. without any mutation in *cyp51A*) was higher than susceptible strains. In parallel studies Paul *et al.* (2013) identified a further ABC transporter gene which they named *abcB* and demonstrated that deletion of *abcB* invariably led to increased susceptibility to azole antifungals and that the transporter was associated with the cell surface. In later studies Paul *et al.* (2017)

investigated the possible importance of *cdr1* (which they termed *abcG1*) expression in azole resistance of *A. fumigatus* isolates which contained the TR₃₄L98H resistance mechanism. Fascinatingly, they found that deletion of *cdr1* (*abcG1*) led to greatly decreased voriconazole resistance in previously resistant TR₃₄L98H isolates. It was therefore argued that *Cdr1* (*AbcG1*) activity was required for elevated azole resistance even in the presence of the TR₃₄L98H mutation.

It was therefore of interest to clarify the importance of *Cdr1* (*AbcG1*) activity in the TR₁₃₀ mutants identified in the present study. A series of *Δcdr1B* mutants were constructed in strains harbouring the TR₁₃₀ X 4R mutation, with knockouts made in two distinct genetic backgrounds harbouring the same mutation. Azole susceptibility testing revealed some level of reduction in the itraconazole MICs in isolates following deletion of *cdr1B*, with the extent being strain dependent. A 2-fold decrease in MIC value was observed for clinical patient-derived *Δcdr1B* transformants, whilst a 4-6 fold reduction in MIC value was seen in the laboratory GM strains, despite the continued presence of the TR₁₃₀ X 4R repeat sequence. Thus, results confirmed the perhaps often overlooked importance of the *cdr1* (*abcG1*) ABC transporter in the azole resistance phenotype of *A. fumigatus*. Nevertheless, the *Δcdr1B* transformants still exhibited MIC values above the clinical breakpoints, thereby illustrating again the importance of the role of TR₁₃₀ X 4R in conferring azole resistance. Results again indicated differences in genetic background between the clinical and laboratory *akuB*^{KU80} strain as discussed above (Section 3.4.4).

In parallel work, the Zn₂- Cys₆ transcription factor *AtrR* has been shown to have a role in antifungal drug resistance in *A. oryzae*, and *A. nidulans* (Hagiwara *et al.*, 2017). *AtrR* has also been shown to play a role in azole resistance of *A. fumigatus* via regulation of genes involved in ergosterol biosynthesis such as *cyp51A*, regulation of virulence and hypoxia adaptation, and regulation of the expression of the ABC transporter *cdr1B* (*abcG1*). Deletion of *AtrR* resulted in an azole-hypersensitive phenotype in a clinical azole-resistant strain of *A. fumigatus* harbouring a *cyp51A*/G54E mutation (Hagiwara *et al.*, 2017). This finding suggests an underlying connection between ABC transporters such as *cdr1B* and genes involved in ergosterol biosynthesis such as *cyp51A* whereby both together may affect azole resistance in *A. fumigatus*.

3.4.6 Evidence of treatment failure of azole resistant *Aspergillus fumigatus* harbouring TR₁₃₀ mutations

Galleria mellonella has been utilized as an appropriate animal model for analysing virulence and pathogenicity in a wide variety of *A. fumigatus* strains (Maurer *et al.*, 2019; Jemel *et al.*, 2020). Forastiero *et al.* (2015) suggested the use of such a ‘mini host’ for *in vivo* work as it provides a convenient correlation with mammalian models and thereby improves the speed of evaluating pathogenicity determinants and the preliminary evaluation of novel antifungal agents. Therefore, *G. mellonella* was used to evaluate both the virulence, and the efficiency of itraconazole treatment of *A. fumigatus* strains harbouring TR₁₃₀ in comparison to WT strains.

Results showed no statistically significant differences in term of virulence between any of the TR₁₃₀ strains (either patient-derived or GM forms) and the WT controls. This indicated that the TR₁₃₀ strains were not inherently more virulent as a result of the TR₁₃₀ mutation and any possible cellular effects. However, a significantly reduced efficacy of azole (itraconazole) treatment was observed in larvae containing the clinical patient-derived isolates with the TR₁₃₀ mutation as compared to the WT azole sensitive control. This was a very important finding as results indicated that the presence of the TR₁₃₀ mutation could lead to azole treatment failure as a result of the elevated azole resistance of TR₁₃₀ mutant strains. Thus, the recommended therapeutic dose (10 mg/kg) of itraconazole was not sufficient to treat *A. fumigatus* infections in the *G. mellonella* model when using the clinically-derived TR₁₃₀ *A. fumigatus* strains (although treatment was more efficient for the GM TR₁₃₀ mutant strains).

The *G. mellonella* model has been used elsewhere to try and evaluate the efficacy of drug treatments for *A. fumigatus* infection. Scientists have suggested different models for treatment such as the use of combination of two different azoles, increasing the dosage of the azole and the use of other compounds beside the azoles. For example, combining two different therapies such as the simultaneous application of Ethylene Glycol Tetra-Acetic Acid (EGTA), which is a calcium chelator, with itraconazole (ITZ) shows higher survival rates in *G. mellonella* larvae infected with azole susceptible *A. fumigatus* compared to single dosages of ITZ (100mg/kg) or EGTA (14mg/kg) (Liu *et*

al., 2015). Additionally, implementing new formulations in the drug composition of antifungal agents and/or higher doses of posaconazole (PSC) and voriconazole (VRC) was found to be effective against *A. fumigatus* infection (Forastiero *et al.*, 2015). It was found that using VRC and PSC at the recommended therapeutic dose (10 mg/kg) and (20 mg/kg), respectively, was not sufficient to treat azole resistant *A. fumigatus* strains infecting *G. mellonella*. However, the use of higher doses of VRC improved outcomes, with increased survival of *G. mellonella* infected with azole resistant *A. fumigatus* strain harbouring the TR₃₄/L98H mutation. Consequently, *G. mellonella* has been used to assess the efficiency of VRZ treatment following infection with azole sensitive and azole resistant strains of *A. fumigatus* (Garcia-Rubio *et al.*, 2018). Larvae infected with susceptible strains showed increased survival after treatment with voriconazole, indicating that voriconazole is effective in managing infections in *G. mellonella*. By contrast, VRZ showed a lowered *in vivo* efficiency in control of strains with moderate resistance, which harboured different SNPs in the *cyp51A* gene, compared to non-resistant controls (Garcia-Rubio *et al.*, 2018).

3.4.7 Evolution of the TR130 resistance mechanism

The origin and evolution of tandem repeat-based resistance mechanisms has been the subject of much debate. For example, there are arguments for and against a unique or multiple origin for the widespread TR₃₄/L98H mutation in *A. fumigatus* (Verweij *et al.*, 2009; Camps *et al.*, 2012b; Hagiwara *et al.*, 2016).

Regarding the origin of the TR₁₃₀ mutation found in the present study, interestingly, six of the isolates harbouring the TR₁₃₀ X 4R had been isolated from two different patients. Five among them were isolated over a period from March 2015 to October 2018 from one patient (code number D48387) and one was isolated in November 2015 from a different patient (code number 08000346). According to the phylogenetic analysis, all of the TR₁₃₀ X 4R isolates were clustered together which might give an indication of possible patient-to-patient transmission as both patients were diagnosed with cystic fibrosis and had been visiting the same hospital clinic for treatment (Prof. D Armstrong, *pers comm*). This would be a highly significant, and concerning, finding as so far there has been no definite evidence of patient-to-patient transmission of *A.*

fumigatus, although there is very recent evidence of environment-to-patient transmission of azole resistant *A. fumigatus* (Rhodes *et al.*, 2022). Meanwhile, isolates containing the other X 3R and X 2R forms of the TR₁₃₀ repeat were also isolated from the same D48387 patient, and these clustered with the other TR₁₃₀ isolates. This suggests a possible explanation for the evolution of the TR₁₃₀ mutation. It is hypothesised to have evolved initially as a 2R form (represented by isolate 158, isolated from the patient in March 2015), then evolved into 3R (represented by isolate 19, isolated from the patient in March 2017) and 4R (represented by isolates C136, C137, 80, 82 and 169, isolated from the patient between October 2015 and October 2018) forms within the same D48387 patient who had a long history of antifungal treatments using various azoles over an extended time period e.g. itraconazole, voriconazole, and caspofungin (Prof. D Armstrong, *pers comm*). Other scenarios are of course possible. A similar report of the evolution of azole resistance has been reported from a 69-year-old man, a former smoker with chronic obstructive pulmonary disease (COPD), who was under treatment with voriconazole (200mg 2×/d), alternating with posaconazole (300mg/d) and amphotericin B. Two azole-resistant *A. fumigatus* isolates were cultured from the patient and it was found that they harboured a novel tandem repeat (TR₁₂₀) without any alteration in the coding region of *cyp51A* (Hare *et al.*, 2017).

3.5 Conclusions

The presence of the TR₁₃₀ itraconazole resistance mechanism in the promoter region of *cyp51A* gene of *A. fumigatus* represents a novel and potentially very significant mechanism of azole resistance in *A. fumigatus*. Unlike previously reported azole-resistant isolates with TR forms (TR₃₄ and TR₄₆), which combined and seem to require alterations in the coding region for full resistance, the TR₁₃₀ appeared to provide the main mechanism of azole resistance in the isolates under study, albeit with some influence of the genetic background of the clinical versus GM strains.

And during the completion of thesis studies a very significant new finding was made. Details of the discovery of the TR₁₃₀ mutation, together with the primers used for amplification of this region, were shared as part of a collaboration between the Nottingham group and a group led by Prof. Matt Fisher (Imperial College London). As

part of a citizen science project, 736 azole resistant *A. fumigatus* were isolated from soil samples throughout the UK during June 2019 and these were later screened for mutations in the *cyp51A* promoter region using the set of primers designed for use in my research. This resulted in the discovery of four azole resistant environmental isolates containing a set of four repeats of the 130 bp sequence in the promoter region of *cyp51A* (Shelton *et al.*, 2022). These TR₁₃₀ X 4R sequences were identical to the ones described in the present study and as first seen in the clinical isolates from 2015-2018.

The presence of this mutation in an environmental setting is a cause of much concern as it suggests the possible start of a global spread of this mutation. To compound the risk, it was found that at least some of the clinical isolates were sexually fertile, which means that gene flow of the TR₁₃₀ mutation may occur via sexual reproduction in the environment (Swilaiman *et al.*, 2020; Verweij *et al.*, 2009). The development of this mutation clinically and environmentally might be linked to two possible reasons, which in both cases are linked to the usage of antifungal drugs. In a clinical setting, TR₁₃₀ might have developed in individuals who are receiving long-term antifungal therapy, leading to selection for this change in the promoter of the target gene. In a similar manner, the usage of azole fungicides in the environment to prevent crop loss might have increased the chance for this modification in the promoter of *cyp51A* again as selective response to azole treatment.

3.6 Future direction:

Strong evidence has been presented in this chapter to demonstrate that TR₁₃₀ is the primary mechanism involved in conferring the azole resistant phenotype in the clinical isolates from which it was first discovered. From this study, it has been shown that introducing the TR₁₃₀ mutation into a sensitive parental strain results in generating azole-resistant lab strains. Moreover, we have shown evidence that the resistant phenotype is linked to a single gene using sexual crossing, which is *cyp51A* in our case. Although we were able to show the effect of tandem repeats in increasing *cyp51A* expression, the initial studies of gene expression and the treatment failure using *Galleria mellonella* as a model for infection have raised many questions that require further investigation, linked to the following experimental work:

- The difference seen amongst patient and lab strains in the azole resistance level as well as differences in the *cyp51A* expression level suggest that patient strains might have an additional mechanism within the *cyp51A* gene or elsewhere in the genome in addition to TR₁₃₀. The sequence analysis showed that the TR₁₃₀ isolates have additional SNPs at various codons (46, 127, 248, 255, 427), which previous studies have suggested do not impact on azole resistance. However, the combination of the TR₁₃₀ and those SNPs requires further analysis. This might include introducing those SNPs as well as the TR₁₃₀ into an azole-sensitive parental strain and then assessing the resistant level and the expression level of *cyp51A*. It will also be of interest to screen the TR₁₃₀ forms more widely against other antifungals such as amphotericin B and Olorofim to assess in more depth differences in resistance level against these drugs.
- The difficulty we faced in detecting an increase in *cyp51A* expression in the TR₃₄ strains is hard to explain. Expression studies were made with eight different azole resistant TR₃₄ strains, with results normalised against more than four WT strains and two different reference genes. We used a previously published pair of primers for both target and reference genes in this study, and they worked perfectly with all isolates with no problems detected even with isolates harbouring TR₄₆. However, it might be worthwhile designing new primers for the expression work and reanalysing this data.
- For the pathogenicity and treatment of *A. fumigatus* infection, work had to be completed in limited time available at the University of Exeter. Looking ahead, it will be vital to evaluate the efficacy of previously recommended different types and ways of treating azole resistant *A. fumigatus* infection. This could include using a high dose of voriconazole or using a combination of two drugs, which it is hoped will provide insights into treatment possibilities of the highly resistant *A. fumigatus* strains harbouring the TR₁₃₀ mutations.
- It will also be of interest to further investigate variation in *cdr1* (*abcG1*) gene activity in global populations of *A. fumigatus* and how this might impact on TR₁₃₀ influenced gene expression of *cyp51A*.

Chapter- 4 Investigation of Non-*cyp51A* Azole Resistance Mechanisms Including use of the BSA Method

4.1 Introduction

The global spread in occurrence of azole resistance in populations of *A. fumigatus* since the early 2000s has been attributed mainly to mutations in the *cyp51A* gene, with more than 30 different mutations in the promoter or coding region of *cyp51A* having been reported in clinical or environmental isolates (Verweij *et al.*, 2020). However, as described in **Section 1.3.2** of the introductory chapter, many surveys of clinical isolates have found that up to 50% of azole resistant isolates have no apparent mutation in *cyp51A* or its promoter region (Fraczek *et al.*, 2013; Chowdhary *et al.*, 2014; Rivero-Menendez *et al.*, 2016; Wei *et al.*, 2017) whilst surveys of environmental isolates have also routinely detected resistant isolates with no apparent causal *cyp51A* mutation (Chen *et al.*, 2020b; Kang *et al.*, 2022). This indicates the presence of other resistance mechanisms that are non-*cyp51A* based. Some possible causal mechanisms have been identified such as mutation in the *hapE*, *hmg1* and *erg6* genes, together with overexpression of the ABC transporter *cdr1B* gene and other efflux pumps (**Section 1.3.2**). However, for the most part the causal basis of resistance in such ‘non-*cyp51A*’ azole resistant isolates of *A. fumigatus* have remained unknown. This poses an obstacle for management of aspergillosis disease because molecular diagnostics are not available to identify such mutants.

Investigating the mechanistic basis of traits such as non-*cyp51A* azole resistance where the genetic basis is unknown poses a major challenge. However, in the case of *A. fumigatus* the recent discovery of a sexual cycle (Dyer and Paoletti, 2005; O’gorman *et al.*, 2008; Paoletti *et al.*, 2005; Pyrzak *et al.*, 2008) has provided a very useful tool for undertaking genetic analysis. The sexual cycle can be used in preliminary studies to determine whether a trait such as azole resistance has a monogenic or polygenic basis (Dyer *et al.*, 2000; Ashton and Dyer, 2016; Ashton *et al.*, 2022). The sexual cycle can also be used in applications such as bulk segregant analysis (BSA) and quantitative trait

loci (QTL) analysis to identify causal genetic elements. Indeed, a form of BSA has recently been developed for genetic analysis in *A. fumigatus* as will now be described.

4.1.1 Bulk Segregant Analysis (BSA)

The BSA technique can be utilized to identify a region of the genome linked to a trait of interest where the genetic basis is unknown and detailed hereditary maps are unavailable (Michelmore *et al.*, 1991). The process involves setting up a sexual cross between two parents who have or do not exhibit the trait of interest. A sufficiently high number of offspring (e.g. 80 to 120) are, then divided into two 'pools' depending on the absence or presence of a particular trait. DNA is then extracted from all the progeny, and the resulting DNA pooled according to the absence or presence of the trait of interest and then processed for further analysis. The classical approach for BSA then used DNA fingerprinting (e.g. RAPD-PCR) and genetic map-oriented methods to identify a chromosomal region linked with, and therefore containing, the desired gene (**Figure 4.1**). However, for this method to work, a densely saturated genetic map was required (Ashton and Dyer, 2016).

More recently the BSA technique has been updated to incorporate analysis of progeny pools using next-generation-sequencing (NGS) approaches (Ashton and Dyer, 2016). This has provided a very powerful method to identify the 'unknown' genetic basis of traits, as NGS can be used to precisely identify SNPs of interest that are only present in DNA pools of the progeny with the trait of interest and therefore linked to the causal gene(s) or other mutation. For example, such BSA involving NGS of progeny pools has been used to identify genes in the filamentous fungi *Sordaria macrospora* and *Neurospora crassa* involved in spore colour and heterokaryon incompatibility (Nowrousian *et al.*, 2012; Heller *et al.*, 2016; Heller *et al.*, 2018).

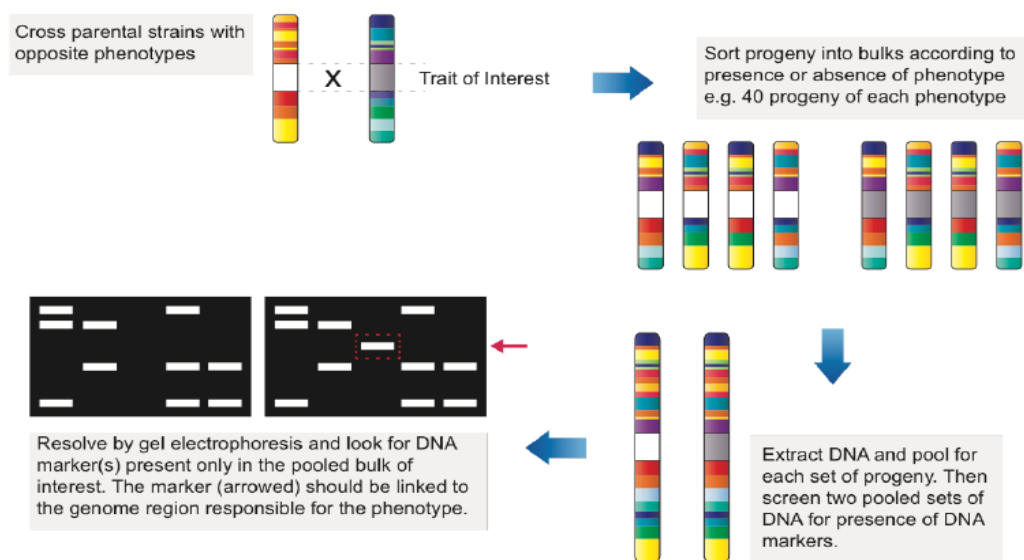


Figure 4-1: Illustration of the bulk segregant analysis (BSA) approach used for identifying a region of the genome linked to a trait of interest. Adapted from (Ashton, 2018).

Most recently, the BSA approach combined with NGS has been used to investigate and successfully locate a gene of interest responsible for an azole resistant phenotype in *A. fumigatus* (Ashton *et al.*, 2022). The rationale and methodology for the BSA method used by Ashton *et al.* (2022), and which is followed in BSA work in this chapter, is illustrated in **Figure 4.2**. Specifically, it was found that BSA could be utilized to identify a genomic region of less than 300 kb in size that contained the gene of interest. To improve the resolution of the BSA technique, Ashton *et al.* (2022) performed multiple rounds of backcrossing and also varied the number of progeny used in the pools in order to increase the isogeneity of progeny pools and therefore more accurately detect a single linked region that would vary between the progeny pools. Utilizing DNA from pools of 40 sensitive and 40 resistant offspring collected from either the initial (F1) or third or sixth backcrosses of *A. fumigatus*, the BSA approach successfully revealed the presence of a series of SNPs between the two pools. A total of 123 candidate genes containing SNPs were identified from the F1 analysis, 46 candidate genes from the third backcross and 20 candidate genes in a single 292 kb region of the genome from the analysis of the sixth backcross. Subsequent BSA analysis was then performed using progeny from a second backcross, but this time pooling a higher number of 80 resistant and 80 sensitive progeny. This identified a relatively low number of variant genes between the two pools (29 genes with SNPs located on the same 292 kb region of the genome). Among the

candidate genes was *cyp51A*, and further bioinformatic investigation determined that a methionine to lysine (M220K) substitution in the CYP51A protein was responsible for the azole resistance phenotype, consistent with the previous findings of (Mellado *et al.*, 2004).

