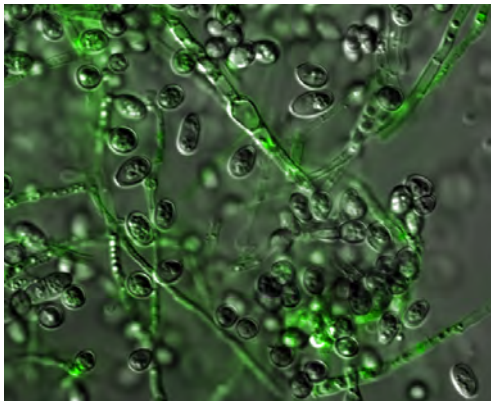
BIOLOGICAL STUDIES INTO *SCEDOSPORIUM AURANTIACUM*, AN OPPORTUNISTIC PATHOGEN COLONISING HUMAN LUNGS



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Doctor of Philosophy

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An experiment is a question, which science poses to Nature and a measurement is the recording of Nature's answer

'Max Planck'

German theoretical physicist

(1858-1947)

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ABSTRACT

Scedosporium aurantiacum is a recently identified highly virulent opportunistic pathogen, which is capable of causing a range of infections in immunocompromised people. *S. aurantiacum* is also known to colonise the respiratory tracts of cystic fibrosis (CF) patients, with a prevalence ranging from 10-17.4% in Australian CF patients. In view of the polymicrobial nature of CF, *S. aurantiacum* may be frequently encountered with the prokaryotic lung inhabitants such as *Pseudomonas aeruginosa.* These mixed bacterial-fungal interactions or interactions between the fungus and the host lungs may impact the outcome of CF.

In this study, a high throughput microtitre plate-based nutrient utilisation assay involving 94 unique substrates was used to characterise four *S. aurantiacum* strains displaying different virulence levels as determined by the *Galleria mellonella* larvae model. This approach unveiled five carbohydrate metabolism pathways and a difference in the sucrose and turanose metabolism between high and low virulence *S. aurantiacum* strains. One high and one low virulence strain were further co-cultured with clinical CF isolates of *P. aeruginosa* in a medium mimicking human CF sputum to explore the mechanisms of interactions between *P. aeruginosa* and *S. aurantiacum* using plate tests and confocal fluorescence microscopy. A genetic transformation system was also developed for *S. aurantiacum* to facilitate detailed observation of the bacterial-fungal interactions. In liquid cultures, the biofilm forming strains of *P. aeruginosa* exhibited a higher inhibitory effect against growth of *S. aurantiacum* compared to the non-biofilm forming strains. These results indicated that the ability to form biofilms might be useful for *P. aeruginosa* to cause inhibition of *S. aurantiacum* growth when in direct contact with the fungus. However, these biofilm forming *P. aeruginosa* strains could inhibit the growth of *S. aurantiacum* strains even in the absence of a direct physical contact *i. e.* in co-cultures involving separating membranes. These results suggest that in addition to biofilm formation, *P. aeruginosa* could also utilise diffusible extracellular metabolites for inhibiting the fungal growth.

Further on, respiratory epithelial cells derived from a lung carcinoma were infected with a high virulence *S. aurantiacum* strain and the response of cells was determined using electron microscopy and transcriptomics-based approaches. *S. aurantiacum* invaded the

respiratory epithelial cells by an initial adherence to the cell surface followed by penetration into the cell using conidial germ tubes. The respiratory cells exhibited a protective response against the fungal infection by up-regulating the inflammatory pathways leading to the release of cytokines.

In conclusion, this work expands the existing knowledge of the emerging fungal pathogen *S. aurantiacum* and thus provides valuable insights into its yet unanswered role in CF, ultimately leading to a better understanding of pathophysiology of the disease.

STATEMENT OF DECLARATION

I certify that the work presented in this thesis has not been submitted as a part of the requirement for a degree or course to any institution or university other than Macquarie University.

I also certify that this thesis is an original piece of research conducted by me between December 2011 and June 2015 and it contains no material previously published or written by any other person except where due reference is made in the text. Biosafety approval (NLRD Ref. 5201200092) was obtained to use *S. aurantiacum* for research purpose.

I consent that a copy of this thesis is available at Macquarie University Library for loan and photocopying forthwith.

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Jashanpreet Kaur

June 2015

LIST OF PUBLICATIONS

The thesis is based on article 1, 2 and 3, presented as Chapter III, IV and V respectively.

1. Kaur, J., Duan, S. Y., Vaas, L. A., Penesyan, A., Meyer, W., Paulsen, I. T. & Nevalainen, H. 2015. Phenotypic Profiling of *Scedosporium aurantiacum*, an Opportunistic Pathogen Colonizing Human Lungs. *PLoS One*, 10, e0122354.
2. Kaur, J., Pethani, B. P., Kumar, S., Kim, M., Sunna, A., Kautto, L., Penesyan, A., Paulsen, I. & Nevalainen, H. 2015. *Pseudomonas aeruginosa* inhibits the growth of *Scedosporium aurantiacum*, an opportunistic fungal pathogen isolated from the lungs of cystic fibrosis patients. *Frontiers in Microbiology*, 6**,** 866.
3. Kaur, J., Kautto, L., Penesyan, A., Paulsen, I. T., Nevalainen, H. Interactions of an Emerging Fungal Pathogen *Scedosporium aurantiacum* with the Human Lung Epithelial cells. Manuscript prepared for submission.

Articles 4 and 5 are also cited in the text and include Kaur, J., as one of the authors.

