Investigating the use of natural products as potential antifungals

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Declaration:

No part of this thesis has been submitted in support of an application for any degree or qualification at the University of Kent or any other University or institute of learning. I can confirm all work submitted is of my own.

Daisy Shaw

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Abstract:

Rhizopus microsporus is a spore-forming filamentous fungus of the order Mucorales. *Rhizopus* species, are responsible for post-harvest rot of many soft fruits and vegetables, and are one of the aetiological agents causing the fatal disease mucormycosis. Given that *Rhizopus spp*. are naturally resistant to several commercial antifungal therapies, it is important that we find novel approaches to control the growth of these fungi. Here we investigate the antifungal activity of secreted metabolites from the Gram-negative bacterium *Pseudomonas aeruginosa* and the plant derived compound madecassic acid from the medicinal herb *Centella asiatica,* against *R. microsporus*. Both *P. aeruginosa* (PA01 and PA14) cells and supernatants displayed significant antifungal activity, killing 96% of the fungal spores, suggest that the bacterium secretes a molecule(s) with significant antifungal potential. Although *P. aeruginosa* secreted siderophores inhibited fungal growth via iron chelation, these siderophores were fungistatic, not fungicidal in the PA14 strain. Screening bacterial mutants in key secondary metabolites including phenazines, rhamnolipids, quinolones and polyhydroxyalkanoates, did not relieve the antifungal activity of the bacterium, suggesting these key virulence factors are not required for this effect. Pre-incubation of supernatant with spores before use as treatment against fresh spores showed removal of antifungal activity, suggesting that the fungus might metabolise or sequester the antifungal molecule. The plant-based molecule, madecassic acid exhibited antifungal activity against *R. microsporus, Candida albicans* and *Cryptococcus neoformans* with concentrations of 250 µM inhibiting growth for all three fungi. Our findings show that both *P. aeruginosa* and madecassic acid have potential for use as antifungal treatments alternative to those currently commercially available.

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Abbreviations:

1D NOESY	One dimensional Nuclear Overhauser Effect Spectroscopy
5MPCA	5-methyl-phenazine-1-carboxylic acid
AHL	Acyl-homoserine lactone
AML	Acute myeloid leukaemia
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
AOX	Alternative oxidase
AQ	Alkyl quinolone
BHB	β-hydroxy butyrate
C. albicans	*Candida albicans*
C. neoformans	*Cryptococcus neoformans*
CFU	Colony-forming units
COPD	Chronic obstructive pulmonary disorder
COVID-19	Coronavirus disease 2019
CT	Computed tomography
DFO	Deferoxamine
DKA	Diabetic ketoacidosis
DMSO	Dimethyl sulfoxide
DSS	Sodium trimethylsilylpropanesulfonate
E. coli	*Escherichia coli*
Fe.DFO	Ferrioxamine
IL-8	Interleukin-8
IV	Intravenous
LB	Lysogeny broth
MIC	Minimum inhibitory concentration
MoA	Mode of action
MOI	Multiplicity of infection
NMR	Nuclear magnetic resonance
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate buffered saline
PCA	Phenazine-1-carboxylic acid
PHA	Polyhydroxyalkanoate
PMS	Phenazine methosulfate
PQS	*Pseudomonas* quinolone signal
R. microsporus	*Rhizopus microsporus*
RNS	Reactive nitrogen species
ROCM	Rhino-orbital-cerebral mucormycosis
ROS	Reactive oxygen species

S. aureus	*Staphylococcus aureus*
SAB	Sabouraud dextrose broth
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SHAM	Salicylhydroxamic acid
T1SS	Type 1 secretion system
T2SS	Type 2 secretion system
T3SS	Type 3 secretion system
T4SS	Type 4 secretion system
T5SS	Type 5 secretion system
T6SS	Type 6 secretion system

CHAPTER ONE - INTRODUCTION

1.1 Fungal infections:

Whilst not as prevalent as their bacterial and viral counterparts, fungal infections are a growing problem in the human population worldwide. Although many fungal species are harmless and live commensally in both humans and animals, opportunistic pathogens can cause devastating consequences in immunocompromised populations[1]. The genus *Candida* is the most common cause of fungal infection in humans[2], and whilst these yeasts are likely the only fungal species to live commensally in the human flora, they also cause numerous infections. Fungi can also cause cutaneous infections in humans, such as ringworm, athlete's foot and nail infections, also known as onychomycosis[3]. Tinea pedis, more commonly known as athlete's foot, is considered the most common type of dermatophytosis[4], with over half of these infections caused by *Trichophyton rubrum*[5]. Some fungal infections are commonly associated with hospital visits, such as the budding yeast *Candida auris*. Since its discovery in 2009, *Candida auris* has been found in six of the seven continents of the world[6], and causes high mortality rates due to its resistance to many antifungal treatments. With hospitals likely acting as a reservoir for *C. auris*, aiding transmission against medically vulnerable individuals, this pathogen has been described as a burden on public health[7].

The top three invasive fungal pathogens are *Aspergillus fumigatus, Candida albicans* and *Cryptococcus neoformans.* Infections from the top ten invasive fungal species carry a higher mortality rate that from tuberculosis or malaria[8]. Invasive mould infections in particular are a significant cause of death in immunocompromised individuals[9], with aspergillosis and

mucormycosis occurring in 10% of patients being treated for haematological malignancies[10]. Cases of these invasive diseases are becoming more of a problem due to advances in treatments and improved prognosis of many illnesses causing an increased population of immunocompromised individuals[9].

1.2 Mucormycosis:

Mucormycosis is an invasive fungal infection caused by several filamentous fungi of the order Mucorales[11], most commonly of the genera *Rhizopus*[12] (47%), *Mucor* (18%), *Lichtheimia* (5%) or *Apophysomyces*[13] (5%)[14] and *Rhizomucor*[14] (4%). Whilst this disease is rare, it is associated with high mortality rates, particularly in patients with underlying conditions, such as poorly controlled diabetes mellitus, haematological malignancies, those who are immunocompromised[15], (but surprisingly not often those with HIV or AIDS[16]), and those undergoing corticosteroid therapy[16]. Otherwise, immunocompetent individuals can become susceptible to mucormycosis as a consequence of injuries causing open wounds and skin trauma[17]. After candidiasis and aspergillosis, mucormycosis is the third most common invasive mycosis to infect patients with haematological diseases[18]. Cases are rarely reported in patients without these predisposing factors. Depending on the route of entry and location of infection within the body, mucormycosis can cause different presentations of disease including rhino-orbito-cerebral, cutaneous, disseminated, pulmonary and even gastrointestinal mucormycosis[19], with symptoms dependent on the type of disease.

1.2.1 Rhino-orbital-cerebral mucormycosis:

Sudden onset rhino-orbital-cerebral mucormycosis (ROCM) is the most common manifestation of the disease, making up 34-39%[15,20] of mucormycosis cases. Rarely associated

with immunocompetent individuals, a predisposing factor for ROCM is commonly uncontrolled diabetes mellitus, due to a hyperglycaemic environment reducing phagocytotic ability, and resulting in glycosylation of iron chelating serum proteins such as transferrin and ferritin[17]. Filamentous structures can penetrate vascular walls, and continue to germinate, leading to ischemic necrosis[21]. Iron is essential for fungal growth, and the acidic environment occurring in cases of diabetic ketoacidosis (DKA) due to a build-up of the ketone body β-hydroxy butyrate (BHB)[17], leads to an increase of free iron in the environment due to dissociation from serum proteins. Patients undergoing treatment for haemochromatosis with the iron chelator deferoxamine (DFO) also have a higher susceptibility to mucormycosis[21]. Formation of ferrioxamine (Fe.DFO), the iron chelated complex formed with DFO, is key to this association. Mucormycosis causing microorganisms from the *Rhizopus* genera are able to bind to this chelated iron, due to ferrioxamine cell surface receptors[17], where it remains associated and can be used as an iron source for the fungus[22]. Fundamentally, Fe.DFO complexes in DFO treated patients act as iron-rich sources, aiding pathogenesis. The fungal pathogens causing mucormycosis rely on binding of fungal spore coat proteins (CotH) to the host GRP78 (glucose-regulator protein 78) for endothelium invasion. Excess glucose, iron and BHB also lead to upregulated expression of these receptors, increasing fungal invasion[17]. Common presentations of ROCM include nasal discharge and/or congestion, headache, pyrexia, blurry vision, and other symptoms commonly associated with a rhinovirus infection[21], as well as some more indicative symptoms, such as the classic hallmark of a black necrotic lesion, although the absence of this does not exclude the possibility of mucormycosis[16]. ROCM is usually identified through endoscopic investigation, looking for necrosis and taking samples for biopsy, as well as computed tomographic (CT) scans for identification of oedema in the oral or cerebral cavities[21].

1.2.2 Pulmonary mucormycosis:

Pulmonary mucormycosis is the second most common manifestation of mucormycosis (24% of cases)[23], with the predisposing factors commonly being haematological malignancies[24], particularly acute myeloid leukaemia (AML)[16] and solid organ transplants[14], with diabetes mellitus not being as strongly associated with this presentation of mucormycosis compared to others[24]. A case study reviewing 929 cases of mucormycosis states pulmonary mucormycosis is associated with 76% mortality rate[23]. Upon investigation, patients most commonly present with pleural and nodule effusion upon radiological investigations, in addition to neutropenic fever[24], and can be differentiated from similar diagnoses, such as invasive pulmonary aspergillosis via the identification of the reversed halo sign on CT scans[18], which can also aid early intervention and management, improving patient outcomes. Initial presentation with pulmonary mucormycosis usually includes pyrexia, dry and non-productive cough[16], pleuritic chest pain[25], dyspnoea and neutropenia[24]. Invasion of blood vessels by fungal hyphae can result in parenchymal necrosis, potentially leading to haemoptysis, although fatalities from pulmonary mucormycosis usually occur via disseminated infection, before respiratory failure presents[25].

1.2.3 Gastrointestinal mucormycosis:

Gastrointestinal mucormycosis, although very uncommon (7% of mucormycosis cases[26]), is associated with a mortality rate up to 85%[16,26], in part due to the difficulty and delay in its diagnosis. Gastrointestinal mucormycosis is acquired through ingestion of foodstuffs contaminated by a mucormycosis causing pathogen. Death in the case of gastrointestinal mucormycosis is most commonly caused by gastrointestinal haemorrhage[16]. Many of the symptoms of gastrointestinal mucormycosis are non-specific, such as nausea, vomiting, and

general abdominal pain. Blood in stools can also occur, and is thought to be attributed to abdominal abcesses[27]. Timely diagnosis, usually requiring surgery or endoscopy for confirmation, is critical for successful treatment, which often involves combination therapy of antifungals with surgical debridement of the lesions[26].

1.2.4 Cutaneous mucormycosis:

Cutaneous mucormycosis can present in varying severities and can be further categorised as localised, deep extension, or disseminated[16], by which point it can be become life-threatening. Cutaneous mucormycosis is obtained by skin inoculation with the responsible pathogen and is thought to represent 19% of mucormycosis cases[28]. Symptoms are typically necrotic skin lesions, swelling, abscesses[21] and ulceration[29]. Primary cutaneous mucormycosis is not uncommon in patients with serious skin wounds or burns[21]. Secondary cutaneous mucormycosis occurs due to dissemination of mucormycosis from another location[29]. Treatment for this clinical form usually involves antifungal treatment using liposomal amphotericin B and posaconazole, with surgical debridement often being necessary. Depending on severity of tissue damage and invasion into muscle fascia, skin grafts may be needed[28].

1.2.5 Disseminated mucormycosis:

Disseminated mucormycosis, although rarer than some other clinical forms (23%), is by far the one with the highest mortality rate (96%)[23]. This manifests after primary infections are left untreated and allowed to spread to other areas of the body, which is most commonly associated with spread of pulmonary mucormycosis in patients with severe neutropenia. The most common site of disseminated disease is the brain, with almost a 100% mortality rate

once cerebral dissemination occurs[30], although a 2009 case study shows a patient with relapsed leukaemia, who developed disseminated mucormycosis infections on the skin, in the lungs and the brain, was successfully treated with a combination of liposomal amphotericin B, posaconazole and isavuconazole[31].

1.2.6 Mucormycosis and COVID-19:

Since the onset of the coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), there has been a surge in the number of cases of mucormycosis as a secondary infection, particularly in India, suffering with a mucormycosis epidemic when they were significantly impacted by their second wave of the pandemic, with the highest case numbers of COVID-19 worldwide[32]. It is now known that recognition of the SARS-CoV-2 B.1.617.2 (Delta) variant spike protein, by ACE2 (angiotensin converting enzyme 2), leads to upregulation and translocation of host GRP78[33], which acts as an important host receptor for endothelial invasion by Mucorales[34]. Additionally, treatment for severe COVID-19 often involves the use of immunosuppressant drugs, such as corticosteroids, to reduce the cytokine storm that occurs as an elevated immune response to infection, increase the risk of infection[35]. Corticosteroid use can also lead to hyperglycemia[36], which, as mentioned previously, aids hyphal invasion[17].

1.3 *Rhizopus microsporus:*

One of the aetiological agents causing mucormycosis is *Rhizopus microsporus*. This is a spore-forming filamentous fungus, that is found ubiquitously in the environment and is considered an opportunistic pathogen to humans and animals in an immunocompromised host[37].

R. microsporus can reproduce via three different methods: vegetative, asexual, and sexual methods. The lifecycle of *R. microsporus* via asexual reproduction is demonstrated below in Figure 1.1. *R. microsporus* starts its life in the form of spores. Under optimal growth conditions, spores begin to swell and once they polarise, germ tubes start to form, before further development into non-septate hyphae. Sporangiophores form from the hyphae and mature to produce spherical sporangia, containing more spores. Once the sporangia have matured, they lyse and release sporangiospores into the environment. Sporangiospores are the transmissible form of this pathogen, with hyphae causing invasion[38].

1.4 Treatment of fungal infections and antimicrobial resistance:

Antimicrobial resistance (AMR) is currently a rising global issue. It is well recognised that we are moving towards a time where many of the antimicrobial treatments we currently use will become redundant against many disease-causing microorganisms[39]. Whilst most of the antimicrobial resistance focus is placed on bacteria and their resistance to antibiotics, fungal pathogens are also becoming increasingly resistant to the antimicrobial agents that were once used to treat them. Antifungal resistance is particularly concerning due to the similarities between fungal species and their eukaryotic hosts, limiting the number of drug targets that can be used[40]. Classes of antifungal agents are not numerous, with echinocandins, polyenes, azoles and flucytosine being the limited treatment options[41]. Some fungal species, such as *C. auris*, are inherently resistant to antifungal drugs, with resistance mechanisms occurring naturally, whilst others develop resistant strains in their once susceptible population, through positive selection of resistance mechanisms[41]. Mucormycosis poses difficulties when it comes to treatment, due to the inherent antimicrobial resistance within the Mucorales. It has been suggested that *M. circinelloides* develops drug resistance through use of RNAi mechanisms[42].

Resistance to azoles is particularly prevalent in this order of microorganisms, with some studies showing Mucorales display complete resistance to the azole posaconazole[43]. An example of a drug target and resistance mechanism involves the biosynthesis of ergosterol, the fungal equivalent of cholesterol. Azoles cause fungistatic effects by inhibiting the lanosterol 14α-demethylase enzyme, which converts lanosterol to ergosterol, for cell membrane integrity maintenance. Fungal resistance against azoles can be achieved via mutations in the *ERG11* gene, which encodes lanosterol 14α-demethylase, changing the affinity of azole binding to the enzyme[44]. Echinocandins are another class of antifungal agents, which work by inhibiting synthesis of the cell wall component β-(1,3)-D-glucan, which is not present in mammalian cells, by binding non-competitively to the Fks1p catalytic subunit of β-(1,3)-D-glucan synthase[45]. Mucorales are known to have resistance against echinocandins[46].

Mutations in the *FSK1*, *FSK2* and *FSK3* genes code which code for Fks1p, prevent binding of echinocandins to this subunit, thus preventing inhibition of β-(1,3)-D-glucan synthesis[45].

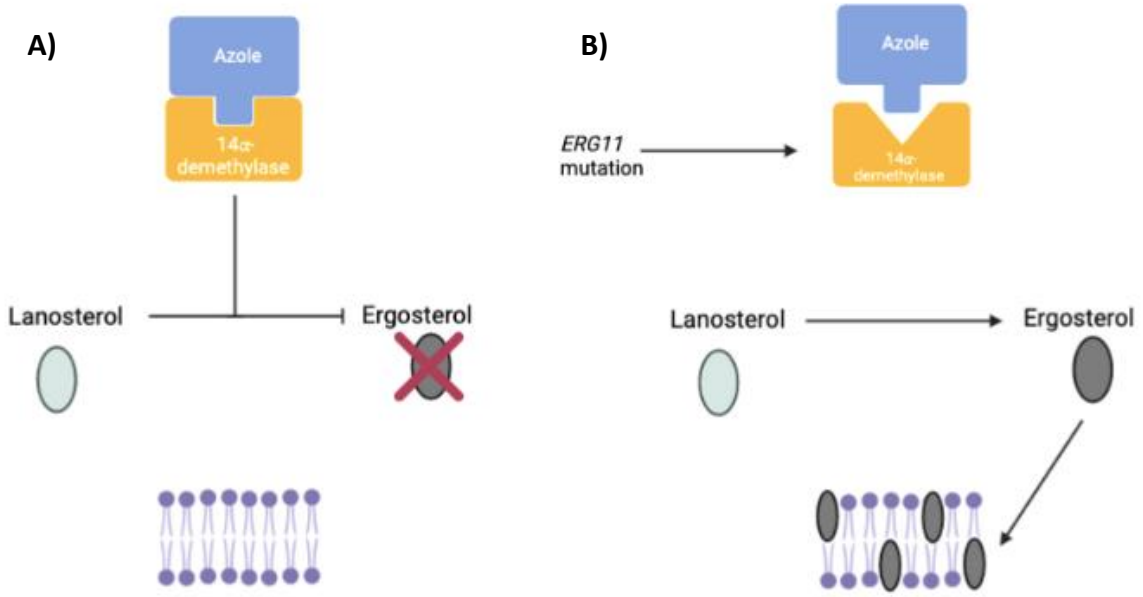

Figure 1.2. Antifungal resistance to azoles. A) In susceptible fungal cells, azoles inhibit the enzymatic activity of lanosterol 14α-demethylase to convert lanosterol to ergosterol. B) Azole resistance mechanisms include mutation of the *ERG11* gene encoding lanosterol 14α-demethylase, changing its binding affinity to azoles, thus preventing inhibition of conversion of lanosterol to ergosterol for cell membrane stability[44]. Created with BioRender.com

Due to antifungal resistance, polyene therapy remains the main choice of treatment for mucormycosis[47], along with surgical debridement where necessary[48]. Intravenous (IV) liposomal amphotericin B is usually the first-choice antifungal agent used in treatment against mucormycosis. In liposomal formulations, the Amphotericin B is incorporated into a liposome bilayer[49], generating a drug with less nephrotoxicity and increased tolerability compared to conventional amphotericin B deoxycholate, which was used more commonly in the past[50]. The mode of action of amphotericin B involves binding of the polyene to ergosterol in the fungal cell membrane, inducing ion channel formation. This leads to loss of protons and cations through the channels, leading to depolarisation of the cell and ultimately cell death.

Amphotericin B can also cause oxidative damage to fungal cells by forming free radicals and increasing membrane permeability, as well as stimulating phagocytic cells in the host immune response[50].

The newly developed triazole, isavuconazole, has been shown to be effective against mucormycosis, although no more than amphotericin B[20]. Of importance is also the administration technique of existing and potential new antifungal treatments. Liposomal amphotericin B is administered intravenously[49], and cannot be absorbed by the body via intramuscular or oral administration[50], which can limit its use in patients without easy access to hospitals, and makes it more difficult to distribute. It is therefore important that we continue to develop novel treatments against mucormycosis, with different modes of administration, especially since its surge of new cases in Indian COVID-19 patients, since their second peak of SARS-CoV-2 infection in the pandemic[51].

It is imperative that we continue to investigate novel therapies and approaches to treatment resistant fungal infections. We already know that bacterially secreted products, such as pyoverdine and pyochelin, can demonstrate fungistatic effects[52], so further investigation of prokaryotes and other natural products are a sensible place to start.

1.5 Aims and objectives:

The aims of this project are to investigate the suitability of natural products for use as novel antifungal agents against *R. microsporus* infection, using both bacterially secreted products, and plant-derived compounds.

CHAPTER TWO – EXPLORING BACTERIA FOR NOVEL ANTIFUNGAL AGENTS

2.1 Introduction:

2.1.1 Bacteria as antifungals:

As already mentioned, antimicrobial resistance is becoming an ever-increasing problem and is becoming a burden on healthcare systems[39]. It is therefore more important now than ever that we explore new strategies for producing and isolating novel antimicrobial agents. It is not a new concept that bacteria have the ability to produce antifungal molecules, with many examples of commercially used antifungals being synthesised by bacteria. The widely used polyene, nystatin, is produced by *Streptomyces noursei*[53], and another polyene, pimaricin, is produced by several *Streptomyces* species, such as *S. natalensis, S. gilvosporeus, S. chatanoogensis* and *S. lydicus*[54]. Of important note is that amphotericin B, the chosen therapy for mucormycosis, is produced by the bacterium *Streptomyces nodus*[55], highlighting the capabilities of bacteria to be used to treat fungal infections, particularly mucormycosis.

2.1.2 *Pseudomonas aeruginosa:*

Pseudomonas aeruginosa is a Gram-negative, aerobic, rod-shaped bacterium, which is known for infecting individuals with cystic fibrosis, burn wounds and those with other predisposing conditions, such as chronic obstructive pulmonary disorder (COPD), or cancer[56]. *P. aeruginosa* is known to form biofilms, and is one of the ESKAPE pathogens, alongside *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Enterobacter* species, which are all highly antibiotic and multi-drug resistant, and major causes of nosocomial infections[57].

P. aeruginosa has been seen to exhibit antimicrobial activity against certain pathogenic fungi, such as *Aspergillus fumigatus, Saccharomyces cerevisiae*[58], some *Candida* species: *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis*, *Candida glabrata*[59]*, Candida kefyr, Candida guilliermondii, Candida lusitaniae and Candida pseudotropicalis*, as well as other bacterial pathogens[58]. Most importantly to this project, *P. aeruginosa* has shown antimicrobial activity against *R. microsporus*[52]. The ability of this bacterium to have this effect on such a range of other microorganisms is potentially due to its common co-existence alongside these species, leading to the need to evolve competitive advantages to outgrow other species within the same niche. In the lungs of cystic fibrosis patients, *A. fumigatus* and *P. aeruginosa* are the most common co-colonising microorganisms[60], and for that reason their interactions have been regularly studied and give indications of potential bacterial mechanisms or virulence factors that *P. aeruginosa* is utilising.