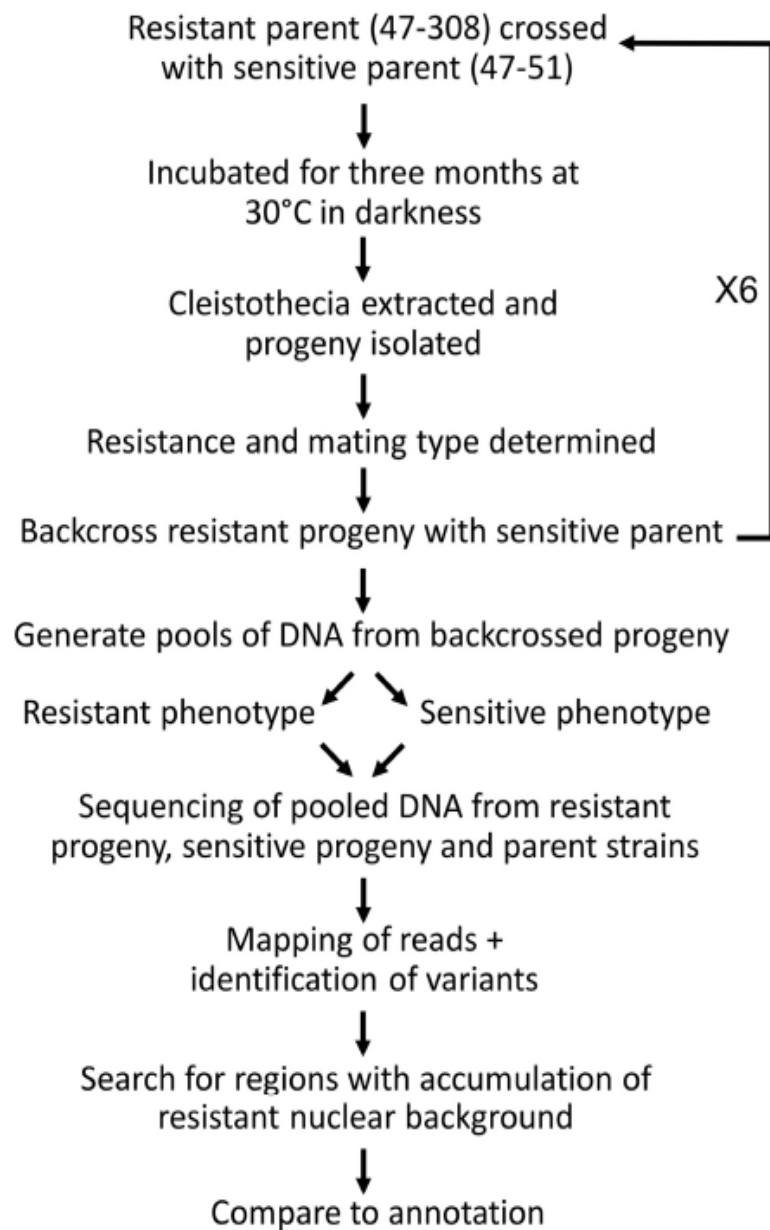


Figure 4-2: BSA rationale and methodology used to identify an ‘unknown’ gene conferring azole resistance in *A. fumigatus*. Adapted from (Ashton *et al.*, 2022).

4.1.2 Chapter Aims

Work in this chapter aimed to identify as far as possible the genetic basis of azole resistance in a series of isolates supplied by collaborators with supposed non-*cyp51A* forms of azole resistance. In particular it was aimed to exploit the sexual cycle of *A. fumigatus* to both identify whether traits were mono- or poly-genic in basis, and then use the BSA method of Ashton *et al.* (2022) to identify a linked genome region with distinctive SNPs and candidate target gene(s) that might be the causal basis of resistance. Research in this chapter therefore had the following specific objectives:

1. To obtain azole resistant isolates of *A. fumigatus* from environmental and clinical isolates that exhibit ‘unknown’ forms of azole resistance. The itraconazole E-test method was used to ensure that the isolates genuinely exhibited resistance to azoles.
2. To ensure that the isolates had unknown azole resistance mechanisms, checks were made that the resistant isolates did not have known changes in the drug target *cyp51A* (mainly because the majority of the resistant isolates show particular genetic changes in this gene) which result in azole resistance. Checks were also made for other known resistance mechanisms, such as a point mutation in *HapE* or *Cox10* genes, via PCR amplification and sequence analysis of these genes.
3. Where an unknown form of resistance could be demonstrated, sexual crossing would then be used: (a) to determine the mono- or poly-genic basis of resistance; (b) to produce pools of progeny for bulked segregant analysis (combined with NGS) to determine the genetic basis of resistance using. It was planned to use a high number of progeny (100 progeny for each pool of sensitive and resistant groupings) collected from a third backcross to improve the resolution of BSA.

4.2 Material and Methods

4.2.1 Strains

A total of 32 supposedly azole resistant strains of *A. fumigatus* were obtained for use in this study as described in (Section 2.1.1). All of these isolates supposedly had non-*cyp51A* forms of resistance to azoles. The first isolate (47-312), which is an environmental isolate, was obtained from a former doctoral student at the University of Nottingham. This isolate was previously crossed with a sensitive isolate (47-54), and offspring were collected, and spore suspensions were stored at -80 °C. A further set of 15 clinical isolates and one environmental isolate were obtained from Imperial College London (through a collaboration with Prof. Matthew Fisher and Dr. Thomas Sewell). Another set of 5 clinical isolates were obtained from Radboud Medical University, Netherlands (through a collaboration with Prof. Paul Verweij). Lastly, 10 azole resistant environmental isolates were obtained from Leibniz Institute for Natural Product Research, Jena, Germany (through a collaboration with Dr. Amelia Barber). Isolates used in this chapter are listed below **Table 4.1**. This table will be updated later in the results section with further details.

Table 4-1: List of supposedly azole resistant *A. fumigatus* isolates obtained from different locations and sources.

Isolates ID	Provider	Source
47-312	Doctoral student at Nottingham (George Ashton)	Environmental
C128, C136, C137, 169, 19, 158, 80, 82, 32	Imperial College London (This set of isolates were analysed in Chapter 3)	Clinical
C127	Imperial College London	Clinical
C129		Clinical
C138		Clinical
C139		Clinical
C117		Clinical
C118		Clinical
C286		Environmental
VIII	Radboud Medical University, Netherlands	Clinical
V138		Clinical
V144		Clinical
V167		Clinical
V186		Clinical
20	Leibniz Institute for Natural Product Research, Jena, Germany)	Environmental
56		Environmental
106		Environmental
176		Environmental
313		Environmental
439		Environmental
486		Environmental
570		Environmental
675		Environmental
684		Environmental

4.2.2 Strain Maintenance and Revival

All *A. fumigatus* isolates used in this study were maintained as described in **Section 2.1.2**.

4.2.3 Antifungal Susceptibility Testing

The E-TEST method was used to determine the antifungal sensitivity of all *A. fumigatus* isolates to the azole antifungals itraconazole and voriconazole as described in **Section 2.2.2.1**.

4.2.4 DNA Extraction

Genomic DNA of isolates was extracted as described previously in **Section 2.2.3**.

4.2.5 PCR Amplification and Sequence Analysis of *cyp51A*, *hapE* and *cox10* Genes

DNA sequence analysis was performed to ensure that the provided azole resistant isolates did not have any known azole resistance mechanisms, such as a point mutation within the coding regions of a target gene *cyp51A*, as described in **Section 2.2.4**. PCR products were resolved in 1% agarose gels by gel electrophoresis to check the amplicon size for each gene.

4.2.6 MAT-typing PCR, and Crossing

The mating type of isolates for sexual crossing was determined using the methods described in **Section 2.2.5**. The crossing protocol was as described in **Section 2.2.7**, using crosses between the candidate resistant isolates and supermater strains as described by (Ashton and Dyer, 2019).

4.2.7 Preparation for Bulk Segregant Analysis

The first step of this method involved a cross between the azole sensitive 47-267 and resistant C286 isolates. After 3-4 months of incubation a mature cleistothecia was identified and around 80 progeny were collected from the first F1 round of crossing. This was followed by three rounds of backcrossing between the original sensitive 47-267 parent and the progeny with the resistant phenotype to reduce genetic differences between the two progeny. Before each round of backcrossing, the mating type and the resistance phenotype was tested to ensure that the backcrossing was correctly performed. After the third backcrossing, over 200 progeny were collected and screened for azole susceptibility. gDNA was extracted from 200 progeny comprising 100 azole resistant and 100 sensitive progeny. The DNA concentration was checked using the Invitrogen™ Qubit™ 4.0 Fluorometer and adjusted to a final concentration of 50 ng μl^{-1} . Based on the presence or the absence of the resistant phenotype, the gDNA was split into two pools (azole resistant or azole sensitive). A final DNA suspension of 100 μl at a concentration of 50 ng μl^{-1} was created for each DNA pool by adding 2 μl of the relevant DNA sample (resistant or sensitive). These two gDNA pools and both sensitive 47-267 and resistant C286 parents was sent to be genome sequenced and compared by

BSA to identify genetic differences, using three replicates for each of the parents and resistant and sensitive pools.

4.2.8 Genome Sequencing and Bioinformatic Analysis by the BSA Approach

Whole genome sequencing and bioinformatic analysis was performed by Edinburgh Genomics at Ashworth Laboratories (University of Edinburgh, UK), following the same analysis pipeline described by (Ashton *et al.*, 2022). Therefore, only a brief description will be provided here as more detailed information can be found in (Ashton *et al.*, 2022). The sequence analysis aimed to identify any SNPs present in the resistant parent/pool of resistant progeny and not present in the sensitive parent/pool of sensitive progeny. The analysis was performed following five main steps. Reads were first put through quality assurance and trimming. The readings were then aligned to a reference *A. fumigatus* sequence provided by the Joint Genomes Institute (JGI), USA (isolate A1163; Genbank accession ABDB000000000) and SNP variants were called from the reference genomes to determine the variant information for each replicate. Third, homozygous variations shared by replicates of each set of three sample replicates were identified by comparing the variant information for each group. After that, in order to identify the variants present in both the resistant strain progeny and parent samples but absent in both the susceptible strain progeny and parent samples, the common variants from each group were then compared to those of every other group using the intersect algorithm of BCF and VCF tools. Finally, specific analysis tools (e.g. SnpEff) were used to compare the projected effects of the chosen variations on the coding areas of the relevant genome sequence against the information already available.

4.3 Results

4.3.1 Antifungal Susceptibility Testing Using E-test

The minimum inhibitory concentration (MIC) point was read after 48 hours of incubation of the isolates with the itraconazole strip. The numbers on the strip correspond to itraconazole concentration in micrograms per millilitre. The point at which the fungal growth stopped was considered as the minimum azole concentration that inhibits fungal growth (**Figure 4.3**).

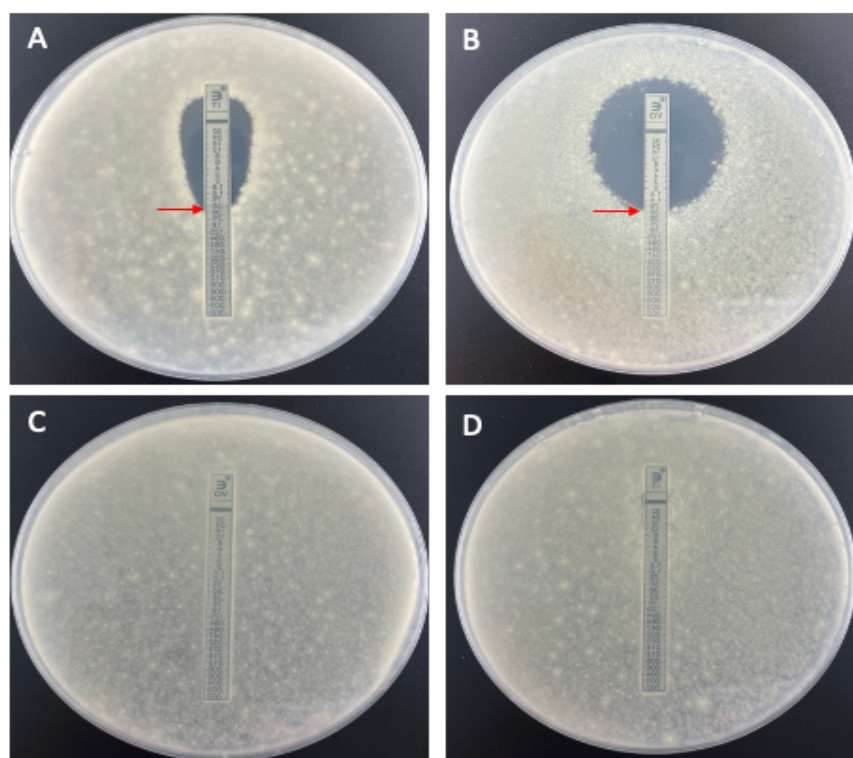


Figure 4-3: Antifungal susceptibility testing using itraconazole and/ or voriconazole E-test strips which have a concentration of 32 µg/ml of the drug at the top of the strip that decreases down the strip towards the bottom. The name of the drugs indicated in the top of each strip, where IT referred to itraconazole (**A** and **C**) and VO referred to voriconazole (**B** and **D**). MIC values were recorded after 48 h incubation of *A. fumigatus* isolates in RPMI media at 37°C. (**A** and **B**) Sensitive *A. fumigatus* isolates where the isolate stopped growing below the recommended breakpoint (2 µg/ml), and the drug was able to inhibit the growth with MIC around 0.50µg/ml indicated with red arrow. This compares with resistant *A. fumigatus* isolates where the isolate shows full growth without any inhibition zone around the strip, with MIC greater than 32µg/ml (**C** and **D**).

Results showed that three of the Imperial isolates (32, C129 and C139), four of Netherland isolates (V111, V144, V167 and V186) and five of German isolates (20, 56, 106, 570, and 675) no longer exhibited the supposed resistant phenotype, with MIC

values lower than the clinical breakpoint values of 2 µg/ml when tested against itraconazole and 1 µg/ml when tested against voriconazole (**Table 4.2**). Moreover, although the MIC value for the environmental isolate 47-312 and some of German isolates (176, 313 and 486) showed elevated resistance for isolates of *A. fumigatus*, at ca. 2 µg/ml against itraconazole and between 0.38-1 µg/ml against voriconazole, those isolates were not considered to be highly resistant. Therefore, those isolates were excluded from this study, except for the Imperial isolate 32 that was used as a control azole sensitive isolate for studies in **Chapter 3**. However, the remaining 16 isolates were highly resistant, with MICs values between ≥ 2 -32 µg/ml against itraconazole or / and voriconazole (**Table 4.2**) and therefore, further analysis was required to choose the best candidate(s) for BSA work.

1.1.1 PCR Amplification and Sequence Analysis of *cyp51A*, *hapE* and *cox10* Genes

PCR was used to amplify the *cyp51A*, *hapE* and *cox10* genes for three environmental isolates (C286, 439 and 684) and 13 clinical isolates (C128, C136, C137, 169, 19, 158, 80, 82, C127, C138, C117, C118 and V138). In addition, a set of primers were used to amplify ca. 655 bp of the promoter region upstream of the starting codon of the *cyp51A* gene. The fragment size of the PCR products of the *cyp51A* promoter for six of imperial clinical isolates (C128, C136, C137, 169, 80, and 82) were much larger than the expected size of 655 bp, with amplicons instead of ca.1,045bp. Moreover, another six isolates showed larger than expected amplicon sizes for the *cyp51A* promoter PCR product, with a small increase in size seen in two Imperial isolates (C117 and C118), one of the German isolates (439) and for the Netherland isolate V138 and larger sizes seen in two Imperial isolates (19 and 158) (**Figure 4.4**). These results indicated that these 12 isolates might have duplication of a specific sequence within the promoter region of *cyp51A*. The remaining isolates (C127, C138, C286 and 684) all showed a fragment size (655 bp) of the expected size for WT *cyp51A*.

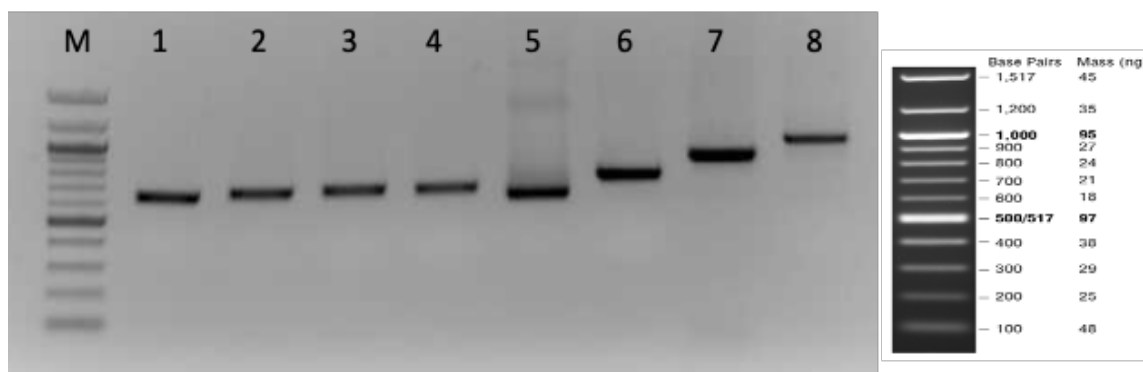


Figure 4-4: Representative gel electrophoresis of PCR products following amplification of the *cyp51A* promoter region using gDNA from different isolates of *A. fumigatus*. Bands with the expected WT size, 655 bp were observed in lanes 1 and 5, representing the WT promoter without any mutation or duplication seen in four isolates (C127, C138, C286 and 684). A small shift in the fragment size of around 689 bp represent isolate with TR₃₄ (lane 2) was seen in the German isolate 439. A slightly larger fragment (around 700 bp – lanes 3 and 4) represent isolates with TR₄₆ seen in (C117, C118 and V138). Lanes 6, 7, 8 showing the TR₁₃₀ duplication with different number of repeats; 2 repeat (lane 6), 3 repeat (lane 7), and 4 repeat (lane 8) seen in set of isolates discussed in **Chapter 3**. Products were run alongside 100bp DNA ladder (M) in a 1% agarose gel.

Sequence analysis of amplicons for the Imperial isolates C128, 158, C136, C137, 80, 82, 19 and 169 has already been described in (**Section 3.3.2 of Chapter 3**) and will not be discussed in the present chapter, except to say that: (a) these isolates were found to exhibit various forms of *cyp51A* TR₁₃₀ that were believed to confer azole resistance; and (b) single nucleotide polymorphisms genotype (SNPs) were present at several codons (46, 127, 248, 255, 427) of *cyp51A* compared to the reference *A. fumigatus* Af293 azole susceptible control strain (Genbank accession number AF338659), but sequences of *cyp51A* for these strains were identical to the azole sensitive A1163 genome sequenced isolate (GenBank accession number AF222068) so were not likely to be associated with the azole resistance phenotype (Howard *et al.*, 2009). Meanwhile, clinical isolate C127 exhibited a unique point mutation in *cyp51A*, namely A1362G. This change would substitute the amino acid glycine for aspartic acid at codon 430 (D430G). This mutation has been previously reported to be linked to voriconazole resistance in a clinical isolate from Japan (Majima *et al.*, 2020). In parallel, *cyp51A* sequence analysis of the C138 clinical isolate revealed a point mutation, C718T, causing a proline to leucine change at position 216 in the CYP51A protein (P216L). This position has been reported to be associated with azole resistance (Camps *et al.*, 2012a).

Sequence analysis of the *cyp51A* promoter revealed the presence of certain known tandem repeat mutations in some isolates. The two Imperial isolates C117 and C118 and the Netherlands isolate V138 were all found to contain the TR₄₆ duplication in the promoter region. This duplication has previously been reported as being associated with the high azole-resistant phenotype (Van Der Linden *et al.* (2013). Meanwhile, the German isolate 439 was found to contain the TR₃₄ duplication in the promoter region. This duplication has been widely reported to be linked to azole resistant in isolates from both an environmental and clinical setting (Fisher *et al.*, 2018).

This analysis left just two remaining environmental isolates that did not appear to show any known form of azole resistant mechanisms, namely C286 from the Imperial set of isolates and 684 from the German group of isolates. As the MIC values for itraconazole and voriconazole for C286 were higher (≥ 8 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$, respectively) than the German isolate (4 $\mu\text{g/ml}$ and 0.25 $\mu\text{g/ml}$, respectively), C286 was therefore chosen as the best candidate for the BSA analysis. A summary of results obtained from sequence analysis of *cyp51A* and its promoter for all isolates used in this analysis is provided in **Table 4.2**.

Finally, sequence alignment of *hapE* and *cox10* gene sequence from isolate C286 against the sensitive control strain Af293 revealed that both had an identical DNA sequence i.e. there were no changes within the codon region that might explain the occurrence of azole resistance in this isolate (data not shown).

Table 4-2: List of *A. fumigatus* isolates with additional information concerning the E-test MIC values determined using two azoles' drugs (itraconazole and voriconazole) and the sequence analysis results from various test genes including *cyp51A*. MIC values were determined after 48 h incubation according to the E-test method.

Isolates ID	Provider	Source	MICs Values (µg/ml)		Sequence details
			ITRA	VORI	
47-312	Doctoral student at Nottingham (George Ashton)	Environmental	2	0.38	WT
C128, C136, C137, 169, 19, 158, 80, 82, 32	Imperial College London (This set of isolates have already been used in Chapter 3)	Clinical	Details provided in Chapter 3		<i>cyp51A</i> /TR ₁₃₀ except isolate 32
C127	Imperial College London	Clinical	24	≥ 2	<i>cyp51A</i> /D430G
C129		Clinical	0.38	0.094	
C138		Clinical	≥ 32	≥ 2	<i>cyp51A</i> /P216L
C139		Clinical	0.50	0.094	
C117		Clinical	≥ 32	≥ 32	<i>cyp51A</i> /TR ₄₆
C118		Clinical	≥ 32	≥ 32	<i>cyp51A</i> /TR ₄₆
C286		Environmental	≥8	2	
VIII	Radboud Medical University, Netherlands	Clinical	0.38	0.094	
V138		Clinical	≥ 2	≥ 32	<i>cyp51A</i> /TR ₄₆
V144		Clinical	0.38	0.094	
V167		Clinical	0.38	0.75	
V186		Clinical	0.38	0.094	
20	Leibniz Institute for Natural Product Research, Jena, Germany)	Environmental	1	0.38	
56		Environmental	1	0.38	
106		Environmental	0.50	0.125	
176		Environmental	2	0.50	
313		Environmental	2	0.50	
439		Environmental	32	1	<i>cyp51A</i> /TR ₃₄
486		Environmental	≥ 2	1	
570		Environmental	0.75	0.125	
675		Environmental	0.50	0.125	
684		Environmental	4	0.25	

4.3.2 Preparation for BSA

4.3.2.1 Sexual crossing, determination of mating type and screening for azole resistance

The multiplex mating-type diagnostics of (Paoletti *et al.*, 2005) revealed that the Imperial azole resistant isolate C286 was of the *MAT1-2* genotype. Crosses were therefore set up with the azole sensitive supermater strain 47-267 (Swilaiman *et al.*, 2020) of the opposite *MAT1-1* mating type. Ascospore progeny (40) were collected after four months incubation and examined for itraconazole susceptibility using an itraconazole E-test. Results showed a clear 1:1 ratio of resistant (20) to sensitive (20) progeny i.e. providing evidence that C286 exhibits monogenic resistance to itraconazole. The result was confirmed using another cross with different azole sensitive supermater partner (47-51) which again showed a similar 1:1 pattern of inheritance, with the susceptibility phenotype stable in both the resistant and sensitive groups of progeny (data not shown).