4. Kamath, K. S., Kumar, S. S., Kaur, J., Venkatakrishnan, V., Paulsen, I. T., Nevalainen, H. & Molloy, M. P. 2015. Proteomics of hosts and pathogens in cystic fibrosis. *Proteomics Clin Appl*, 9, 134-46.
5. Perez-Bercoff, A., Papanicolaou, A., Ramsperger, M., Kaur, J., Patel, H. R., Harun, A., Duan, S. Y., Elbourne, L., Bouchara, J. P., Paulsen, I. T., Nevalainen, H., Meyer, W. & Huttley, G. A. 2015. Draft Genome of Australian Environmental Strain WM 09.24 of the Opportunistic Human Pathogen *Scedosporium aurantiacum*. *Genome Announc*, 3.

PRESENTATIONS AND AWARDS

(*Oral Presentation)

1. Kaur, J*., Vaas, L. A., Duan, S. Y., Meyer, W. & Nevalainen, H. Analysis of *S. aurantiacum* Strains Exhibiting Different Virulence using a Phenotype Microarray. *4th International Workshop on 'Pseudallescheria/Scedosporium infections', Innsbruck, Austria, 2013.*

2. Kaur, J*., Penesyan, A., Vaas, L. A., Teo, J., Paulsen, I. T., Meyer, W. & Nevalainen, H. Phenotype Profiling of *Scedosporium aurantiacum*, an Opportunistic Pathogen Colonising Human Lungs. *ASM NSW-ACT Becton Dickinson student award, Sydney, Australia, 2014.*

3. Kaur, J., Penesyan, A., Vaas, L. A., Teo, J., Paulsen, I. T., Meyer, W. & Nevalainen, H. Phenotype Profiling of *Scedosporium aurantiacum*, an Opportunistic Pathogen Colonising Human Lungs. Poster presentation. *International Mycological Congress, Bangkok, Thailand, 2014.*

Awards

1. Postgraduate Research Fund Award ($5000), Macquarie University, Sydney, Australia, **2013**.

2. Finalist for the ASM NSW-ACT Becton Dickinson Student Award, Sydney, Australia, **2014**.

LIST OF ABBREVIATIONS

Abbreviations frequently used in the text are as follows:

ACN	Acetonitrile
AIDS	Acquired Immunodeficiency Syndrome
BLAST	Basic Local Alignment Search Tool
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CHS	Chitin Synthase
CAL	Calmodulin
CLSM	Confocal Laser Scanning Microscopy
CFU	Colony Forming Units
DAPI	4’,6-Diamidino-2-Phenylindole
DAVID	The Database for Annotation, Visualisation and Integrated Discovery
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EF1α	Elongation Factor 1- α
EORTC /MSG	European Organisation for Research and Treatment of Cancer/Mycoses Study Group
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GO	Gene Ontology
gpdA	Glyceraldehyde-3-Phosphate Dehydrogenase gene
HBE	Human Bronchial Epithelial
HIV	Human Immune Virus
hph	Hygromycin Phosphotransferase gene
IL	Interleukin

IPA	Ingenuity Pathway Analysis
ITS	Internal Transcribed Spacer
LB	Luria Bertani
MAPK	Mitogen Activated Protein Kinase
MCS	Multiple Cloning Site
MLST	Multilocus Sequence Typing
MOI	Multiplicity of Infection
NF-kB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PEG	Polyethylene Glycol
pki	Pyruvate Kinase gene
PM	Phenotype Microarray
PRM	Peptidorhamnomannan
psl	Polysaccharide Synthesis Locus
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
QS	Quorum Sensing
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SCFM	Synthetic Cystic Fibrosis Medium
SDA	Sabouraud’s Dextrose Agar
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
TEM	Transmission Electron Microscopy
TLR	Toll-like Receptors
UHPLC	Ultra High Performance Liquid Chromatography
YFP	Yellow Fluorescent Protein

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1

INTRODUCTION

1.1. Fungi as Disease Causing Agents

Invasive fungal infections are the major cause of morbidity and mortality worldwide and despite efforts to completely eradicate them, they continue to emerge and re-emerge with an even greater frequency and cause significant burden on human and animal health (Rappuoli, 2004). Reports have suggested that around one and a half million people are killed every year due to the invasive fungal infections compared to other diseases such as tuberculosis (Barberan *et al*., 2014) or malaria (Harrison, 2007). While most of the fungal infections are contained effectively by the human immune system, the current incidence of fungal infections is mainly because of the improvements in diagnostic tools and techniques, and rise in patients at risk such as those with malignancies, HIV (human immune virus) and AIDS (acquired immunodeficiency syndrome) infections (Eggimann *et al*., 2003; Byrnes *et al*., 2011). In addition to that, modern immunosuppressive medical interventions including prolonged use of antineoplastic agents, broad-spectrum antibiotics, adrenal corticosteroids, and prosthetic devices and grafts as well as more aggressive surgery are also known to contribute to fungal infections (Enoch *et al*., 2006). In humans, fungi can cause a wide variety of diseases ranging in severity from catheter related fungemia, pneumonia and septicaemia, to more localised infections in skin, lungs and paranasal sinuses, to extensive haematogenous dissemination (Pfaller and Diekema, 2004; Rippon, 1982; Walsh and Groll, 1999). Although the incidence of invasive fungal infections (mycoses) is much lower than the superficial fungal infections (*e. g.* skin and nails), yet invasive diseases are of great concern owing to the high mortality rates associated with them (Brown *et al*., 2012).

Human pathogenic fungi are a morphologically, clinically and ecologically diverse group (Bowman *et al*., 1992). Examples of the most common fungal pathogens affecting humans include *Candida albicans*, *Aspergillus fumigatus*, *Fusarium* spp., *Cryptococcus neoformans*, *Coccidoides immitis*, *Histoplasma capsulatum* and *Blastomyces dermatidis* (Anaissie *et al*., 1989). Overall, these fungal pathogens can be divided into two broad classes, primary pathogens and opportunistic pathogens. Primary fungal pathogens usually have an environmental origin and are capable of infecting individuals who have been exposed to large doses of the fungus (*e.g. C. immitis* and *H. capsulatum*). Opportunistic pathogens are known to cause invasive fungal infections in people with an impaired or damaged immune system (Fig. 1-1). They can exist either in the environment (*e. g. C.*

neoformans, A. fumigatus) or as commensals in healthy individuals (*e. g. C. albicans*) (van Burik and Magee, 2001).