2.1.3 Known metabolites secreted by *P. aeruginosa:*

2.1.3.1 Phenazines:

There are many speculated bacterial molecules that are thought to be responsible for inhibiting growth of fungi. One of which is 5-methyl-phenazine-1-carboxylic acid (5MPCA), known to be a precursor of the blue pigmented phenazine pyocyanin, which has previously exhibited antifungal activity against *C. albicans.* The structural analogue of 5MPCA, phenazine methosulfate (PMS), is usually used in laboratory experiments, as 5MPCA is unstable under physiological conditions. Figure 2.1 below shows the phenazine pathway. Bacterially secreted phenazines, which are nitrogen containing heterocyclic compounds[61], have been seen to have important interactions with fungal microorganisms and accumulate in the fungal cell wall by

covalently binding to fungal amines, generating reactive oxygen species (ROS)[62] and reactive nitrogen species (RNS)[61].

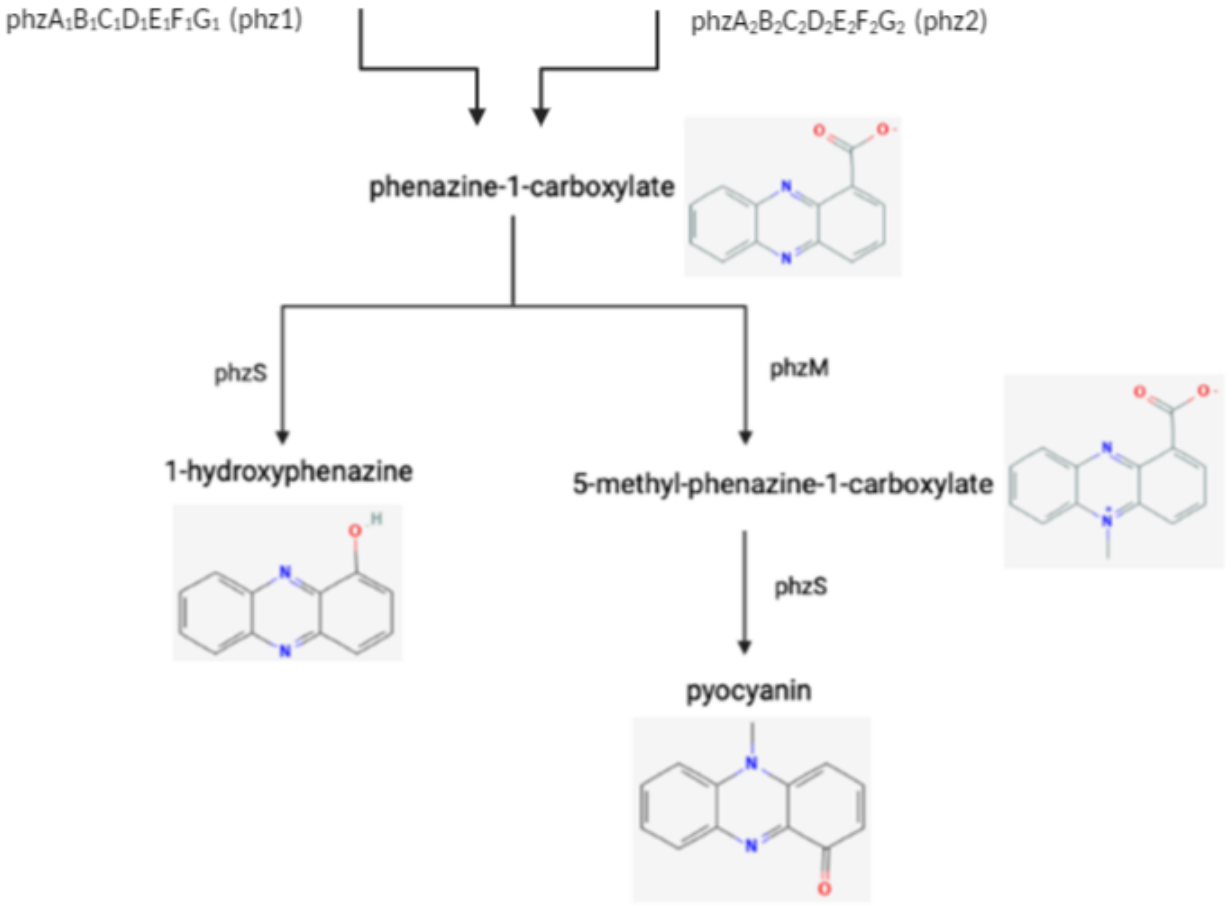

Figure 2.1. Phenazine pathway in *P. aeruginosa*. 5-methyl-phenazine-1-carboxylate (5MPCA) is a precursor of pyocyanin. Diagram created with BioRender.com
PubChem Identifier: CID 7097402
URL: https://pubchem.ncbi.nlm.nih.gov/compound/phenazine-1-carboxylate
PubChem Identifier: CID 135412648
URL: https://pubchem.ncbi.nlm.nih.gov/compound/1-Hydroxyphenazine
PubChem Identifier: CID 50986167
URL: https://pubchem.ncbi.nlm.nih.gov/compound/5-Methylphenazine-1-carboxylate
PubChem Identifier: CID 6817
URL: https://pubchem.ncbi.nlm.nih.gov/compound/Pyocyanine

2.1.3.2 Quinolones:

The quorum sensing abilities of *P. aeruginosa* are important to its virulence gene regulation, with its key quorum sensing molecules being alkyl quinolones (AQ) and acyl-homoserine

lactones (AHL), which induce activity of the transcriptional regulators LasR and RhlR[61]. *Pseudomonas* quinolone signal (PQS), which is regulated via quorum sensing genes, is known to have many roles, including regulating expression of other virulence factors, induction of oxidative stress and iron chelation[63,64]. PQS therefore is important for sustained *P. aeruginosa* infection and it is no surprise that this molecule is often found in the lungs of Cystic Fibrosis patients[64].

2.1.3.3 Rhamnolipids:

Mono- and di-rhamnolipids are glycolipid biosurfactants produced by *P. aeruginosa* and another class of molecules regulated by quorum sensing. Rhamnolipids can be a useful defence against other bacterial species, causing disorganisation of plasma membranes, and importantly promote *P. aeruginosa* swarming motility[61]. These molecules have been seen to be toxic against *Aspergillus fumigatus*[60] by causing thickening of the fungal hyphal wall and inhibition of β 1-3 glucan synthase activity. The effects of rhamnolipids against *A. fumigatus* are thought to be fungistatic rather than fungicidal[61].

2.1.3.4 Toxins:

P. aeruginosa secretes a plethora of other enzymes and toxins that could exhibit antimicrobial effects against other species that may provide competition to its survival. Important examples include exotoxin A, elastase, phospholipase C and exoenzyme S[65]. Elastase, which is regulated by the previously mentioned LasR and RhlR, exerts virulence by degradation of host immune cells and catabolism of the extracellular matrix protein eslatin[61]. Exotoxin A, which is injected into neighbouring cells via the type 3 secretion system (T3SS), inhibits elongation factor 2 (EF2) within the host, and therefore prevents protein synthesis within the cell[66].

2.1.4 Bacterial secretion systems:

P. aeruginosa makes use of a range of bacterial secretion systems. The type one, three, four (and potentially six) secretion systems (T1SS, T3SS, T4SS and T6SS) use a one-step process for release of molecules and compounds, whilst the type two and five secretion systems (T2SS and T5SS) use a two-step process[67], requiring Sec or Tat secretion pathways for the transport of substrates across the inner membrane, before T2SS or T5SS transportation across the outer membrane[68].

T1SS, which uses an outer membrane protein, ATP-binding cassette transporter and an adaptor protein, is responsible for secretion of the alkaline protease AprA and the AprX protein, for which the function is still unknown[61]. T2SS is responsible for the transport of numerous metabolites across the bacterial membrane, including elastase, phospholipase C, alkaline phosphatases, proteolytic enzymes and ADP-ribosyl transferases[69]. T3SS utilises injection of effectors into host cells via an injectisome[67], and these effectors include ExoS, ExoT, ExoU and ExoY[61], of which ExoU has been identified as the most biologically toxic, exhibiting phospholipase A2 activity[70]. T4SS, which can be categorised as effector translator systems and conjugation machines, is involved in the translocation of DNA and proteins to target cells[71]. It is thought that *P. aeruginosa* does not use the typical T4SS[67], although in other bacteria, this system is involved in horizontal gene transfer[69]. T5SS is involved in the transport of EstA (glycerol esterase A)[72], which is thought to be important for rhamnolipid production[69]. T6SS is the most recently discovered secretion system in *P. aeruginosa* and is also thought to transfer effectors via injection into the host cell[67], with its main roles being in biofilm formation and virulence[69].

2.1.5 Aims and objectives:

The aims and objectives of this chapter are to reproduce previous data showing that *P. aeruginosa* wild type strains have fungistatic and fungicidal effects against *Rhizopus microsporus,* investigate potential pathways responsible for antifungal activity and identify a mode of action (MoA) of antifungal activity.

2.2 Materials and methods:

2.2.1 Media and strains:

LB broth was made using 25 g/L powder (Sigma-Aldrich), and when required, supplemented with 2% Agar (Melford). SAB broth was made using 30 g/L powder (Sigma-Aldrich). SAB agar was made using 65 g/L powder (Merck), and for viability counts, was supplemented with 50 mg/ml filter sterilised tetracycline hydrochloride, for a final concentration of 50 µg/ml. M9 minimal media was made using M9 salts (6.6 g/L Na_2HPO_4, 3.3 g/L KH_2PO_4, 1.1 g/L NH_4Cl, 0.55 g/L NaCl, 1mM $MgSO_4$, 0.1mM $CaCl_2$), 4 g/L glucose and 10 ml/L thiamine. PBS was made using one Oxoid tablet (pH 7.3±0.2 at 25c, sodium chloride 8.0 g/L, potassium chloride 0.2 g/L, di-sodium hydrogen phosphate 1.15 g/L, potassium dihydrogen phosphate 0.2 g/L) per 100ml.

The *R. microsporus* strain used in this study was a clinical isolate obtained from the Birmingham Trauma Centre[52]. All *P. aeruginosa* strains used in this study are listed in Table 2.1.

Table 2.1. *Pseudomonas aeruginosa* strains used:

NAME	DESCRIPTION	FUNCTION AFFECTED	STRAIN	Source
PA01WT ATCC 15692	Wild type	N/A (wild type)	N/A (wild type)	[73]
PA14 Newman WT	Wild type	N/A (wild type)	N/A (wild type)	[74]

ΔrhlA	*rhlA* knockout mutant	Removal of gene that synthesises 3-(3-hydroxyalkanoyloxy) alkanoic acids	PA14 WT	[75]
ΔrhlC	*rhlC* knockout mutant	Removal of gene catalysing formation of di-rhamnolipids	PA14 WT	[75]
Tn *ΔrhlA 1020*	*rhlA* mutant obtained through transposon mutagenesis	Removal of gene encoding rhamnosyltranferase chain A	PA14 WT	[76]
Tn *ΔrhlA 1021*	*rhlA* mutant obtained through transposon mutagenesis	Removal of gene encoding rhamnosyltranferase chain A	PA14 WT	[76]
Tn *ΔrhlB*	*rhlB* mutant obtained through transposon mutagenesis	Removal of gene encoding rhamnosyltranferase chain B	PA14 WT	[76]
ΔpqsA-C	Mutant lacking *pqsA-C* genes	Quinolone signal biosynthesis prevented	PA14 WT	[76]

Tn *ΔpqsA-C*	*pqsA-C* mutant through transposon mutagenesis	Quinolone signal biosynthesis prevented	PA14 WT	[76]
Tn *ΔpqsA*	*pqsA* mutant through transposon mutagenesis	Quinolone signal biosynthesis prevented	PA14 WT	[76]
Tn *ΔpqsH*	*pqsH* mutant through transposon mutagenesis	Quinolone signal biosynthesis prevented	PA14 WT	[76]
ΔphaG	*phaG* mutant	Removal of gene encoding (R)-3-hydroxyacyl-CoA	PA14 WT	[76]
ΔphaC	*phaC* mutant	Removal of gene encoding poly(3-hydroxyalkanoate) synthase 2	PA14 WT	[76]
ΔpvdA / ΔphZ1/2 / ΔpchE	Triple knockout mutant of *phZ1/2* operons, and *pvdA* and *pchE* genes	Phenazine-1-carboxylate, pyoverdine and pyochelin not produced	PA14 Newman WT	[74]

ΔaprD	Type 1 secretion system knockout	Removal of gene encoding alkaline protease secretion protein AprD	PA14 WT	[76]
ΔxcpT	Type 2 secretion system knockout	Type 2 secretion system	PA14 WT	[76]
ΔpscD	Type 3 secretion system knockout	Type 3 secretion system via disruption of inner ring of the T3SS needle structure[69]	PA14 WT	[76]
ΔfimX	T4BP knockout	Type 4 pili assembly	PA14 WT	[76]
ΔclpV2	Type 6 secretion system knockout	Type 6 secretion system	PA14 WT	[76]
ΔhsiC3	Type 6 secretion system knockout	Type 6 secretion system	PA14 WT	[76]

2.2.2 Sub-culturing of *Pseudomonas aeruginosa*:

Bacterial strains were stored at -80℃ in 25% glycerol, and then streaked onto solid LB agar and incubated overnight at 37℃. Bacterial colonies were transferred to a flask of LB liquid broth and cultured overnight at 37℃ in a shaker incubator. PA01 M.W. and PA14 Newman strains were used as the wild type strains.

2.2.3 Sub-culturing of *Rhizopus microsporus*:

SAB agar plates were inoculated with *Rhizopus microsporus* stocks stored at -80°C in 25% glycerol. Plates were incubated at room temperature, upside down, until use at ~13-15 days. Further sub-cultures were performed by flooding the spore plates with 10 ml PBS and transferring 100 µl of PBS-spore suspension onto a new SAB agar plate, spreading across the plate and then incubating as mentioned previously.

2.2.4 Harvesting *Rhizopus microsporus* spores:

Plates containing ~13-15-day old spores were flooded with 10 ml PBS, using a plate spreader to cover the whole plate. Suspension was transferred to a falcon tube and centrifuged at 3000 rpm for 3 minutes. Supernatant was disposed of, and spore pellet was resuspended in 10 ml PBS. Using a haemocytometer, an initial spore count was obtained.

2.2.5 Separation of bacterial cells and supernatant:

After being incubated overnight at 37°C, 200 rpm in LB broth, 8-10 ml of bacterial sample was transferred to a falcon tube and centrifuged for 10 minutes at 4000 rpm. Supernatant was poured off and filter sterilised with a 0.2 µm filter to remove any remaining bacterial cells. Bacterial pellet was resuspended in 10 ml PBS and 1 ml was transferred to a 1.5 ml Eppendorf, before centrifuging for 1 minute at 13,000 rpm. Washing and resuspension in PBS was repeated 3 times.

2.2.6 OD_{600} bacteria:

The washed bacterial cell samples were diluted 1:10 in PBS, and their optical density at 600 nm was measured via spectrophotometry, using PBS as a blank. The desired MOI of

fungi:bacteria was 1:100, so based on 10,000 fungal spores per ml, and 10^6 CFU/ml needed, the target OD_{600} value was 8.16 x 10^{-4} [77]. The volume of bacteria required to add to the assay was calculated using $\frac{(target\ OD600)\times(1*10\text{^}-3)}{Current\ OD600\ of\ culture}$.

2.2.7 Spore viability assay:

1x10^4 spores/ml of *Rhizopus microsporus* were added to wells of a 24 well plate, containing either bacterial cells, or 50% bacterial supernatant, with a 50:50 SAB:LB media control and a positive fungal germination control. T0 controls were set up the same as positive controls but plated out at a 1:100 dilution (in PBS) onto SAB agar (containing 50 mg/ml Tetracycline). The 24 well plate was incubated statically overnight at 37℃. Plates were then observed under the light microscope and the level of germination was determined. Wells without excessive hyphal germination were plated out onto SAB agar (containing 50 mg/ml Tetracycline), both undiluted and diluted 1:10 with PBS. These plates were incubated upside, overnight, at room temperature. The following day, plates were viewed over a light box and the number of visible spores were counted as a check for viability and compared to the viability of the T0 control plates.

2.2.8 Addition of iron to spore viability assay:

10 mM stock solution of $FeCl_3$ was made and was diluted with PBS to give a 100 µM working solution, which was added to wells of the 24 well plate assay and left to incubate at 37℃ overnight. Iron spiked wells were compared to those prepared as shown in the previous step.

2.2.9 6-hour incubation assay:

24 well plate spore viability assays were set up as described previously, with extra wells containing spores, but no supernatant or bacterial cell treatment. The plate was incubated for 6 hours, to begin the germination process, before adding treatment to the additional wells and leaving for the remainder of the 24 hours. Just as T0 controls were used, for the 6-hour incubation assay T6 controls were also plated out and used as a comparison.

2.2.10 Pre-incubation of supernatant with spores:

PA14 was grown overnight in LB media at 37℃, 200 rpm. Culture was centrifuged at 4000 rpm for 10 minutes and supernatant was removed, and filter sterilised using a 0.2 µm filter. A 10 ml pre-incubation culture was made using 50% supernatant and 50% SAB:spores suspension, with spores at a final concentration of $1x10^4$ CFU/ml, using either dormant spores, or spores killed via autoclaving. Co-culture was incubated statically at 37℃ for 24 hours, before filter sterilising supernatant with a 0.2 µm filter to remove fungal spores. Supernatant was then used as above in the standard spore viability assay.

2.2.11 XTT assay:

A Roche Cell Proliferation Kit II (XTT) was used to perform cell viability staining on fungal spores and hyphae. Labelling reagent and electron-coupling reagent were thawed at 37℃ in a water bath. Per 96 well plate, 5 ml labelling reagent was mixed with 0.1 ml electron-coupling reagent, and 50 µl of this was added to each well. The plate was incubated for a further 4 hours at 37℃, before measuring absorbance values of samples at 450 nm and 630 nm.

2.2.12 Organic extraction of secreted products – ethyl acetate extraction:

50 ml *P. aeruginosa* PA14 was cultured overnight in liquid LB at 37℃, 200 rpm. After transferring overnight culture to a falcon tube, it was centrifuged for 10 minutes at 4000 rpm and supernatant was separated from bacterial cell pellet. Supernatant was filter sterilised, using a 0.2 µM filter, and then added to ethyl acetate in a 1:1 ratio to separate hydrophobic and hydrophilic molecules. After leaving to separate, both layers were transferred to separate falcon tubes. The tube containing the hydrophobic fraction was left in a fume hood to allow the ethyl acetate to evaporate, before resuspending in 100% DMSO to obtain the same concentration as in the original supernatant sample. This process was also repeated using LB media, as a control to ensure ethyl acetate treatment does not impact fungal growth.

2.2.13 Organic extraction of secreted products – chloroform extraction:

The same process was repeated as above in the ethyl acetate extraction, but filter sterilised supernatant was added to chloroform in a 1:1 ratio. After leaving to separate, the different layers were transferred to their own falcon tubes and the elution containing the chloroform was left to evaporate in a fume hood. The samples were resuspended in 100% DMSO to achieve the same concentration as in the original supernatant sample. This was again repeated with LB media, as a control to ensure chloroform does not impact fungal growth.

2.2.14 Organic extraction of secreted products – amberlite extraction:

50 ml of *P. aeruginosa* PA14 was cultured overnight in LB media at 37℃, 200 rpm in a shaker incubator. Overnight culture was transferred to a falcon tube and centrifuged for 10 minutes at 4000 rpm. Supernatant was removed from the bacterial pellet and filter sterilised using a 0.2 µM filter. 10 ml of supernatant was added to 1g Thermo Fisher Scientific Amberlite® XAD-

4 in a 15 ml falcon tube and inverted several times, before leaving on a shaker incubator for 10 minutes. The sample was centrifuged for 3 minutes at 4000 rpm and the hydrophilic supernatant was pipetted off and kept. The pellet of amberlite beads was resuspended in 10 ml 100% ethanol and inverted continuously for 1 minute to desorb the hydrophobic molecules from the amberlite beads. This was again centrifuged for 3 minutes at 4000 rpm and the supernatant was removed and left to evaporate in a fume hood, before resuspending in DMSO, maintaining the same concentration as is normally added to the assay.

For rotary evaporation, the above was repeated, but scaled up for use with 800 ml *P. aeruginosa* overnight culture. Amberlite XAD-4 beads were added to the supernatant at a concentration of 100 g/L and incubated overnight at 4°C. Supernatant was removed from the amberlite beads using a syringe and filter paper. Amberlite beads were washed with ethanol at concentrations of 10%, 50% and 100%, via column chromatography. Respective elutions were dried down using a rotary evaporator and resuspended in 5-10 ml 100% ethanol. Fractions were spun in a vacuum centrifuge for 10 hours, at room temperature, until all ethanol was evaporated. Samples were resuspended in 1.5 ml ethyl acetate and 10 µl was added to 1 ml wells containing $1x10^4$ CFU/ml *R. microsporus* dormant spores in 50:50 SAB:LB media. A control containing 10 µl ethyl acetate with spore suspension was also used to determine if the solvent itself had an effect.

Spore viability assays were set up using a negative media control, a positive control using spores with no treatment and wells containing the hydrophilic supernatant, the hydrophobic supernatant and the two combined back together, as well as the corresponding LB extraction elutions as controls. Assays were also set up with amberlite beads added directly to wells

after incubation with supernatant, instead of eluting, evaporating, and resuspending in DMSO. Wells were observed under a light microscope after 24 hours for signs of fungal growth inhibition. OD_{600} plate readings were also taken.

2.2.15 Respirometry:

An Oroboros Oxygraph-2k respirometer with 2 ml capacity chambers was loaded with 1 ml SAB:spore suspension and 1 ml LB (control) or 1 ml sterile PA14 supernatant (antifungal treatment), with a final concentration of $1x10^6$ CFU/ml of *R. microsporus* dormant spores and incubated at 37℃.

To allow initial germination of spores to begin, an alternative method was also used. 2 ml $1x10^6$ CFU/ml spores suspension in SAB media was added to each chamber and incubated at 37℃ until readings became stable. 1 ml of supernatant (and a 1 ml LB control) were injected, displacing 1 ml of the spores suspension. The samples were left to incubate at 37℃ to observe the effect of supernatant treatment on mitochondrial respiration rate.