In preparation for the first backcrossing, it was necessary to determine the mating type of the collected progeny using the multiplex mating-type PCR diagnostic. Although the PCR sometimes produced multiple products for each isolate, the strongest band was always observed where the predicted *MAT* products would be located (see **Figure 4.5**).

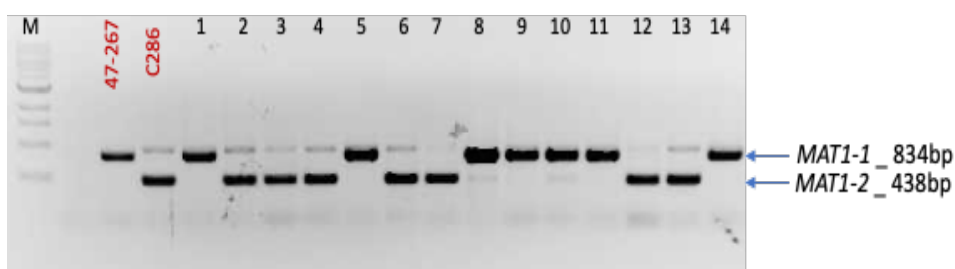


Figure 4-5: Representative results of a multiplex PCR assay to determine the mating type of the parental strains and progeny of *A. fumigatus* used for the BSA work. Amplicon with the expected size 834 bp for *MAT1-1* gene was observed in the PCR products of 47-267 and in lanes 1, 5, 8, 9, 10, 11 and 14 using gDNA of some progeny. The remaining lanes show PCR products of 438 bp in size representing *MAT1-2* gene as seen in the parental C286 product and products in lane 2, 3, 4, 6, 7, 12, 13. Products were run alongside 1kp ladder (M) in 1% agarose gel.

Around five azole resistant progeny of the *MAT1-2* genotype were selected for backcrossing with the parental azole sensitive isolate 47-267, building in some

redundancy of crosses to try and ensure at least some crossing success. After the first successful backcross, 40 progeny were again collected, and the azole susceptibility phenotype was tested using E-test, and progeny mating type determined to allow setting up of the second backcross. A similar procedure was followed for the third round of backcrossing. Around 200 progeny were collected from this third and final backcross and the resistant phenotype was tested using a primary resistant screening whereby progeny were cultured on ACM plate and then transferred to ACM supplemented with 8 µg/ml itraconazole. It was easier to screen around 32 progeny per plate in this one-step system, to distinguish between the resistant and the sensitive progeny, than the more time-consuming E-test methodology. This also helped to ensure that a correct 1:1 segregation pattern of sensitive to resistant phenotype was seen in the progeny, confirming that the resistant trait is linked to a single gene (**Figure 4.6**).

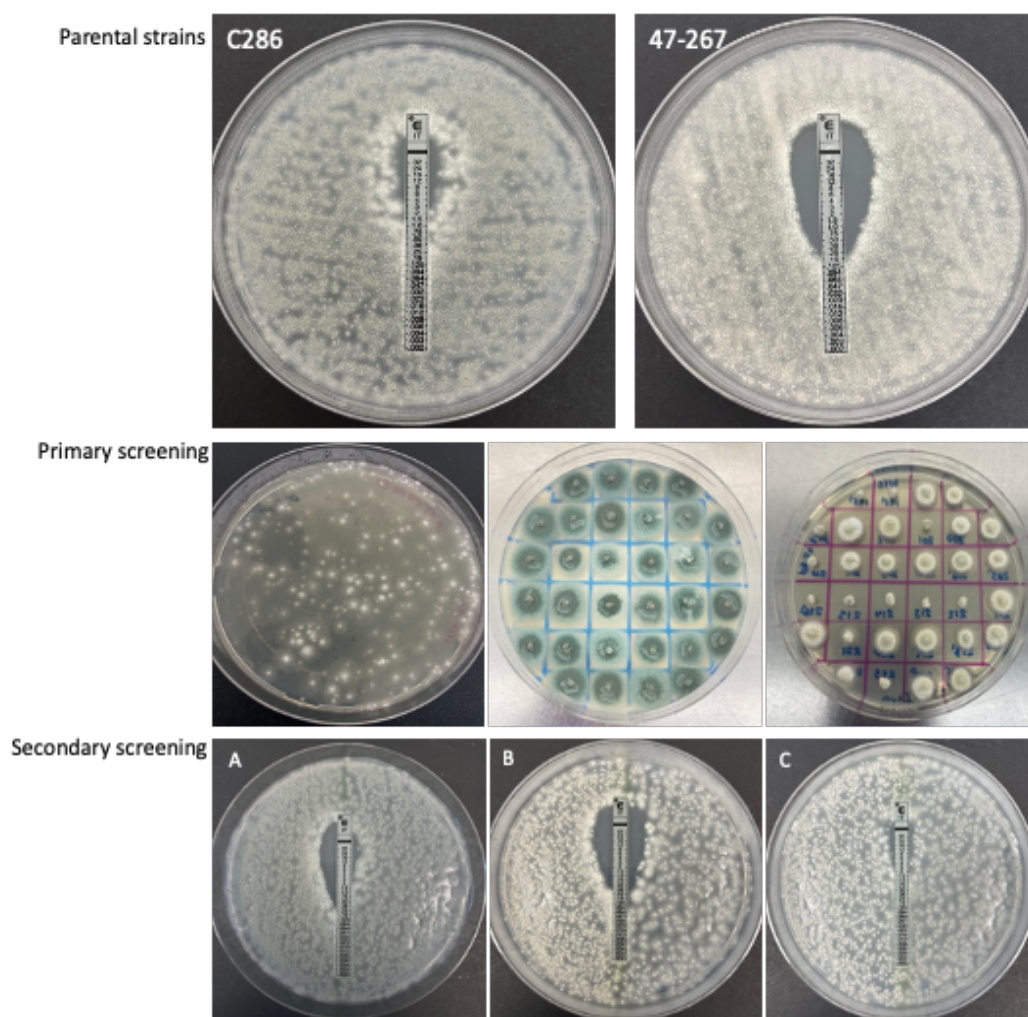


Figure 4-6: Representative images of itraconazole resistant screening of *A. fumigatus* isolates. Top plates show the E-tests to determine itraconazole MIC for parental strains used in the BSA work; itraconazole resistant strain C286 which was able to grow around the higher concentration in the top of the strip with MICs value of $\geq 8\mu\text{g/ml}$, itraconazole sensitive strain 47-267 which stop growing at concentration of $0.25\mu\text{g/ml}$. The second row of plates represent the early screening of progeny, where the first plate show ascospores growing on MEA and ready to be collected. The plate on the middle show progeny after being transferred to ACM and grow for 24 h at 37°C . The last plate showing the same set of progeny growing on ACM supplemented with $8\mu\text{g/ml}$ itraconazole demonstrating that some showed inhibition of growth in the presence of itraconazole (sensitive progeny) and some were able to grow (resistant progeny). Samples of the progeny were screened again using itraconazole E-test as shown in the last row for the secondary screening where each plate contain two progeny inoculated side-by-side either side of the E-test strip. (A) Show plate with sensitive progeny with inhibited growth zone (right side of the plate) resistant progeny (left side of the plate). (B) Show plate with two sensitive progeny with inhibited zone around the strip in each side. (C) Show plate with two resistant progeny showing full growth around the strip in each side.

4.3.3 Bulk Segregant Analysis

Triplicate DNA samples from the azole resistant C286 and sensitive 47-267 parents, and azole resistant and sensitive progeny pools were submitted for genome sequencing in February 2022. Unfortunately, due to delays in the bioinformatic analysis at Edinburgh the sequence data and bioinformatic analysis was only returned in the final month of the present thesis submission. Therefore, only a preliminary analysis of data was possible as will now be described.

The BSA comparison of genomic data from the resistant parent and progeny pool versus the sensitive parent and progeny pool revealed 636 SNPs and 57 INDELS (insertion/ deletion) that differed between the two groups (**Tables 4.3 and 4.4**). This number was higher but comparable to the number of SNPs reported in the study of Ashton *et al.* (2022) who found 170-561 SNPs between the resistant and sensitive groupings depending on the number of backcrosses and size of progeny pools used. We were therefore anticipating to observe a similar result as that of Ashton *et al.* (2022) whereby SNPs would be clustered in one linked region that would contain the candidate gene(s) or other mutations of interest (**Figure 4.7**). However, and very surprisingly, the SNP locations were found to be dispersed throughout the genome with no clear single region. Instead there were multiple regions with clusters of variants present (**Figure 4.8**). There was one region on chromosome DS499598 showing a particularly high number of variants (151 variants), which offered some promise (**Figure 4.8.A and Table 4.3**). However, there was also clustering of SNPs evident elsewhere, such as chromosome DS499594 with a single high peak at position ca. 4,000,000 showing an especially high number of variants per 100 kb (**Figure 4.8.B and Table 4.3**). Moreover, by looking at the INDEL data, similar to the SNP data this did not reveal any single region but rather INDELs were spread throughout the chromosomes (**Table 4.4**). There were nevertheless again some ‘more clustered’ regions and these generally matched the same regions as the SNP data e.g. DS499598 showed the highest number of variants. Due to the late arrival of sequence data no further analysis was possible in this thesis.

Table 4-3: Summary of SNPs variant rate by chromosome according to BSA of progeny from cross 47-267 x C286.

Chromosome	Length	Variants	Variants rate
DS499594	4,896,001	51	96,000
DS499595	4,759,548	63	75,548
DS499596	4,263,309	45	94,740
DS499597	3,791,214	39	97,210
DS499598	2,613,036	151	17,304
DS499599	2,209,696	32	69,053
DS499600	1,927,435	60	32,123
DS499601	1,844,026	34	54,236
DS499602	1,571,256	10	157,125
DS499603	656,735	24	27,363
DS499604	482,145	66	7,305
DS499606	17,043	25	681
DS499611	8,530	1	8,530
DS499614	4,622	8	577
DS499616	3,974	12	331
DS499617	3,954	3	1,318
DS499622	3,157	4	789
DS499623	3,003	3	1,001
DS499630	1,526	1	1,526
DS499636	1,307	3	435
DS499644	1,161	1	1,161
Total	29,062,678	636	45,696

Table 4-4: Summary of INDELs variant rate by chromosome according to BSA of progeny from cross 47-267 x C286.

Chromosome	Length	Variants	Variants rate
DS499594	4,896,001	6	816,000
DS499595	4,759,548	3	1,586,516
DS499596	4,263,309	4	1,065,827
DS499597	3,791,214	3	1,263,738
DS499598	2,613,036	23	113,610
DS499599	2,209,696	5	441,939
DS499600	1,927,435	5	385,487
DS499602	1,571,256	1	1,571,256
DS499603	656,735	3	218,911
DS499606	17,043	2	8,521
DS499616	3,974	1	3,974
DS499620	3,415	1	3,415
Total	26,712,662	57	468,643

4.4 Discussion

There have been many reports of azole resistant isolates of *A. fumigatus* which supposedly do not show a *cyp51A*-based resistance mechanism (Fraczek *et al.*, 2013; Rivero-Menendez *et al.*, 2016; Hagiwara *et al.*, 2018; Sharma *et al.*, 2019; Furukawa *et al.*, 2020). It was therefore of much interest to try to gain information about the genetic basis of such ‘unknown’ forms of azole resistance. Notably, if many of these ‘unknowns’ shared a common mechanism which could be identified then it might be possible to design a molecular diagnostic(s) to monitor the presence of such resistant isolates, of great potential use in disease management. To this end 32 isolates of *A. fumigatus* with supposed non-*cyp51A* based resistance to azoles were examined in the present study, being obtained from a range of different research groups in Europe.

4.4.1 Isolate Screening and Identification of Candidates for BSA Studies

4.4.1.1 Screening for Resistant Isolates

There is no standardised method for antifungal susceptibility testing of filamentous fungus, despite the fact that a broth microdilution adaption of the NCCLS reference method for yeasts has demonstrated acceptable intra-laboratory and interlaboratory reliability (Dannaoui *et al.*, 2001). As discussed earlier (see **Chapter 3**) the E-test method is argued to provide a reliable and accurate method to determine the azole resistance MIC level of isolates of *A. fumigatus*. This method was therefore used to screen all 32 test isolates with two different azole antifungal drugs, namely itraconazole (ITZ) and voriconazole (VOR). It was found that the MIC values for 12 of the isolates was lower than 2 µg/ml in the case of itraconazole and lower than 1 µg/ml against voriconazole, which are the recommended break points for *A. fumigatus* isolates to be considered as resistant isolates. Therefore, these isolates were deemed sensitive and they no longer exhibited the supposed resistance phenotype despite being described as highly resistant by our collaborators who supplied the strains. Furthermore, the ITZ MIC value for a further four isolates was only around 2 µg/ml, which is not deemed to be highly resistant, which requires itraconazole MICs of >8 µg/ml (Alcazar-Fuoli *et al.*, 2006).

Hence, 16 of these isolates (i.e. 50%) were excluded from any further analysis. Importantly this indicated that many supposed ‘azole resistant’ isolates of *A. fumigatus* might only exhibit transient high azole resistance and that the phenotype can be lost during subsequent sub-culture. This suggests some form of transient gene expression and/or epigenetic change rather than a stable mutational change as seen in the *cyp51A*-based mutations (both in the coding and promoter regions) which lead to stable, high level resistance to azoles. The MIC values for the remaining 16 isolates were higher than the clinical breakpoints for itraconazole and voriconazole and these were therefore further investigated.

4.4.1.2 Checking for Known Azole Resistance Mechanisms

Most studies of azole resistance in *A. fumigatus* have reported that the primary mechanism of resistance is due to specific point mutation in the *cyp51A* gene coding region or a duplication in its promoter region (Snelders *et al.*, 2011). More rarely, other studies have reported a point mutation in *hapE* or *cox10* genes, which is also associated with the resistance phenotype (Camps *et al.*, 2012c; Wei *et al.*, 2017). Therefore, it was necessary to check for such known mechanisms in our resistant isolates, despite the fact that the isolates supplied by the collaborators had supposedly already been examined for the presence of the many of the same mutations i.e. these checks were undertaken for our own certainty, especially given the experience of Ashton *et al.* (2022).

Sequence analysis of the remaining highly resistant 16 isolates showed that six of the isolates contained mutations in the *cyp51A* gene already known to cause elevated resistance to azole antifungals. This firstly comprised two isolates (C127 and C138) with point mutations in the coding region of *cyp51A*. The presence of point mutations within the coding region of *cyp51A* has been reported to induce changes in the channels that allow access of azole antifungals to the heme molecule binding target site. Thus, the C138 strain had a known P216L mutation which has been found to be associated with the resistant phenotypes because the 216 amino acid is located close to the opening of the ligand access channel, which appears to affect the docking of azole molecules (Camps *et al.*, 2012a). Meanwhile, the other four isolates all showed TR mutations in the promoter region of *cyp51A* that have already been shown to be linked to azole resistance. Three isolates (C117, C118, V138) exhibited a TR₄₆ in the promoter region and one isolate (439) included a TR₃₄ present in the promoter region of *cyp51A*.

Although disappointing, these findings were again of importance as they showed that even highly reputable research groups might by accident incorrectly analyse isolates of *A. fumigatus* and state that they do not have *cyp51A*-based resistance mechanisms, when in fact this is not the case. Indeed, the present experience replicates that of Ashton *et al.* (2020).

Of the remaining ten isolates, eight were subsequently found to contain the novel TR₁₃₀ mutation as presented in **Chapter 3**. Thus, screening of the 32 ‘unknown’ isolates did have at least one very valuable outcome. This left only two remaining isolates with a truly ‘unknown’ mechanism(s) of azole resistance. The environmental isolate C286 showing the highest level of resistance to itraconazole was chosen as the best candidate for further sexual crossing and BSA work.

4.4.2 Sexual Crossing and Bulk Segregant Analysis

Crossing between the *MAT1-2* azole resistant isolate C286 and two independent *MAT1-1* azole sensitive isolates revealed a clear 1:1 segregation for resistance and sensitivity in the offspring, consistent with monogenic inheritance (Ashton and Dyer, 2016). This meant that the azole resistance genetic trait in C286 was ideal for investigation via bulk segregant analysis as the next step. Huge efforts were thus made over a two year period to choose the best azole resistant candidate for the BSA work, undertake the long process of serial backcrosses, screen progeny for the resistance phenotype, extract gDNA from over 200 progeny to obtain the progeny pools etc. The hope was to identify a single region of the genome that as linked to the azole resistance trait, and then examine this region for candidate gene(s) or other mutations of interest. However, although attempts were made to increase the resolution of the BSA and to minimize the variation by increasing the number of progeny to 100 in each pool, the primary BSA output showed a distribution of SNPs linked to resistance throughout the whole genome instead of being clustered in a single region as seen in the previous study (Ashton *et al.*, 2022). Although some clustered regions could be identified, further analysis was not possible for this thesis due to lack of time. Various hypothesis can be suggested as to why the results showed a different pattern compared to the similar work of (Ashton *et al.*, 2022). Some possible explanations are listed below:

- (a) It is possible that this phenotype has a polygenic basis where multiple regions/genes might be involved in conferring the resistant phenotype in this isolate. And yet crossing work showed a clear monogenic basis, hence a very unlikely explanation.
- (b) There has been insufficient crossover/recombination occurring to break up the other markers that BSA relies on. However, high progeny numbers were used in each pool (100 in each resistant vs sensitive) and three rounds of backcrossing, which was more than enough to yield only a single region with multiple SNPs in the study of (Ashton *et al.*, 2022). Hence again an unlikely explanation, although conceivable if a low rate of recombination in this particular cross.
- (c) There has been some error in implementation of the BSA bioinformatic workflow given that the bioinformatic analysis was done by a different bioinformatic team than the original Nottingham Deep Seq group. Although again unlikely because most of the information of the analysis pipeline was discussed with the team and more clarification was provided by Nottingham team and shared with the Edinburgh group, but this must be considered as a possible factor. For example, in the case of the Nottingham workflow the main output were figures showing the positions of the individual ca. 500 SNPs that had been found. By contrast, the Edinburgh output provided figures showing variants per 10-100 kb along the chromosome position i.e. a more sliding window approach rather than showing the individual SNPs.
- (d) There is an outside chance that the mechanism of resistance is due to a zero-coverage region present in either the resistant or sensitive groupings. Investigation of such zero-coverage regions was included in the Edinburgh analysis. However, limited time availability prevented us looking into these data.

Although the BSA study has so far failed to identify the causal gene/mechanism in isolate C286, it is at least possible to speculate about the possible basis of resistance. For example, in *Candida* spp., most reports of azole drug resistance have been linked with greater efflux of azole drugs as a result of the overexpression of various kinds of efflux pumps (Cowen *et al.*, 2002; Sanglard and Odds, 2002; Prasad and Kapoor, 2005). By contrast, previous reports of azole drug resistance in *A. fumigatus* have generally

been attributed to changes in the target enzyme (*cyp51A*) both for laboratory mutants and also for clinical strains. Despite the fact that analysis of the *A. fumigatus* genome has revealed the presence of over 40 ATP binding cassette transporter (ABC) homologs as well as over 100 major facilitator transporter genes (five times greater than the number in yeast) (Tekaia and Latge, 2005), there have only been a very limited number of descriptions of isolates which overexpress efflux pumps as an azole resistance mechanism (Mellado *et al.*, 2007). Indeed, these have almost all been for *A. fumigatus* mutant strains created in the laboratory rather than natural clinical or environmental isolates (Nascimento *et al.*, 2003). Just one clinical strain (strain AF72) has been found that overexpresses the *atrF* gene (by five times), which encodes an ABC transporter efflux pump. And this overexpression only occurred when it is grown in the presence of itraconazole (Slaven *et al.*, 2002).

Other mechanisms of resistance to azole antifungals include gene or even whole chromosome duplication, a process referred to as gene amplification in mammals (Sionov *et al.*, 2013). The former mechanism has been observed in certain *A. nidulans* triazole resistant strains where an increase in *cyp51* copy number in the host genome, or introduction of a high-copy number plasmid with the *cyp51* coding sequence, has been shown to result in elevated resistance to azole antifungals (Oshero *et al.*, 2001). This mechanism has been observed in *C. glabrata* (Marichal *et al.*, 1997) and also in engineered laboratory strains of *A. niger* (Van Den Brink *et al.*, 1996).