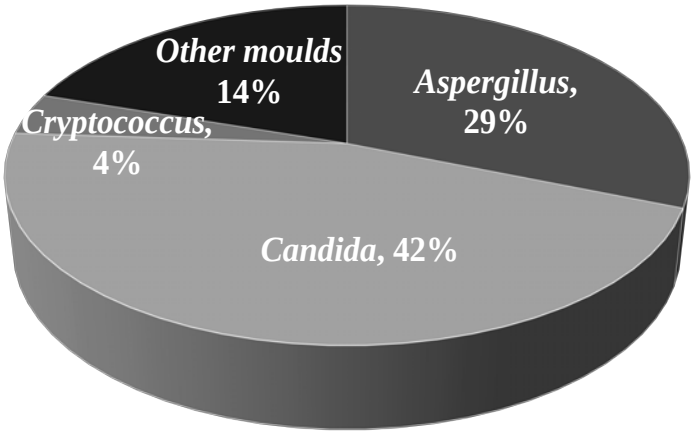


Fig. 1-1. Major pathogens involved in invasive fungal infections among solid-organ transplantation population and hematopoietic stem cell recipients. Image has been modified from (Pfaller and Diekema, 2004).

The yeast *Candida* is the most dominant group of opportunistic pathogenic species that causes life threatening invasive fungal infections in patients who are severely immunocompromised (Pappas *et al*., 2003; Trick *et al*., 2002; Wisplinghoff *et al*., 2004). Almost all *Candida* species are capable of causing human infection, but *C. albicans* is the most frequently encountered species in clinical cases including those from intensive-care and hematology patients (Pfaller and Diekema, 2004). Apart from *C. albicans*, other invasive yeast species such as *Cryptococcus* spp., have also emerged as a significant fungal pathogen with an expanding geographic range and environmental niche. The pathogenic *Cryptococcus* species complex consisting of *C. neoformans and C. gattii*, is capable of causing infection in both immunocompromised and healthy individuals (Byrnes *et al*., 2011; Sorrell, 2001). *C. neoformans* is usually acquired by inhalation and causes cryptococcosis (Byrnes *et al*., 2011).

In recent years, filamentous fungi (moulds) are also increasingly isolated from the clinical specimens worldwide. *Aspergillus* spp. are the most commonly isolated invasive filamentous fungi that are known to cause a broad range of diseases in humans with an impaired immune system. *Aspergillus* spp. can cause a variety of diseases ranging from

local hypersensitivity reactions to fatal systemic mycosis (Barnes and Marr, 2006; Hope *et al*., 2005). Although the genus *Aspergillus* encompasses around 19 known species, most of the human infections are caused by *A. fumigatus*, *A. flavus* and *A. niger* (Baddley *et al*., 2001; Perfect *et al*., 2001). The most predominant *A. fumigatus* species releases large amounts of conidia and is commonly found in the environment. Studies have reported an estimate of 1 to 100 colony-forming units of *A. fumigatus* per cubic metre of air. Therefore, it is likely that humans can inhale a minimum of a hundred conidia each day (Latge 1999, 2001). *A. fumigatus* infections in haemopoetic stem cell recipients are associated with high mortality rates (Marr *et al*., 2002).

While *Aspergillus* remains the most frequent agent of fungal infections, the incidence of infections due to non-*Aspergillus* moulds has increased as well. Three most common non-*Aspergillus* moulds capable of causing infections in the human host include *Scedosporium* spp., *Fusarium* spp., and *Rhizopus* spp. (Brown *et al*., 2012). According to a multi-centre study of mould infections in heart and liver transplant recipients, the highest amount of cases was attributed to *Aspergillus* followed by *Scedosporium* spp., *Rhizopus* spp. and *Fusarium* spp. (Enoch *et al*., 2006).

Members of *Scedosporium* spp. have emerged as significant fungal pathogens because of the increasing incidences of infections caused by these agents in recent years. *Scedosporium* spp. are capable of producing a range of serious cutaneous/sub-cutaneous and/or disseminated infections in immunocompromised hosts (Lamaris *et al*., 2006; Steinbach and Perfect, 2003; Walsh and Groll, 1999). Cortez *et al.* (2008) reported around 435 infection cases attributed to *Scedosporium* spp. in a review of the medical literature. Almost all the *Scedosporium* species were capable of causing opportunistic infections in patients with suppressed immune systems such as those with advanced HIV infection, primary immune deficiencies or solid organ or hematopoietic stem cell transplant recipients (Castiglioni *et al*., 2002; Husain *et al*., 2005; Jayamohan and Ribes, 2006; Lamaris *et al*., 2006; Lopez *et al*., 2001; Marr *et al*., 2002; Panackal and Marr, 2004; Revankar *et al*., 2002; Steinbach and Perfect, 2003). *S. apiospermum* is a medically important species of genus *Scedosporium* that was initially recognised as the main etiologic agent amongst *Scedosporium* spp. in France and Australia (Gompels *et al*., 2002; Jabado *et al*., 1998; Phillips *et al*., 1991; Pistono *et al*., 1989; Santos *et al*., 2000). However, with the progression of time, increased incidences of *Lomentospora prolificans* colonisation were observed in patients with lung disease thereby making it the second

important human pathogen in Australia (Cooley *et al*., 2007). Recently a large number of studies in Australia have emphasised the emergence of a newly discovered highly virulent member named *S. aurantiacum*, which shares similar epidemiological features with *S. apiospermum* (Gilgado *et al*., 2005; Harun *et al*., 2010b). *S. aurantiacum* infections have been mainly found to affect people with diabetes, solid tumours, chronic lung diseases and stem cell transplants with no reports of mortality so far (Heath *et al*., 2009). Some studies in Japan also indicate the presence of *S. aurantiacum* in the central nervous system of near-drowning tsunami survivors (Nakamura *et al*., 2013).