2.2.16 Nuclear Magnetic Resonance Spectroscopy:

Four different samples: an M9 media blank, a known antifungal supernatant sample, supernatant treated with amberlite XAD-4 resin, and supernatant pre-incubated with dormant *R. microsporus* spores, were analysed using one-dimensional (1D) 1H NMR spectroscopy on a 600 MHz AVANCE III spectrometer equipped with a QCI-P cryoprobe (Bruker) at 298 K with a transmitter frequency of 600.05 MHz locked to D_2O (5% v/v), with a total sample volume of 650 ml, with 0.1% w/v DSS (sodium trimethylsilylpropanesulfonate) reference. Tuning and shimming were carried out automatically for each sample as was the

90° pulse calibration. The receiver gain was limited to a maximum value of 128. Data used for metabolite concentration analysis were drawn from presat-NOESY (noesygppr1d) spectra with a mix time (Tmix) of 100 ms using industry standard parameters compatible parameters compatible with the Chenomx metabolite library (CHENOMX 9.0 https://www.chenomx.com; accessed July 2022). The presat-NOESY was measured using 512 scans and 8 dummy scans with a spectral width of 15.98 ppm (9590.75 Hz), giving an acquisition time of 1.71 seconds, a relaxation delay of 3 seconds was used, and the data size was 32,768 points giving a total recycle time of 4.7 seconds. For all experiments, the water resonance was optimised (automatically) for maximum suppression (o1p was ~4.699 ppm), the field strength of the presaturation was 140.25 Hz (standard Bruker parameters) and presaturation was applied both during the D1 delay and the mixing Tmix.

2.2.17 Statistical analysis:

Prism GraphPad was used for producing all graphs and performing all statistical analysis. One-way analysis of variance (ANOVA) tests were performed for each data set, using α=0.05 as the significance threshold, with the null hypothesis (H_0) that there was no significant difference between groups and the alternate hypothesis (H_1) that one or more groups significantly differ. Standard deviation was used for error bars on graphs.

2.3 Results:

2.3.1 Germination of *R. microsporus* is inhibited by *P. aeruginosa*, independent of cell-to-cell contact:

Hyphal formation and germination are key to growth of *R. microsporus*, so compounds that delay or reduce this germination could be good candidates for novel antifungal treatments. To identify whether *P. aeruginosa* produces any molecules that are capable of inhibiting *R. microsporus* germination, dormant fungal spores were incubated with either bacterial cells or supernatant from two strains of *P. aeruginosa* (PA01 and PA14). Both bacterial strains, and both cells and supernatant, were able to inhibit fungal germination, and therefore growth of *R. microsporus* (Figure 2.2), suggesting that the bacterial supernatant contains secreted molecules that exhibit potential antifungal activity.

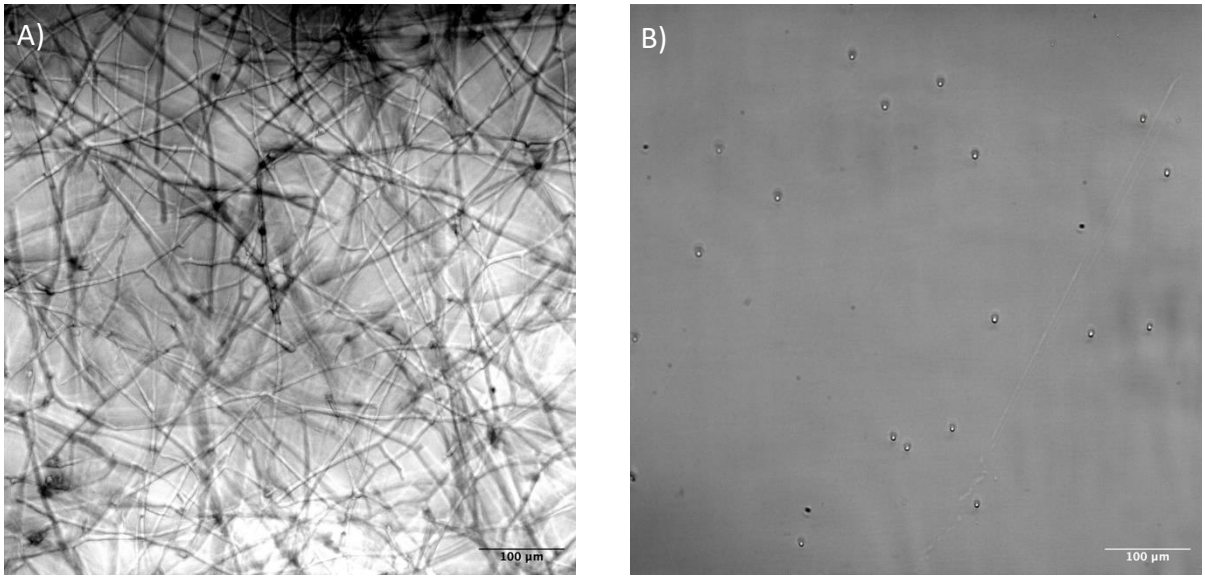

Figure 2.2. *Pseudomonas aeruginosa* secretes a molecule(s) that inhibits the growth of *Rhizopus microsporus*. Dormant *R. microsporus* spores were incubated in 50% bacterial supernatant for 24 hours and fungal growth was observed via brightfield microscopy. A) *Rhizopus microsporus* spores incubated in 50:50 SAB:LB produce hyphae after 24 hours. B) Spores incubated with 50:50 SAB:bacterial supernatant for 24 hours are inhibited (average size = 5.2 µm). Images were taken at 200x magnification.

2.3.2 *P. aeruginosa* kills *R. microsporus*:

To determine whether *P. aeruginosa* just inhibited the growth of the fungus (fungistatic), or whether the bacterium displayed fungicidal activity, the treated spores were then plated out on fresh agar plates to determine the viability of the spores. Both PA01 and PA14 cells and supernatant had a significant killing effect on *R. microsporus* (Figure 2.3). Treatment with PA01 cells and supernatant, and PA14 cells and supernatant leads to 88.17%, 79.89%, 94.94% and 96.08%, respectively. This tells us that both strains have fungicidal activity, and that the molecule is likely secreted by the bacterium.

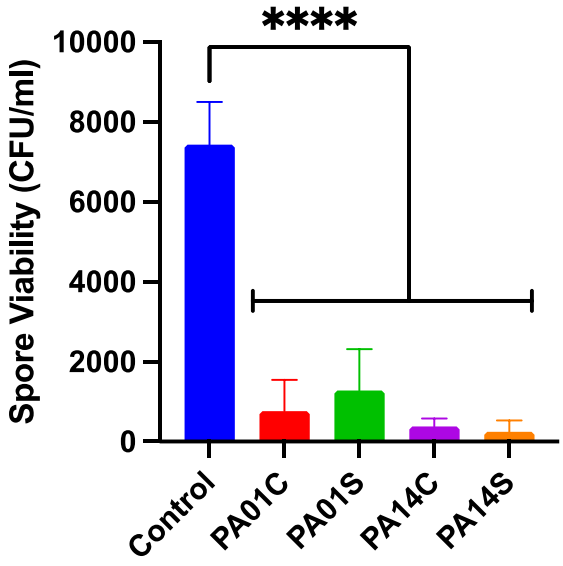

Figure 2.3. Treatment with *P. aeruginosa* wild type strains kills *R. microsporus* spores. Dormant *R. microsporus* spores were incubated with either cells or sterile supernatant of two bacterial strains (PA01 and PA14). Data was plotted as mean average (n=4) and standard deviation as error bars. Data was analysed using one way ANOVA with Dunnett's multiple comparisons test.
C = cells, S = supernatant, **** = p<0.0001

2.3.3 The antifungal effect of *P. aeruginosa* against *R. microsporus* is independent of iron restriction:

R. microsporus germination relies heavily on extracellular iron[52], as does the growth of all microbes. Bacterially secreted siderophores, such as pyoverdine and pyochelin, are known iron chelators which remove extracellular iron. To confirm that it is not iron sequestration leading to the killing of spores (as we know this can inhibit germination), samples were spiked with 100 µM iron. Upon plating out for viability, there was no significant difference in the killing effect of *P. aeruginosa* cells and supernatant when used to treat spores in the absence and presence of exogenous iron (Figure 2.4). Iron treated samples led to 91.23%, 98.28%, 88.4% and 98.04% spore killing after treatment with PA01 cells and supernatant, and PA14 cells and supernatant, respectively. From this we can conclude that whilst iron sequestration can inhibit fungal growth, it is not the key mechanism affecting spore viability in this instance.

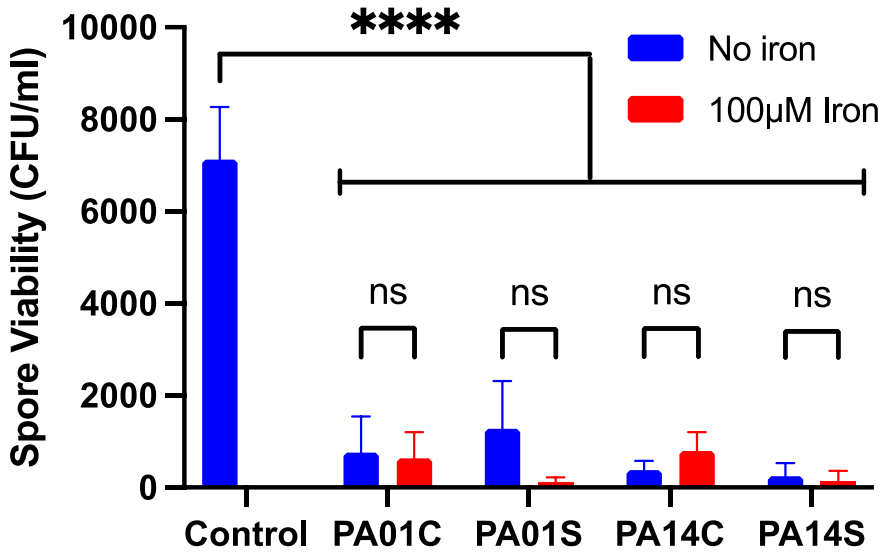

Figure 2.4. The antifungal activity of *P. aeruginosa* against *R. microsporus* is independent of iron restriction by bacterial siderophores. 100 µM Fe(III)Cl_3 was used to spike incubations of *R. microsporus* treated with either cells or supernatant of two bacterial strains (PA01 and PA14). Data was plotted as mean average (n=4), with standard deviation as error bars. Data was analysed using two-way ANOVA with Dunnett's multiple comparisons test. C = cells, S = supernatant, ns = not significant, **** = p<0.0001.

2.3.4 Antifungal activity is also effective against spores after 6 hours of germination:

To investigate whether the antifungal activity of *P. aeruginosa* is only effective against dormant spores, or whether cells that have undergone a level of germination are also susceptible to killing, *R. microsporus* spores were allowed to germinate for 6 hours at 37°C in SAB media, leading to formation of hyphae before addition of either bacterial cells or supernatant (Figure 2.5). Addition of *P. aeruginosa* cells or supernatant led to 92% and 99.7% fungal killing respectively. This suggests the antifungal activity exhibited against dormant spores is also exhibited against spores germinated for a short period of time. To ensure that the perceived antifungal effect was not due to germinated cells not being transferred on to

the agar plates, T6 samples were plated on to agar and compared to T0 counts. Mean spore counts for T0 samples were 3422 CFU/ml compared to 3800 CFU/ml for T6 samples. This confirms that low spore viability in 6-hour experiments is due to bacterial antifungal activity, rather than unsuccessful inoculation of agar plates.

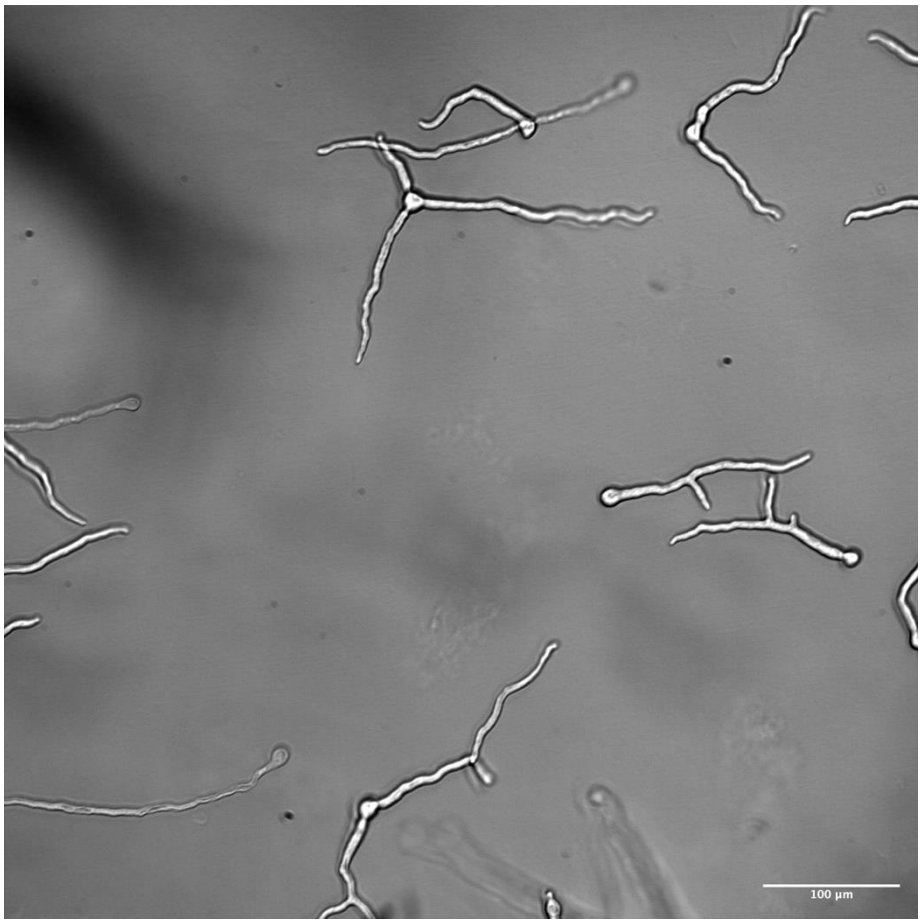

Figure 2.5. Incubation for 6 hours leads to hyphal formation of *R. microsporus* spores. *R. microsporus* dormant spores were incubated in SAB media statically for 6 hours at 37℃. Image taken at 200x magnification.

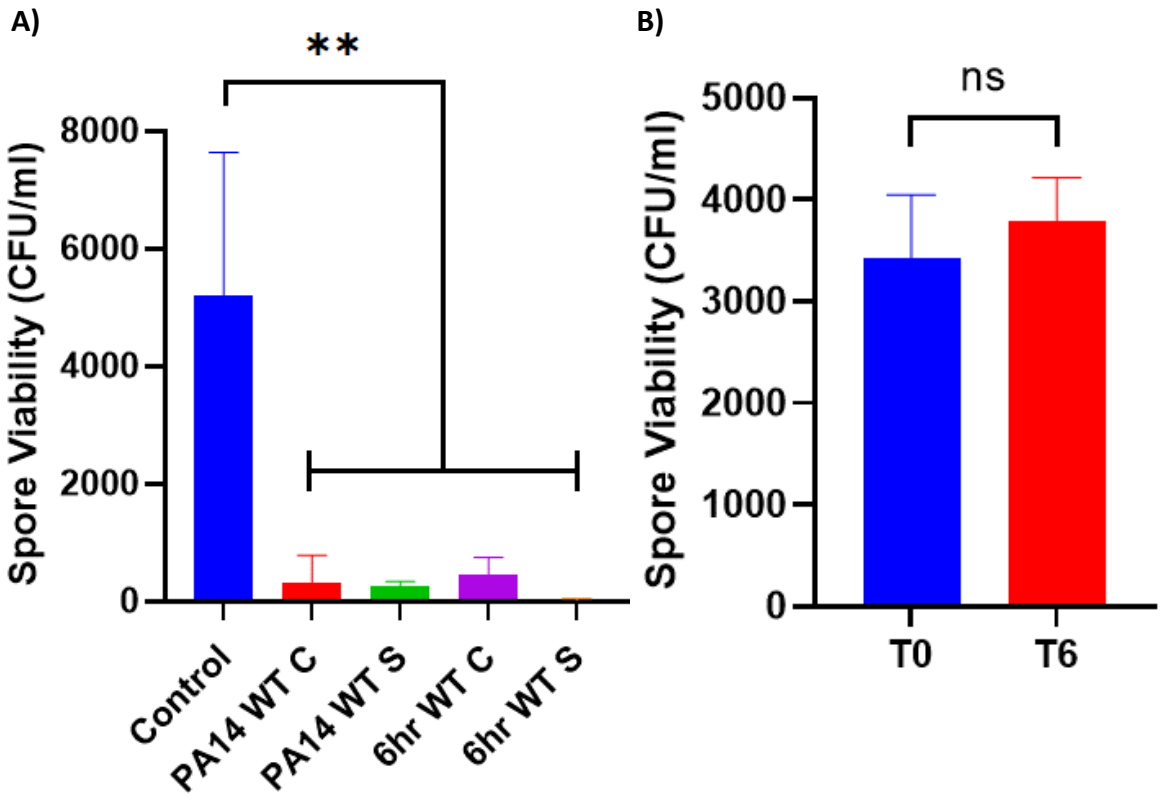

Figure 2.6. Bacterial treatment is effective against fungal cells after 6 hours germination. *R. microsporus* dormant spores were either treated immediately with bacterial cells or supernatant (PA14) or left for 6 hours to begin germination before addition of cells or supernatant. A) Spore viability with treatment added immediately and after 6 hours. B) Comparison of control cell counts at time point zero (T0) and time point 6 hours (T6). Data was plotted as mean average (n=3) and standard deviation as error bars. Data was analysed using one-way ANOVA with Dunnett's multiple comparisons test showing a significant difference between the control and all treated samples, and a one-way ANOVA and Tukey's multiple comparisons test, showing no significant difference between wildtype and mutant samples. ** = $p<0.01$, ns = not significant

2.3.5 PMS induces cell stress at high concentrations:

5MPCA (5-methyl-phenazine-1-carboxylate) is a precursor of pyocyanin, a pigmented phenazine secreted by *P. aeruginosa*, responsible for the characteristic blue-green colouring of this strain, and has been seen to exhibit antifungal activity against other fungal species[78]. PMS (phenazine methosulfate) is a more biologically stable analogue of 5MPCA, which was used to test the effect of this compound on spore viability. PMS was added to the spore viability assay at increasing concentrations, to investigate its effect on *R. microsporus* growth

and germination. Lower concentrations of PMS (100 µM to 400 µM) led to a change in fungal morphology, with spores appearing swollen and granulated (Figure 2.7), suggesting induction of cell stress. At higher concentrations of PMS (500 µM to 1000 µM), light microscopy shows complete inhibition of germination (Figure 2.7). When plated out for viability, concentrations from 400 µM to 3000 µM had a significant impact on spore viability in comparison to the T0 control (Figure 2.8), with all concentrations leading to >96% killing of spores. These results suggest that high concentrations of PMS have an antifungal effect equivalent to crude supernatant, whilst lower concentrations induce prolonged stress to fungal cells before killing occurs.

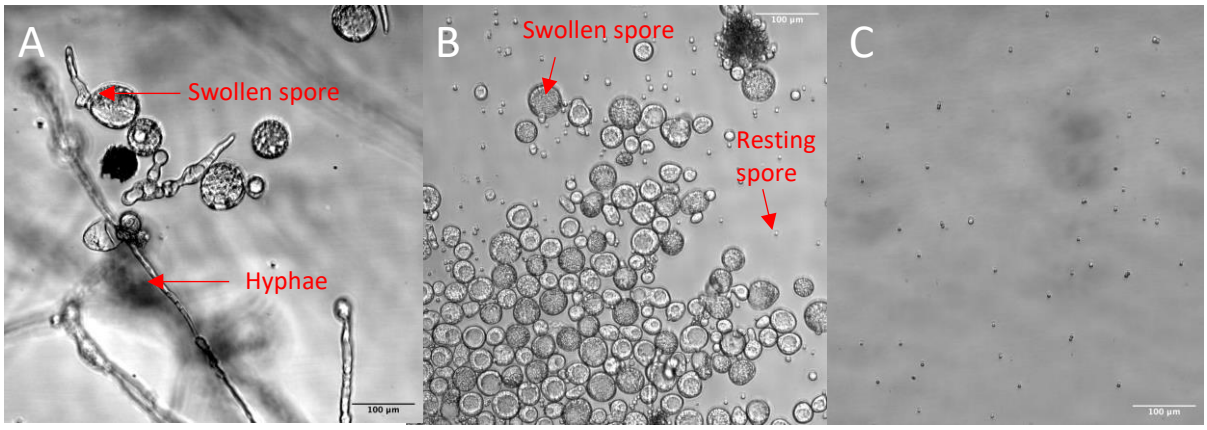

Figure 2.7. Addition of PMS to dormant *R. microsporus* spores leads to altered morphology at low concentrations and inhibition at higher concentrations. Dormant *R. microsporus* spores were treated with increasing concentrations of PMS (100 µM to 3000 µM) and observed via brightfield microscopy. A) Swollen *R. microsporus* spores and some hyphal formation in the presence of 100 µM phenazine methosulfate (PMS). B) Mixture of swollen and resting *R. microsporus* spores in 300 µM PMS. C) *R. microsporus* spores, not undergoing germination (resting spores), in 1000 µM PMS, with an average spore size of 6 µm. Images were taken at 200x magnification.

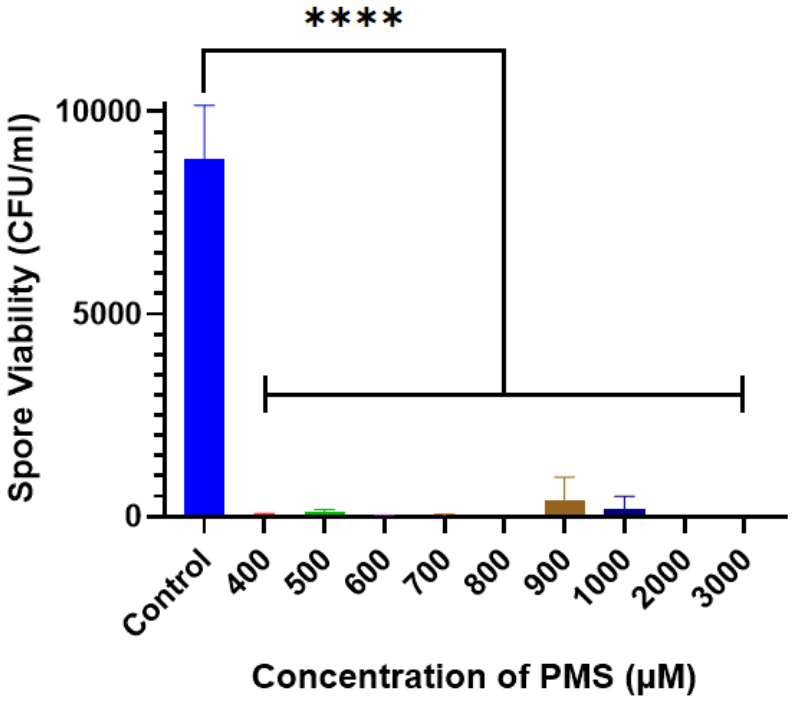

Figure 2.8. Addition of PMS leads to killing of dormant *R. microsporus* spores. Concentrations of 400 µM to 3000 µM of PMS were added to dormant spores. Data was plotted as mean average and standard deviation as error bars. Data was analysed using one-way ANOVA with Dunnett's multiple comparisons test showing a significant difference between the control and all treated samples, and a one-way ANOVA and Tukey's multiple comparisons test, showing no significant difference between different concentrations of PMS. (n=1 [2000, 3000 µM], n=2 [400, 600, 700, 800, 900 µM], n=3 [500 µM]), n=4 [1000 µM]). **** = p<0.0001

2.3.6 Triple knockout mutant of pyoverdine, pyochelin and phenazine-1-carboxylate still exhibits antifungal activity:

As shown in the results for 2.3.5, the phenazine PMS exhibits antifungal activity against *R. microsporus* spores. As we do not know the concentration of the antifungal molecule that is responsible for killing in the supernatant of the parental strain, it is difficult to know if the concentrations of PMS used are comparable to the concentration of the analogue (5MPCA) produced in the supernatant, and whether it is even secreted into the bacterial supernatant in the absence of fungal cells[78]. Therefore, a strain of PA14 lacking phenazine production was used in the spore viability assay to determine the importance of the phenazine pathway for

antifungal activity. This strain is a knockout mutant of the *phZ1/2* operons (containing seven genes each which encode enzymes for the production of phenazine-1-carboxylic acid (PCA)) [78,79], and the *pvdA* and *pchE* genes, which encode the siderophores pyoverdine and pyochelin. This strain was validated by the visible loss of pigmentation in comparison to the parental strain, indicating successful removal of the phenazine pathway and therefore production of pyocyanin. Treatment of spores with bacterial cells and supernatant from this strain exhibited 92.21% and 91.99% killing, respectively. As all mutants from this selection were as effective at killing *R. microsporus* as the parental control strain of PA14 (Figure 2.9), this confirms that the antifungal molecule is neither a phenazine nor a siderophore.