4.5 Conclusions and Future Direction

Of the 32 supposed azole resistant strains of *A. fumigatus* obtained from collaborators for study with non-*cyp51A* forms of resistance, unfortunately around 93% of isolates were eliminated as they showed only low levels of azole resistance or exhibited known mutations in *cyp51A* conferring azole resistance. Only two isolates had truly unknown resistant mechanism(s) and the one (C286) that showed the highest resistant level was chosen for BSA work. We had anticipated to identify a single linked region from the sequencing analysis. However, this was not the case. Given that this is a potentially very informative isolate as it was obtained from an environmental rather than clinical source, and that the result presented in this chapter are only preliminary,

future work is highly recommended to discover the genetic basis of azole resistant in this isolate. Possible future directions for research might include the following:

- 1- Given the possibility of a polygenic trait, it is essential to go back to the F1 progeny as well as the various progeny collected from each backcross and screen all of them again for the resistance phenotype.
- 2- It might be necessary to do further rounds of backcrossing and use techniques such as random amplification of polymorphic DNA(RAPD) PCR (Murtagh *et al.*, 1999) to ensure that sufficient recombination had occurred within collected progeny prior to making pools of resistant and sensitive progeny.
- 3- The available sequence information might need further analysis using a different workflow such as a sliding window analysis to narrow the variations. Indeed, there is other published work where fungal NGS data has been analysed used a sliding window approach to systematically move across the whole genome looking for positions where the BSA sequence data from the resistant versus sensitive groupings showed high divergence. For example, Pomraning *et al.* (2011); Heller *et al.* (2016); Heller *et al.* (2018); Gonçalves *et al.* (2019) were all able to successfully identify regions of the genome with a target gene of interest in studies with *Sordaria* and *Neurospora* spp.
- 4- Further investigation of the the genes and regions effected by the 636 SNPs identified is required, as it is possible that these include a clear candidate causal gene e.g. a transport pump or regulator in the ergosterol biosynthesis pathway.
- 5- Given that whole genome sequence data is now available for the azole resistant parent and progeny, and the sensitive parent and progeny pools, it is possible to repeat the BSA here in Nottingham with the same team who performed the previous bioinformatic analysis presented in (Ashton *et al.*, 2022).

Chapter- 5 Exploitation of Sexual Reproduction to Analyse Pathogenicity Traits and the Population Biology of *Aspergillus fumigatus*

Work in this chapter aims to exploit the newly discovered sexual cycle of *A. fumigatus* (O’gorman *et al.*, 2008), to try and gain insights into the evolution of pathogenicity and population biology of the species. These two discrete areas were investigated separately and therefore this chapter will be divided into two parts. Each part will contain its own introduction, aims, methods, results, and discussion.

5.1 Part 1: *Aspergillus fumigatus* Response to Hypoxia and Reactive Oxygen Species (ROS)

5.1.1 Introduction:

Aspergillus fumigatus is an opportunistic fungal pathogen that can cause severe disease in humans, particularly in the respiratory system. The species is responsible for >90% of all invasive aspergillosis infections, showing 50–95% mortality rates in immunocompromised patients (Abad *et al.*, 2010). The particular symptoms of *Aspergillus* infection are influenced by host immunity and the existence of underlying pulmonary conditions. Invasive or allergic fungal diseases may develop due to *Aspergillus* colonization of the pulmonary environment. The goal of medical treatments is to reduce symptoms and avoid disease progression. However, this can be difficult due to the restricted availability of classes of antifungal medication and the possibility of increased disease progression when using immunosuppressive drugs. Lung colonization is a crucial first step in the progression of many *Aspergillus* disorders (Engel *et al.*, 2020).

Many studies have shown that *A. fumigatus* has a multifactorial basis of resistance to the immune system, relying for example on the structure of the invasive propagules, evasion of the immune system, high growth capacity and metabolic flexibility, oxidative

stress adaptation, and ability to cause damage to the host to increase its virulence (Abad *et al.*, 2010). It is known that *A. fumigatus* produces detoxifying antioxidants that can engage with several stress response pathways (Brown and Goldman, 2016).

In particular, several studies have found that the pathogenicity of a given isolate of *A. fumigatus* is often correlated with its ability to live in a low oxygen environment (Kowalski *et al.*, 2016; Kowalski *et al.*, 2019; Kowalski and Cramer, 2020). Kowalski *et al.* (2016) found that under normal and low oxygen conditions, the commonly used laboratory strains AF293 and CEA10, which differ significantly in virulence, show noticeably divergent growth characteristics in liquid culture. Compared to AF293, CEA10 has higher hypoxia fitness at <0.2% oxygen.

Moreover, changes in *A. fumigatus* colony morphology can reflect underlying changes in cell morphology, which are often caused by genetic and environmental factors. Certain clinical (patient-derived) strains of *A. fumigatus* have been shown to exhibit colony morphology changes under hypoxic conditions that have been associated with worsened clinical outcomes (Kowalski and Cramer, 2020). Kowalski *et al.* (2016) identified a significant association between *A. fumigatus* virulence and fitness under low oxygen *in vitro*. Furthermore, Kowalski *et al.* (2019) identified two distinct morphological features of colonies of certain strains of *A. fumigatus*, namely increased colony furrowing and percentage of vegetative (white, non-conidiating) mycelia, which was associated with growth under low oxygen conditions. This was referred to as the hypoxia-locked morphotype (H-MORPH). H-MORPH strains were shown to cause extensive fungal lesions in a mouse model *in vivo*, accompanied by severe inflammation and enhanced pathogenicity (Kowalski *et al.*, 2019; Kowalski and Cramer, 2020). Therefore, when designing treatment plans or identifying novel intervention targets in colonized individuals, an understanding of in-host fungal adaptability and interaction may be useful in averting the development of *Aspergillus* disease and the resulting lung damage (Engel *et al.*, 2020).

5.1.1.1 Reactive Oxygen Species (ROS): Sources and Role Against Fungal Infection

Reactive oxygen species (ROS) are by-products of normal cellular functions involving oxygen metabolism. While these by-products are called oxygen free radicals,

such as hydroxyl radical ($\text{OH}^\cdot$) and superoxide anion ($\text{O}_2^\cdot$), they also include certain non-radicals, such as hydrogen peroxide (H_2O_2), that are categorized as oxidizing agents, and some non-radicals that can easily be converted into radicals (Halliwell, 2006; Aguirre *et al.*, 2006). The progressive addition of electrons to molecular oxygen results in the formation of $\text{O}_2^\cdot$, H_2O_2 , and $\text{OH}^\cdot$. While routine by-products such as H_2O_2 and $\text{O}_2^\cdot$ are not highly reactive, the potential free radical damage risk is significantly greater with $\text{OH}^\cdot$ (Phaniendra *et al.*, 2015). The main source of ROS production is electron leakage out of the respiratory chain as part of the molecular reduction of oxygen to water, creating $\text{O}_2^\cdot$. Additional ROS sources include microsomal metabolism and the final stage of the phagocytic process involved with killing intruding microorganisms, called a respiratory burst (Temple *et al.*, 2005).

Among other factors, ROS production increases in host cells exposed to pathogens as a result of cellular processes and ROS enzyme-mediated processes. Neutrophils, macrophages, and other phagocytic cells often play a significant role in ROS production which are toxic to most fungal pathogens (Brown *et al.*, 2009; Brown and Goldman, 2016). This toxicity is usually the result of host-mediated responses to fungal infection by microorganisms such as *A. fumigatus*. Following infection, oxidative stress to *A. fumigatus* occurs due to ROS production from the host defence system leading to cell injury, altered gene expression, lipid peroxidation, and post-translational protein modifications (Warris and Ballou, 2019).

ROS created by phagocytes have been assumed to play a fundamental role in killing microbes. However, some *A. fumigatus* strains appear to show partial resistance to ROS, which might lead to the ability to adapt to oxidative stress within the host, with serious consequences for disease control (Van De Veerdonk *et al.*, 2017). Consequently, molecules and proteins associated with ROS resistance have been considered putative fungal virulence factors (Lambou *et al.*, 2010). Indeed, the fungus' ability to adapt to oxidative stress in some cases, and the possible occurrence of drug resistance, make fungal infections even more dangerous. Furthermore, an interplay between various factors can increase the survival chances of *A. fumigatus* in the host. For example, the fungus can produce various toxins that can aid microbial resistance by damaging host cells and reducing the immune response (Brown *et al.*, 2009; Abad *et al.*, 2010; Brown and Goldman, 2016).

5.1.1.2 Menadione as ROS Regenerating Agent Through Redox Cycling

Cellular oxygen reduction processes create O_2^- via the loss of one electron and leakage from the mitochondrial electron transport chain (Halliwell, 2006). While the O_2^- is not highly reactive, it can act as a precursor for other ROS through its conversion to H_2O_2 via dismutation and the creation of highly reactive OH^- as a byproduct of metal-catalysed reactions. O_2^- production can be greatly enhanced by redox-cycling drugs such as paraquat and especially menadione since they enhance the transfer of electrons to molecular oxygen (Morano *et al.*, 2012). Because of this reactive characteristic, menadione is often used as an O_2^- inducer in studies of oxidative stress responses and ROS metabolic processes. However, the precise mechanism by which menadione mediates cellular O_2^- generation remains unclear, an uncertainty that also applies to the complex mechanisms by which cells respond to ROS (Zhou *et al.*, 2021).

Menadione, which has the formal chemical nomenclature 2-methyl-1,4-naphthoquinone, is derived synthetically from vitamin K1 and is also known as vitamin K3. Its effects include enhancing apoptosis (cell death) in various tumour cells. However, the process by which menadione is reduced in the presence of enzymes such as microsomal nicotinamide adenine dinucleotide (NADH) phosphate (NADPH)-cytochrome P450 reductase and mitochondrial NADH-ubiquinone oxidoreductase, producing unstable semiquinone, remains unclear. This chemical conversion creates a negative environment that is further damaging through hydroquinone oxidation in the presence of oxygen, resulting in the production of the ROS radical O_2^- (Kawamura *et al.*, 2006). Because NADPH is a key mitochondrial enzyme, the rapid reaction involved in the redox cycle can quickly deplete the mitochondria of nitrite reductase, disrupting its use in other essential cell functions, resulting in the cell becoming unstable and dying (Zhou *et al.*, 2021). However, the two-electron NADPH process involving quinone oxidoreductase significantly limits ROS by-product production, which tends to be greater with one-electron chemical processes via redox cycling (Criddle *et al.*, 2006).

Menadione can be used as a ROS generating/regenerating agent to mimic ROS activity and examine oxidative stress *in vitro* (Criddle *et al.*, 2006). Menadione, at appropriate levels, can serve as a redox-active agent, and at high levels, it can harm cells by inducing toxic oxidant stress. ROS stress associated with menadione is associated with depolarization and morphological fragmentation of mitochondria (Loor *et al.*,

2010). In the context of *A. fumigatus*, increased menadione levels have been shown to destroy the pathogen due to oxidant stress (Hartmann *et al.*, 2011).

5.1.1.3 Resistance Mechanisms to Oxidative Stress in *Aspergillus fumigatus*

Melanin is a key factor in the protective system used by *A. fumigatus* against the host's immune system. In addition to offering protection from UV light, facilitating survival on and around the host, melanin masks the fungal pathogen to many immune system components and serves as a reducer of the potential oxidative stress damage caused by the immune system response. Given that melanin offers *A. fumigatus* these advantages, disruptions to melanin biosynthesis in the fungus enhances the potential for treating the invasive pathogen (Sugui *et al.*, 2008; Brown and Goldman, 2016). Although previously the green pigment 1,8-dihydroxynaphthalene (DHN)-melanin thought to play key role in shielding *A. fumigatus* conidia against peroxide, which was based on the observation that the ATCC 46645 mutant strain, which has mutations in the *pksP* gene of the DHN-melanin production pathway, is more ROS sensitive than the wild type. However, recent report has shown that deletion of the *pksP* gene in *A. fumigatus* strain CEA10 did not affect sensitivity against ROS reagents (H₂O₂ and superoxide) (Keizer *et al.*, 2022). In their work, a mutation in the *cat1* gene was discovered by genome sequencing of the ATCC 46645 *pksP* mutant strain. This change is most likely the result of the UV mutagenesis method used previously, which may account for the enhanced sensitivity to H₂O₂. Therefore, Since DHN-melanin plays no part in the defence against superoxide or H₂O₂ attacks, conidia cannot survive when these ROS attacking them (Keizer *et al.*, 2022).

Other examples of fungal pathogen resistance to ROS effects can be seen in the detoxification response. *A. fumigatus* has been found to possess multiple ROS detoxification enzymes, including five catalases and four superoxide dismutases. This multiplicity helps the fungus react effectively to the variable impacts of multiple members of the oxidative stress response, creating significant redundancy that enhances its functionality and virulence (Brown and Goldman, 2016). However, the absence of catalase A (*catA*) makes it more susceptible to H₂O₂, unlike the absence of catalases 1

(*cat1*) and 2 (*cat2*). However, virulence was unaffected in the absence of *catA* but significantly reduced in the absence of *cat1* and *cat2* (Paris *et al.*, 2003).

A characteristic of *A. fumigatus* superoxide dismutases (sods) is that *sod1* and *sod2* are expressed in its conidia, while *sod3* is expressed in its mycelia. Interestingly, while the genus shows low *sod4* expression, its deletion is lethal (Brown and Goldman, 2016). Moreover, when *sod1* and *sod2* were deleted, the fungus became more sensitive to ROS, and the loss of *sod3* caused minor growth delays at higher temperatures but no significant loss of virulence (Lambou *et al.*, 2010). Therefore, the varying effects of different components of the oxidative stress response implies a high degree of redundancy, with increased potential protective functionality (Brown and Goldman, 2016).

A promising insight that could improve the effectiveness of treating *A. fumigatus* infections is the finding that some strains lacking the transcription factor *SebA* showed increased sensitivity to oxidative stress and heat shock and various growth defects in nutrient-poor environments, similar to the effects created by the immune response (Brown and Goldman, 2016). *SebA* accumulates in the cell nucleus during heat shock and oxidative stress, regulating response genes. Eliminating *SebA* can therefore significantly reduce fungal pathogen virulence by disrupting multiple cellular response pathways (Dinamarco *et al.*, 2012).

5.1.1.4 Establishment and Adaptation of *Aspergillus fumigatus* in Patients with Cystic Fibrosis (CF)

Patients with cystic fibrosis (CF) often have a history of chronic fungal colonization, with isogenic *A. fumigatus* isolates occasionally present in their sputum for extended periods. However, current knowledge and understanding of the pathophysiology of fungal persistence in the lung environment is limited (Engel *et al.*, 2020). Verweij *et al.* (2016b) suggested that *A. fumigatus* can survive by adjusting its biology and genome to the conditions of the human lung. The extensive biochemical adaptability of the fungus is the foundation for physiological alterations. Moreover, its ability to produce genetic variety through spontaneous mutations or recombination, and the following selection of genotypes best suited to its new environment, is the basis for the genomic alterations (Verweij *et al.*, 2016b).

CF is caused by flaws in the human CF transmembrane conductance regulator (hCFTR), leading to a thicker mucus layer covering lung epithelial cells (Carsin *et al.*, 2017). This thicker mucus layer leads to the blockage of mucociliary clearance, an essential natural defence system against inhaled germs and microbes. Studies have shown that decreased hCFTR activity reduces the ability of innate immune cells to destroy microorganisms in the lung effectively. The outcome of these host-microbe interactions is a loss in lung function due to progressive fibrotic tissue damage, recurrent inflammation, and steep oxygen and osmolarity gradients (Ross *et al.*, 2021).

The most frequent filamentous fungal species isolated from CF patients is *A. fumigatus*, with ~60% returning a positive culture for this fungus (Ross *et al.*, 2021). With documented extremes of 1–25% and significantly lower numbers of child compared to adult patients, the incidence rate of allergic bronchopulmonary aspergillosis is 1–2% amongst asthmatic patients and 8–10% among CF patients (Carsin *et al.*, 2017). Therefore, *A. fumigatus* isolates that have colonized the CF lung must be able to tolerate various difficult, stressful conditions such as exposure to low oxygen (hypoxia) and high levels of ROS and inflammation produced by the immune system (Ross, 2022).

Most phenotypic characterization studies of *A. fumigatus* CF isolates have involved determination of susceptibility to antifungal drugs. Longitudinal studies addressing the genotypic and CF-relevant phenotypic traits of persistent *A. fumigatus* isolates are rare. A recent study by Ross *et al.* (2021) described the presence of an ‘AF100’ series of isolates, which were a longitudinal collection of *A. fumigatus* isolates obtained over a 4.5 year period from a single CF patient and studied for their genetic diversity. Two lineages of persistent isolates were identified by whole-genome sequencing and phylogenetic analysis from 29 isolates collected at 12 distinct periods. In this selection of isolates, there was evidence of positive natural selection in one lineage comprised of five persistent isolates. In contrast to non-persistent and reference isolates, isolates from this persistent lineage produced conidia with an increase in osmotic stress resistance and a greatly enhanced growth ability under normoxia (normal oxygen condition) and oxidative stress-inducing culture conditions (Ross *et al.*, 2021). This study found evidence that a particular allele of *pbs2*, a mitogen-activated protein kinase (MAPK) kinase associated with the high-osmolarity glycerol (HOG) MAPK pathway, most likely

evolved *in vivo* during growth in the CF airway might partially contribute to the conidial phenotypes. Furthermore, this particular *pbs2* allele enhanced hyphal osmotic and oxidative stress tolerance under low oxygen conditions at the expense of conidial oxidative stress sensitivity under standard atmospheric oxygen levels (Ross *et al.*, 2021). A better understanding of the pathogenesis and virulence mechanisms of *A. fumigatus* in CF patients would be expected to aid the selection of antifungal medications.

5.1.1.5 Aims

The primary aim of work in this section of the current chapter was to examine the genetic basis of resistance to the ROS-generating agent menadione as a representative of the wider genetic basis of oxidative stress resistance in *A. fumigatus*. This investigation was made possible by the combination of the ability to exploit the sexual cycle of *A. fumigatus* to perform classical genetic analysis (Ashton, 2016; Ashton and Dyer, 2019), together with the setting up of a collaboration with the group of Prof. Robert Cramer (Dartmouth College, USA) who kindly supplied clinical isolates of *A. fumigatus* from ongoing studies such as those of Kowalski *et al.* (2019) and (Ross *et al.*, 2021).

5.1.2 Material and Methods

5.1.2.1 Strains

Six clinical *A. fumigatus* strains isolated from one CF patient were obtained from the collaboration with the Dartmouth research group, USA and used in this chapter. Two strains, AF100-8A (henceforth termed 8A) and AF100-9A (henceforth termed 9A) were found to be more susceptible to oxidative stress than the other four strains supplied, AF100-2A, AF100-3A, AF100-6A and AF100-7A (Robert Cramer personal communication) and were all part of the AF100 series of isolates reported by Ross *et al.* (2021). Robert Cramer and his group generously advised on the media and oxidative stress bioassays used in this study.

5.1.2.2 Genomic DNA (gDNA) Extraction, MAT-typing PCR, and Crossing

It was necessary to extract gDNA and determine the mating type for sexual crossing using the methods described in (Sections 2.1.4 and 2.1.5). The crossing protocol described in (Section 2.1.8) was then used to establish crosses between the new isolate and super-mater isolates as described by (Ashton and Dyer, 2019).

5.1.2.3 Media

The primary media used in the oxidative stress assay was Aspergillus Minimal Media (AMM) supplemented with 30 μ M menadione when required. The AMM used in this assay is a modified version of the usual AMM made with a filtered sterilized glutamine-based nitrogen supply with 1% glucose, salt solution, and trace elements as mentioned in Section 2.1.3. This modified AMM was used because it simplifies distinguishing between strain morphology and hypoxia growth variability and mimics the host's nutrient-poor microenvironment (Kowalski *et al.*, 2016; Ross *et al.*, 2021).

5.1.2.4 Oxidative Stress Bioassay

For each strain, three plates were prepared with AMM as a control, and three plates with AMM supplemented with 30 μ M menadione (Sigma). Conidia were collected from 7-day-old cultures and counted using a haemocytometer. Spore suspensions were then diluted using sterile double-distilled water (ddH₂O) with 0.05% Tween 80 to a

concentration of 1×10^3 in 2 μl (500 spores/ μl). Under sterile conditions, 2 μl of the spore suspension was dripped onto the centre of plates and left until dry. Plates were then sealed with parafilm and incubated at 37 °C with 5% atmospheric CO₂ for 72 hours in the dark in a Sanyo MCO-15AC CO₂ incubator consistent with the normoxia growth conditions of (Kowalski *et al.*, 2016; Ross *et al.*, 2021).

The colony diameter of three technical replicates of each condition per strain was measured after 72 hours of incubation by averaging two perpendicular diameter measurements per plate. To compare isolate susceptibility to the superoxide generating compound menadione, the ratio of colony diameters with (treated) and without (untreated) menadione stress was calculated (T:U ratio). The data were then exported to GraphPad Prism (GraphPad v9.3.1 for Macintosh; San Diego, CA, USA) for further analysis.

5.1.3 Results

5.1.3.1 Determination of mating type—multiplex PCR Assay

Two of the USA clinical isolates were found to be *MAT1-1*, and the remaining four were of the *MAT1-2* genotype (**Figure 5.1**).

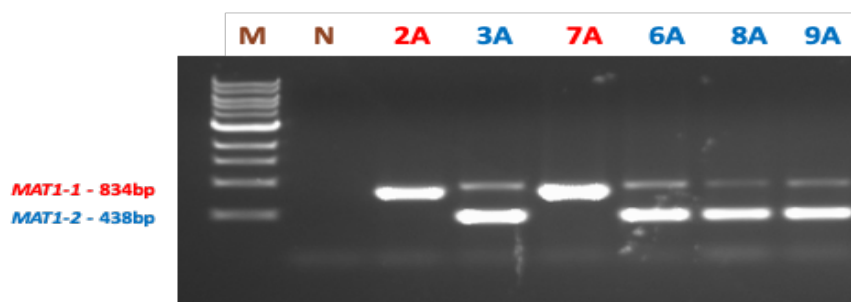


Figure 5-1: Representative multiplex PCR assay results to determine the mating type of *A. fumigatus* isolates. Amplicons with the expected size (834 bp) for MAT1-1 were observed in lanes 2 and 4, representing isolates AF100-2A and AF100-7A. MAT1-2 was observed in lanes 3, 5, 6, and 7 with the expected size (438 bp) representing isolates AF100-3A, AF100-6A, 8A, and 9A. Products were run alongside a 1 kb DNA ladder (M) on a 1% agarose gel.