1.1.1. *Scedosporium aurantiacum*: An Emerging Fungal Pathogen

Originally, *Scedosporium* spp. comprised only two major clinically important species: *S. apiospermum* (teleomorph of *S. boydii*) and *L. prolificans*. *S. aurantiacum* is an ascomycetous fungus and has been recently added to the group as a subset of *S. apiospermum* isolates in Australia based on the morphologic and phylogenetic studies (Rainer *et al*., 2000; Gilgado *et al*., 2005; Gilgado *et al*., 2008; Ramsperger *et al*., 2014). The classification of different *Scedosporium* species is shown in Table 1-1.

Table 1-1. Classification of *Scedosporium* spp. based on the phylogenetic studies carried out by Gilgado and colleagues (2005).

Clade	Organism
I	*S. aurantiacum*
II	*S. minutispora*
III	*S. dehoogi*
IV	*S. apiospermum sensu stricto*
V	*S. boydii*, *S. ellipsoidea* and *S. augusta*

Similarly to many other fungi, *S. aurantiacum* is an opportunistic pathogen that affects mainly immunocompromised people (Cortez *et al*., 2008). It has been known to cause a wide range of life threatening invasive diseases such as malignant otitis externa,

osteomyelitis, invasive sinusitis, keratitis and pneumonia (Cortez *et al*., 2008). Treatment of *Scedosporium* infections is generally limited by the high resistance of *S. aurantiacum* to the most commonly used antifungal agents (Lackner *et al*., 2012 and 2014). Recently, *S. aurantiacum* has gained attention worldwide due to its association with non-opportunistic infections reported in various body sites such as lungs and upper respiratory tracts of patients with cystic fibrosis (Cortez *et al*., 2008; Harun *et al*., 2011; Blyth *et al*., 2010b; Pihet *et al*., 2009). The true clinical relevance of *S. aurantiacum* in CF and non-CF patients is somehwat debatable due to the general interpretation of positive respiratory tract cultures as colonisation rather than disease (Blyth *et al.* 2010c). Nevertheless, these reports reflect the increasing prevalence of *S. aurantiacum* as a significant fungal pathogen especially in Australia.

1.1.2. Cystic Fibrosis

Cystic Fibrosis (CF) is a fatal genetically inherited autosomal recessive disorder which is known to affect a large number (approximately 1 in 3000 births) of European Caucasian population in different parts of the world (O'Sullivan and Freedman, 2009). This disease was first described in 1930, and was mainly characterised by malabsorption of fat and protein, congenital steatorrhea, growth failure and broncho-pulmonary infections in the affected individuals (Hide and Burman, 1969). The disease affects several organs in the human body such as secretory cells, sinuses, lungs, pancreas, liver and reproductive tract (Ratjen and Doring, 2003; Tsui, 1992). However, the major cause of mortality in cystic fibrosis patients was identified as the progression of lung disease, caused by persistent respiratory infections. It was later in 1946, when the genetic basis of CF was revealed with the identification of a 230 kb gene present in chromosome 7, called *CFTR* (transmembrane conductance regulator) gene. This gene encodes a 1480 amino acids long polypeptide transmembrane regulator protein that controls the transport of sodium and chloride in the epithelial cells, sweat ducts, airways and pancreatic ducts (Collins, 1992; Riordan *et al*., 1989; Rommens *et al*., 1989; Zielenski *et al*., 1991; Quinton, 1999). With over more than a thousand different mutations identified in the *CFTR* gene, Phe508del or F508del that cause deletion of phenylalanine at position 508 is found in approximately 70% of the CF patients (Ratjen and Doring, 2003; Tsui, 1992). Apart from regulating the chloride channel, this gene is also known to control a number of processes such as intracellular vesicle transport,

ATP channels, and acidification of intracellular organelles, inhibition of sodium transport through sodium channels and inhibition of calcium–activated chloride channels (Mehta, 2005; Reisin *et al*., 1994; Schwiebert *et al*., 1995; Stutts *et al*., 1995; Vankeerberghen *et al*., 2002).

Several hypotheses have been proposed to explain the involvement of *CFTR* gene in the progression of the CF disease (O'Sullivan and Freedman, 2009). Among them, the most appropriate is the salt hypothesis which explains that defects in the *CFTR* gene lead to abnormal bicarbonate-chloride exchange which ultimately causes accumulation of a thick and hyper-viscous layer of mucus outside the cells as seen in Fig. 1-2 (Quinton, 1999; Gibson *et al*., 1970; Smith *et al*., 1996; Zabner *et al*., 1998; Quinton, 2008). Defective mucociliary clearance and inhibition of natural antimicrobial peptides (β-defensins) by increased chloride concentration in the periciliary layer provide ideal conditions for the persistence of pathogenic microbes (bacteria and fungi) invading the cystic fibrosis lung (Goldman *et al*., 1997; Gaillard *et al*., 2010). In addition to causing direct harm to the host cells, pathogen invasion also activates the host immune response leading to inflammation, which causes further deterioration of the lung infrastructure (Kamath *et al*., 2015).