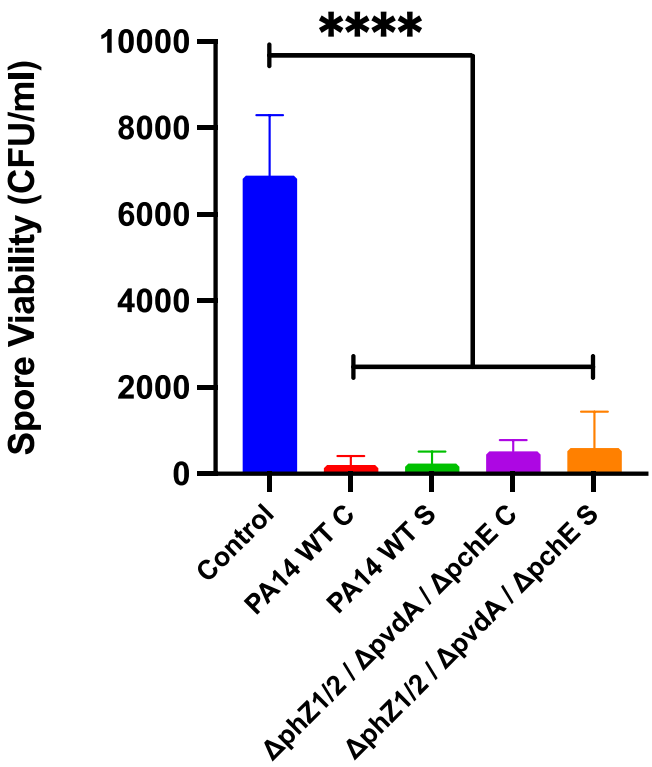

Figure 2.9. A *P. aeruginosa* phenazine-siderophore triple mutant still exhibits antifungal activity against *R. microsporus*. Dormant *R. microsporus* spores were incubated with a triple *P. aeruginosa* mutant strain, lacking the *phz1 and phz2 operons*, and the *pvdA* and *pchE* genes. Data was plotted as mean average (n=4) and standard deviation as error bars. Data was analysed using one-way ANOVA with Dunnett's multiple comparisons test showing a significant difference between the control and all treated samples, and a one-way ANOVA and Tukey's multiple comparisons test, showing no significant difference between wildtype and mutant samples. C = cells, S = supernatant, **** = p<0.0001

2.3.7 Mutants defective in rhamnolipid biosynthesis still exhibit antifungal activity:

The rhamnolipids which are secreted by *P. aeruginosa* have been shown to inhibit the growth of some filamentous fungi, although it has not been noted whether this effect is fungistatic or fungicidal[61]. A preliminary metabolomic experiment indicated that the rhamnolipids were enriched in *P. aeruginosa* supernatants that display antifungal activity (Hall unpublished observation). Therefore, to investigate whether the rhamnolipids are molecules of interest to this study, a selection of mutants defective in rhamnolipid biosynthesis were tested for their antifungal activity. Deletion of the three keys genes involved in rhamnolipid biosynthesis (*rhlA*, *rhlB* and *rhlC*) did not perturb the ability of *P. aeruginosa* to kill *R. microsporus* spores (Figure 2.10). Therefore, we can conclude that the antifungal molecule is not a rhamnolipid.

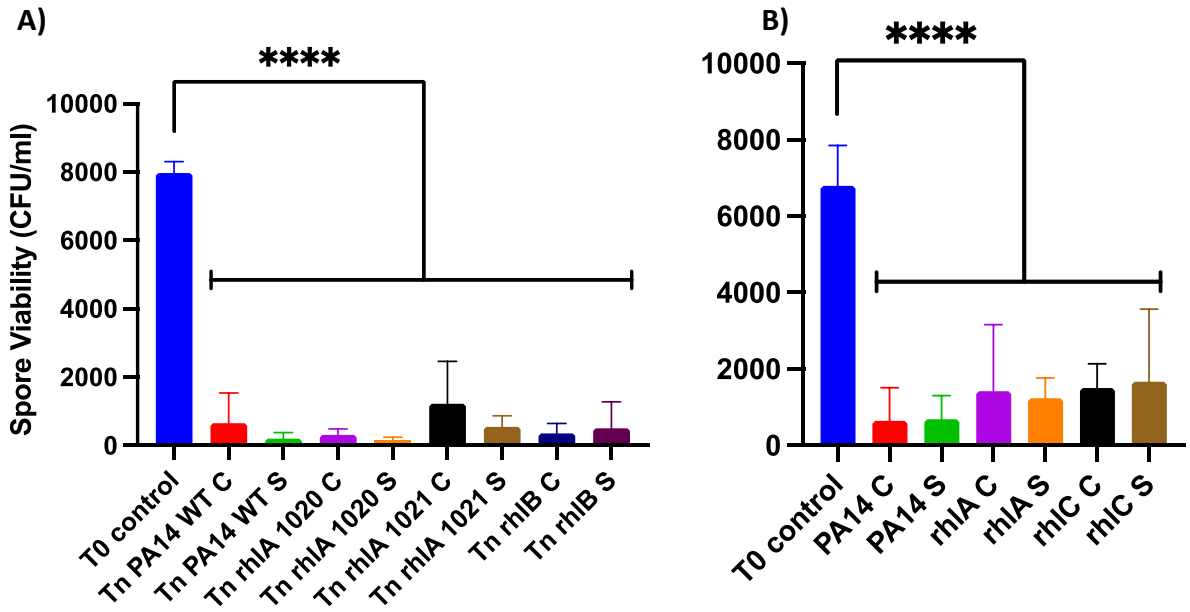

Figure 2.10. Rhamnolipid mutants still exhibit antifungal activity against *R. microsporus*. Dormant *R. microsporus* spores were incubated with bacterial cells or supernatant of a selection of strains lacking *rhlA, rhlB* and *rhlC* genes. A) Spores treated with mutant strains obtained via transposon mutagenesis. B) Spores treated with mutant strains obtained via traditional methods. Data was plotted as mean average (n=4, n=3 for graph A and B respectively) and standard deviation as error bars. Data was analysed using one-way ANOVA with Dunnett's multiple comparisons test showing a significant difference between the control and all treated samples, and a one-way ANOVA and Tukey's multiple comparisons test, showing no significant difference between wildtype and mutant samples. C = cells, S= supernatant, **** = $p<0.0001$

2.3.8 Quinolone signal knockout mutants still exhibit antifungal activity:

The same preliminary metabolomic experiments also suggested that the *Pseudomonas* Quinolone Signal (PQS) may be of interest for this investigation and the antifungal activity of *P. aeruginosa* (Hall unpublished observation). Strains of bacteria with removal of PQS genes obtained via transposon mutagenesis were tested as a treatment against dormant *R. microsporus* spores. Results showed that all Tn mutants exhibited similar levels of antifungal activity to the parental control strain. Strains for transposon mutants Tn *pqsA-C,* Tn *pqsA* and Tn *pqsH* allowed survival of 7.35%, 33.69% and 14.52% for cell treatment and 13.59%, 29.90% and 7.17% for supernatant treatment, respectively, whilst samples treated with the PA14 parental strain averaged 23.04% and 2.49% (for cells and supernatant respectively). As no effect was seen with these Tn mutants, a strain independently confirmed to be lacking the *pqsA-C* gene was tested. Fungal spores cultured with this bacterial strain or supernatant displayed only 8.44% and 13.65% survival respectively. These results suggest that although PQS is an important molecule for *P. aeruginosa* quorum sensing, it is not the key pathway involved in the antifungal activity we see here against *R. microsporus*.

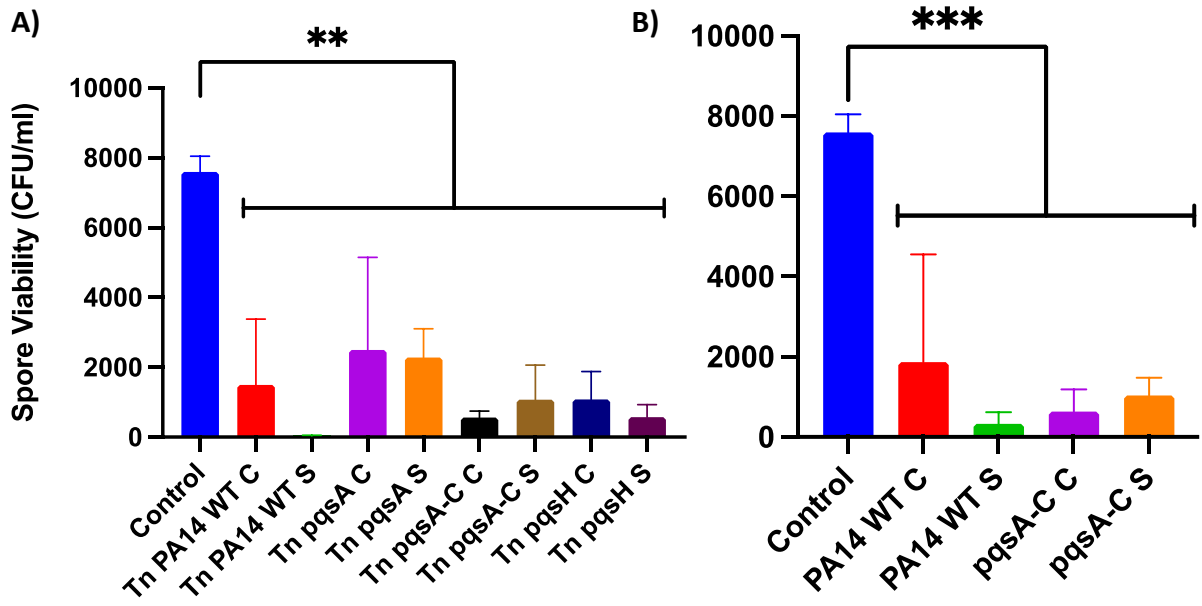

Figure 2.11. Mutants of the *Pseudomonas* quinolone signal still exhibit antifungal activity against *R. microsporus*. Dormant *R. microsporus* spores were treated with either bacterial cells or supernatant from a selection of mutant strains lacking *pqs* genes. A) Spores treated with mutant strains obtained via transposon mutagenesis. B) Spores treated with mutant strains obtained via traditional methods. Data was plotted as mean average (n=3) and standard deviation as error bars. Data was analysed using one-way ANOVA with Dunnett's multiple comparisons test showing a significant difference between the control and all treated samples, and a one-way ANOVA and Tukey's multiple comparisons test, showing no significant difference between wildtype and mutant samples. C = cells, S = supernatant, *** = p<0.001, ** = P<0.01

2.3.9 Pha mutants still exhibit antifungal activity:

Polyhydroxyalkanoate (PHA) derivatives have previously been shown to exhibit bioactivity against bacteria and other fungal species[80], so strains of *P. aeruginosa* deficient in polyhydroxyalkanoate pathways were tested for antifungal activity. Knockout mutants for the genes *phaC* and *phaG*, encoding polyhydroxyalkanoate synthase[81] and (R)-3-hydroxyacyl carrier protein-CoA transferase[82], respectively, were used as treatments against *R. microsporus* dormant spores. Removal of polyhydroxyalkanoate synthase prevents polymerisation of monomers into PHA polymers[81] and (R)-3-hydroxyacyl-ACP-CoA tranferase

prevents formation of the PHA precursor (R)-3-hydroxyacyl-CoA[82], thus both prevent PHA production. These mutants showed no significant difference in antifungal activity in comparison to the PA14 parental strain (Figure 2.12), exhibiting 89.28%, 83.29%, 79.42% and 85.52% killing for *ΔphaC* cells and supernatant, and *ΔphaG* cells and supernatant. This suggests that polyhydroxyalkanoates are not the compounds responsible for the antifungal activity of *P. aeruginosa* against *R. microsporus.*

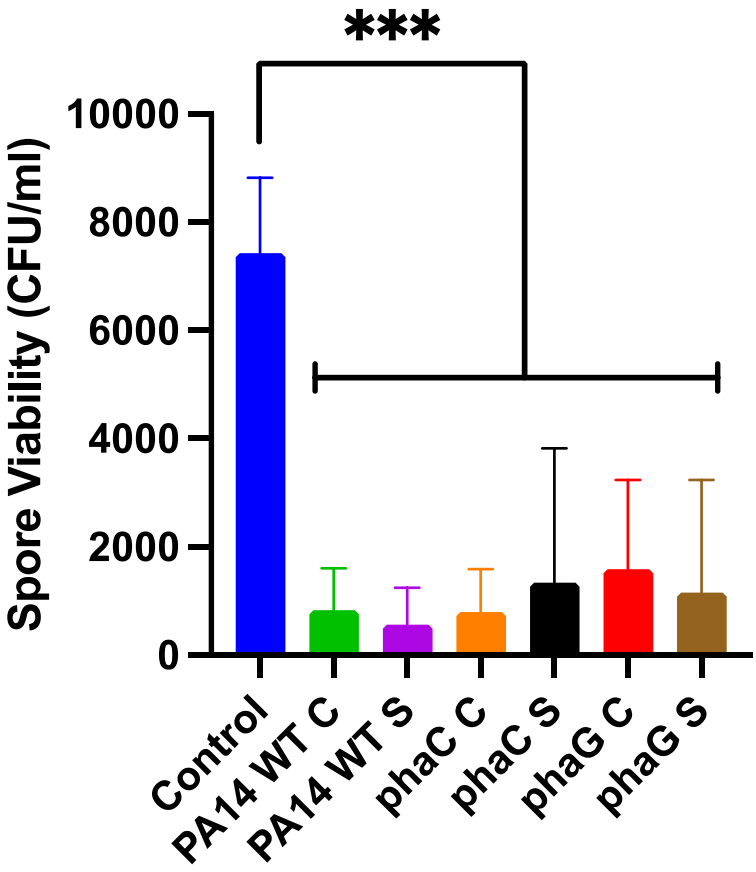

Figure 2.12. *P. aeruginosa* polyhydroxyalkanoate (PHA) mutants still exhibit antifungal activity against *R. microsporus.* Dormant *R. microsporus* spores were treated with either bacterial cells or supernatant from a selection of mutant strains lacking *pha* genes. Data was plotted as mean average (n=4) and standard deviation as error bars. Data was analysed using one-way ANOVA with Dunnett's multiple comparisons test showing a significant difference between the control and all treated samples, and a one-way ANOVA and Tukey's multiple comparisons test, showing no significant difference between wildtype and mutant samples. C = cells, S = supernatant, *** = $p<0.001$

2.3.10 Secretion system mutants still exhibit antifungal activity:

Due to the number of secreted secondary metabolites produced by bacteria, these prokaryotes have evolved many secretion systems which are used for secretion of metabolites out of the cell for virulence, communication and nutrient acquisition[72]. *P. aeruginosa* takes advantage of the bacterial secretion systems T1SS, T2SS, T3SS, T5SS and T6SS[67], which are used for secretion of different families of molecules. Many of the molecules secreted via these systems hold biological importance. Therefore, to identify whether any of these well-established secretion systems are responsible for the secretion of the antifungal molecule against *R. microsporus,* a selection of mutants deficient in these secretion systems were tested. The mutants used were as follows: *aprD* (T1SS), *xcpT* (T2SS), *pscD* (T3SS), *fimX* (T4BP), *clpV2* (T6SS) and hsic3 (T6SS). None of the six secretion system mutants screened (both bacterial cells and supernatant) displayed a significant difference in spore killing ability in comparison to the PA14 wild type strain (Figure 2.13), and all were statistically significantly different from the control. This suggests that the antifungal molecule is not being secreted via any of the secretion systems tested for, and perhaps a different approach is required.

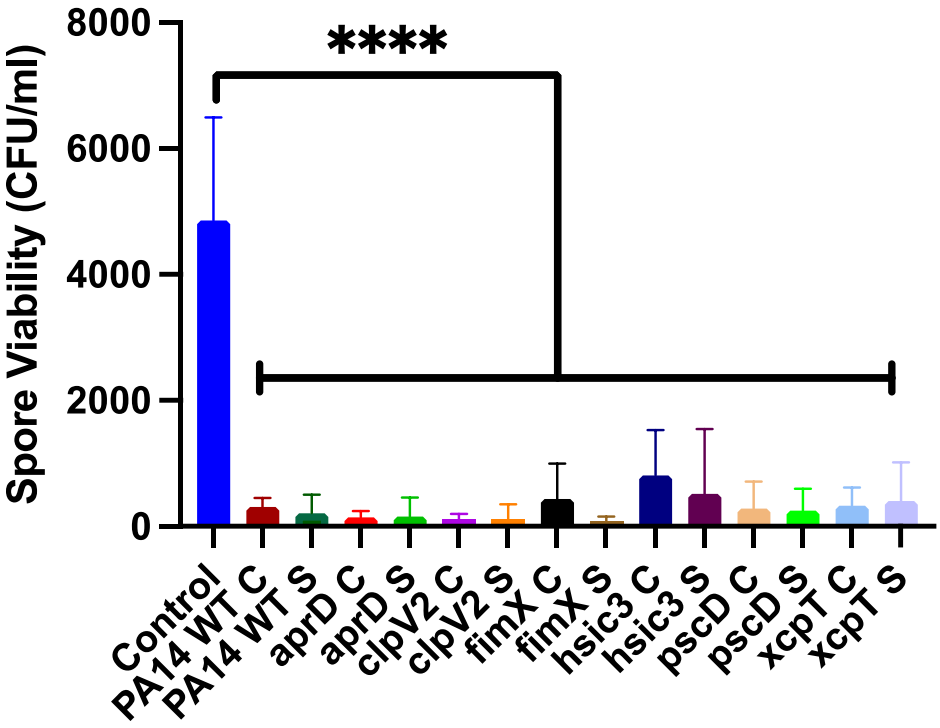

Figure 2.13. All secretion system mutants still exhibit antifungal activity against *R. microsporus*. Dormant *R. microsporus* spores were treated with cells and supernatant for a selection of mutant strains lacking certain bacterial secretion systems. Data was plotted as mean average (n=4) and standard deviation as error bars. Data was analysed using one-way ANOVA with Dunnett's multiple comparisons test showing a significant difference between the control and all treated samples, and a one-way ANOVA and Tukey's multiple comparisons test, showing no significant difference between wildtype and mutant samples.C = cells, S = supernatant, **** = p<0.0001

2.3.11 Bacterial supernatant pre-incubated with fungal spores loses its antifungal activity:

Previous investigations have shown that pre-incubation of supernatant with dormant spores overnight removed the antifungal activity upon filter sterilisation and re-introduction into the assay[77]. Here these findings were reproduced, showing that although pre-incubation with spores does not fully remove antifungal activity, it only results in 47.29% killing, compared to 97.52% by the wild type PA14 supernatant. To determine whether spores are metabolising the antifungal molecule or whether it is simply binding to their surface, this was repeated

using dead spores (heat-killed via autoclaving). Supernatant pre-incubated with dead spores still exhibited antifungal activity against fresh dormant spores in the assay, although this activity was not as significant as the crude antifungal supernatant (Figure 2.14). This difference in activity between supernatant pre-incubated with dormant and dead spores suggests that during this pre-incubation period, it is the metabolism or sequestration of the antifungal molecule by spores that leads to its removal from the supernatant, rather than simple surface binding.

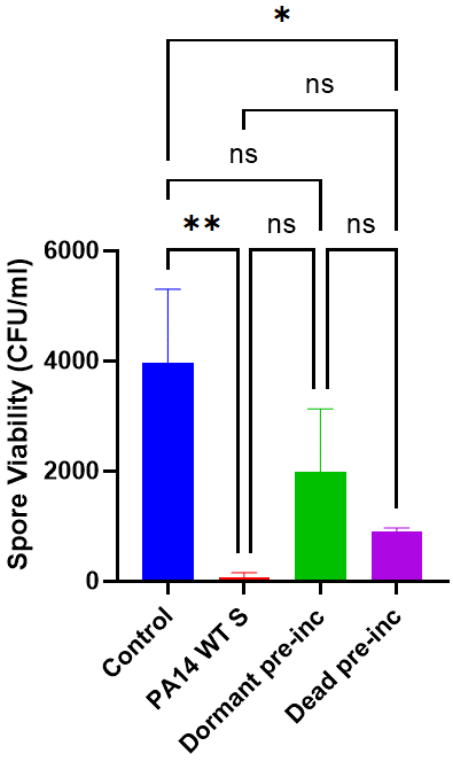

Figure 2.14. Pre-incubation of supernatant with *Rhizopus microsporus* cells before treatment removes antifungal activity. Sterile PA14 wild type supernatant was pre-incubated with either dormant or dead (heat-killed) *R. microsporus* spores. Data was plotted as mean average (n=3 for WT and dormant spores, n=2 for dead spores) and standard deviation as error bars. Data was analysed using one-way ANOVA with Dunnett's multiple comparisons test showing a significant difference between the control and all treated samples, and a one-way ANOVA and Tukey's multiple comparisons test, showing no significant difference between wildtype and mutant samples. S = supernatant, ns = not significant, * = $p<0.05$, ** = $p<0.005$

2.3.12 Fractions from ethyl acetate and chloroform extractions lose their antifungal activity:

A series of fractionation techniques were used to try and separate the antifungal component from the remainder of the supernatant. The organic solvents chloroform and ethyl acetate were used separately to fractionate molecules by hydrophobicity and identify the hydrophobicity of the antifungal molecule. These organic solvents separated supernatant into a hydrophobic fraction, for which the organic solvent was evaporated off, and a hydrophilic fraction. Both hydrophilic and hydrophobic fractions exhibited no inhibitory effect against *R. microsporus* when added to dormant spores, i.e., *R. microsporus* underwent hyphal germination in both conditions. When both fractions were recombined, antifungal activity was not restored, which suggests that these methods could be degrading the antifungal molecule, reacting and changing the molecule, or the molecule itself is volatile and being lost in the evaporation process. Due to the fungi undergoing full hyphal germination, samples were not able to be plated out for viability.