5.1.3.2 Crossing Results

All crossing attempts were successful. The two *MAT1-1* strains, AF100-2A and AF100-7A, produced cleistothecia with the 47-55 and 47-190 *MAT1-2* super-mater strains. The four *MAT1-2* strains, AF100-3A, AF100-6A, 9A, and 8A, similarly produced cleistothecia with 47-51, 47-59, 47-169, 47-187 and 47-267 *MAT1-1* super-mater strains. While all crosses were successful, only two were used for further analysis in this study, namely 8A crossed with 47-51, and 9A crossed with 47-59.

5.1.3.3 Oxidative Stress Response to Menadione

5.1.3.3.1 *Characterization of the Parental Strain Phenotypes*

Strains 8A, 9A, 47-51 and 47-59 were screened under oxidative stress by incubation on AMM supplemented with 30 μ M menadione at 37°C with 5% CO₂ in the dark. The two strains 9A and 8A had previously been reported to show highly similar phenotypic profiles under CF-relevant stress, such as low oxygen (hypoxic; 1% O₂, 5% CO₂, and 94% N₂) and oxidative stress (Ross *et al.*, 2021). Colonies of the 9A and 8A strains showed distinct morphological features such as the presence of white non-conidiating

mycelium and a markedly increased in number of furrows compared to the ‘normal-looking’ colonies of the control parental strains 47-59 and 47-51 under both control and menadione treatment conditions (**Figures 5.2.A and 5.3.A**). While all strains could grow in the presence of menadione, there was a significant reduction in growth in the presence of 30 μ M menadione compared to untreated conditions for all four strains (**Figures 2.B and 3.B**). However, strains 9A and 8A were more resistant, showing significantly less reduced growth (T:U of 0.59 and 0.58, respectively) than the control 47-59 and 47-51 strains (T:U of 0.44 and 0.43, respectively) i.e. indicating that strains 9A and 8A are relatively more resistant to the oxidative stress generated by menadione (**Figures 2.C and 3.C**).

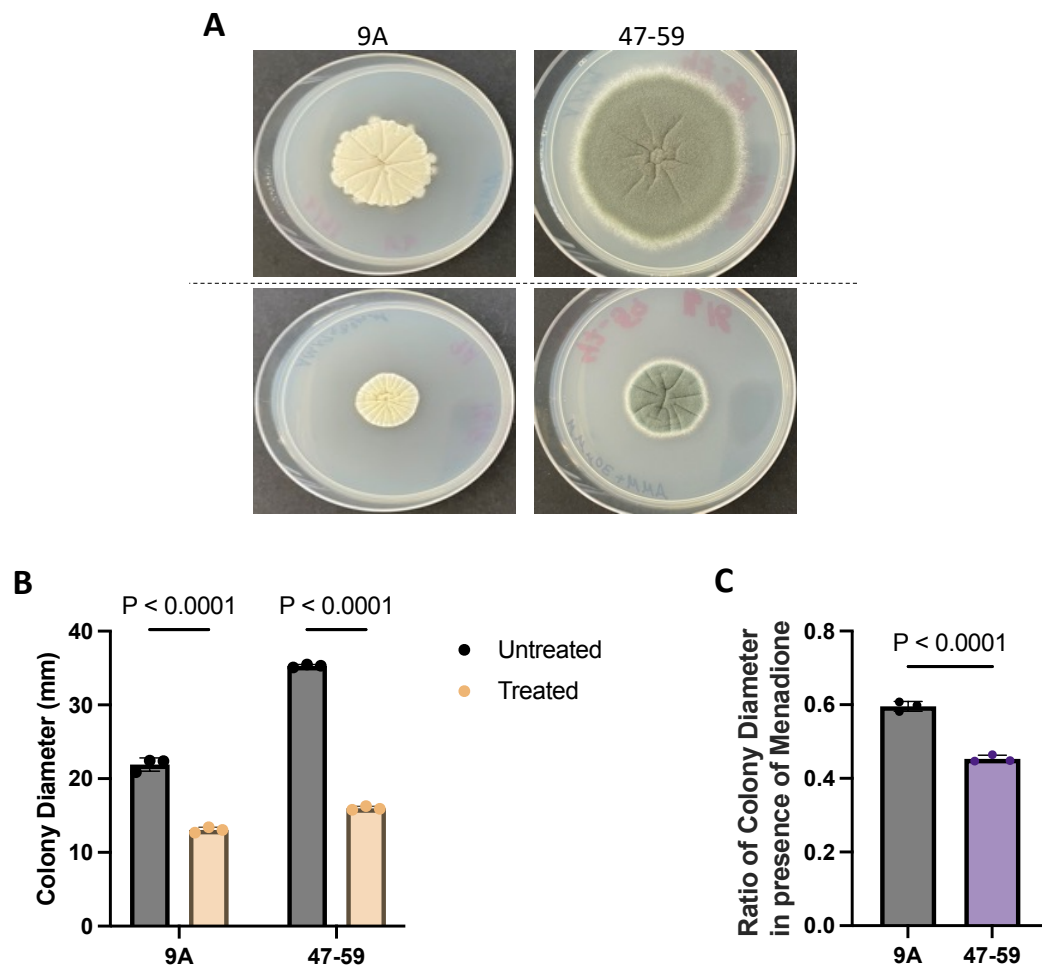


Figure 5-2: Phenotypic characterization of *A. fumigatus* strain 9A and parental strain 47-59 under control and oxidative stress conditions. **(A)** Single colony images highlighting parental strain morphology on AMM alone (top images) and supplemented with 30 μ M menadione (bottom images). **(B)** Colony diameter was calculated using the average perpendicular colony measurement of three technical replicates per condition without (untreated) and with (treated) 30 μ M menadione to induce oxidative stress, showing a significant growth reduction when treated with superoxide generator agent menadione. **(C)** Comparison of T:U ratios of the two parental strains under oxidative stress conditions with 30 μ M menadione shows that 9A has significantly greater growth than 47-59. Error bars represent standard deviations; for colony diameter, calculated per replicate, significance was assessed via two-way ANOVA using the Šidák multiple-comparisons test to compare treated versus untreated for each isolate; for T:U ratios, significance was assessed via one-way ANOVA using the Šidák multiple-comparisons test to compare the two isolates. Created using GraphPad v9.3.1 for Macintosh. Results with $P < 0.05$ were considered statistically significant.

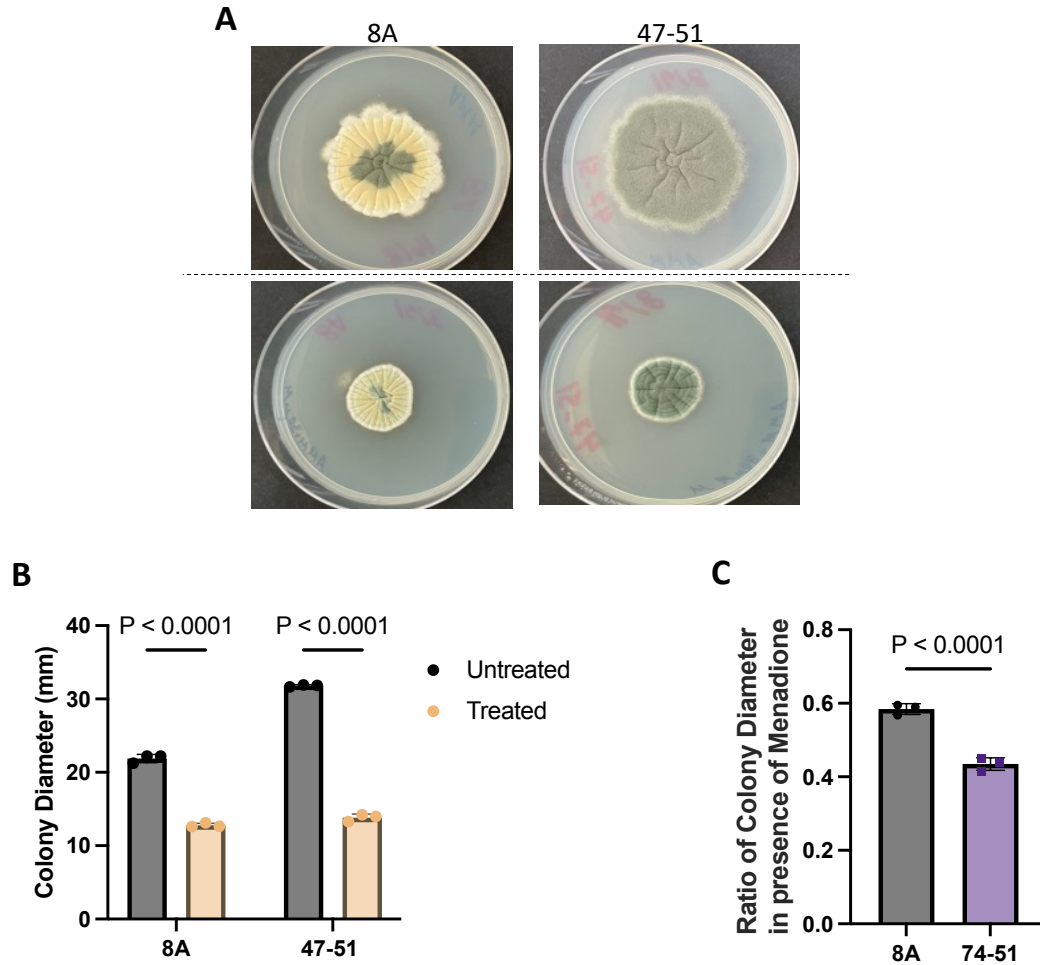


Figure 5-3: Phenotypic characterization of *A. fumigatus* strain 8A and parental strain 47-51 under control and oxidative stress conditions. **(A)** Single colony images highlighting morphology of parental strains on AMM alone (top images) and supplemented with 30 μ M menadione (bottom images). **(B)** Colony diameter was calculated using the average perpendicular colony measurement of three technical replicates per condition without (untreated) or with (treated) 30 μ M menadione to induce oxidative stress shows a significant growth reduction when treated using superoxide generator agent menadione. **(C)** Comparison of T:U ratios of the two parental strains under oxidative stress conditions using 30 μ M menadione shows that 8A has significantly greater growth than 47-51. Error bars represent standard deviations; for colony diameter, calculated per replicate, significance was assessed via two-way ANOVA using the Šidák multiple-comparisons test to compare treated versus untreated for each isolate; for T:U ratios, significance was assessed via one-way ANOVA using the Šidák multiple-comparisons test to compare the two isolates. Created using GraphPad v9.3.1 for Macintosh. All results with $P < 0.05$ were considered statistically significant.

5.1.3.3.2 Characterization of Offspring Phenotypes

While the two strains, 8A and 9A, were previously reported to be more susceptible to oxidative stress than certain control strains (Ross *et al.*, 2021), in our study they appeared more resistant to menadione than the WT parental 47-51 and 47-59 isolates. Therefore, efforts were made to analyse the offspring from crosses between these

isolates. A total of 40 progeny from each of the crosses 9A $\times$ 47-59 and 8A $\times$ 47-51 were collected and screened under oxidative stress conditions with exposure to 30 μ M menadione. Analysis of individual progeny from each cross was performed with progeny identified by a subsequent serial number.

5.1.3.3.2.1 9A $\times$ 47-59 Cross

All progeny from this cross showed a near unique colony morphology with striking variations in colouration and furrow numbers. (**Figure 5.4.A**) shows examples of offspring colony morphology under the control and oxidative stress treatment conditions, with additional images of other offspring provided in **Supplementary 1**. All progeny showed a significantly reduced colony size ($P < 0.0001$) in the presence of 30 μ M menadione except for progeny number 26, indicating that this sexual offspring is highly resistant to menadione (**Figure 5.4.B**). The degree of growth reduction in the presence of menadione was confirmed by the T:U ratio of each progeny (**Figure 5.4.C**). There was a spectrum of menadione resistance in the progeny, ranging from low growth in sensitive progeny (T:U of ~ 0.39), such as progeny number 25, and high growth in the highly resistant progeny 26 (T:U of ~ 0.92). A comparison of T:U ratios using one-way analysis of variance (ANOVA) between parental strains 9A and 47-59 and their offspring showed no significant difference in growth in the presence of menadione for 22 of the offspring compared to 9A, indicating they had similar menadione resistance levels to 9A with a T:U ratio range of 0.56–0.70. Moreover, six of the progenies showed no significant difference in the growth in the presence of menadione compared to 47-59, indicating they had similar menadione resistance levels to this parental strain with a T:U ratio range of 0.39–0.55. By contrast, the other progeny were more resistant to menadione with a T:U ratio greater than both parental strains. Thus, results showed a continuous variation in the oxidative stress response for all progenies with possibly three different levels of resistance evident: low as seen in the 47-59 parent, medium as seen in the 9A parent, and high, which was only seen in the sexual offspring.

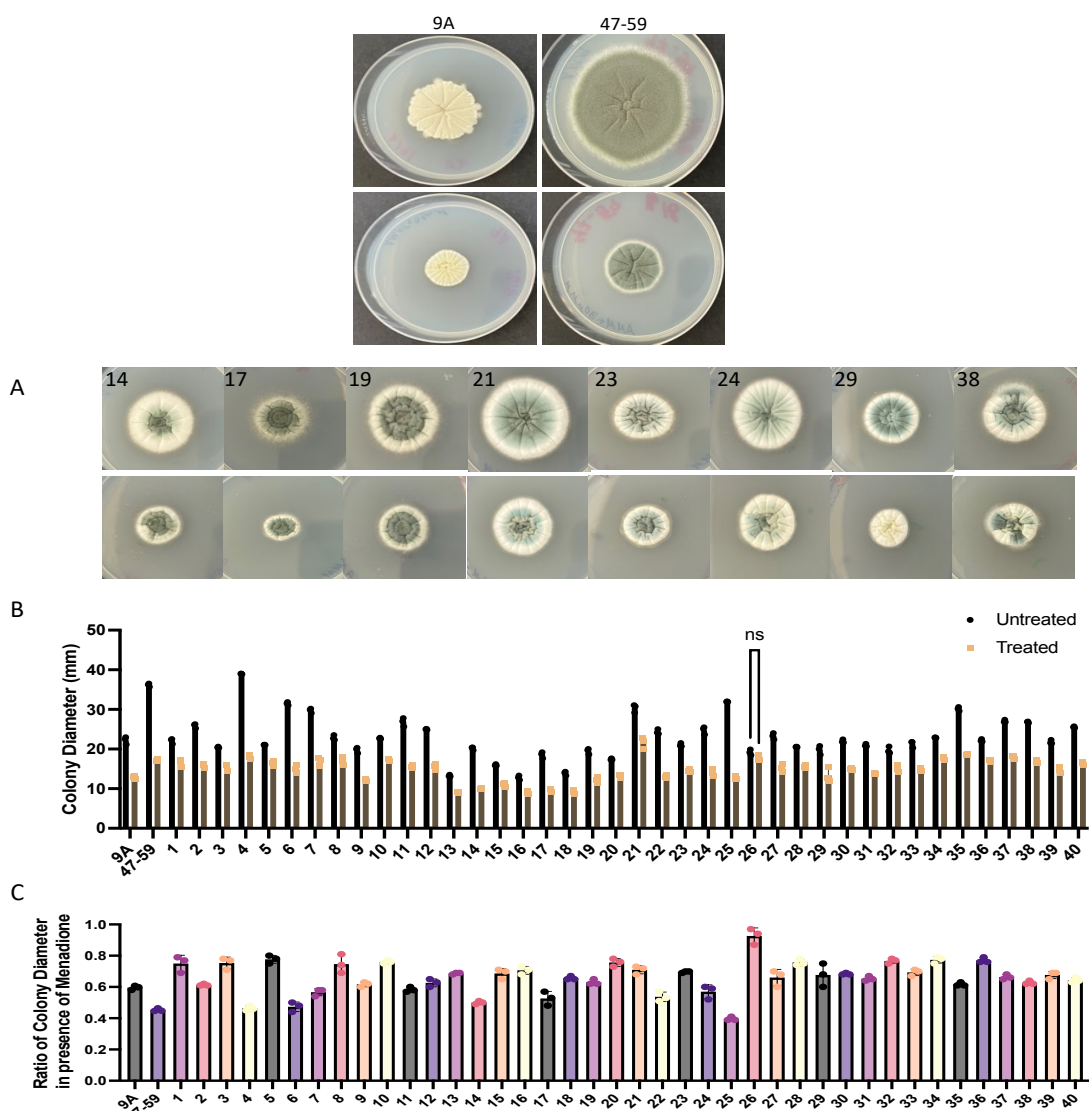


Figure 5-4: Phenotypic characterization of progeny from the 9A × 47-59 cross under oxidative stress conditions (top images show a single colony of the two parental strains). **(A)** Single colony images showing examples of progeny morphology on AMM alone (top images) or AMM supplemented with 30 μM menadione (bottom images); numbers in the top left corner of each image indicate progeny number. All progeny showed a unique colony morphology with striking variations in colouration and furrow numbers. **(B)** Colony diameter was calculated using the average perpendicular colony measurement of three technical replicates per condition without (untreated) and with (treated) 30 μM menadione to induce oxidative stress. Results showed a significant growth reduction of all progeny when treated with superoxide generator agent menadione except for number 26. **(C)** Comparison of T:U ratios between the two parental strains and all 40 progenies under oxidative stress conditions using 30 μM menadione shows a continuous phenotypic spectrum. Error bars represent standard deviations.

5.1.3.3.2.2 8A × 47-51 Cross

Most progeny from this cross showed a colony morphology under oxidative stress similar to that of the 47-51 parental strain, albeit that some progeny showed increased an increased number of furrow numbers. However, five progeny were almost identical

to the 8A parental strain colony morphology, with a white to yellowish colour and mainly non-conidiating mycelium (see progeny numbers 7, 31, and 36 in **(Figure 5.5.A)**). Some progeny produced white non-conidiating mycelium only under oxidative stress conditions, such as numbers 38 and 40 (**Figure 5.5.A** (bottom image)). In addition, white edges with some sections or breaks in colonies were observed in some progeny. Further images of other offspring are provided in **Supplementary 2**. The presence of 30 μ M menadione resulted in significantly reduced colony size in all isolates ($P<0.0001$; **Figure 5.5.B**) as evidenced by the T:U ratio of each progeny (**Figure 5.5.C**). There was a spectrum of menadione resistance in the progeny ranging from highly sensitive progeny such as numbers 13 and 26 (T:U of ~ 0.32 – 0.34) through to more highly resistant progeny such as number 7 (T:U of ~ 0.61), while others showed an intermediate response. A comparison of T:U ratios using one-way ANOVA between the parental strains 8A and 47-51 and offspring showed no significant difference in growth in the presence of menadione for seven of the offspring compared to 8A, indicating they had similar menadione resistance to 8A with a T:U ratio range of 0.51–0.61. Notably, four progeny shared similar colony morphology with the 8A parental strain. Moreover, 31 of the progeny showed no significant difference in growth in the presence of menadione compared to 47-51, indicating they had similar menadione resistance to this parental strain with a T:U ratio range of 0.35–0.50. Progeny numbers 13 and 26 were highly sensitive to menadione with significant differences compared to the sensitive parent strain 47-51 ($P<0.0001$ and $P=0.0184$, respectively). Thus, results showed a continuous variation in the oxidative stress response for all progeny with three different resistance levels possibly evident: low in two offspring, medium as seen in the parental strain 47-51, and high as seen in the parental strain 8A.

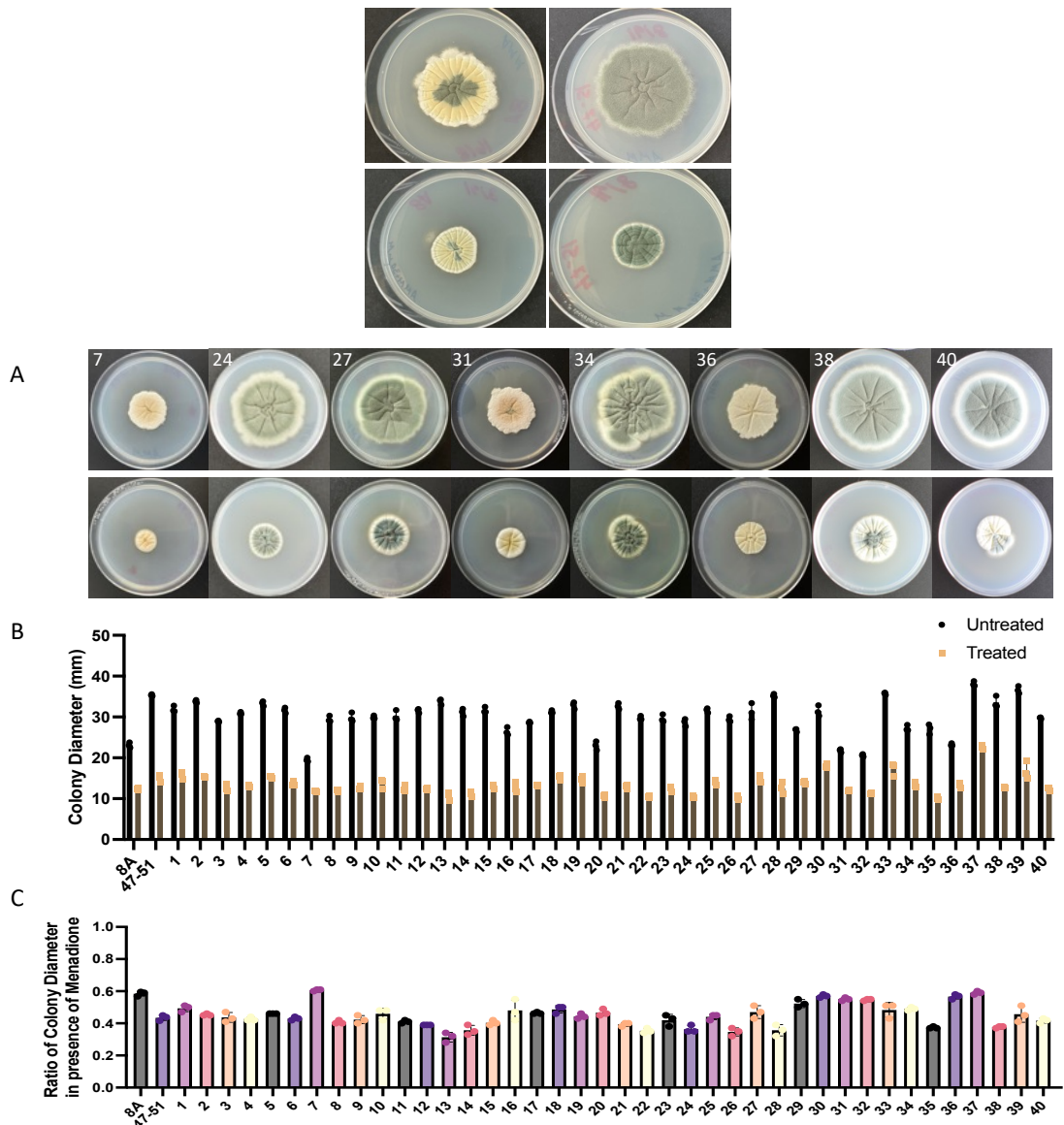


Figure 5-5: Phenotypic characterization of progeny from the 8A $\times$ 47-51 cross under oxidative stress conditions (top images show a single colony of the two parental strains). (A) Single colony images showing examples of progeny morphology on AMM alone (top images) or AMM supplemented with 30 μ M menadione (bottom images); numbers in the top left corner of each image indicate progeny number. All progeny showed a unique colony morphology with striking variations in colouration and furrow numbers. (B) Colony diameter was calculated using the average perpendicular colony measurement of three technical replicates per condition without (untreated) or with (treated) 30 μ M menadione to induce oxidative stress. (C) Comparison of T:U ratios between the two parental strains and all 40 progenies under oxidative stress conditions using 30 μ M menadione shows a continuous phenotypic spectrum. Error bars represent standard deviations.