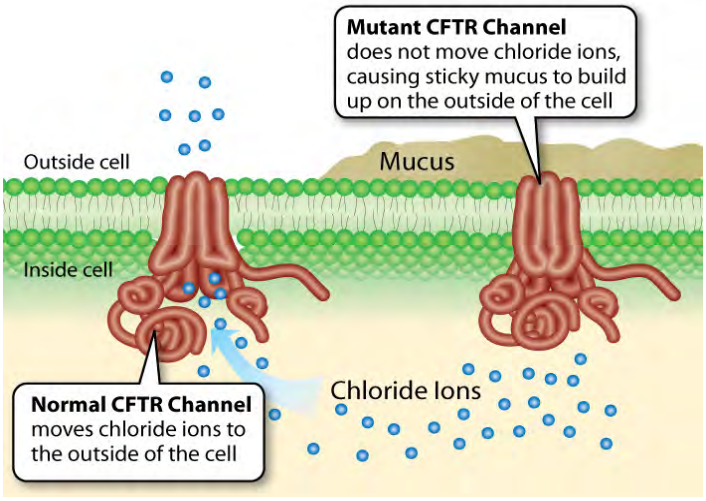


Fig. 1-2. CFTR channel during the healthy and cystic fibrosis conditions. Image has been reproduced from (http://cyfbin.weebly.com/cyfb.html).

While bacteria remain the main infectious agents involved with CF (Sibley *et al*., 2008), a wide range of fungal species is also known to inhabit the respiratory tracts of CF patients.

The most frequently encountered fungal species include *Aspergillus* spp., *Candida* spp., and *Scedosporium* spp. (*L. prolificans, S. apiospermum, S. boydii* and *S. aurantiacum*) (Liu *et al*., 2013; Cooley *et al*., 2007; Pihet *et al*., 2009). However, the isolation frequency of these fungal species has varied according to the studies. Since most of the CF epidemiological studies are focussed on *Aspergillus*, the prevalence of non-*Aspergillus* species especially *Scedosporium* remains underestimated (Bakare *et al*., 2003). While *S. apiospermum* is the major lung coloniser in Europe (France and Germany), its sibling species *S. aurantiacum* represents the majority (14.7%) of the Australian CF isolates (Delhaès *et al*., 2008; Heath *et al*., 2009). According to a study conducted by Blyth *et al.* in 2010, *S. aurantiacum* was the most frequent coloniser of respiratory tracts of CF patients in Sydney (Blyth *et al*., 2010a,b). These reports reflect the increasing prevalence of *S. aurantiacum* as a significant fungal pathogen especially in Australia. This is particularly interesting since *S. aurantiacum* is a recently identified species and only a small number of clinical *S. aurantiacum* isolates have been described so far (Gilgado *et al*., 2005).

1.1.3. Geographic Distribution and Ecological Niche of *S. aurantiacum*

Scedosporium spp. are ubiquitous in nature. Summerbell *et al.* (1989) were the first to reveal that soil is the main reservoir for *Scedosporium* spp. as they identified *Scedosporium* spp. from the soil of potted plants present near the hospitals. Following this, a report published by Guarro *et al.* (2006) indicated that members of *Scedosporium* spp. including *S. apiospermum* can be isolated from a wide range of human impacted environments including agricultural and garden soil, sewers, polluted ponds and sediments. Kaltseis *et al.* (2009) carried out an extensive study in Europe to determine the occurrence of *Scedosporium* spp. in both natural habitats and areas with high human impact including agricultural soil, urban playgrounds and industrial areas. They calculated the isolation frequency of *Scedosporium* species in various habitats as shown in Fig. 1-3 to identify the differences in the ecological preference of individual species. According to their study, *S. apiospermum* was the most frequently isolated species overall and *S. aurantiacum* was isolated from all the ecological niches with an equal frequency.

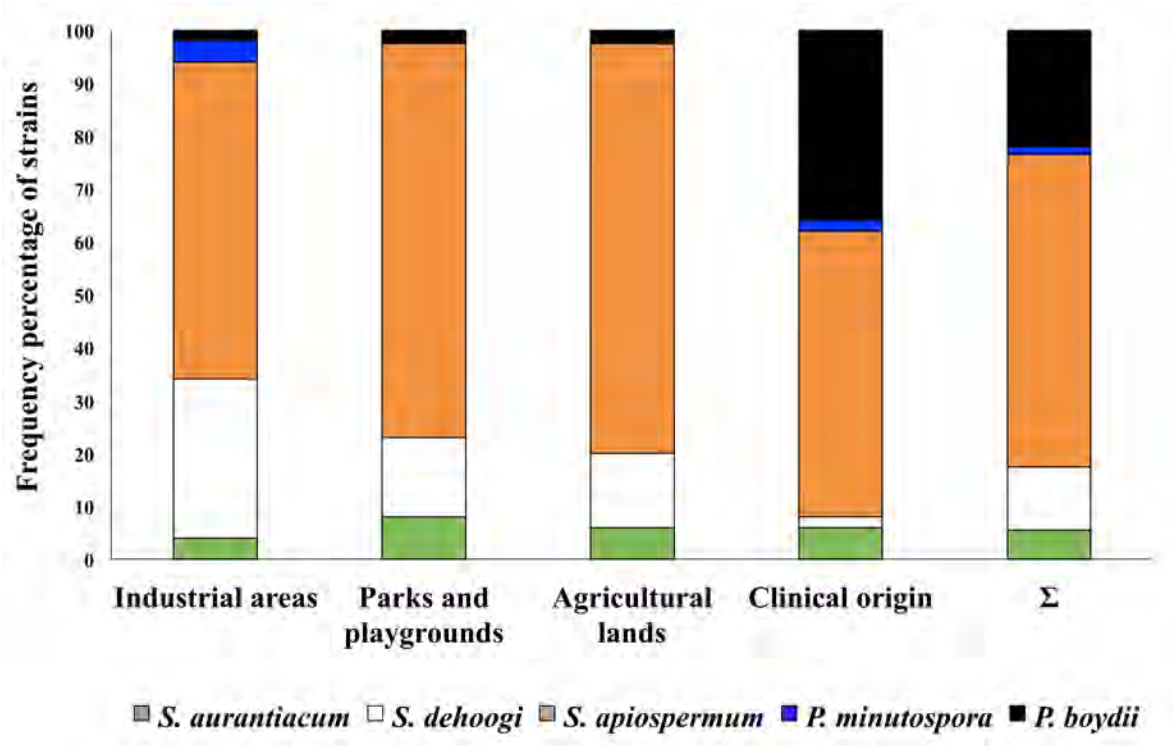


Fig. 1-3. Isolation frequency of different strains of *Scedosporium* across different environments such as industrial areas, parks and playgrounds, agricultural lands and hospitals. Image has been modified from (Kaltseis *et al*., 2009).