2.3.13 Bacterial supernatant incubated with amberlite XAD-4 loses its antifungal activity, but activity cannot be recovered from other fractions:

Amberlite XAD-4 is a hydrophobic resin with a surface area of 750 m^2/g and a pore size of 100 Å[83]. As organic extraction techniques were unsuccessful, this was another method used to separate crude supernatant into two fractions, containing either hydrophilic or hydrophobic compounds, with hydrophobic compounds being adsorbed to the resin and hydrophilic compounds remaining within the supernatant. When bacterial supernatant treated with amberlite XAD-4 is added to dormant *R. microsporus* spores, sometimes inhibition occurs, whilst at other times, full hyphal germination is seen, with two biological repeats showing full hyphal germination and three biological repeats showing some level of inhibition of growth.

This difference suggests that differing amounts of the siderophores, which we know are capable of inhibiting growth, are being produced with unavoidable small fluctuations in growth conditions. Amberlite XAD-4 beads were added directly as beads to dormant spores in the assay. This method showed no inhibitory or antifungal activity against *R. microsporus*, even after recombining beads with the hydrophilic fraction, suggesting that if the antifungal molecule is hydrophobic and binding to amberlite, binding affinity to beads is stronger than the effect of the molecule on spores.

A more established technique of column chromatography was used with 10%, 50% and 100% ethanol solutions, as explained in the materials and methods section. Addition of each of these samples into the spore viability assay showed no sign of restoring the antifungal activity, even when all three ethanol elutions were recombined with the hydrophilic elution. Results showed increased hyphal germination when amberlite beads (post-incubation with supernatant) were added to fungal cultures and when these beads were added to cultures alongside the amberlite treated supernatant. OD_{600} readings (Figure 2.15) reflect this, although presence of amberlite beads themselves would have affected these values. As there is not a way to standardise the number of beads added into the assay, this is perhaps not the most accurate way to quantify the findings.

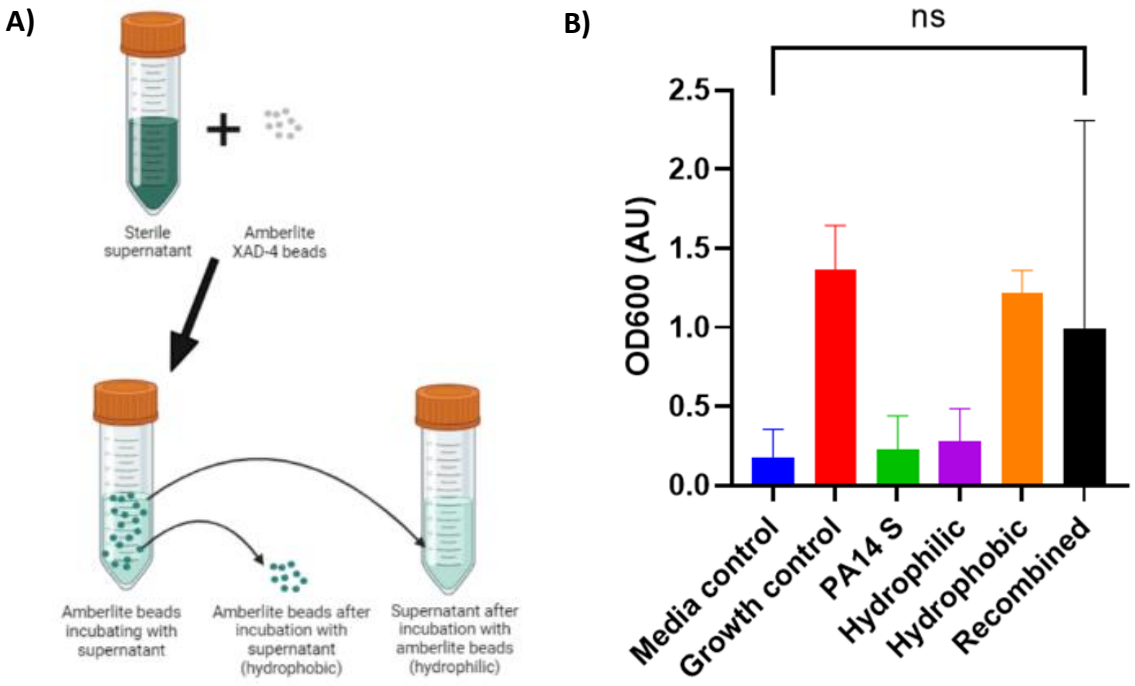

Figure 2.15. Bacterial supernatant incubated with amberlite XAD-4 loses its antifungal activity, but it cannot be recovered from other fractions. A) Sterile PA14 supernatant was incubated with XAD-4 amberlite beads – once removed amberlite beads were assumed to hold hydrophobic compounds, whilst the remaining supernatant was assumed to contain hydrophilic compounds. Dormant *R. microsporus* spores were treated with wild type supernatant, amberlite treated supernatant, amberlite beads, and amberlite supernatant and beads recombined. B) Optical density was read at 600 nm. Data was plotted as mean average (n=3) and standard deviation as error bars. Data was analysed using one-way ANOVA.
ns = not significant, PA14 = spores treated with wild type supernatant, Hydrophilic = spores treated with hydrophilic fraction of supernatant after incubation with amberlite beads, Hydrophobic = spores treated with amberlite beads and hydrophobic compounds bound to

2.3.14 *P. aeruginosa* still exhibits antifungal activity when grown in minimal media:

As attempts to identify the secreted molecule via organic extraction had been unsuccessful due the fact that the active molecule could not be recovered, an alternative approach which was not dependent on the use of solvents was employed. Nuclear Magnetic Resonance (NMR) is a technique that identifies compounds via excitation of nuclei using a magnetic field. The 1D NOESY technique used here reveals the proximity and spatial interaction between protons, which leads to signals being plotted as peaks on spectra, which can be recognised

and pieced together to identify structures of compounds. NMR requires no manipulation of the supernatant increasing the chance of identifying the active molecule(s). However, as no fractionation is required, NMR will pick up all molecules present in the sample. As the concentration of the active compound is unknown, the NMR spectrum of nutrient rich media may be too complex to permit identification of compounds of low concentration, with minimal media providing greater power to identify molecule of low concentration. Therefore, first we needed to determine if the antifungal molecule is produced and secreted by *P. aeruginosa* in minimal media. M9 minimal media was chosen due to its regular and successful use as a minimal media for this type of NMR spectroscopy. *P. aeruginosa* grew to the same optical density in M9 media as in LB and did not require additional incubation time to reach this. Then to identify whether the antifungal molecule was present, bacterial cells and supernatant from M9 media overnight cultures were used in the spore viability assay, using the same method as the original spore viability assay using LB. This data was compared to cells and supernatant from the standard LB media overnight cultures. Both the bacterial cells and the M9 supernatant exhibited antifungal activity comparable to *P. aeruginosa* grown in LB (Figure 2.16). Therefore, the antifungal compound is present under these growth conditions.

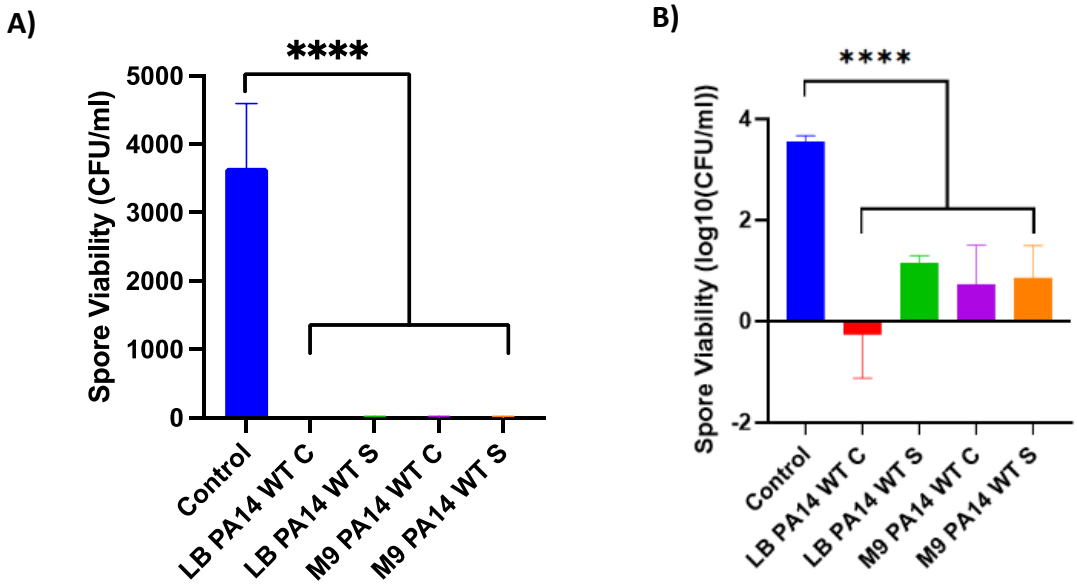

Figure 2.16. *Pseudomonas aeruginosa* still exhibits antifungal activity against *R. microsporus* when grown in M9 minimal media. PA14 wild type *P. aeruginosa* was grown in either LB broth or M9 minimal media, and cells and supernatant were used to treat *R. microsporus* dormant spores, to compare antifungal activity. A) Data was plotted as mean average (n=4), with standard deviation as error bars. B) The log10 values of viability data was calculated and plotted on a linear scale. Original data was analysed using one-way ANOVA and Dunnett's multiple comparisons test showing a significant difference between the control and all treated samples, and a one-way ANOVA and Tukey's multiple comparisons test, showing no significant difference between samples grown in LB or M9 media. **** = $p<0.0001$

2.3.15 NMR spectra shows differences between minimal media before and after bacterial growth:

To identify the secreted molecule with antifungal activity, M9 media, untreated M9 supernatant, and M9 supernatant that had been treated with amberlite, were analysed by 1D presat NOESY NMR. To identify whether the NMR was able to detect molecules secreted by *P. aeruginosa*, the NMR spectrum of the M9 supernatant was first compared to the M9 media. Peaks at ~5.23 and ~4.64 show a reduction in concentration in the bacterial supernatant sample (Figure 2.17). This suggests that the compounds that these peaks correspond to are

consumed or metabolised by the bacteria during growth and may be important nutrients for bacterial growth. On the other hand, some compounds present themselves after bacterial growth and are likely to be secretory products released into the supernatant. For example, there are two additional doublets and a potential triplet in the active supernatant compared to the media control in this region of the spectra (Figure 2.18). Therefore, we can see that NMR is able to identify differences in two different samples of the supernatant, and we can see from both Figures 2.17 and 2.18 that components are metabolised and secreted.

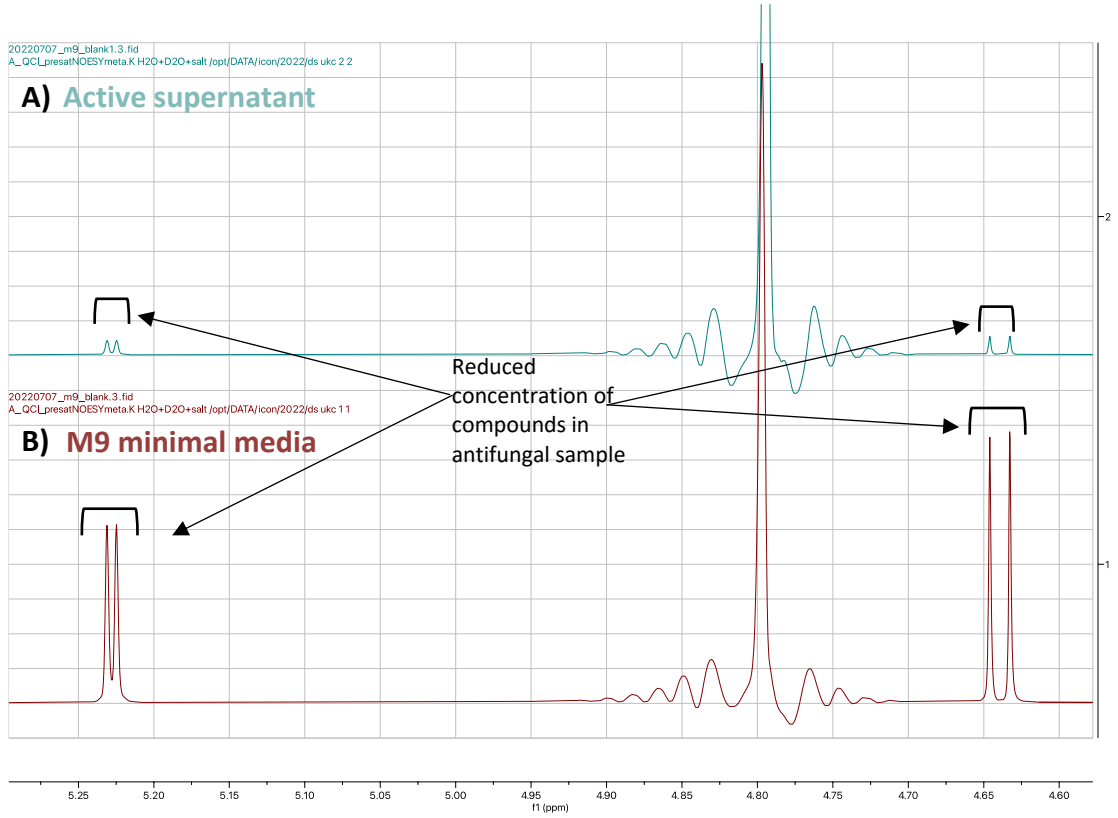

Figure 2.17. Bacteria use and metabolise molecules found in blank M9 minimal media. NMR spectra for active supernatant and M9 minimal media blank were compared to identify differences in the molecular components. A) Spectrum of the active supernatant sample that shows antifungal activity against *R. microsporus*. B) Spectrum of the M9 minimal media used for the bacterial overnight culture.

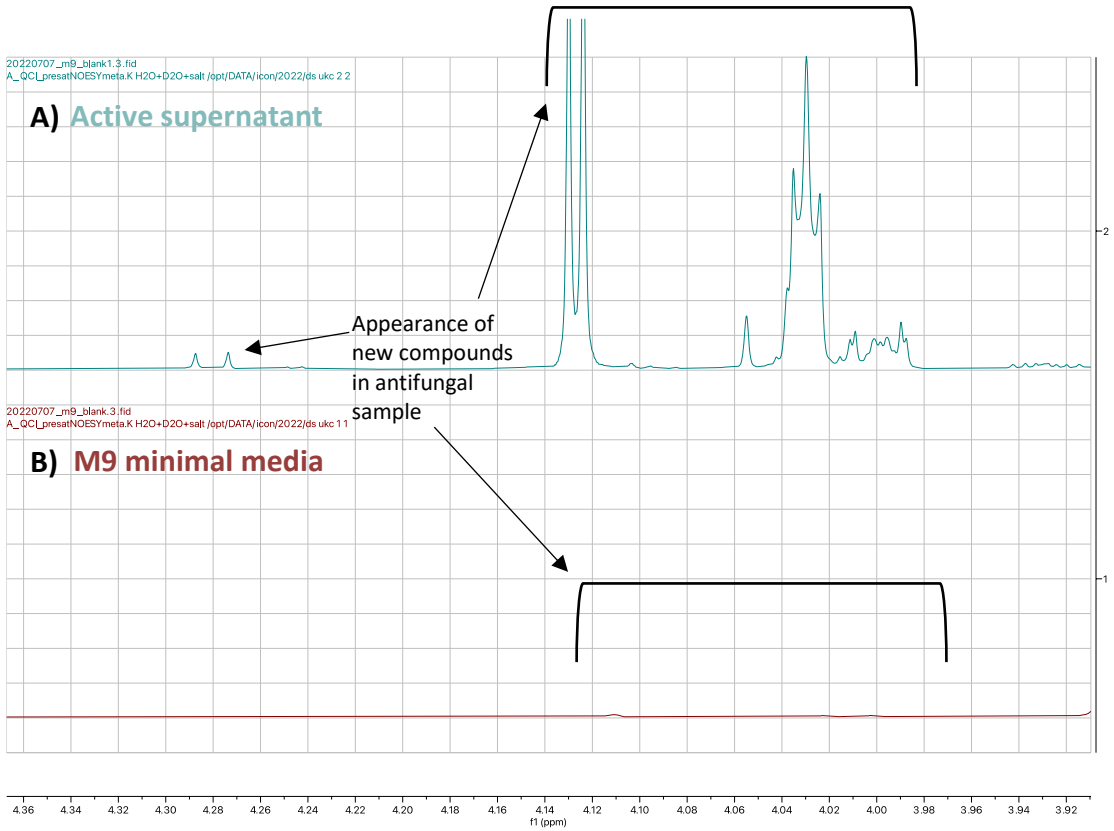

Figure 2.18. Bacteria secrete and change molecules into their growth media. NMR spectra for active supernatant and M9 minimal media blank were compared to identify differences in the molecular components. A) Spectrum of the active supernatant sample that shows antifungal activity against *R. microsporus*. B) Spectrum of the M9 minimal media used for the bacterial overnight culture.

2.3.16 NMR shows amberlite XAD-4 treatment removes aromatic compounds:

Previous experiments have shown that treatment of the bacterial supernatant with amberlite XAD-4 removes the antifungal activity. Therefore, to confirm that amberlite is removing molecules and to identify the types of molecules the resin binds to, the NMR spectrum of the M9 supernatant was compared to the spectrum of the amberlite treated supernatant. Incubation with amberlite XAD-4 led to the removal of almost all of the aromatic compounds from the supernatant (Figure 2.19). Furthermore, there are a few unidentified peaks in the amberlite treated sample, which are not present in the original supernatant, suggesting these are either new compounds or as a result of amberlite reacting with the sample. Overall, these

results show us that aromatics, which are known hydrophobic compounds, are removed via this method of treatment, and confirm amberlite XAD-4 resin is leading to adsorption of hydrophobic components of the supernatant.

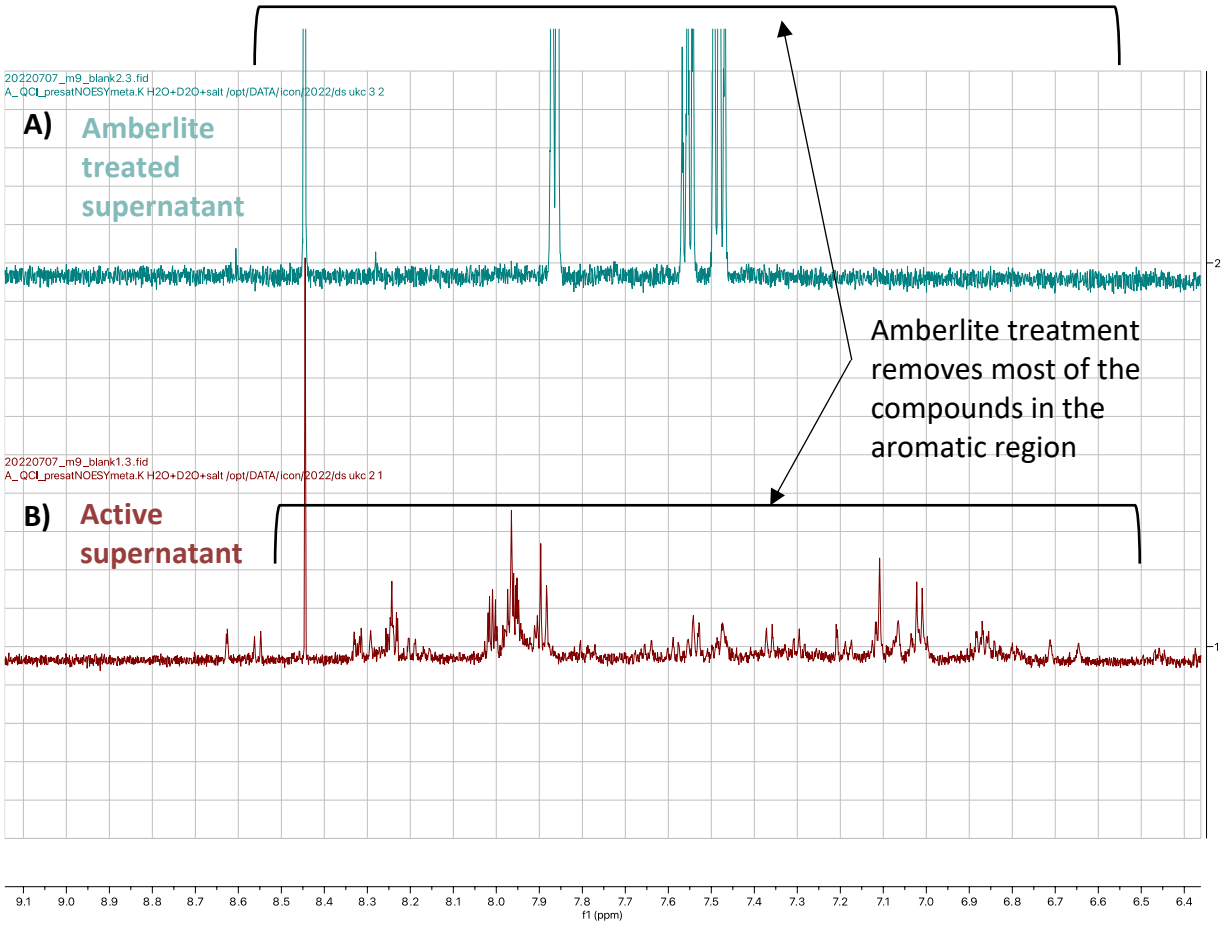

Figure 2.19. Amberlite treatment of supernatant removes most of the aromatic compounds. NMR spectra for supernatant treated with amberlite beads and antifungal supernatant were compared to identify differences in the molecular components. A) Spectrum of the amberlite treated supernatant sample. B) Spectrum of the active supernatant that is antifungal against *R. microsporus.*

2.3.17 NMR shows pre-incubating supernatant with spores changes the spectral profile:

Supernatant pre-incubated with spores, which we know removes antifungal activity, was used for NMR, allowing comparison to active supernatant, with the expectation that the molecule(s) responsible for antifungal activity will be removed from the spectrum. Spectra A, B and C in Figure 2.20 represent supernatant pre-incubated with dormant spores, supernatant treated with amberlite XAD-4, and the active antifungal samples, respectively. It can be seen that concentrations of glucose in the active and amberlite treated samples are similar, but after pre-incubation with spores, glucose concentration is reduced by a factor of ~3.75, showing that before fungicidal effects of the supernatant kill spores, they are metabolically active and consuming glucose.