5.1.3.3.3 Inheritance of Menadione Resistance

Analysis of progeny from the two crosses 9A $\times$ 47-59 and 8A $\times$ 47-51 showed the presence of sensitive and more resistant menadione phenotypes, together with some intermediate (medium) resistance. However, it was challenging to distinguish between

the resistance levels, particularly medium and high. To better understand the inheritance of menadione resistance, progeny from both crosses were sorted according to resistant level to allow a better direct comparison as shown in **(Figures 5.6.A and 5.6.B)**. The pattern of T:U growth ratios failed to show a clear 1:1 segregation of the trait between the parents, instead a continuous spectrum was present indicating a likely polygenic genetic basis. It was noted again that certain highly resistant progeny were evident from each cross (number 26 with a T:U of ~0.92 from the 9A cross and number 7 with a T:U of ~0.61 from the 8A cross), showing the power of sexual recombination to create novel variation within offspring.

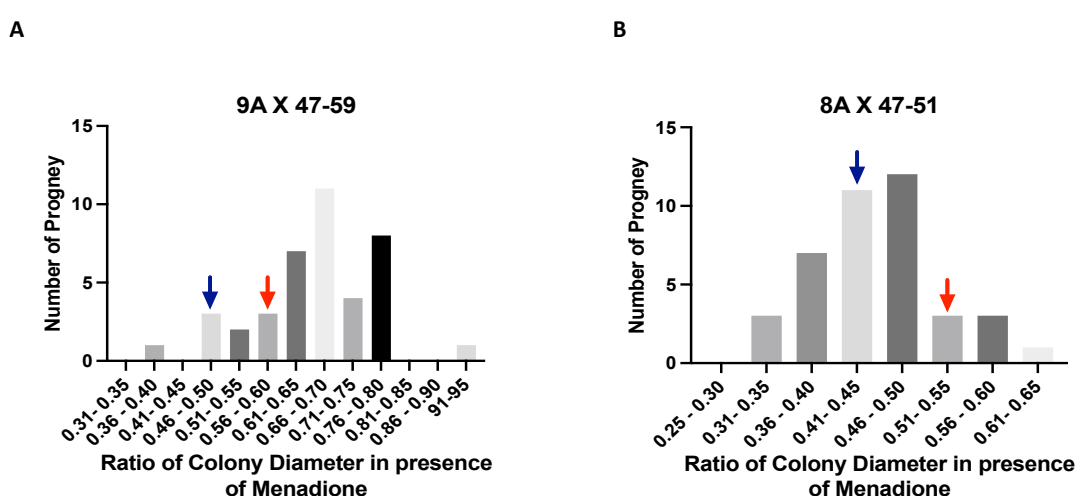


Figure 5-6: Distribution of T:U ratios of colony diameter for progeny from crosses involving *A. fumigatus* clinical isolates 9A and 8A and supermatr WT reference strains. **(A)** Progeny of 9A crossed with 47-59, where the red and blue arrows indicate the T:U ratios of 9A and 47-59, respectively. **(B)** Progeny of 8A crossed with 47-51, where the red and blue arrows indicate the T:U ratios of 8A and 47-51, respectively.

5.1.4 Discussion

The ability to resist oxidative stress encountered during host response to infection can be considered an important pathogenicity trait for opportunistic fungal pathogens. In the case of *A. fumigatus*, various factors are already known to contribute to resistance to ROS such as the presence of melanin and a series of ROS detoxification enzymes (Brown and Goldman, 2016), which are likely to apply to isolates of *A. fumigatus* in general. In a recent investigation of a series of clinical isolates obtained from one CF patient, Ross *et al.* (2021) reported the discovery of an interesting set of *A. fumigatus* isolates some of which were found to possess unique phenotypes in response to CF-

relevant stresses, including reduced growth under normoxic conditions (21% O₂, 5% CO₂, and 74% N₂), increased growth under hypoxic conditions (1% O₂, 5% CO₂, and 94% N₂), resistance to the antifungal voriconazole, susceptibility to the superoxide generating compound menadione, and sensitivity to sorbitol mediated osmotic stress. Thus, these isolates might have genetic traits of interest linked to altered susceptibility to ROS and pathogenicity. Following discussions, two of these isolates, strains 9A and 8A, were obtained and attempts made to investigate the genetic basis of menadione resistance in these isolates. We had been informed that conidia of 9A and 8A showed increased sensitivity to menadione which was proven in the same study, but that conversely 9A hyphae were resistant to menadione under hypoxia and normoxia conditions (Ross *et al.*, 2021) Prof. Robert Cramer, personal communication).

These two strains were evaluated in our lab for menadione response under 5% atmospheric CO₂ to mimic the normoxia conditions of Ross *et al.* (2021). Two environmental wild-type (WT) supermater strains (47-51 and 47-59) were included for comparison. It was found that the WT strains showed higher growth rates and asexual sporulation than the clinical isolates under control conditions, but when menadione was incorporated into the growth media the WT strains showed a significantly greater decrease in growth than the clinical strains (**Figures 5.2 and 5.3**) i.e. the clinical strains appeared to harbour some genetic trait(s) providing enhanced resistance to menadione. Sexual crosses were therefore set up to investigate the possible genetic basis of the menadione resistance phenotype i.e. to determine how many genes might be involved in controlling menadione resistance in *A. fumigatus* given that segregation patterns in sexual progeny can be used to evaluate whether an observed characteristic is monogenic or polygenic in nature (Ashton, 2016; Ashton *et al.*, 2022). Progeny analysis from two separate crosses between the clinical isolates 9A or 8A, with elevated resistance to menadione, and two supermater strains with lower resistance to menadione, revealed a continuous spectrum of menadione resistance when using the treated:untreated (T:U) growth ratios as a measure (**Figure 5.6**). This indicated a polygenic basis of the resistance trait and that multiple genes might be involved in the menadione resistance phenotype in those two isolates. Of particular interest was the appearance of a single, highly menadione resistant progeny isolate '26' from the cross 9A x 47-59. This is a possible example of transgressive segregation, whereby sexual recombination can lead

to new extremes within the progeny, with important evolutionary potential (Rieseberg *et al.*, 1999).

Inspection of the progeny from the crosses also revealed considerable morphological diversity amongst some of the ascospore offspring. Screening of progeny from crosses involving a parental strain with H-MORPH (9A and 8A) and a parental strain lacking this colony morphology feature (47-51 and 47-59) showed that ~12.5% of progenies from the 8A $\times$ 47-51 cross produced white colonies (i.e. a lack of asexual sporulation) with significant furrow numbers on AMM with and without menadione. Moreover, 5% of progeny from the same cross produced white colonies only under oxidative stress conditions (i.e. in the presence of menadione). However, it was difficult to discern any particular pattern of inheritance, again suggesting a polygenic basis for these character traits. By contrast, almost all progeny from the 9A $\times$ 47-59 cross showed a similar morphology with low sporulation and significant furrow numbers on AMM with and without menadione. These macroscopic abnormalities, such as numbers of furrows, white colour, and low sporulation under laboratory conditions mimicking host conditions, provide an insight into the ability of *A. fumigatus* to adapt to host stress conditions. The presence of progeny with striking H-MORPH colony morphology, combined with elevated menadione resistance, highlights the ability of fungi to produce offspring better adapted to the host environment. Kowalski *et al.* (2019) have suggested that changes in colony morphology, such as H-MORPH, are an indication of the effect of oxygen on the biofilm architecture. In their study, they identified an oxygen-mediated *A. fumigatus* colony morphology mechanism associated with accelerated disease development and virulence regulated by the *hrmA* gene. In addition, other molecular mechanisms have been reported to influence *A. fumigatus* colony morphology. Genes that influence colony shape and cell wall production have been identified using reverse genetic techniques. These include 1,3-glucan glycosyltransferase *gel2*, chitin synthases *chsC* and *chsG*, and the polysaccharide synthase *cpsA* that helps maintain cell wall integrity (Kowalski and Cramer, 2020). With the appearance of H-MORPH in sexual offspring and their highly menadione-resistant progeny, further studies are needed to better understand the implications for fungal pathogenic mechanisms.

5.1.5 Conclusion and Future Work:

A. fumigatus spores cause >300,000 invasive infections and >3,000,000 chronic disorders annually. Inhaling these spores can cause various illnesses, depending on the host's immune system, with high morbidity and mortality reported worldwide. The development of effective therapeutic approaches for the clinical management of fungal infections has been hampered by an incomplete knowledge of *A. fumigatus* pathophysiology and host response (Ortiz *et al.*, 2022). Here, we used sexual crosses to investigate the genetic basis of resistance to oxidative stress caused by the superoxide generator agent menadione. The initial results based on the analysis of 80 progeny from two different crosses under oxidative stress conditions indicate that multiple genes are likely contribute to this phenotype. Moreover, many progeny showed the very interesting H-MORPH colony morphology which was previously reported to be associated with increased *A. fumigatus* virulence. Therefore, further studies are needed to understand these phenotypic aspects and their association with *A. fumigatus* virulence.

Future directions:

- 1- The skewed distribution of menadione resistance among progenies suggests the presence of additional genetic components responsible for increased resistance in some progeny. These genetic components might involve two major genes, or one major gene with different modifier genes with small effect sizes, in this phenotype. Therefore, additional crosses might be useful to determine how many genes are involved. For example, these crosses could involve a backcross between highly resistant progeny from the first cross and parental strain 9A or 8A. It is expected that no sensitive strains would be produced in the progeny because the parental 9A or 8A genes would be allelic if the same single resistance gene were present in both parental isolates. This pattern would contrast with the expected polygenic resistance model outcome, which would anticipate sensitive strains among the recombinant progeny because the parental mutations would occur at more than one locus. Indeed, some crosses have already been performed, and offspring collected. Those offspring were initially grown on ACM under normal conditions and showed slow growth and low

sporulation, which is intriguing but time limits precluded further study (see **Supplementary 3**).

- 2- It may be possible to develop morphotype markers for disease progression and better patient care by understanding how fungal macroscopic morphotypes reflect *in vivo* phenotypic changes in human fungal infections. Therefore, the macroscopic morphology of all colonies should be identified and quantified using Fiji (ImageJ) software as previously described (Kowalski *et al.*, 2019), whereby the colony perimeter can be selected, the percentage vegetative mycelia (white, non-conidiating mycelia; PVM) can be quantified, and furrow numbers can be counted, facilitating the assessment of which progeny can be considered H-MORPH. Kowalski *et al.* (2019) suggest that strains with >3 furrows and a PVM >40% should be considered H-MORPH (see **Supplementary 4**).

5.2 Part 2: Population Biology of *Aspergillus fumigatus*

5.2.1 Introduction:

5.2.1.1 Discovery of Clade A and Clade B within global *Aspergillus fumigatus* populations

The evolution of antifungal drug resistance is jeopardizing our ability to suppress and cure fungal infections. Azole-resistant *A. fumigatus* isolates have been reported mainly in Europe but have also emerged worldwide in the last decade (Verweij *et al.*, 2020). Those resistant isolates have been reported from both clinical and environmental settings, which suggests that resistance is occurring as a result of selection pressure brought on by the pervasive use of azole antifungal drugs in agriculture (Abdolrasouli *et al.*, 2015). Therefore, there is a need to investigate the probable connection between the evolution and spread of a wide spectrum of azole resistant isolates in the environment and the rising clinical incidence of azole-resistant aspergillosis. Indeed, very recently evidence has been reported showing the infection of patients by azole-resistant isolates of *A. fumigatus* thought to have arisen in the environment (Rhodes *et al.*, 2022). Thus, there is a need to understand the underlying population biology of *A. fumigatus* upon which azole resistance might evolve and spread.

Various studies have been made to investigate the population genetic structure of the *A. fumigatus* using a variety of molecular markers. One analysis using whole genome sequencing of 24 *A. fumigatus* isolates, including azole-resistant and susceptible clinical and environmental strains obtained from India, the Netherlands, and the United Kingdom showed that clinical and environmental isolates could not be distinguished according to phylogenetic analysis. The TR₃₄/L98H allele was found in a variety of genetic backgrounds from both clinical and environmental sources (Abdolrasouli *et al.*, 2015). In the same study, it was demonstrated through population genomic analysis that *A. fumigatus* was panmictic, with the same level of genetic variation found between continents and between nations. However, there was a noteworthy exception, where isolates from India containing TR₃₄/L98H were found to form a distinct, closely related group despite coming from both environmental and clinical sources. One year later, the 24 *A. fumigatus* genomes were then merged with 17

genomes from clinical isolates in Japan. In this study it was found that, with the exception of one isolate which clustered with the Indian isolates, that the bulk of Japanese isolates grouped together with the single global population of *A. fumigatus* according to phylogenetic analysis (Meis *et al.*, 2016). The widespread frequency of the TR₃₄/L98H allele was suggested to reflect a recent selective sweep of the genotype combined with possible recombination into a variety of genetic backgrounds (Abdolrasouli *et al.*, 2015). By contrast, a more recent study by Sewell *et al.* (2019) demonstrated that global populations of *A. fumigatus* can be divided into two broad clades and that the *cyp51A* alleles TR₃₄/L98H and TR₄₆/Y121F/T289A are unequally distributed across these two clades. This study involved hierarchical clustering and multivariate analysis of 4,049 *A. fumigatus* isolates collected from different location around the world and genotyping of nine microsatellite loci using analysis of short tandem repeats of *A. fumigatus* (Sewell *et al.*, 2019).

Most recently, key findings have emerged from a whole genome sequencing analysis of 218 *A. fumigatus* isolates from clinical and environmental sources collected from different sites across England, Wales, Scotland, and the Republic of Ireland, some of which displayed an azole resistant phenotype. This phylogenomic study indicated that the *A. fumigatus* population was not panmictic, but rather again demonstrated the presence of two different and distinct phylogenetic clades (Verweij *et al.*, 2020; Rhodes *et al.*, 2022). The population was arranged into a typical 'dumbbell' in the unrooted phylogenetic tree with two clades evident plus evidence of restricted inter-clade hybridization. "Clade-A" contained 123 isolates, while "Clade-B" contained 95 isolates. The vast majority of isolates exhibiting azole resistance were grouped inside Clade-A (hence Clade 'A' for azole resistant) with 80 percent of isolates (n=99/123) demonstrating polymorphisms within *cyp51A* leading to an azole resistance trait. A similar trend was observed for phenotypically characterized resistance, with roughly 78 percent of itraconazole-MICs values above diagnostic thresholds categorized as Clade A. By contrast, 86 percent of isolates (n = 82/95) in Clade B had an azole-susceptible phenotype.

Applications such as discriminant principal component analysis (DAPC), principal component analysis (PCA), *fineStructure*, and STRUCTURE were used to individually confirm the presence of the two clades. The associated analyses also provided evidence

of the presence of significant genetic sub-clustering inside Clade-A, for example clonal genetic variants such as ‘Clade A_A’. Moreover, Clade A could be classified into two main sub-clusters, named Clusters 3 (Clad A_A) and 1, according to further interpretation employing STRUCTURE, PCA, and DAPC. Cluster 3 contained all the TR₃₄/L98H genetic variations, whereas Cluster 1 constituted isolates with a wide range of *cyp51A* genetic variants. Isolates with the TR₄₆/Y121F/T289A genetic variations were observed in both in A Clade Cluster 1 and also Clade B, however not in Clade A Cluster 3. Correspondingly, a novel polymorphism TR₃₄/L98H/T289A/I364V/G448S was found only in Clade A Cluster 3 as well as an unrelated lone TR₃₄ (**Figure 5.7**). These findings indicate that TR-linked azole resistance has evolved only a few times, consistent with findings and the proposal of Camps *et al.* (2012), with limited recombination resulting in so far only limited homogenization of the alleles throughout the phylogeny of *A. fumigatus* (Rhodes *et al.*, 2022).

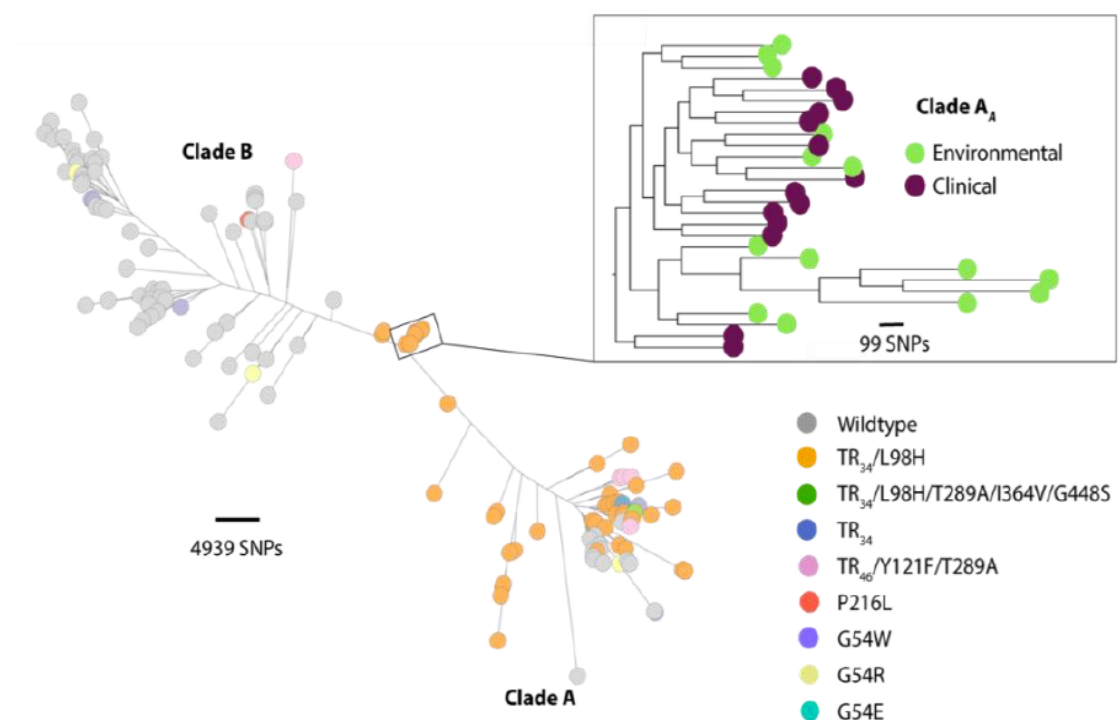


Figure 5-7: Unrooted phylogenetic tree of 218 isolates from the UK whole genome sequencing study showing the genetic relationship between the isolates and the identified clades A and B. Adapted from (Rhodes *et al.*, 2022).

The discovery of these two clades and the variation within one clade according to the presence or absence of the azole resistance phenotype have raised several questions with respect to the global population of *A. fumigatus*. It is believed that *A. fumigatus*

reproduces primarily by asexual means in the environment, producing clones with a high degree of genetic identity. However, it is possible that through sexual recombination that gene flow of azole-resistance alleles may take place into various genetic backgrounds (Rhodes *et al.*, 2022).

5.2.1.2 Aims

As the reasons behind the presence of the different Clade A and Clade B populations of *A. fumigatus* was not clear, it was of great importance to investigate whether sexual crossing was possible between the two groups. This is of major medical significance given possible gene flow of azole resistance alleles between the Clades. In parallel work, the ability for Clade A and Clade B mating partners to successfully complete the sexual cycle and produce cleistothecia has been recently proven by work undertaken by another member of the Nottingham group (F Stanford, unpublished data), demonstrating that genetic transfer between the two clades is possible. This suggests that the Clade A and B structure might be due to limited effective recombination between the two clades in the natural environment and/or some other physiological differences between the clades. Therefore, the main aim of this part of the chapter was to investigate whether the Clade A azole resistant isolates might exhibit lower sexual fertility compared to azole sensitive clade A isolates, as this finding would help explain the limited effective recombination between azole resistant Clade A isolates and Clade B isolates. This work was made possible by the use of sexual crossing between supermater isolates from Clades A and B, and azole resistant and sensitive isolates from Clade A which were kindly provided through a collaboration with the Imperial College (London) group lead by Prof. Matt Fisher.