As the majority of reports published from different parts of the world indicated environment as a source of *Scedosporium* spp., a survey was carried out in the greater Sydney area to estimate the occurrence of members of the *Scedosporium* spp. complex in the Australian environment (Harun *et al*., 2010a; Kaltseis *et al*., 2009). The major sampling locations included areas impacted by high human activity (Circular Quay, Darling Harbour, Hyde Park and Royal Botanic Gardens) and sub-urban areas (Blue Mountains) (Fig. 1-4). Fungal burden in soil samples was expressed as CFU/g (colony forming units per gram) of dry weight of soil. The survey revealed that the frequency of *Scedosporium* isolates ranged from 60-905 CFU/g dry weight of soil samples recovered from areas with high human impact (Circular Quay and Darling Harbour) and 0-260 CFU/g dry weight in the suburban areas (Fig. 1-4). In particular, *S. aurantiacum* was the most dominating environmental species comprising 54.6% of the total isolates recovered from various sites in the greater Sydney region. *S. aurantiacum* was found mainly in the urban areas whereas *L. prolificans* was isolated from all the environmental sites (Harun *et al*., 2010a).

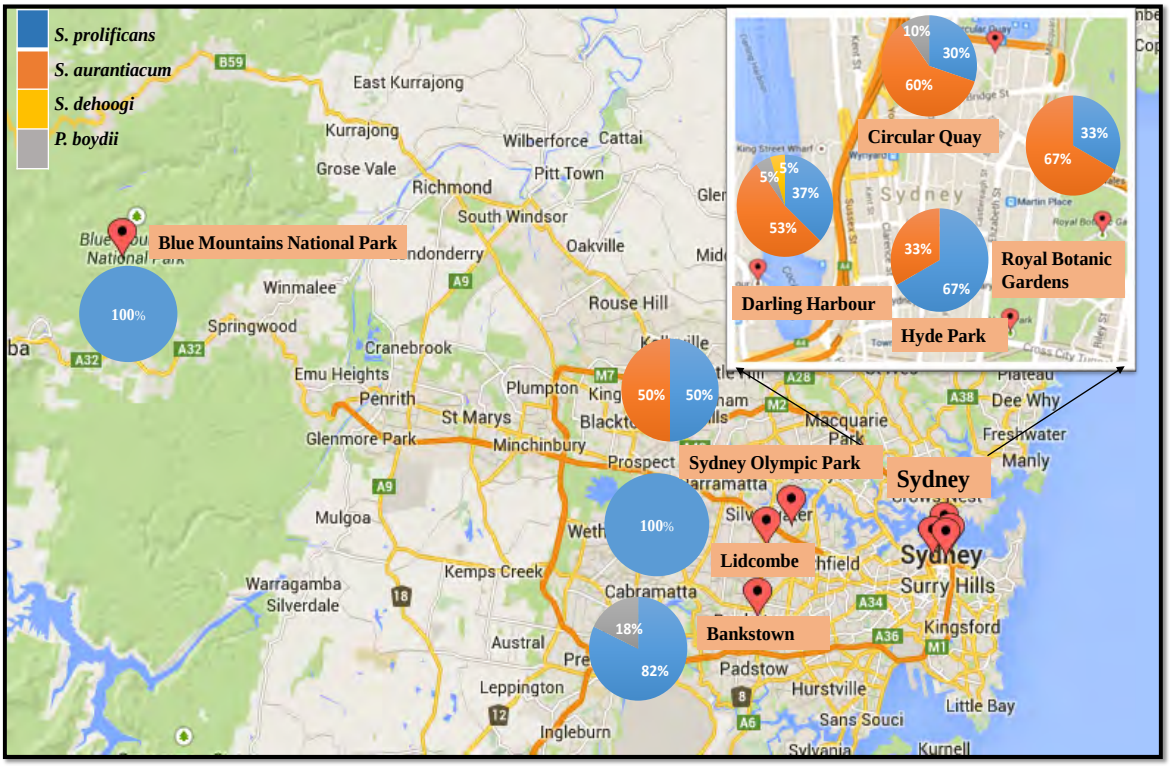


Fig. 1-4. Geographical distribution of different *Scedosporium* species across different locations in Sydney. Image has been modified from (Harun *et al*., 2010a; Kaltseis *et al*., 2009; Blyth *et al*., 2010a).

It was concluded from the study that the increased incidences of *S. aurantiacum* infections in Australia might be linked to the high prevalence of *S. aurantiacum* in the Australian environment (Blyth *et al*., 2010b; Delhaès *et al*., 2008; Heath *et al*., 2009). Members of *Scedosporium* spp. are also capable of adapting to harsh environmental conditions as an increased recovery of *Scedosporium* spp. has been noticed from the lands contaminated with organic compounds including hydrocarbons, industrial waste and crude oil produced as a result of human activities (Guarro *et al*., 2006). The survival of this fungus in hydrocarbon-contaminated soils can be attributed to their ability to utilise natural gas and polycyclic aromatic compounds for growth (Davies *et al*., 1973; Garcia-Pena *et al*., 2001; Janda-Ulfig *et al*., 2008).