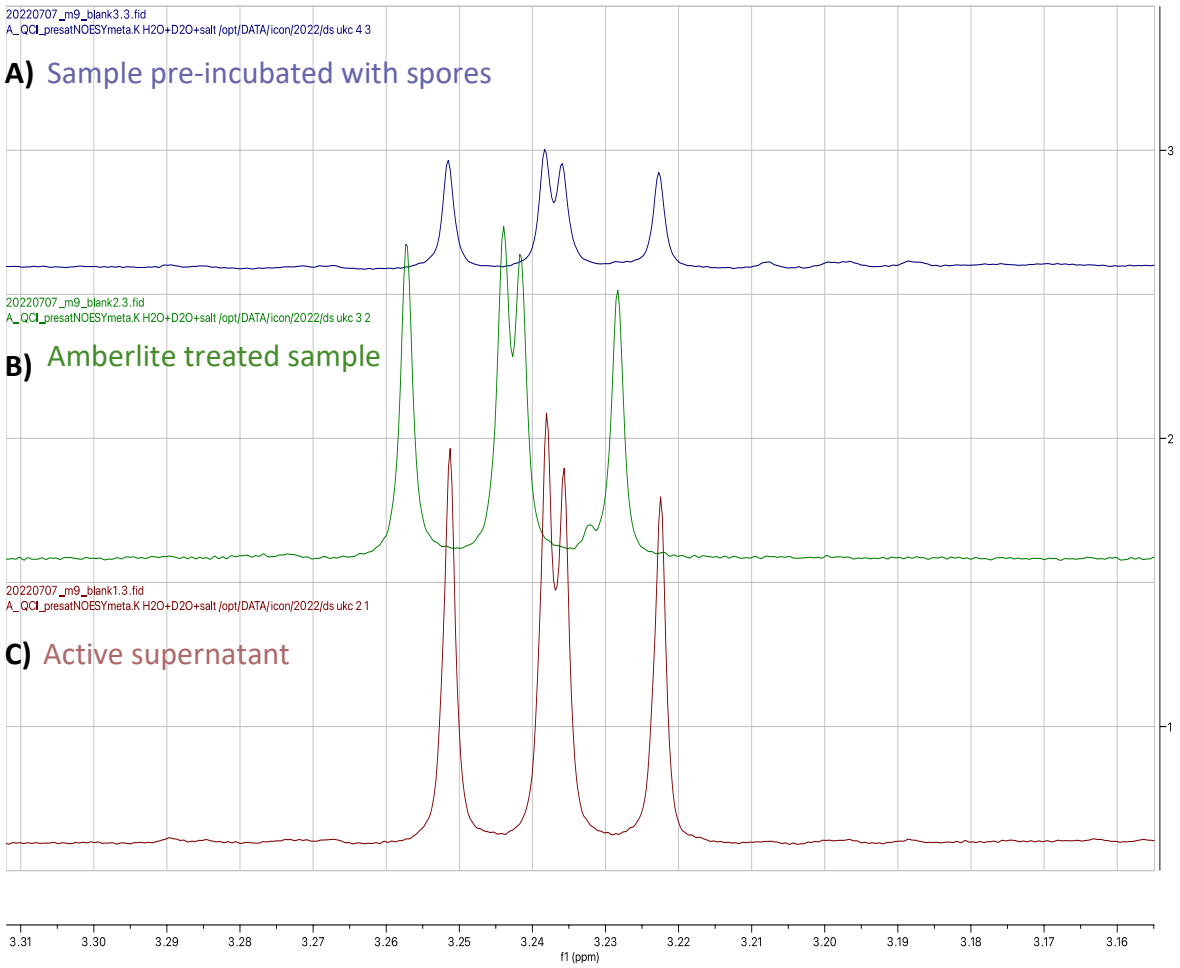

Figure 2.20. Pre-incubating supernatant with spores changes the NMR spectral profile. NMR spectra of different samples were overlaid to compare the concentrations of different compounds. A) Supernatant pre-incubated with dormant spores. B) Supernatant treated with amberlite XAD-4. C) Crude supernatant that has antifungal activity.

2.3.18 NMR sugar regions show spores metabolise sugars before antifungal activity occurs:

If we compare the remainder of the sugar region of the spectra, we can see clear reductions in concentrations of these sugars in the pre-incubated samples compared to the active sample. In Figure 2.21 below, spectra B and C show the sample pre-incubated with spores and the active sample, respectively, with spectra A showing a visual representation of the difference in concentration between these two samples. After pre-incubation with spores, glucose is reduced, as is mannitol. This shows us that spores are undergoing metabolism once

added to antifungal supernatant before they are killed and that the antifungal effect is not immediate.

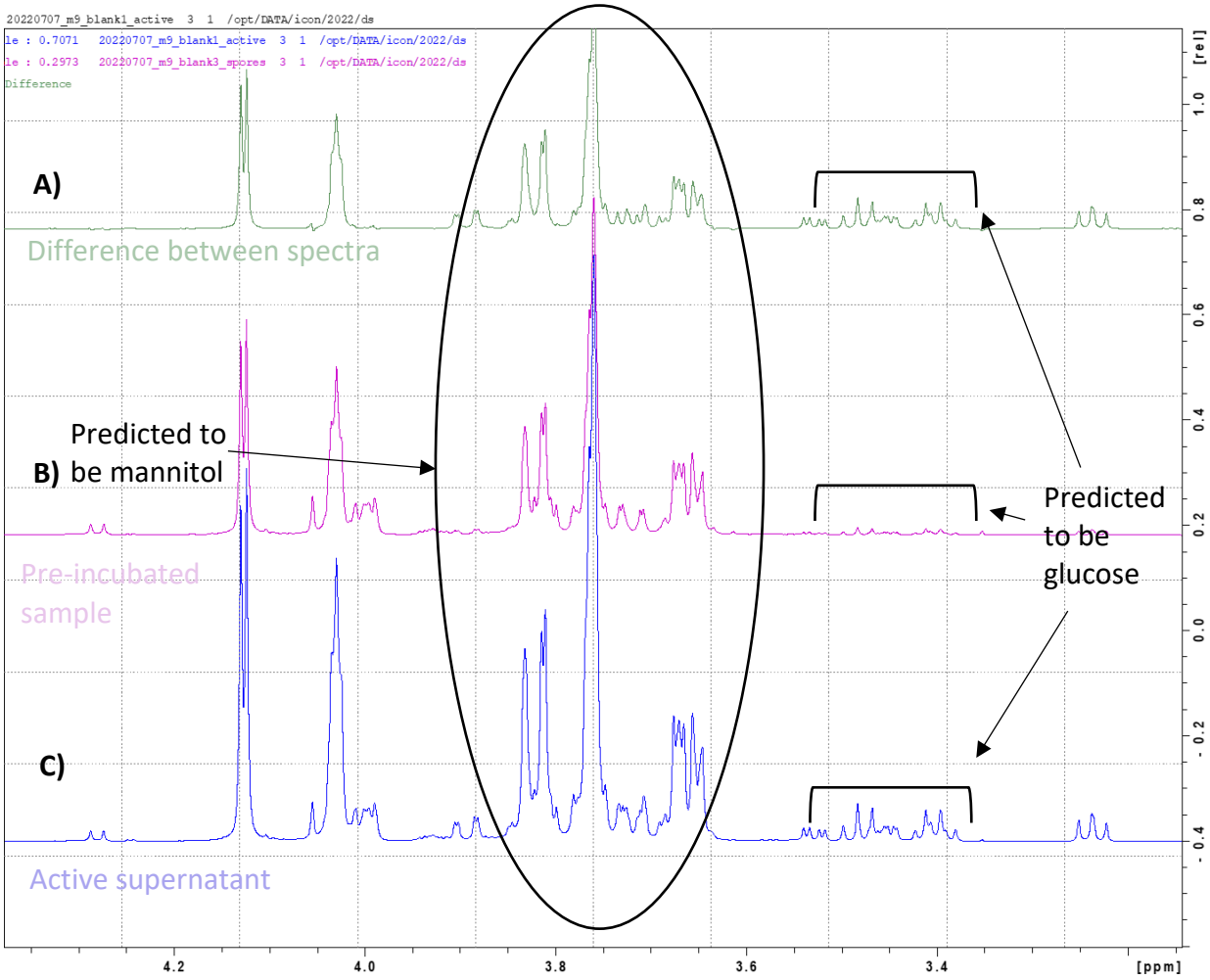

Figure 2.21. Comparison of active supernatant with sample pre-incubated with spores shows spores metabolise sugars. Spectra of a sample pre-incubated with spores and the active supernatant in the sugars region were compared, and a spectrum of the differences in peak concentrations was made. A) The difference between spectra B and C, B) supernatant samples pre-incubated with spores) and C (antifungal supernatant sample).

2.3.19 Pre-incubation of supernatant with spores leads to changes in aliphatic compounds:

Comparison of spectra in the aliphatic region shows that after pre-incubation with spores, supernatant contains some compounds that are not present in the active sample. This can be seen below in Figure 2.22, where spectra A represents the difference between the samples, spectra B is the sample of supernatant pre-incubated with dormant *R. microsporus* spores and spectra C is the supernatant sample known to have antifungal activity against *R. microsporus*. We can see reductions in signal intensity of unidentified peaks at ~3.4 ppm and ~1.2 ppm, which suggests spores are using these compounds before antifungal supernatant kills them. These spectra also show the appearance of an unidentified triplet at ~1.1 ppm in the sample after pre-incubation with dormant spores, suggesting fungal metabolism is leading to conversion of molecules to aliphatics.

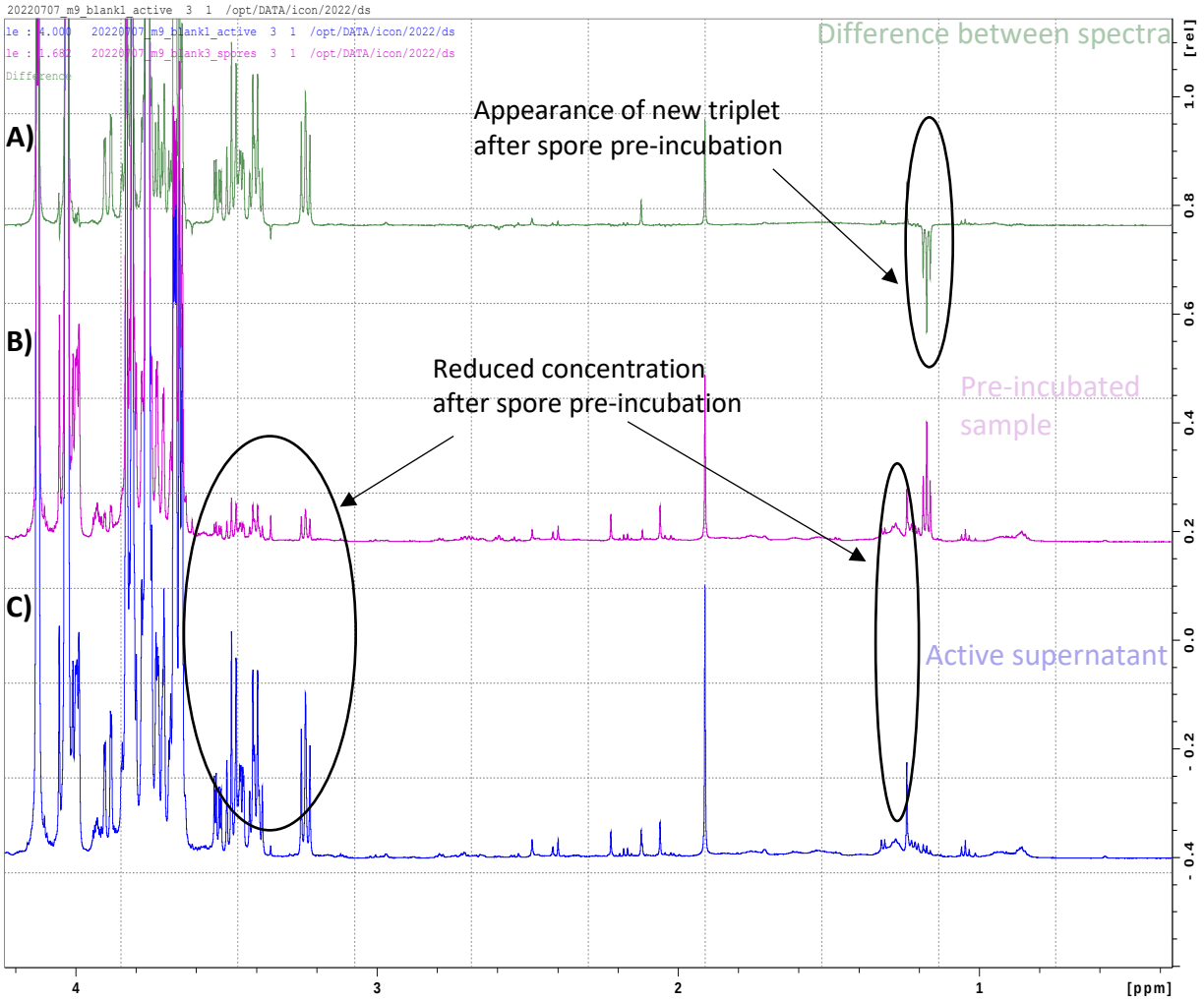

Figure 2.22. Pre-incubation of active supernatant with spores leads to changes in aliphatic compounds. Spectra of a sample pre-incubated with spores and the active supernatant in the aliphatic region were compared, and a spectrum of the differences in peak concentrations was made. A) The difference between spectra B and C, B) supernatant samples pre-incubated with spores) and C (antifungal supernatant sample).

2.3.20 Chenomx NMR Suite cannot predict secondary metabolites:

Chenomx NMR Suite was used to identify possible metabolites of interest and was useful for predicting some of the compounds mentioned in the NMR spectra above. This gives us some information about the way fungal spores are behaving before the antifungal effects of supernatant induce cell death. However, the program only has the capacity to identify a set list of primary metabolites, which are unlikely to be responsible for the antifungal activity of

the supernatant. Seeing as the molecule(s) or compound(s) responsible for antifungal activity is likely to be a secondary metabolite, this programme has limited capabilities for predicting compounds from these spectra.

2.3.21 Supernatant indirectly inhibits mitochondrial respiration of spores:

As the molecule(s) or compound(s) responsible for the antifungal activity of *P. aeruginosa* against *R. microsporus* has still not yet been identified, a different approach was taken, trying to determine the MoA of killing, or at least rule some modes out. *P. aeruginosa* has previously been seen to induce mitochondrial dysfunction in human intestinal epithelial cells[84], so the possibility of *P. aeruginosa* targeting mitochondrial respiration in *R. microsporus* was investigated. Respirometers, like the name suggests, monitor the levels of mitochondrial respiration of the sample provided, by measuring the amount of O_2 uptake by cells, so this technique was used to determine if introduction of supernatant would have an observable effect on fungal respiration, in a short time span.

Initial data collected via respirometry suggested that inhibition of spore germination is not directly related to mitochondrial respiration, as addition of supernatant causes a steady decrease in O_2 flow per cell (Figure 2.23), as opposed to a sharp decline, which would be expected if mitochondrial respiration was targeted directly. This suggests that instead of showing that mitochondrial respiration is being inhibited, it is just a representation of respiration decreasing because of reduced fungal viability. This was only completed to N=1, adding supernatant and spores directly to the chamber at the same time. It was decided that it would be best to allow spores to begin germination in the chambers before adding the supernatant, in hope to see whether there is a sudden impact on respiration or not, however

this posed difficulties as filling only half the volume of the chamber allows more excess oxygen, preventing the readings from stabilising.

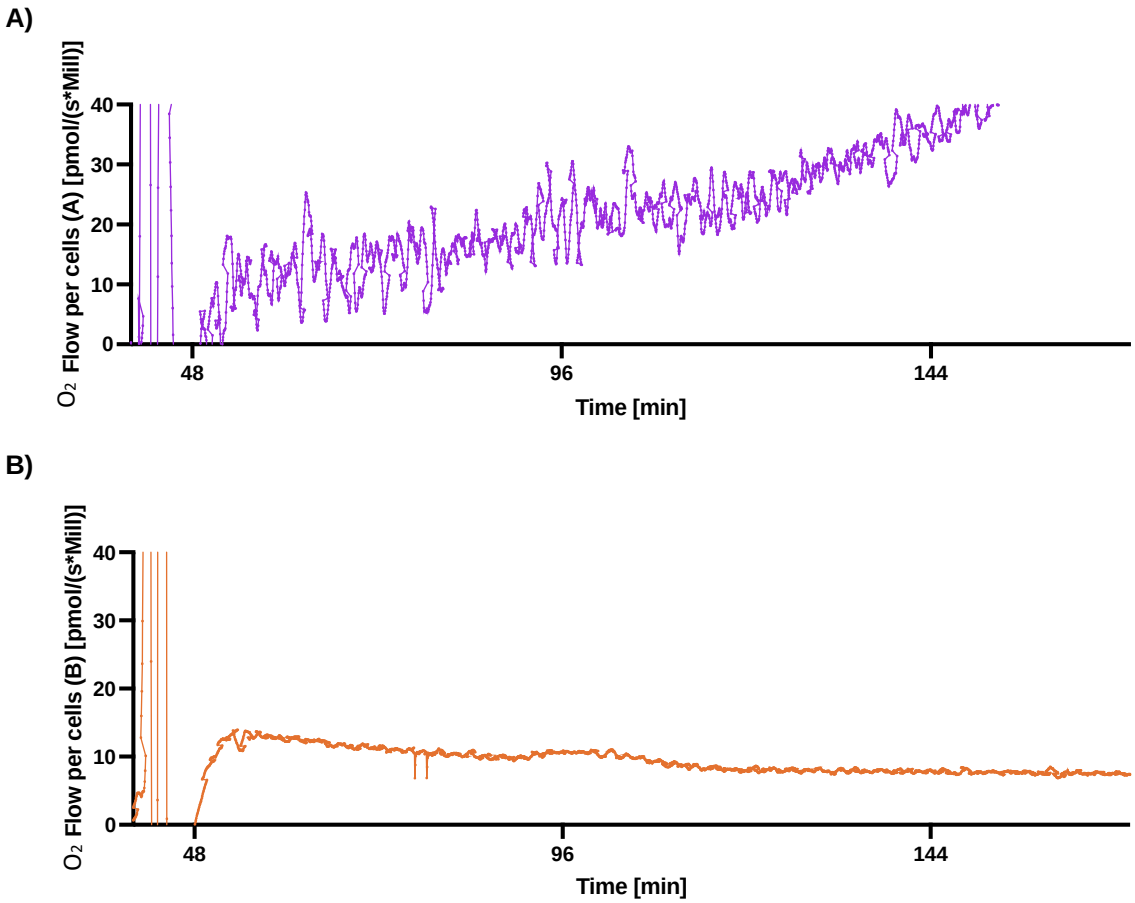

Figure 2.23. *Pseudomonas aeruginosa* supernatant indirectly inhibits spore respiration. Dormant *R. microsporus* spores were incubated at 37℃ in a respirometer with A) 50:50 SAB:LB or B) 50:50 SAB:supernatant. N=1

2.4 Discussion:

The aim of this project was to investigate the interaction between *P. aeruginosa* and *R. microsporus*, mainly to explore the antifungal potential of *P. aeruginosa.* It was confirmed, as previously seen[52], that the PA01 and PA14 wild type strains of *P. aeruginosa* are able to inhibit germination and survival of *R. microsporus* spores at the dormant stage of the lifecycle. Insignificant differences between the use of bacterial cells and sterile supernatant strongly suggests that antifungal activity is independent of cell-to-cell contact between the two microorganisms. However, from the results obtained it is not possible to fully rule out the possibility that the bacterial cells and the supernatant exhibit two different modes of action (MoA). For example, it is possible that the supernatants contain a secreted secondary metabolite that exhibits antifungal activity, but when bacterial cells are co-existing with fungal spores, chemical signalling may lead to a direct cellular interaction that induces inhibition of fungal growth and survival. This is not the first time bacteria have been observed to have antifungal activities. The Gram-negative bacteria *Klebsiella pneumoniae* has been shown to have inhibitory effects on another filamentous fungus, *Aspergillus fumigatus*, whilst increasing production of specific mycotoxins known as gliotoxins[85]. Interestingly, *P. aeruginosa* cells have been shown to also exhibit this effect on *A. fumigatus*, however when *P. aeruginosa* supernatant is used, fungal growth increases and gliotoxin production decreases[86]. In addition to this, the lactic acid producing bacteria *Lactobacillus plantarum* has been shown to inhibit growth of *Penicillium commune,* which causes spoilage issues in the food industry[87]. It is therefore not solely between *P. aeruginosa* and *R. microsporus* that this type of bacterial-fungal interaction occurs.

Iron is critical for fungal germination, and the PA01 strain of *P. aeruginosa* has been shown to inhibit the growth of *R. microsporus* through the sequestration of iron using its high affinity siderophores[52]. Therefore, iron spiking was used to investigate the possibility of iron sequestration, by the iron chelators pyoverdine and pyochelin, as being key in the inhibition of germination and killing of spores. As shown in the results, iron spiking with 100 µM $Fe(III)Cl_3$ does not reduce the inhibitory effect of the PA14 bacterial cell and supernatant treatment, and spores are still not viable once plated out onto rich media agar, suggesting that whilst iron restriction has been identified as important for the activity exhibited by PA01[52], this is not the case for PA14. In agreement with this, mutants defective in the biosynthesis of the two key siderophores (pyoverdine and pyochelin) exhibited similar levels of antifungal activity as the parental control strain. Therefore, taken together these data suggest that *P. aeruginosa* does not kill *R. microsporus* through sequestration of free iron.

P. aeruginosa has been shown to exhibit toxicity towards other fungi. One molecule that has been identified as exhibiting antifungal activity is 5MPCA[78], in particular, against *Candida albicans*[62]. 5PMCA is a precursor of pyocyanin in the *P. aeruginosa* phenazine pathway. As explained previously, PMS is a biologically stable analogue of 5MPCA. Addition of PMS directly to the assay suggested PMS provides a biological stress to fungal cells, at least to those in the dormant stage of the lifecycle. Significant spore swelling and granulation was observed at lower concentrations of PMS (100-300 µM), and at higher concentrations, PMS inhibited growth (400-1000 µM). The concentration of phenazines in bacterial culture supernatants is estimated to be in the range of 1-100 µM[88], suggesting that the concentrations of PMS that exhibit antifungal activity are super physiological, and may not reflect conditions in the actual assay, although the effect on morphology observed here is interesting nonetheless. In

agreement with this, mutants defective in phenazine biosynthesis still exhibited antifungal activity. These mutants included the removal on the *phzA1-G1 (phz1) and the phzA2-G2 (phz2)* operons from the PA14 strain, which both encode enzymes for the production of the 5MPCA precursor PCA[79], meaning that phenazine production is disrupted early on in the pathway.