5.2.2 Material and Methods:

5.2.2.1 Strains:

A total of 28 *A. fumigatus* isolates from Clade A were used in this study, 10 of which were azole sensitive and 18 were azole resistant. More detailed information of each isolate is provided in **Table 5.1** including the mating type, year of sampling, source and any genetic marker(s). In addition, four supermater (SM) *A. fumigatus* strains from the Nottingham culture BDUN collection were used as the opposite mating partner in crosses. Those four isolates were 47-51 (*MAT1-1*; Clade A), 47-55 (*MAT1-2*; Clade B), 47-169 (*MAT1-1*; Clade B) and 47-190 (*MAT1-2*; Clade B), which were drawn from both Clade A and B (F Stamford, J Rhodes, M Fisher, PS Dyer, unpublished results).

Table 5-1: Details of Clade A azole resistant and sensitive *A. fumigatus* isolates used in sexual crosses with Clade A and B supermater strains.

Clade A/Cluster 3					
2xMAT1-2 (Susceptible)					
Code	ID	year	<i>cyp51A</i> marker	Mating type	Source
C103	MRL_05	2015	TR34/L98H	<i>MAT1-2</i>	Clinical
C368	ENV-S-14	2016	TR34/L98H	<i>MAT1-2</i>	Environmental
4xMAT1-2 (Resistant)					
Code	ID	year	<i>cyp51A</i> marker	Mating type	Source
C8	PHW_155	2015	TR34/L98H	<i>MAT1-2</i>	Environmental
C12	PHW_225	2015	TR34/L98H	<i>MAT1-2</i>	Environmental
C16	PHW_373	2015	TR34/L98H	<i>MAT1-2</i>	Environmental
C18	PHW_423	2015	TR34/L98H	<i>MAT1-2</i>	Environmental
4xMAT1-1 (Resistant)					
Code	ID	year	<i>cyp51A</i> marker	Mating type	Source
C161	CXH_07	2017	TR34/L98H	<i>MAT1-1</i>	Clinical
C78	DUB_39	2014	TR34/L98H	<i>MAT1-1</i>	Environmental
Clade A/Cluster 1					
4xMAT1-1 (Susceptible)					
Code	ID	year	<i>cyp51A</i> marker	Mating type	Source
C38	ML	2014	Wildtype	<i>MAT1-1</i>	Clinical
C220	47-51	2005	Wildtype	<i>MAT1-1</i>	Environmental
C223	47-59	2005	Wildtype	<i>MAT1-1</i>	Environmental
C196	CBS 129448	1984	Wildtype	<i>MAT1-1</i>	Environmental
4xMAT1-2 (Susceptible)					
Code	ID	year	<i>cyp51A</i> marker	Mating type	Source
C221	47-52	2005	Wildtype	<i>MAT1-2</i>	Environmental
C246	47-154	2012	Wildtype	<i>MAT1-2</i>	Environmental
C603	IGF11997	2015	Wildtype	<i>MAT1-2</i>	Clinical
C201	CBS 499.64	1964	Wildtype	<i>MAT1-2</i>	Environmental
4xMAT1-1 (Resistant)					
Code	ID	year	<i>cyp51A</i> marker	Mating type	Source
C86	DUB_47	2016	TR34/L98H	<i>MAT1-1</i>	Environmental
C341	ComPos-1	2017	TR34/L98H	<i>MAT1-1</i>	Environmental
C364	ENV-S-2	2016	TR34/L98H	<i>MAT1-1</i>	Environmental
C365	ENV-S-3	2016	TR34	<i>MAT1-1</i>	Environmental
4xMAT1-2 (Resistant)					
Code	ID	year	<i>cyp51A</i> marker	Mating type	Source
C6	PHW_107	2015	TR34/L98H	<i>MAT1-2</i>	Environmental
C7	PHW_120	2015	TR34/L98H	<i>MAT1-2</i>	Environmental
C25	PHW_53	2015	TR34/L98H	<i>MAT1-2</i>	Environmental
C29	PHW_577	2015	TR34/L98H	<i>MAT1-2</i>	Environmental
4xTR46 (Resistant)					
Code	ID	year	<i>cyp51A</i> marker	Mating type	Source
C79	DUB_40	2014	TR46/Y121F/T289A	<i>MAT1-2</i>	Environmental
C89	DUB_50	2015	TR46/Y121F/T289A	<i>MAT1-2</i>	Environmental
C93	DUB_54	2015	TR46/Y121F/T289A	<i>MAT1-2</i>	Environmental
C370	CYP-15-2	2015	TR46/Y121F/T289A	<i>MAT1-2</i>	Clinical

5.2.2.2 Crossing

Crossing was performed according to (Ashton and Dyer, 2019) as described previously (**Section 2.2.7**) with three technical replicates per cross. Control crosses between SM was included to ensure that suitable conditions were present to induce the sexual cycle. Each isolate was crossed with two different SM strains of the opposite mating type as indicated in the crossing plan (**Table 5.2**). If cleistothecia were observed, then the number of fruiting bodies was counted per cross as described by (Ashton and Dyer, 2019), using a hoovering technique to ensure accurate enumeration.

Table 5-2: Planned crosses between supermater (SM) and suitable Clade A azole sensitive or resistant partners.

Crossing Partner Super mater (SM)	Control SM crosses	Clade A azole sensitive	Clade A azole resistant (TR34)	Clade A azole resistant (TR46)
47-51 (<i>MAT1-1</i>)	47-55, 47-190 (<i>MAT1-2</i>)	C221, C246, C603, C201, C103, C368 (<i>MAT1-2</i>)	C6, C7, C25, C29, C8, C12, C16, C18 (<i>MAT1-2</i>)	C79, C89, C93, C370 (<i>MAT1-2</i>)
47-169 (<i>MAT1-1</i>)	47-55, 47-190 (<i>MAT1-2</i>)	C221, C246, C603, C201, C103, C368 (<i>MAT1-2</i>)	C6, C7, C25, C29, C8, C12, C16, C18 (<i>MAT1-2</i>)	C79, C89, C93, C370 (<i>MAT1-2</i>)
47-55 (<i>MAT1-2</i>)	47-51, 47-169 (<i>MAT1-1</i>)	C38, C220, C223, C196 (<i>MAT1-1</i>)	C86, C141, C364, C365, C161, C78 (<i>MAT1-1</i>)	
47-190 (<i>MAT1-2</i>)	47-51, 47-169 (<i>MAT1-1</i>)	C38, C220, C223, C196 (<i>MAT1-1</i>)	C86, C141, C364, C365, C161, C78 (<i>MAT1-1</i>)	

5.2.3 Results

After three months of incubation at 30°C in the dark, each sexual cross was examined for cleistothecia formation under the dissecting microscope. As expected, control crosses using pairings between SM strains produced high number of cleistothecia. When observing crosses between one of the SM and isolates from either clade A susceptible or clade A resistant, the number of cleistothecia formed showed a wide variation between isolates from the same Clade A group even when they were crossed to the same SM. Moreover, some isolates failed to form cleistothecia with one or both SM. For example, sample C196 was only able to produce very low numbers of cleistothecia when crossed with 47-55 and no cleistothecia at all when crossed with 47-190. In another example, three isolates (C93, C12, C341) totally failed to produce any cleistothecia with any suitable SM. By contrast, some crosses yielded very high numbers of cleistothecia e.g. two of the TR₃₄ azole resistant isolates (C364 and C365) and one of the TR₄₆ azole resistant isolates (C370) showed high fertility in crosses to one or both SM strains (**Figure 5.8**). However, overall there was no clear pattern or differentiation in fertility (i.e. ability to form cleistothecia) between the Clade A azole sensitive and azole resistant isolates.

In order to fully compare the fertility of the Clade A azole resistant and azole sensitive groups, numbers of cleistothecia formed from each cross were pooled into either SM, Clade A susceptible, Clade A TR₃₄ resistant and Clade A TR₄₆ resistant groupings as shown in (**Figure 5.9**). A one-way ANOVA showed that there were no significant difference in fertility of the different Clade A resistant groups harbouring the TR₃₄ and TR₄₆ mutations compared to Clade A sensitive group, indicating that the presence of the distinct azole resistant grouping within Clade is not due to lowered fertility of the resistant isolates. Thus, the ability to form cleistothecia when most isolates from different groups were crossed with a suitable partner provided clear evidence of the potential for gene flow between the Clades A and B.

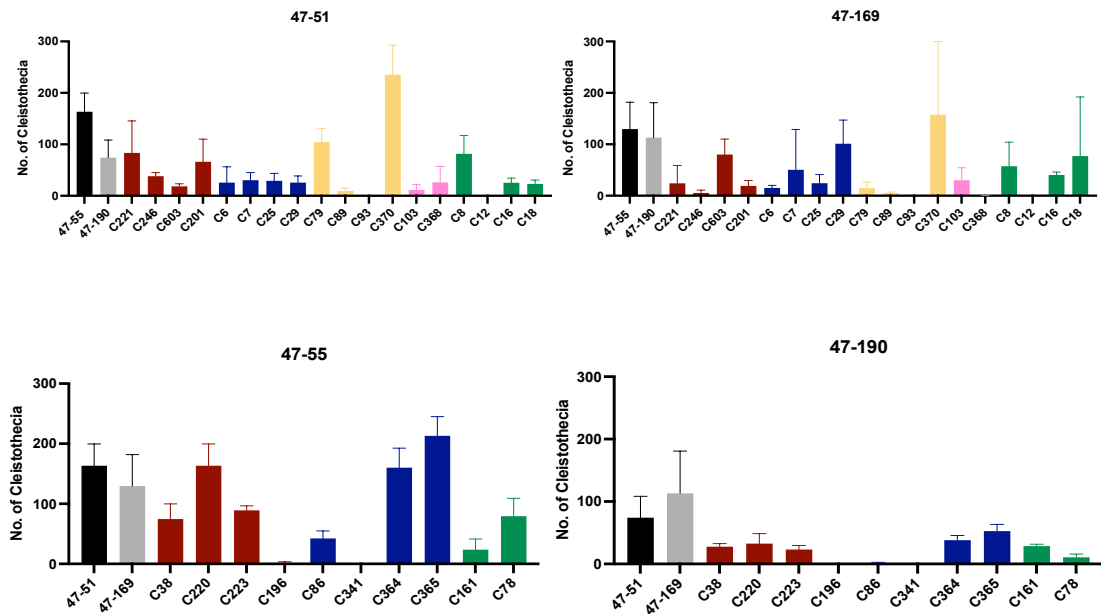


Figure 5-8: Production of cleistothecia of *A. fumigatus* per 9 cm diameter plate in sexual crosses between supermater (SM) strains and isolates of the opposite mating from various Clade A groupings. Bar colouring indicates the different groups of isolates. Black and Gray bars represent the SM strains, red bars represent the clade A/ cluster 1 azole susceptible stains, blue bars represent the Clade A/ cluster1 azole resistant TR₃₄ strains, yellow bars represent the clade A/ cluster1 azole resistant TR₄₆ strains, pink bars represent the clade A/ cluster3 azole susceptible strains and green bars represent the clade A/ cluster3 azole resistant strains. Error bars represent the number of cleistothecia counted from three technical replicates per sample.

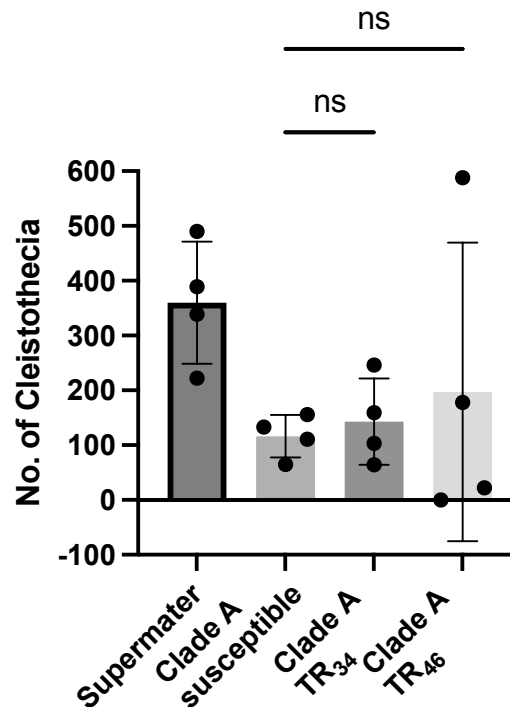


Figure 5-9: Fertility comparison between the different *A. fumigatus* population groups. Number of cleistothecia was generated by pooling the data from isolates involved in this crossing work from each group. Statistical analysis was performed using a one-way ANOVA with Dunnett multiple comparison. No significant difference in fertility was detected between the azole susceptible and azole resistant groups.

5.2.4 Discussion, including Conclusions and Future Work

The population genetic structure of *A. fumigatus* has been thoroughly investigated using a variety of molecular markers to clarify the genetic diversity and life history of this human fungal pathogen (Sewell *et al.*, 2019). This has led to the identification of two major subgroupings of the fungus, termed Clade A and Clade B, in global populations of the fungus (Verweij *et al.*, 2020; Rhodes *et al.*, 2022; Sewell *et al.*, 2019). A recent study by (Rhodes *et al.*, 2022) showed that the bulk of environmentally occurring azole-resistance alleles segregated within Clade A and showed signs of selection at several loci, some of which are known to adapt in response to selection by fungicides. These findings opened multiple questions relating to the partition observed in *A. fumigatus* populations and the possible emergence and spread of azole resistant genetic markers between isolates.

In the present work, attempts were made to address gaps in our knowledge concerning the possibility of gene flow between the different groups (i.e. azole resistant

and sensitive) within both clades. This was achieved by using sexual crosses to determine the fertility of crosses between various members of the two clades. Results showed that crosses between azole sensitive and resistant strains from Clade A, and supermater strains from Clade A and B were all able to produce comparable numbers of cleistothecia, with no significant differences in fertility between Clade A azole sensitive and Clade A resistant TR₃₄ or Clade A resistant TR₄₆ resistant strains. Therefore, the different distribution of the TR₃₄ and TR₄₆ alleles both within Clade A and between Clades A and B seems not to be related to a lack of sexual fertility between the groups. Instead results indicate that genetic transfer between the two groups is possible under the right condition and in the presence of a suitable mating partner. Therefore, the maintenance of the resistance groupings within Clade A is probably due to historical subdivision and limited sexual recombination of the TR₃₄ allele (as the dominant resistant marker) since it first emerged.

Future directions:

It will now be of great interest to undertake further sexual crossing work such as:

- 1- Confirmation that the azole resistance TR₃₄ retains efficiency in sexual crosses between Clade A and Clade B isolates.
- 2- Further investigation of the apparent hybrid grouping Clade A_A, is this indeed derived from an historic mating between Clade A and Clade B isolates?
- 3- Is there any linkage between azole resistance and increased fitness in Clade A azole resistant isolates? This can be investigated by studying segregation patterns of cross between a highly fit azole resistant isolate and a lower fitness azole sensitive isolate.

Chapter- 6 Concluding Discussion

Fungi have co-existed with animals and people for millions of years, and while interactions have been largely confined to non-living tissues, there is increasing evidence that living tissues are a new niche for fungi to explore. The push of humanity to expand across the globe has brought a wider range of fungi and people into more frequent contact, and the recent COVID-19 pandemic combined with improved and heroic medical procedures have opened doors to more frequent chances of fungal infection. There is no path to totally eliminating the risk of fungi attacking human tissues. The only proper path is that of identifying the potential dangers, defining effective methods to counteract the threats, and implementing preventive and therapeutic protocols that make the best use of effective treatments to try to prolong their long-term use. It is a challenge with no ultimate victory, but with the hope of frequent and consistent success.

Aspergillosis denotes a wide variety of infections that are caused by fungi of the genus *Aspergillus*. The diseases mostly affects people with suppressed immunity such as cancer, AIDS and transplant patients (Dagenais and Keller, 2009). Exposure to this fungus is unavoidable because it is found in decaying leaves, grain crops, and composts with a very high spore load released into air columns. Everyday exposure to this pathogen is hardly ever a concern for individuals with healthy immune systems. Occasionally, aspergillosis infections manifest themselves through acute pulmonary aspergillosis, allergic bronchopulmonary aspergillosis, or aspergilloma (Kwon-Chung and Sugui, 2013). Invasive aspergillosis (IA) is the most acute form of aspergillosis that arises when the diseases spread rapidly from the lungs, which act as the portal of entry to the heart, skin, and brain. Those diseases are common among patients with a suppressed immune system. This disease is of concern because it is the major cause of fatality in patients suffering from hematological malignancies such as leukemia, solid-organ and hematopoietic stem cell transplant patients (Dagenais and Keller, 2009; Kwon-Chung and Sugui, 2013). Therefore, with the increase in numbers of fungal infections reported in immunosuppressed patients, there is an urgent need for more understanding of how to deal with microbial pathogens including fungi, bacteria, and viruses. There is indeed an increasing awareness of the importance of research into

fungal pathogenicity. However, there are still many areas of research that need to be undertaken in order to improve our understanding of these pathogens and their impact on human, animal, and plant life.

As a result of the perpetual presence of *A. fumigatus* within the environment as well as the close vicinity of patients at risk from IA, it is an urgent matter to understand the evolutionary origins and mechanisms of the growing azole resistance in *A. fumigatus*. Consequently, there is a hope that the repeated induction or spread of azole resistance may be averted in order to enable future treatment of *A. fumigatus* IA. Moreover, as the incidence of azole resistance in fungi species increases, it is important to understand the mechanisms of this resistance to both improve upon contemporary azole prescriptions and enhance the fight against fungal related diseases.

Summaries of work conducted and ideas for future work have been already provided within each chapter of the present thesis, this will not be discussed further. However, throughout my research and work on this project, every day was irradiating me with a new challenge combined with valuable knowledge. Therefore, a few final personal comments will be provided as follows.

6.1 What Did we Learn from the Recent Viral Pandemic?

With the fact that over 180 million people have been infected with the COVID-19 disease worldwide and over 3.9 million have died as a result (Ezeokoli *et al.*, 2021), which seems a scary number, I was really surprised by the fact that this number does not truly represent deaths due to COVID-19 infection alone. There have been a growing number of publications reporting co-infection with both COVID-19 and also fungal infection by *Aspergillus* spp. which has been termed ‘COVID-19-associated pulmonary aspergillosis’ (CAPA) (Arastehfar *et al.*, 2020; Costantini *et al.*, 2020). This should not come as a surprise, because co-infections with *Aspergillus* spp. and influenzas known as ‘Influenza-associated pulmonary aspergillosis’ (IAPA) have been known for many years. Nevertheless, the number of reported cases of CAPA (4% to 34% of all hospital COVID-19 cases) seems to be much greater than historic IAPA rates in the very short period of time since this pandemic started (Ezeokoli *et al.*, 2021).

So, the challenge presented by this pandemic teaches us a good lesson that host factors, medical procedures, and type of treatments, are all linked to risk factors for fungal co-infections. From this it can be seen that there is a significant need to create cutting-edge plans to reduce nosocomial secondary fungal infections in the hospital setting.

6.2 Cleverness of Fungi

The discovery of the novel TR₁₃₀ mutation in this thesis provides further evidence that the majority of azole resistant *A. fumigatus* isolates exhibit a mechanism linked to alterations in the Cyp51A drug target. A sign of the possible environmental spill over and spread of this mutation has been already detected as it was shown to be present in two different patients between 2015-2018, as well as being found in four isolates in 2019 from the citizen science soil sampling project of (Shelton *et al.*, 2022). Therefore, there is an urgent need to highlight that care must be taken when handling soil, given that most people are not aware of the risk that such azole resistant isolates might be present in their garden soil and compost. More importantly, the widespread use of azoles fungicides to control plant pathogen and prevent fungal decay of structural materials has been suggested to be the primary cause for the widespread appearance of resistant isolates in the environment (Verweij *et al.*, 2020).

The newly discovered TR₁₃₀ and other similar relatively large tandem repeats must now be considered as one of possible mutations that ‘clever’ fungal pathogens might use to escape from attack by antifungals drugs. Moreover, whenever diagrams are made describing mechanisms of azole resistant in *A. fumigatus*, the TR₁₃₀ now needs to be included as one of the important mechanisms given that isolates harboring this mechanism have been detected in both environmental and clinical setting. As well as being highly resistant to itraconazole, TR₁₃₀ isolates show fair resistance to voriconazole beyond the recognized clinical break point. **Figure 6.1** shows an updated version of the previously used (**Figure 1.3**) showing azole resistant mechanisms in *A. fumigatus*.