1.1.4. Cultivation and Identification of *S. aurantiacum*

According to EORTC/MSG (European Organization for Research and Treatment of Cancer/Mycoses Study Group) guidelines, the diagnosis of fungal infections relies on histological or cytological evidence in samples from normal body sites and isolation of the fungal species in culture (De Pauw *et al*., 2008). *S. aurantiacum* infections have been found in various body sites such as blood, skin and sub-cutaneous tissue, bone, ear, lung and sinuses (Heath *et al*., 2009). However, the recovery of *S. aurantiacum* in a routine fungal culture media such as PDA (Potato Dextrose Agar) and/or SDA (Sabouraud's Dextrose Agar) is problematic and can be masked by the overgrowth of faster growing fungi such as *Aspergillus* spp. and *Candida* spp. often leading to underestimation of their presence in the clinical samples (Cimon *et al*., 2000). Similarly, staining procedures involving Grocott methamine silver (GMS) or Periodic acid-Schiff (PAS) staining can also lead to misidentification due to similarity in the histopathological appearance of the members of *Scedosporium* spp. and other fungi such as *Aspergillus* spp. and *Fusarium* spp. (ESCMID, 2003; Cortez *et al*., 2008; Kimura *et al*., 2010). These limitations can be overcome by the use of semi-selective isolation media containing components such as dichloran, benomyl, penicillin, chloramphenicol and streptomycin that can reduce the growth of bacteria and non-*Scedosporium* moulds (Henson, 1981; Ramsperger *et al*., 2014). Rainer *et al.* (2008) were the first to introduce a *Scedosporium* selective medium called SceSel+ with an addition of benomyl to increase the recovery of *Scedosporium* spp. by inhibiting the growth of other fungi such as *Candida* and *Aspergillus* spp. The method currently recommended for the identification of the members of *Scedosporium* species in the laboratory involves the use of a combination of semi selective media *i. e.* SceSel+ and standard mycological media such as PDA and /or SDA (Blyth *et al*., 2010a).

1.1.4.1. Macroscopic Features

The optimum temperature for the growth of all the members of *Scedosporium* spp. on PDA and/or SDA is 25°C but they are also capable of tolerating higher temperatures *i. e.* 40 and 45°C (Cortez *et al*., 2008). The growth characteristics and colony morphology of the clinically important *Scedosporium* species are described in table 1-2.

Table 1-2. Colony morphology and characteristics of various *Scedosporium* species grown on PDA. Table has been revised from (Ramsperger *et al.*, 2014).

Species	**Colony morphology on PDA plates at 25 °C**				**Growth at temperature**	
	Colour	**Texture**	**Reverse**	**Yellow diffusible pigment**	**40 °C**	**45 °C**
S. boydii/P. boydii	White-grey to dark grey or smoky brown	Downy to cottony	Dark brown to grey-black	V	+	-
S. apiospermum	White-grey to dark grey or smoky brown	Downy to cottony	Dark brown to grey-back	V	+	-
S. aurantiacum	Yellow-grey and brown-grey with white margins	Dense, cotton to lanose (woolly)	Orange-brown to brown	+	+	+
S. dehoogi	White to pale-grey	Cottony	Colourless	-	-	-
S. prolificans	Olive-grey to black, white coloured mycelial tuffs	Downy to cottony	Dark brown-grey-black	-	+	V

+ growth present, - growth absent, V variable

The colonies of *S. aurantiacum* range in colour from yellow to brown grey and are dense, cottony which follow concentric pattern of growth with an irregular white margin as shown in Table 1-2. *S. aurantiacum* can be distinguished from other *Scedosporium* species based on its ability to tolerate high temperature (45°C) and production of yellow diffusible pigment that is usually observed at the reverse of the culture plate (Fig. 1-5) after few days of incubation (Gilgado *et al*., 2005; Lackner *et al*., 2012). *S. aurantiacum* can also tolerate high amount of $MgCl_2$ (5%) compared to NaCl (Gilgado *et al*., 2008).