Therefore, taken together, we can conclude that whilst phenazines have antifungal abilities at high concentrations, which could be further investigated in the future, it is not these molecules that we are responsible for the antifungal activity we are observing in this assay. It was for this reason that the activity of lower concentrations of PMS were not tested, as our aim here was to identify the molecule responsible for the antifungal activity in this particular assay, but further investigations in the future to determine the effect of lower concentrations of PMS could be interesting.

Aside from the phenazine knockout, mutant strains of *P. aeruginosa* PA14, from three other pathways of interest (rhamnolipids, PQS and PHAs), were used in comparison to the known antifungal wild type PA14 strain. Previous metabolomic studies have highlighted rhamnolipids as potential molecules of interest in the killing of filamentous fungi by *P. aeruginosa*, and these molecules have in fact been seen to exhibit antifungal activity against other Mucormycetes, such as *Mucor circinelloides*[89]. Rhamnolipids are glycolipid biosurfactants naturally produced by *P. aeruginosa*, often best produced in nitrogen limiting conditions, due to the importance of the carbon to nitrogen ratio[90]. Rhamnolipids have been seen to aid virulence through different methods, with certain mutant strains of *P. aeruginosa* having shown virulence to other bacterial species, such as *Staphylococcus aureus*, via delivery

of toxins and secondary metabolites into the cells by rhamnolipid-formed micelles[91]. The results in this project showed that rhamnolipid mutants exhibited the same level of antifungal activity as the parental strain, indicating that rhamnolipids are not having an antifungal effect on *R. microsporus.* These findings do not disregard the evidence that these molecules have antifungal abilities, however, do show that the concentrations secreted by *P. aeruginosa* are not significant enough to induce this effect on spores.

The *Pseudomonas* quinolone signal pathway has an important role in quorum-sensing, communication, and evasion for this bacterium, with PQS molecules, which are produced during the stationary phase of growth, signalling to the population when antibiotic or bacteriophage treatment are detected in the environment[64]. Our findings show that PQS is not responsible for the antifungal activity of *P. aeruginosa* against *R. microsporus,* as mutants defective in this pathway still exhibited the same antifungal effect as the parental strain. PQS still has an important role within this bacterium. A study into the relevance of PQS in *P. aeruginosa* virulence showed that a hypervirulent strain produced significantly more PQS than the wild type, despite no external cell stress[64], showing that although PQS may not be the responsible molecule for the antifungal interaction between *P. aeruginosa* and *R. microsporus* that is studied in this project, these molecules have a key role in the virulence of *P. aeruginosa*.

Polyhydroxyalkanoates (PHAs) are known to serve as intracellular carbon and energy reserves in both Gram-negative and Gram-positive bacteria, including *P. aeruginosa.* PHAs have the invaluable potential to transform the production of bioplastics[92], but have also been seen to exhibit antimicrobial activity. Compounds synthesised from the PHA derivative (R)-3-

hydroxyoctanoic acid have shown MIC values of 0.1-6.3 mM against the fungal pathogens *C. albicans* and *Microsporum gypseum*[80]. Despite this significant activity against other fungal pathogens, PHA mutant strains of *P. aeruginosa* still exhibited the same level of antifungal activity against *R. microsporus* as the parental strain.

In the hope to gain a clearer idea about how antifungal activity is occurring in this interaction, or the classification of the antifungal molecule, six different *P. aeruginosa* knockout strains, of five different bacterial secretion systems, were tested for antifungal activity against *R. microsporus* and compared against the wild type PA14 strain. It is thought that the Type III Secretion System (T3SS) is the most important secretion system for the virulence of *P. aeruginosa* against human hosts. This secretion system operates via injection of effectors into host cells via an injectisome[67]. Type VI Secretion System (T6SS),

also exerts virulence via injection[69]. As previously mentioned, we predict that the antifungal molecule of interest is secreted by *P. aeruginosa* into the supernatant independent of cell-to-cell contact, despite the fact that we cannot definitively rule out the hypothesis that there is a direct fungal-bacterial interaction when bacterial cells are used as a treatment. The fact that the knockout mutant strains for these two secretion systems still exhibit the same level of antifungal activity as the parental strain further supports the argument that inter-microbial contact is not a requirement for this effect. Interestingly, T3SS has actually been shown to be involved in bacterial-fungal symbiosis between *Burkholderia rhizoxinica* and *R. microsporus*[93], although it is unclear if this system is involved in the interaction between *P. aeruginosa* and *R. microsporus*.

The Type I Secretion System (T1SS) assists one-step transport of adhesins, proteases and lipases, amongst other compounds, such as the alkaline protease virulence factor AprA and the protein of unknown function AprX[67]. T1SS allows transport across both the inner and outer membranes of the bacteria via its inner membrane ATP-binding cassette (ABC) transporter, membrane fusion protein (MFP) and outer membrane (OM) protein[68,69]. Significant spore killing via the knockout mutant of this secretion system suggests that the types of molecules mentioned above, which are secreted via T1SS, are not involved in the antifungal activity seen in this project by *P. aeruginosa.*

The Type II Secretion System (T2SS) does not span both the inner and outer bacterial membranes, so requires transport via the Sec or Tat secretion pathways to transfer substrates across the inner membrane, where they can then be transported across the outer membrane by the T2SS machinery[68]. It has been observed that the type II secretion system in the bacteria *Burkholderia rhizoxinica* is responsible for secretion of enzymes that lead to chitin degradation in *R. microsporus* hyphae[94]. Two T2SS systems exist in *P. aeruginosa*; the Xcp system and the Hxc system. The Xcp system is responsible for secretion of exotoxin A, alkaline phosphatase, elastase, lipases and phospholipase C, whereas the Hxc system is needed for secretion of LapA, an alkaline phosphatase with low molecular weight[95]. As the T2SS mutant used in this project was a knockout for the *xcpT* gene (which still exhibited significant killing ability), it means that the effect of removing the Hxc system was not investigated.

It is thought that *P. aeruginosa* does not utilise the Type IV Secretion System (T4SS) like other Gram-negative bacteria do[67], but does take advantage of type IV pili (T4P). The fimX mutant used in this project removes one of the genes encoding assembly of T4P, which is key to the

bacteria's biofilm formation. It has been seen that removal of T4P reduces colonisation of *P. aeruginosa* in cystic fibrosis patients[96], which we can predict in turn leads to a reduction in its virulence. It could be expected that use of the T4P knockout strain would lead to a reduction in antifungal activity when using bacterial cells, although we know that sterile bacterial supernatant also upholds the same antifungal effect as the bacterial cells themselves. This indicates that biofilm formation is not required to have an antifungal effect against *R. microsporus*. This further solidifies the idea that activity is due to presence of an antifungal molecule or compound in the supernatant and cell-to-cell interactions are not required.

Our library of secretion system mutants does not contain a Type V Secretion System (T5SS) knockout mutant, meaning that the effect of this system was not investigated in this project, although it is thought that *P. aeruginosa* does make use of this secretion system[67]. LepA, a large exoprotease, is secreted via T5SS[67]. It is thought that LepA creates a pro-inflammatory response in human bronchiole epithelial cells via induction of interleukin-8 (IL-8)[97], with this inflammatory effect suggesting LepA may be important for virulence in human hosts, although it is unclear whether this would be important for virulence against other microorganisms. T5SS is also likely to be responsible for secretion of the esterase EstA (hydrolyses glycerol esters), which is important in production of rhamnolipids[69]. We already know from this project, rhamnolipids are not involved in the antifungal effect of *P. aeruginosa* against *R. microsporus*. These results showing continued antifungal activity after secretion system knockouts could suggest that *P. aeruginosa* is able to upregulate alternative systems to produce secreted factors when their usual pathway is prevented, therefore still achieving fungal inhibition and killing.

Results showed that first pre-incubating sterile supernatant overnight with dormant *R. microsporus* spores, before adding to the assay, removed the antifungal activity. Simple binding of the molecule to the spore surface was ruled out by experiments proving that spores used for the pre-incubation needed to be viable (section 2.3.10), suggesting that the antifungal molecule is being metabolised or sequestered by *R. microsporus*. Production of molecules by *R. microsporus* is not well studied in comparison to those of other fungi, although it is known to secrete the anticancer compounds rhizoxins[98], although these are unlikely to be important for the susceptibility of the fungus to the effects of *P. aeruginosa.*

When crude supernatant was incubated with amberlite XAD-4 resin, causing hydrophobic molecules to bind to the resin, the hydrophilic elution sometimes displayed inhibitory effects, but was shown not to be antifungal upon plating out of spores. This is likely due to the presence of the hydrophilic iron chelators pyoverdine and pyochelin in this fraction, which we know have an inhibitory effect on growth[52], but clearly do not kill fungal spores. This alone would suggest that the antifungal molecule is in the hydrophobic fraction of the supernatant, however from the results we know that we were unable to induce fungal killing with this sample, even once recombined with the hydrophilic fraction.

There are a few possible explanations for this inability to restore antifungal activity, which could be attributed to the methods used to carry out this fractionation process. Firstly, the time it took for the organic solvent to evaporate using the standard fume cupboard reached up to seven days, suggesting it is possible that the antifungal compound is not stable at room temperature for this length of time, perhaps breaking down or degrading (if found in the hydrophobic elution). However, as mentioned above, the far quicker method of using rotary

evaporation showed the same effect, so it could be possible that the antifungal molecule or compound shares a similar volatility with the chloroform, ethyl acetate or ethanol solvents it was dissolved in, meaning it would have been lost in the evaporation process, no matter which technique was used. It could be possible that the interaction between the desired compound and the extraction method (either organic solvent or hydrophobic resin) is too strong for the elution methods used. Lastly, it is always a possibility that the organic solvents used, as well as the amberlite XAD-4 resin, changes the structure or activity of the compound, meaning it is no longer antifungal after this treatment. As the compound we are trying to extract is unknown, it is difficult to identify how each method is going to affect the bioactivity. Due to not knowing the nature of the molecule(s) fresh supernatant was always used, and therefore the effect of temperature, freeze-thaw cycles and lyophilisation on the antifungal activity were not determined.

NMR data provided initial findings that can be used for further investigation. There were some clear differences between some of the samples used. It is clear that treatment with amberlite XAD-4 resin removes the majority of aromatic compounds from the supernatant, as peaks that are in this region of the spectra disappear after treatment. On the other hand, a few unidentified peaks in the amberlite XAD-4 treated sample, which are not present in the active sample, indicate that amberlite XAD-4 beads either deposit extra molecules into the supernatant, or a chemical reaction is occurring between a bacterially secreted molecule and the beads, leading to a change in chemical structure. Although abilities of Chenomx NMR Suite were limited for what we wanted to achieve, it did provide some insight into how our dormant spores behave. Reductions in glucose concentration in supernatant after inoculation with spores overnight shows several things. Firstly, it suggests the killing effect of supernatant

is not immediate, allowing significant metabolism of glucose by the spores before their viability is affected. Secondly, it shows that nutrient sensing signals that activate germination are induced even in M9 minimal media, or spores are actively consuming glucose whilst in their dormant state.

It is possible that the technique of respirometry will provide more useful evidence about whether or not *P. aeruginosa* PA14 supernatant is a respiratory inhibitor once the compound or molecule has been isolated and the method of introduction to fungal cells can be optimised.

CHAPTER THREE – NOVEL PLANT COMPOUNDS AS ANTIFUNGALS

3.1 Introduction:

3.1.1 Plant compounds as antimicrobials:

In a world where antimicrobial resistance is becoming a burden to healthcare, due to misuse and overuse, it is important that we continue to search for alternative therapeutic methods to tackle this major health threat[39]. One option for this is exploring the use of plant-derived and other natural compounds as antimicrobial agents, which are a promising alternative to current commercially used products[99]. It has been found that natural products do not disrupt the normal microflora[100], often exert low toxicity to host cells[101] and usually have high specific activity, with the hope that by creating novel antimicrobials via these sources, susceptibility is retained. Fungal pathogens in particular are difficult to treat, not only because of emerging resistance, but also due to cell wall similarities with mammalian cells, increasing toxicity effects and making it more difficult to find effective antifungals that do not have adverse side effects on the human host[99].

An example of a known antimicrobial natural compound is propolis, produced by bees from plant resin for maintenance of the hive, along with other uses[101]. Propolis has been used by humans for millennia for its antibacterial, antiviral, and antifungal activity and also has anti-inflammatory, antitumour and antioxidant properties[102]. Many food types produce secondary metabolites with wide spectrum antimicrobial activity[103], such as cinnamaldehyde from cinnamon oil, which has been shown to have strong antimicrobial activity against endodontic pathogens such as *C. albicans, Fusobacterium nucleatum, Porphyromonas endodontalis* and *Prevotella intermedia*[104]. Cranberry juice is another widely used treatment for urinary tract

infections[105], showing antimicrobial activity against both *Escherichia coli and Staphylococcus aureus*, thought to be attributed to high concentrations of phenolic phytochemicals[106]. Plants can therefore be considered as an alternative source for extracting compounds with antimicrobial potential.

3.1.2 Madecassic acid:

Madecassic acid is a biologically active pentacyclic triterpenoid (Figure 3.1) derived from *Centella asiatica,* a medicinal plant indigenous to Southeast Asia[107]. Madecassic acid is known to have antioxidant, anti-diabetic, anti-inflammatory and wound-healing properties, and more recently, has been identified *in vivo* as an anti-tumour compound[108]. Madecassic acid does this by inducing apoptosis in cancerous colon cells[109], and is thought to have these effects at concentrations of around 99 µM[110] Although madecassic acid has not been well studied as an antimicrobial agent, another active compound extracted from *C. asiatica*, asiatic acid, has been shown to exhibit antibacterial and antifungal activity[111], and synergistically aids efficacy when used in combination with established antifungals, such as fluconazole[112].

Here we investigate the potential cytotoxicity of madecassic acid *in vitro* against the fungal pathogens *R. microsporus, C. albicans* and *C. neoformans*.

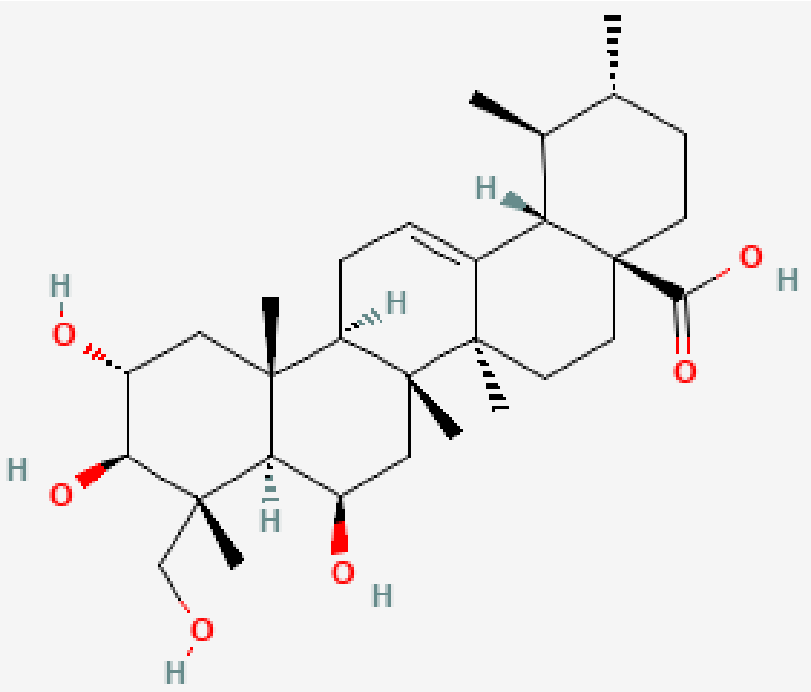

Figure 3.1. 2D structure of the pentacyclic triterpenoid madecassic acid

PubChem Identifier: CID 73412
URL: https://pubchem.ncbi.nlm.nih.gov/compound/Madecassic-acid

3.1.3 Aims and objectives:

The aims of this chapter are to investigate whether madecassic acid has the potential to be used as an antifungal agent against *Rhizopus microsporus,* as well as other fungal pathogens (*C. albicans* and *C. neoformans*). Potential modes of action of activity are also investigated.

3.2 Materials and methods:

3.2.1 Media and strains:

3.2.2 MIC assay:

Madecassic acid (gift from Geraud Sansom, University of Kent) was stored at -20℃ as a 55.5 mM stock in DMSO. This was thawed, and a fresh stock of 1000 µM was made in SAB media for each assay. A 96 well plate was prepared with 100 µl SAB media in each well, before adding 100 µl of madecassic acid solution to the first well and mixing. 100 µl was transferred from the first well to the next well, mixed and this was repeated with all subsequent wells, creating a 1 in 2 dilution gradient, leaving a 0 µM drug well and a SAB media negative control well.

C. albicans and *C. neoformans* were sub-cultured in SAB overnight at 37℃, 200 rpm, and *R. microsporus* spores were harvested from ~13-15-day old plates by flooding with 10 ml PBS. Samples were transferred to falcon tubes and centrifuged for 3 minutes at 3000 rpm. Supernatant was poured off and cell pellets were resuspended in 10 ml PBS. Cells were counted on a haemocytometer and resuspended in SAB media at a stock concentration of $2x10^4$ CFU/ml, to achieve a working concentration of $1x10^4$ CFU/ml in each sample well. Then, 100 µl of the relevant fungal cell suspensions were added to the wells. MIC assay plates were incubated for 24 hours at 37℃. Optical density readings at 600 nm were taken of the 96 well plates using a BMG Labtech FLUOstar Omega plate reader. For those concentrations that inhibited fungal growth, these samples were plated out onto SAB agar (with 50 mg/ml Tetracycline) to test for viability. 100 µl inhibited *R. microsporus* was spread on a plate to test for viability.

3.2.3 XTT assay:

A Roche Cell Proliferation Kit II (XTT) was used as a test for viability. After thawing at 37℃, 5 ml labelling reagent was mixed with 0.1 ml electron-coupling reagent per 96 well plate. MIC assays were set up as mentioned previously, and after 24 hours incubation, 50 µl of XTT reagent was added to each well of the assay. The plate was incubated for a further 4 hours, before the optical density of samples were measured at 450 nm and 630 nm. Data was analysed using the following formula: Absorbance=$A_{450-620nm}$−mean(A_{blank}).

3.2.4 Respirometry:

An Oroboros Oxygraph-2k respirometer was used, set to 37℃, and each chamber was loaded with 2 ml of 1x10^6 CFU/ml *R. microsporus* resting spores in SAB media. Germination was allowed to begin and once O_2 flow was consistent, 500 µM of madecassic acid was injected into one chamber, and the other was left as a control sample. After re-stabilisation of respiration, the complex III (cytochrome c reductase) inhibitor, antimycin A, was added to both chambers (2 µl 2 mM) and the effect was observed in both the madecassic acid treated sample, and the control sample. Salicylhydroxamic acid (SHAM), an alternative oxidase inhibitor, was then added (4 µl 0.5 M) and the effect was observed.

3.3 Results:

3.3.1 Madecassic acid inhibits *R. microsporus* growth at 250 µM:

To investigate the minimum inhibitory concentration (MIC-90) of madecassic acid to inhibit growth of *R. microsporus* germination, spores were incubated with the compound at increasing concentrations (0-500 µM). This was recorded visually via light microscopy and via

optical density at 600 nm. Both methods showed madecassic acid to have an MIC-90 of 250 µM against *R. microsporus* (Figure 3.2). Aside from inhibiting growth, morphology of *R. microsporus* spores was otherwise not affected by the presence of madecassic acid at any concentration.

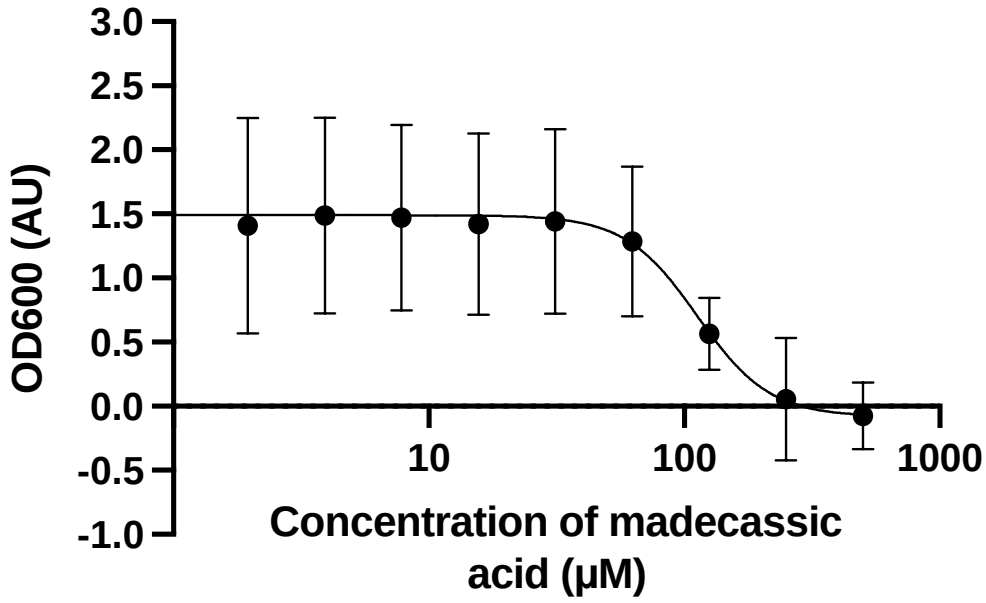

Figure 3.2. Madecassic acid inhibits the growth of *R. microsporus* dormant spores at 250 µM. Dormant *R. microsporus* spores were treated with increasing concentrations of madecassic acid (0-500 µM), and the optical density at 600 nm was read. Data was plotted using a log-10 scale, using standard deviation for error bars (n=7).

3.3.2 Madecassic acid kills *R. microsporus* resting spores at 250 µM:

To investigate if the inhibitory effect seen by madecassic acid against *R. microsporus* is just that, inhibitory, or whether fungal cells were being killed, inhibited samples were plated onto SAB agar to determine if they were viable or not. Observation of plates after overnight incubation at room temperature confirmed that samples of *R. microsporus* that appeared to have been inhibited, were also not viable, and this was significantly different from the control sample (500 µM $p<0.0004$, 250 µM $p<0.0006$). We can therefore conclude that at 250 µM, madecassic acid is fungicidal against *R. microsporus* (Figure 3.3).