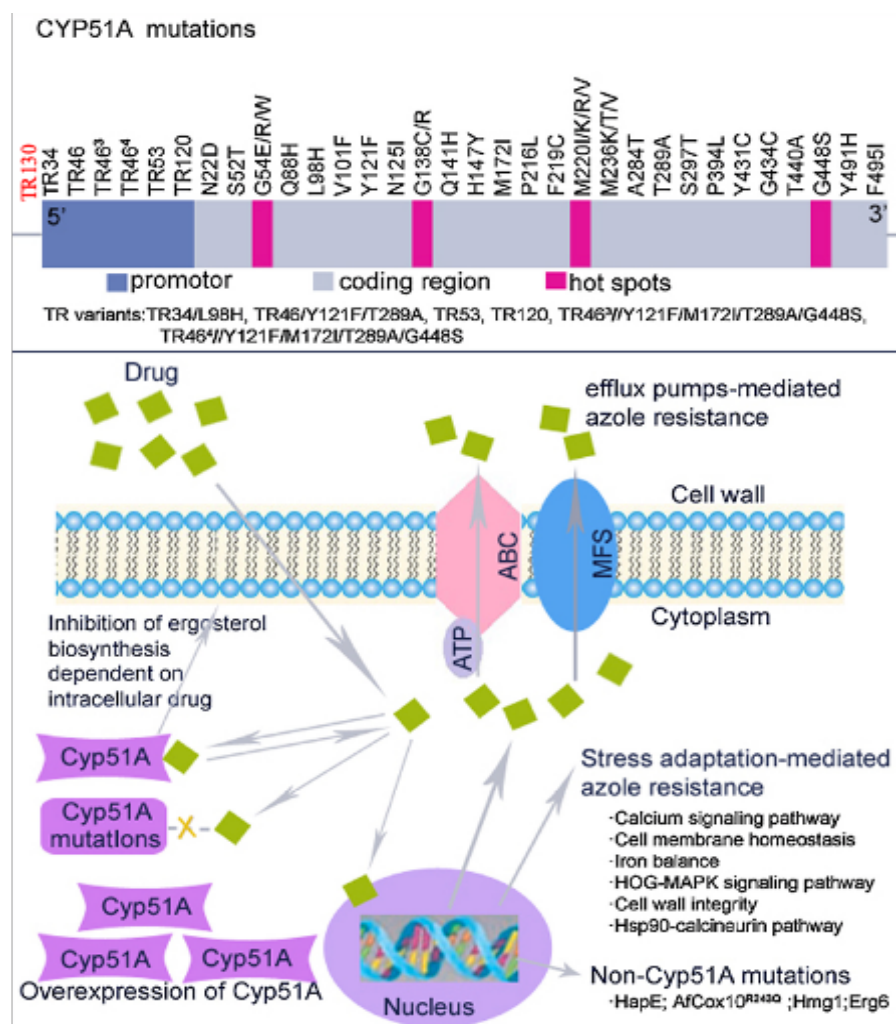


Figure 6-1. Updated version of diagram showing azole resistant mechanisms in *Aspergillus fumigatus* where TR₁₃₀ is included in red. Adapted from (Chen *et al.*, 2020a).

6.3 Final Thought

From my personal perspective, research in this project has shown the need to be ever vigilant and surveillant of *A. fumigatus* populations to watch out for the evolution of new antifungal resistance forms as well as emergence of enhanced pathogenicity in subsets of isolates. This is mainly because this is such a prevalent worldwide species, combined with the fact that it is an unusually thermotolerant fungus, meaning that *A. fumigatus* is a particular risk to human and other animal life and well being. It is argued that fungal plant pathogens have posed a great threat to the quality and quantity of plant, animal and human life since the beginning of the agrarian revolution, but the risk posed by opportunistic fungal animal pathogens should also not be underestimated.

One very interesting final outstanding thought which might be true or not is the fungal infection-mammalian selection (FIMS) theory. According to the FIMS hypothesis a fungal bloom that occurred after the meteorite impact disaster at the end of the Cretaceous period favorably benefited mammals to take over from ectothermic reptiles, see **(Figure 6.2)** [adapted from (Casadevall and Damman, 2020)]. Proponents of this theory have argued that various pieces of evidence support their hypothesis such as the higher body temperature of mammals protecting them from fungal infection while ‘cold blooded’ dinosaurs (less able to regulate body temperature) would be more prone to infection, combined with the fact that less vegetation was available for them to eat would mean that weakened dinosaurs would be a good target for fungal infection (Casadevall and Damman, 2020). By way of a parallel example, in the present day there are many cases reported of fungal infection arising from environmental disturbance. For example, here in Nottingham there was a significant rise in numbers of fungal infections reported in Nottingham City Hospital linked to the building of a new route for the Nottingham tram (personal communication from a consultant clinical scientist, infection control specialist advisor working on the same hospital). Therefore, it is important for all human beings to pay attention to all the actions they do that can harm the environment, as this may inadvertently lead to damage that might affect human, animal and plant life in more general.

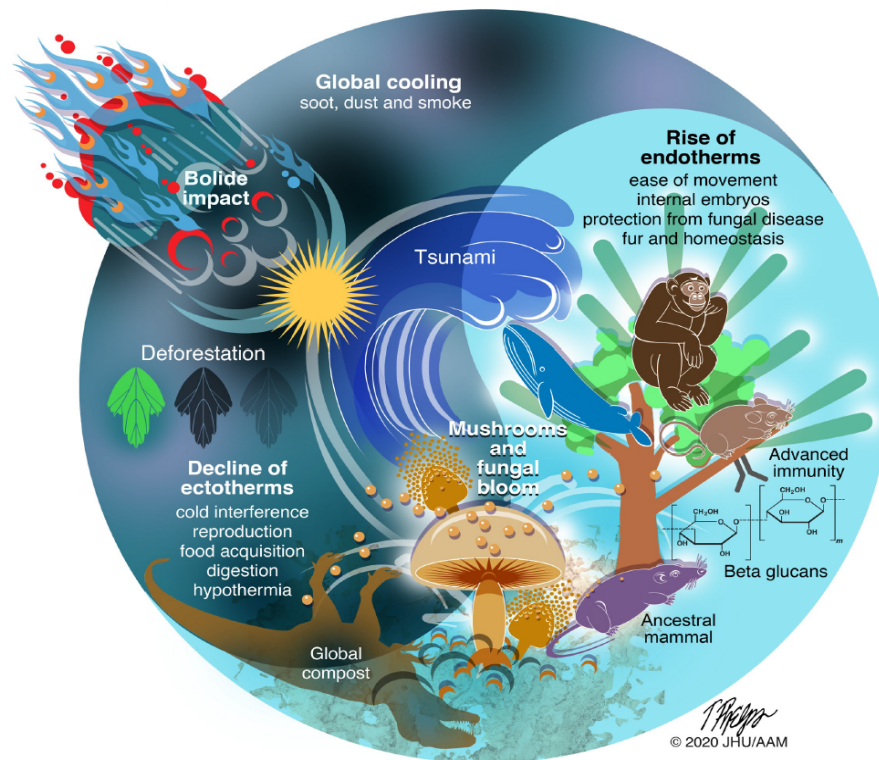


Figure 6-2. Representative schematic of the hypothesized events leading to the fungal selection of mammalian endotherms after the Chicxulub disaster. Adapted from (Casadevall and Damman, 2020).

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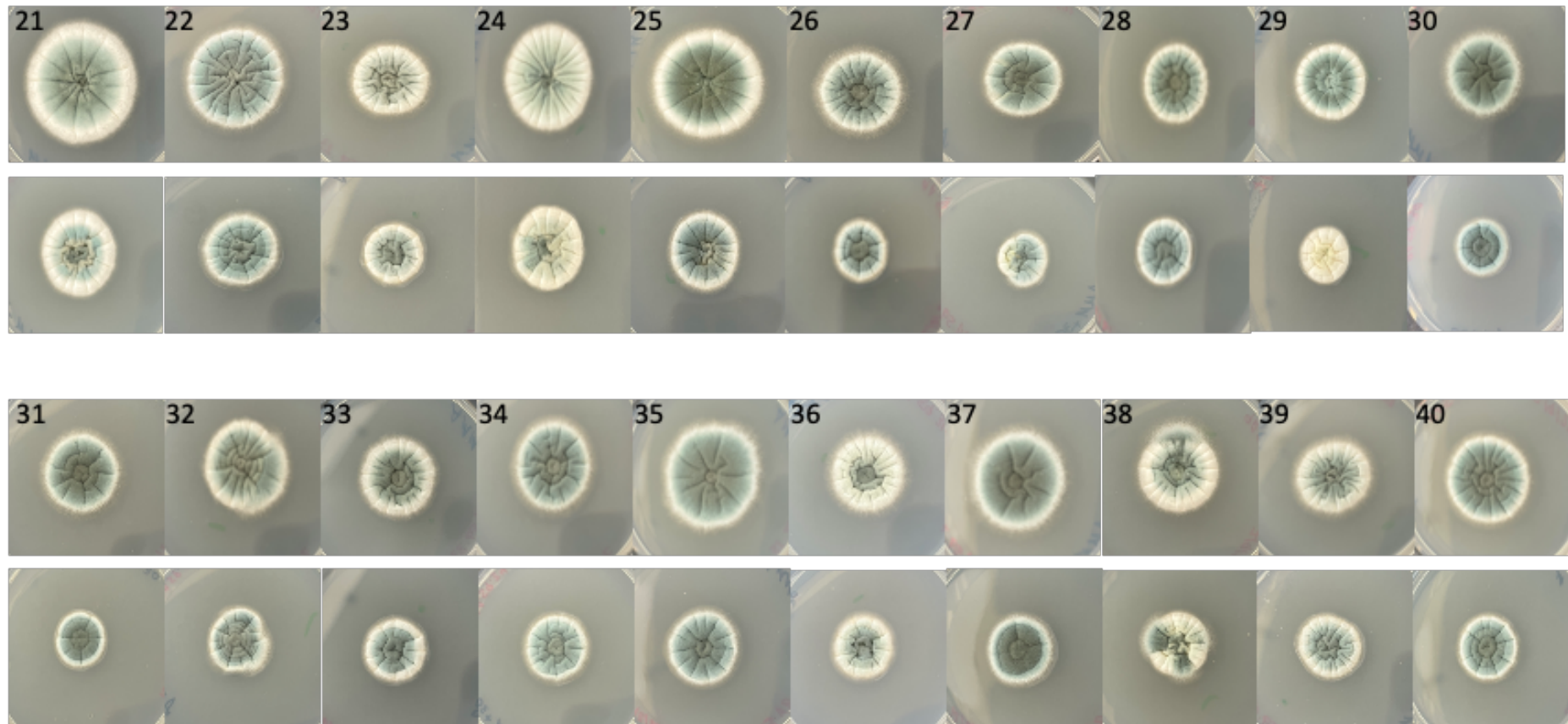
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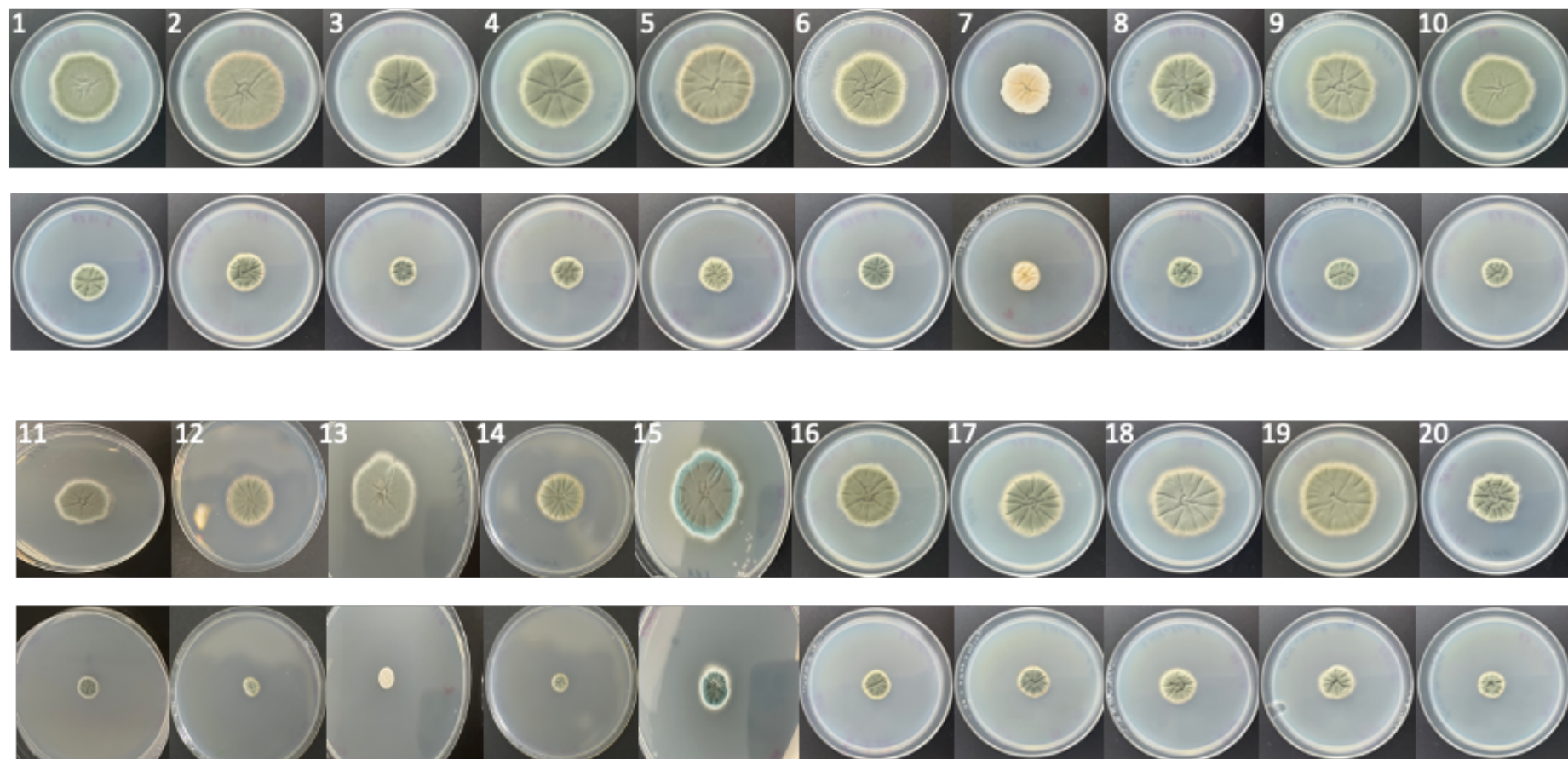
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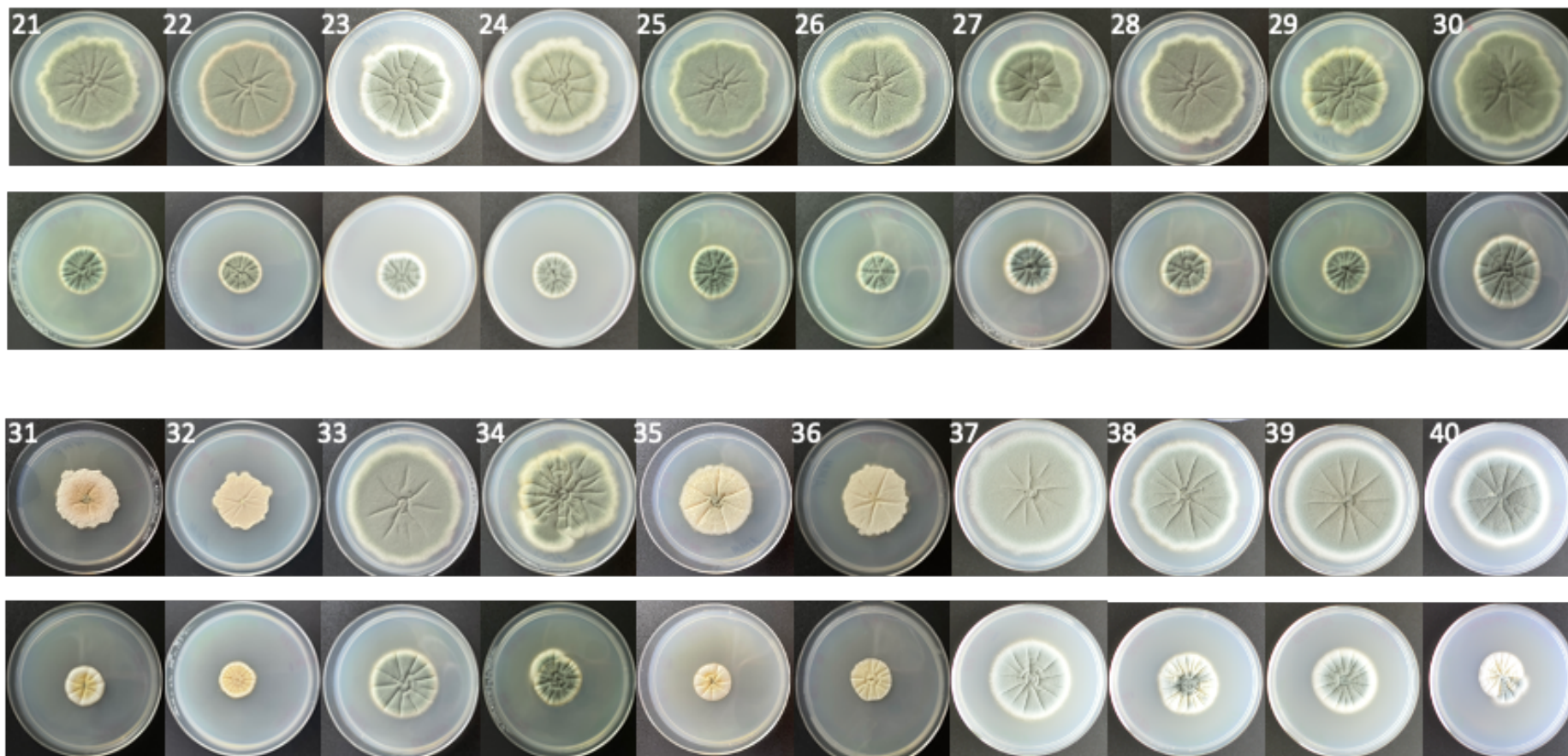
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Supplementary

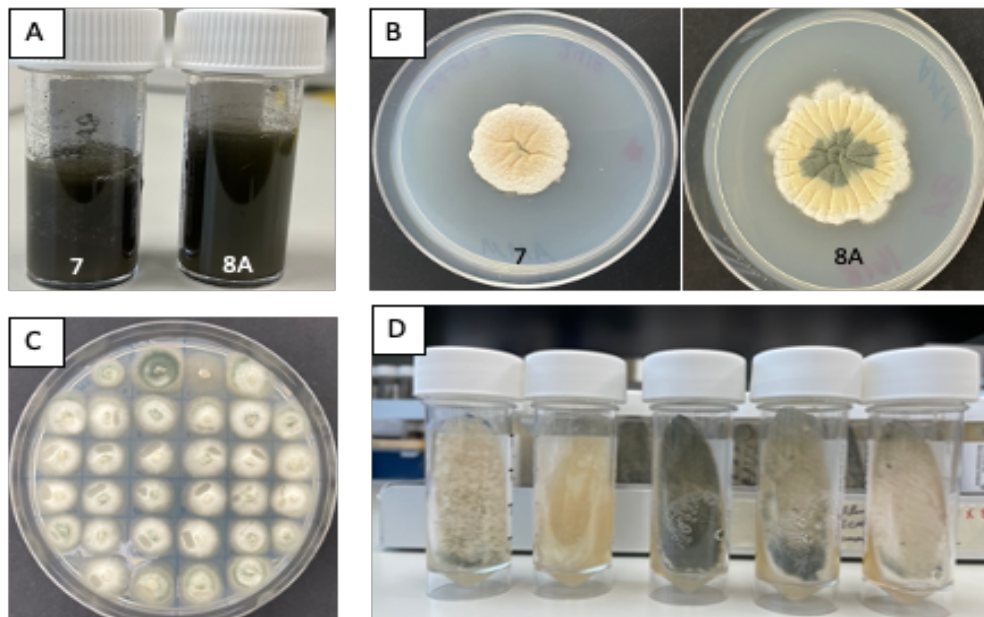


Supplementary 1: Single colony images showing morphology of 20 progeny from the cross 9A X 47-59 on AMM alone (top images) or AMM supplemented with 30 μ M menadione (bottom images); numbers in the top left corner of each image indicate progeny number.

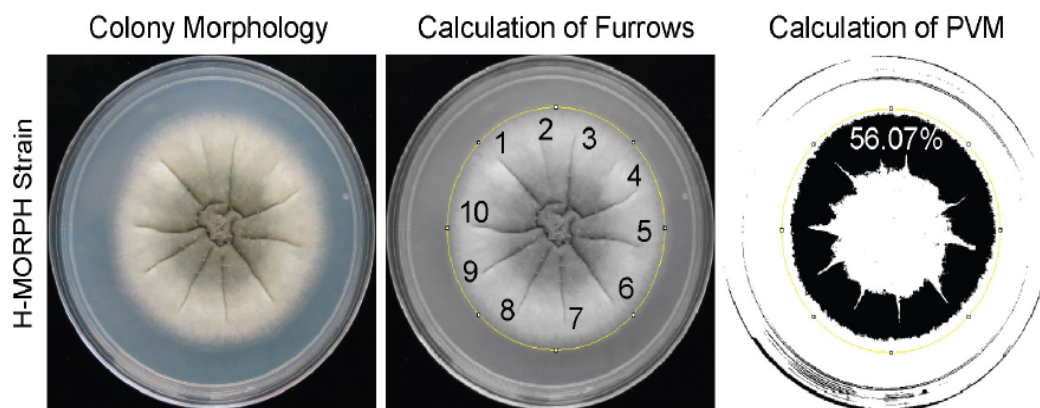




Supplementary 2: Single colony images showing morphology of all 40 progeny from the cross 8A X 47-51 on AMM alone (top images) or AMM supplemented with 30 μ M menadione (bottom images); numbers in the top left corner of each image indicate progeny number.



Supplementary 3: low sporulation and white non-conidiating mycelium of *A. fumigatus* progeny on ACM from a backcross between progeny number 7 and parental strain 8A. (A) universal containers contained spores suspension collected from a culture grown in ACM of the two strains: progeny number 7 and parental strain 8A showed the ability of both strains to produce spores on ACM. (B) morphology of progeny number 7 and parental strain 8A on AMM. (C) 36 collected offspring on ACM 9cm plate showing low sporulation and white non-conidiating mycelium after incubation at 37 °C for 2 days. for almost all progenies. (D) ACM slopes of cultured offspring again showing low sporulation and white non-conidiating mycelium after incubation at 28 °C for 7 days.



Supplementary 4: Example identification and quantification of the horological characteristics of interest: furrowing and percentage vegetative mycelia (white, non-conidiating mycelia; PVM) in order to identify H-MORPH. Adapted from