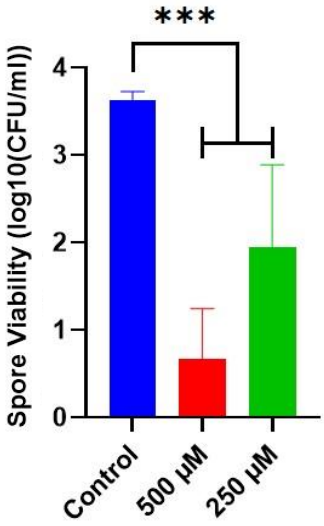

Figure 3.3. Inhibitory concentrations of madecassic acid are also fungicidal against *R. microsporus.* Samples at inhibitory concentrations of madecassic acid were plated out on SAB agar to test for viability. Data was plotted as the log10 value of the mean average (n=3) and standard deviation as error bars. Data was analysed using one-way ANOVA using the original data. *** = $p<0.005$

3.3.3 Madecassic acid does not inhibit germination through direct inhibition of mitochondrial respiration:

Studies of madecassic acid in bacteria have shown that this compound may be exerting an antibacterial effect via inhibition of respiration (Shepherd unpublished observation), so it was of interest to investigate whether mitochondrial respiration could be a key target for madecassic acid in its action against fungal pathogens.

Experiments found that addition of madecassic acid to spores does not lead to a decrease in respiration rate, suggesting that inhibition of growth is not due to mitochondrial respiration inhibition. Addition of antimycin A to the control spores (spores with no treatment) leads to a sudden drop in respiration rate as cytochrome c reductase is inhibited. The lack of recovery suggests an alternative oxidase (AOX) is not being used here. Addition of antimycin A to spores treated with madecassic acid has no impact on respiration rate. This suggests that madecassic acid either competitively inhibits activity of antimycin A, or degrades the molecule. Addition of SHAM to treated spores also has no impact on respiration rate, potentially for similar reasons (Figure 3.4). This result was seen in two of the three biological repeats that were carried out.

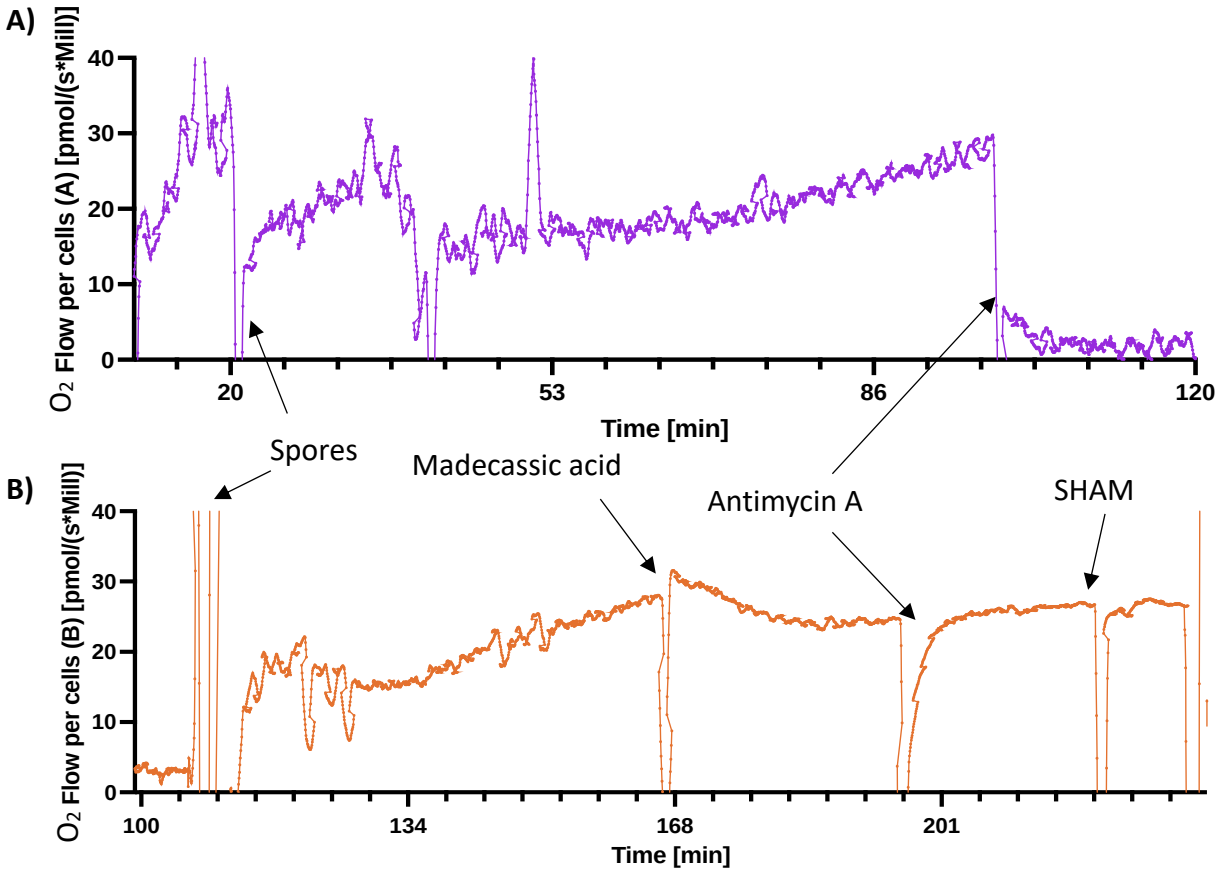

Figure 3.4. The pentacyclic triterpenoid plant compound, madecassic acid, does not inhibit the germination of *R. microsporus* resting spores via inhibition of mitochondrial respiration. Dormant spores were incubated at 37°C in a respirometer, before addition of 500 µM madecassic acid to the test chamber, followed by the respiratory inhibitors antimycin A and SHAM. A) Control sample containing dormant spores, but no addition of madecassic acid. B) Treated sample, containing dormant spores, which were then treated with madecassic acid.

3.3.4 Madecassic acid is not inhibiting activity of respiratory inhibitors due to pH effects:

To rule out the possibility that the inhibitory effect of madecassic acid against the respiratory inhibitors (antimycin A and SHAM) is just due to the pH effect of the acid pH was measured after addition of madecassic acid to SAB media. Using standard pH strips, it was seen that SAB media alone had a pH of around 5.5, which was unchanged upon addition of 500 µM madecassic acid (the highest concentration used), suggesting that any prevention of inhibitory activity is not due to pH degradation of these molecules.

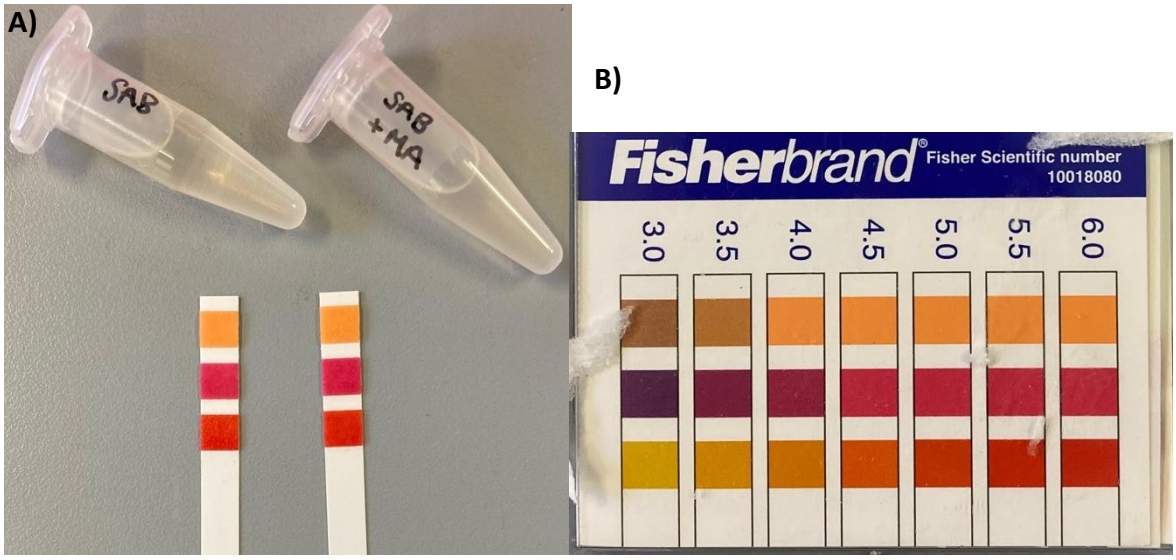

Figure 3.5. Madecassic acid does not inhibit the activity of respiratory inhibitors antimycin A and SHAM, via pH effects. Standard SAB media and SAB media with 500 µM madecassic acid, were tested for pH. A) The comparison of pH between standard SAB media (pH ~5.5) and SAB media with addition of 500 µM (pH ~5.5), and B) The manufacturer's pH reference.

3.3.5 Madecassic acid also has an inhibitory effect on the growth of *Candida albicans* and *Cryptococcus neoformans*:

To determine whether madecassic acid exhibits antifungal activity against other common fungal pathogens, and has potential as a broad-spectrum therapy, inhibitory experiments were repeated with other fungal species, which are structurally and morphologically different from *R. microsporus*. Increasing concentrations of madecassic acid (0-500 µM) were tested for antifungal activity against the yeast *C. albicans* and the encapsulated yeast *C. neoformans*. The MIC-90 for both was found to be 250 µM (Figure 3.6), although data collected for *C. neoformans* was variable due to conditions used not being optimal.

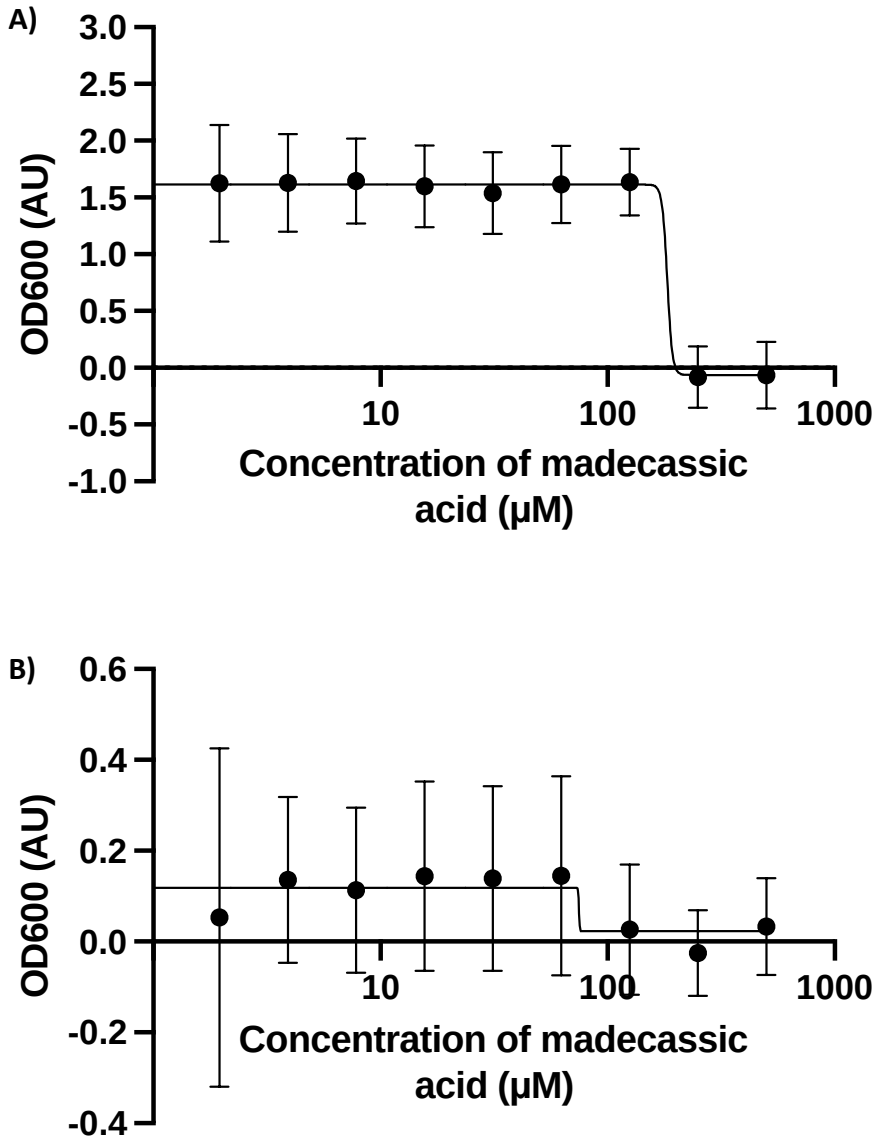

Figure 3.6. Madecassic acid is inhibitory against *C. albicans* and *C. neoformans* at an MIC of 250 µM. Both *C. albicans* and *C. neoformans* were treated with increasing concentrations of madecassic acid (0-500 µM). A) The effect of increasing concentrations of madecassic acid on the growth of *Candida albicans*. B) The effect of increasing concentrations of madecassic acid on the growth of *C. neoformans.* Data was plotted using a log-10 scale, using mean average readings and standard deviation for error bars (n=7).

3.4 Discussion:

Due to increasing emergence of antimicrobial resistance, it is important that we find novel approaches to treat microbial infections. Many plants produce compounds that have previously been seen to exhibit antimicrobial activity against certain microorganisms[99]. An example of a plant already known to produce compounds with therapeutic potential is *Centella asiatica*, a Southeast Asian plant, which amongst other molecules, produces an anti-inflammatory, anti-diabetic and antioxidant compound called madecassic acid[108]. Madecassic acid is a plant derived pentacyclic triterpenoid, which has previously been seen to have other therapeutic uses, for example as an anti-tumour compound[108]. There is current research taking place to investigate the effect of madecassic acid against bacterial cells, but not much is known about its activity against fungal pathogens. We were kindly gifted a small amount of madecassic acid (Geraud Sansom, University of Kent) in order to begin a pilot investigation into the antifungal potential of this compound. Whilst focusing on *R. microsporus* (filamentous fungus) throughout this project, the antifungal activity of madecassic acid against *C. albicans* (yeast) and *C. neoformans* (encapsulated yeast) was also explored, due to their differences in structure and to determine if this compound could have potential as a broad-spectrum therapy against a range of fungal pathogens.

Experimental results showed that incubation of high concentrations of madecassic acid with fungal cells had inhibitory effects on the growth of all three fungal species at 250 µM. However, slow and inconsistent growth of *C. neoformans* suggested that growth media and conditions used in this case were not ideal for this fungus, although this assay could be optimised in the future to further investigate the effect on this species. Although investigations into the antimicrobial properties have not been published before, apoptosis of

cancer cells has been reported with concentrations of 99 µM or less[110], suggesting that an MIC-90 of 250 µM is fairly high, so might not be safe for therapeutic use.

Inhibited samples of *R. microsporus* were plated out on agar to test for viability, indicating whether the madecassic acid was just inhibiting growth, or whether it also led to cell death. Negligible survival of spores after madecassic acid treatment suggests its effect is fungicidal. Although a fairly reliable method to determine viability status of microbial cells, it is necessary to pose the question: can we really ever determine the difference between a dead cell and one which has entered a permanent state of dormancy? Of course, there are cases where you can definitively classify a cell as dead. For example, cell lysis or apoptosis significantly changes cellular morphology. Other causes of cell death might leave the cell membrane intact, whilst no metabolism is occurring within the cell. Incubation at room temperature on SAB agar was used throughout these investigations for viability counts, but this of course could give different counts for viability than using a different medium or incubation temperature, which could reactivate dormant cells that might be assumed dead in different conditions.

Respirometry was used to investigate the effect that madecassic might be having on the mitochondrial respiration of our main fungal pathogen of interest, *R. microsporus*. Experiments showed interesting findings, suggesting that madecassic acid was not having a direct inhibitory effect on mitochondrial respiration, despite previous experiments suggesting that the spores are in fact killed by the compound. This adds weight to the argument that it is difficult to fully confirm death of spores as opposed to inhibition, with us assuming here that cells were killed, but they are still respiring. Madecassic acid was shown to be impacting the activity of known respiratory inhibitors. As expected, addition of antimycin A to a control

sample of germinating spores led to a drop in respiration, which did not recover, suggesting AOX is not being utilised. Addition of antimycin A to a sample of spores treated with madecassic acid does not inhibit germination as expected, and the same result is seen when SHAM is added. The effect of pH on the inhibitors was ruled out, shown by madecassic acid not changing the pH of the sample. Other possible reasons could be that madecassic acid is causing degradation or reacting with the inhibitors in another way, or is actually competitively binding to the respiratory complexes, but not causing inhibition itself. Regardless of the reason, this shows us that madecassic acid is having an effect on mitochondrial respiration, even if this is not the key MoA used for its antifungal ability. It is important to recognise these interactions, as use of this compound with antimycin A and SHAM impacts their ability to inhibit fungal growth. Only inhibitors of complex III and AOX (antimycin A and SHAM, respectively) were used in these experiments, however respiratory inhibitors targeting different complexes of the electron transport chain could be investigated further, to gain a better understanding of the interaction of madecassic acid with this pathway. Other respiratory inhibitors of interest could include rotenone or honokiol, which inhibit complex I[113]. It could be of interest to see if madecassic acid also has the same effect on these inhibitors as it does with the ones we tested, particularly as they target a different respiratory complex.

CHAPTER FOUR – SUMMARY

4.1 Conclusions:

The aims of this project were to investigate the potential of *P. aeruginosa* and the plant-derived compound, madecassic acid, to be used as novel antifungals against the infectious agents causing mucormycosis. We confirmed that *P. aeruginosa* exhibits fungicidal activity against *R. microsporus* via secretion of a molecule(s) or compound(s) into the supernatant. The identity of the molecule(s) is still unknown, however, we have identified that PQS, PHAs, rhamnolipids and phenazines are not responsible for this activity.

Madecassic acid also shows fungicidal effects against *R. microsporus* and has potential as a broad-spectrum antifungal treatment due to its activity against *C. albicans* and *C. neoformans* as well. Although not clear, it seems madecassic acid has some effect on mitochondrial respiration in *R. microsporus*, whereas *P. aeruginosa* supernatant seems to have no direct effect on respiration. This indicates that these methods are using different MoAs to exhibit antifungal activity, and therefore can both be taken forward for further investigation as potential new antifungal therapies.

4.2 Limitations:

There are several limitations within this study, which are important to recognise when we look at the findings. Respirometry was utilised to test if the antifungal activity of *P. aeruginosa* supernatant was affecting mitochondrial respiration of spores, either confirming or ruling this out as a mode of action. The respirometry data shown in Figure 2.23 was obtained by adding spores and supernatant (or spores and LB for the control) directly into the chamber. This did

not allow spores to begin germination before addition of the antifungal treatment, so different approaches were then tried thereafter. It is therefore necessary to optimise a method that allows addition of antifungal treatment after spores have been allowed to begin germination. Using half of the chamber volume to allow germination and then adding the remainder of supernatant after proved difficult due to the excess oxygen this allowed to fill the chamber. It was therefore decided that this could be better investigated once the antifungal molecule(s) has been isolated and can be tested at a high concentration.

An argument that could be made is that there is the possibility that rather than secreting something into the supernatant that is antifungal, *P. aeruginosa* could be depleting the growth media of nutrients that *R. microsporus* cannot survive without, hence all *P. aeruginosa* strains are showing a significant reduction in spore viability in comparison to when fresh media is being used for the control. This is of course something very difficult to rule out without having a mutant strain that does not have this antifungal ability to confirm otherwise, however these assays have previously been carried out with other bacterial species, such as DH5α *Escherichia coli* which does not exhibit the antifungal activity seen with *P. aeruginosa*.

As mentioned in the discussion section of chapter three, it is difficult to know if we can really determine the difference between dead spores and those that have entered a permanent or extended state of dormancy. Many popular cell stains, such as the XTT stain used, detect respiration, and again, cells could be lying dormant, with very low levels of respiration. One cell stain that may be more accurate is the FUN-1 stain. Due to time restrictions, use of this stain with *R. microsporus* spores was not optimised, but this is a stain that could be used going

forward. Propidium iodide is a fluorochrome stain that binds DNA when the plasma membrane of a dead cell becomes permeable[114], and can therefore be used as a more accurate viability stain. *P. aeruginosa* secretes a significant amount of extracellular DNA into the supernatant, so upon removal of this, propidium iodide has the potential to be a useful stain to test viability after treatment with supernatant. At the end of the day, the interest behind these investigations is the potential for antifungal treatments to a microorganism causing a disease associated with high mortality (mucormycosis)[15]. The importance in this case is not whether the pathogen is killed or solely in a prolonged dormant state, but more about whether these compounds could be used as therapies to prevent disease progression.

Due to time constraints, *P. aeruginosa* mutants were not validated via PCR, although certain mutants were confirmed via their expected change in pigmentation (such as phenazine mutants no longer producing pyocyanin, and therefore no longer appearing blue). Other mutants were independently validated from the labs they originated from, and certain mutants had been validated via secretome analysis. If time allowed, ideally all mutants would be screened and validated via PCR.

Another limitation that was encountered was regarding the Chenomx NMR Suite program that was used to identify metabolites from NMR spectra. This program has a limited number of compounds in its database, all of which are primary metabolites. As we are working with bacterially secreted products, we can assume that the molecule responsible for antifungal activity is a secondary metabolite, but it seems that Chenomx NMR Suite does not extend to molecules of this type. In fact, it does not seem that there are any NMR metabolomics programs that can currently be used for identification of biological molecules, however this

program was used to identify some primary metabolites that appear after bacterial growth and incubation with spores, and provides interesting information nonetheless.

4.3 Further investigations and directions for the project:

In addition to the investigations carried out in this study, there are other experiments that could enhance and deepen our understanding of the science we have found here. Ultimately, the main aim is to identify the molecule(s) or compound(s) secreted by *P. aeruginosa* responsible for its antifungal activity against *R. microsporus.* Further investigations that build on what we have already found could include repeating more NMR scans, potentially even comparing the amberlite eluted fraction to the amberlite treated supernatant and other samples, as this comparison was not made. Use of a gentler ion exchange resin may also be necessary, due to the difficulties seen recovering the antifungal activity when using amberlite. We can also utilise NMR to identify peaks of molecules and compounds we already know are not responsible for antifungal activity, and therefore narrow down the peaks that are left for further identification. NMR could also be used to investigate how long it takes for spores to stop consuming glucose by carrying out runs of samples of several timepoints, to indicate how long it takes for the antifungal effects of *P. aeruginosa* to halt spore growth.

The role of ROS was not studied in this project, but it is well established that the formation of ROS can have an important role in antifungal activity, leading to cell damage and eventually apoptosis[115]. It could be possible that ROS formation is contributing to the antifungal activity of both *P. aeruginosa* and madecassic acid on *R. microsporus*, and could therefore be a useful next step to identify a MoA, particularly once the compound(s) responsible for the activity of *P. aeruginosa* has been identified.

In this study, only the ratio of one part bacterial supernatant to one part *R. microsporus* was investigated. Although it is difficult to determine the concentration of the antifungal compound(s) without identifying the compound itself first, different ratios of bacterial supernatant to *R. microsporus* could be investigated to see if the same antifungal effect is observed with smaller volumes of supernatant (i.e. whether the compound(s) is potent or secreted at high concentrations).

This project has identified the potential of both *P. aeruginosa* and madecassic acid as future antifungal agents against one of the aetiological agents for mucormycosis, *R. microsporus,* which is otherwise highly resistant to many existing commercial antifungals.

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