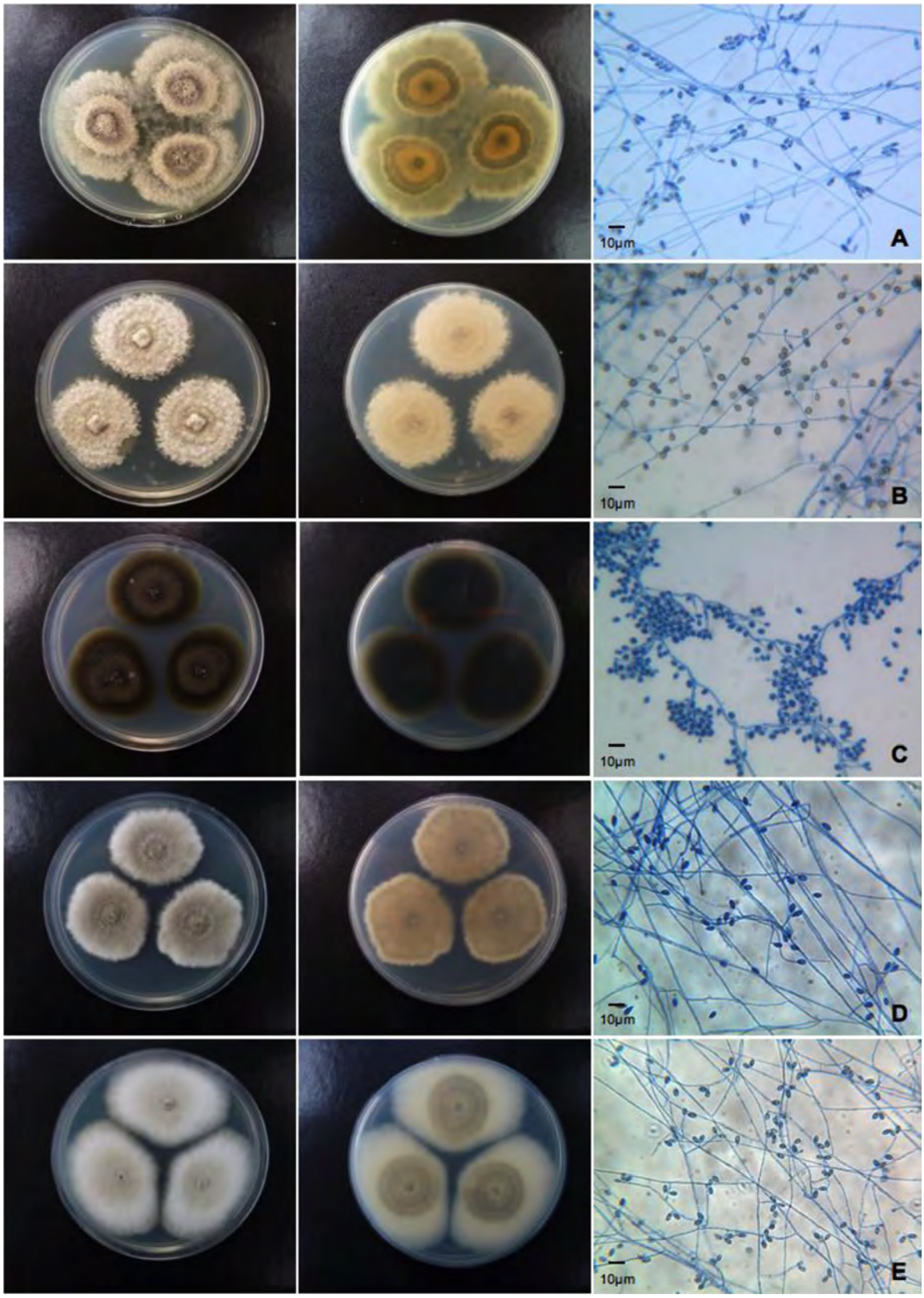


Fig. 1-5. Colonies of different species of *Scedosporium* on Sabouraud dextrose agar. Light microscopy images (magnification 400x) from solid cultures of A) *S. aurantiacum*, B) *S. apiospermum*, C) *L. prolificans*, D) *S. dehoogi*, E) *S. boydii*. Scale bar = 10 µm. Image is used with permission from Ramsperger *et al.* (2014).

1.1.4.2. Microscopic Features

The members of *Scedosporium* spp. are usually characterised by the presence of an asexual reproductive stage called *Scedosporium* anamorph. While the appearance of colonies on PDA plates is important for identifying *Scedosporium*, morphology of the conidia and conidiophores is the most useful feature for discriminating between different *Scedosporium* species (Gilgado *et al.*, 2008). Gilgado and colleagues were the first to study the microscopic features of *S. aurantiacum* (Fig. 1-6) and they described that *S. aurantiacum* conidia could be formed from three different structures 1) conidiophores, 2) synnemata, or 3) undifferentiated hyphae. The conidia-bearing hyphae called conidiophores were either present as solitary or in a group. Solitary conidiophores were often reduced to a lateral cylindrical or flask shaped projection called as conidiogenous cell and produced sub-hyaline, smooth walled, obovoid, or sub-cylindrical conidia that were 5 to 14 µm long and 2 to 5 µm wide. The size and shape of *S. aurantiacum* conidiogenous cells and conidia were similar to those of *S. apiospermum*. A group of erect conidiophores called synnemata were also found to produce apical or lateral conidia in two isolates *i. e.* IHEM 15458 and RKI 2782/95. The conidia produced from synnemata were usually cylindrical or claviform, 6 to 12 µm long and 3 to 5 µm wide. Undifferentiated hyphae produced abundant conidia in all isolates, which ranged from sessile, solitary, lateral, brown, smooth and thick walled, to usually obovoid, 6 to 10 µm long and 3 to 5 µm wide. The presence of a teleomorph (sexual reproductive stage) is unknown in *S. aurantiacum* (Gilgado *et al.*, 2005).

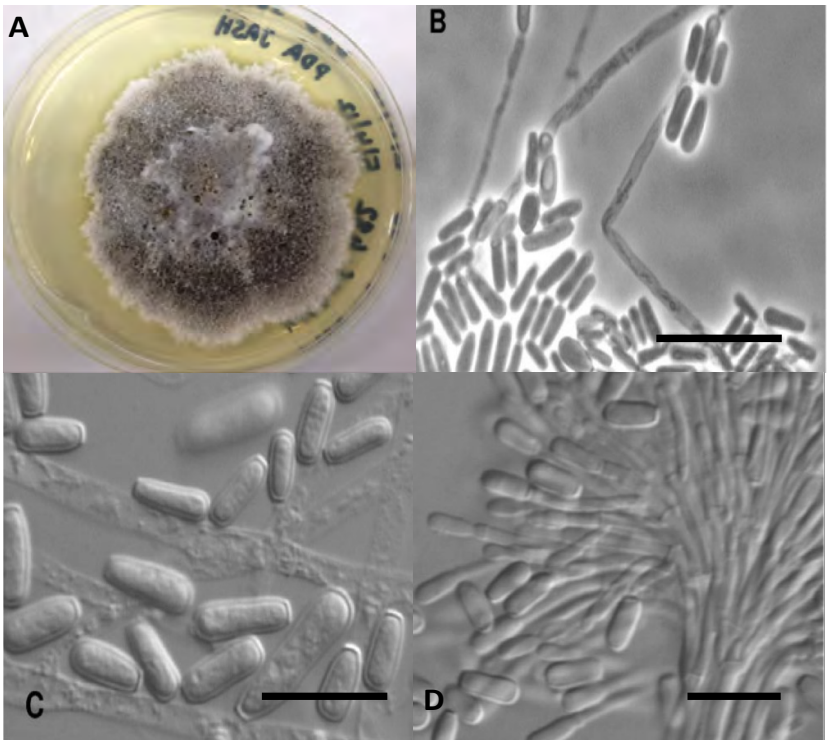


Fig. 1-6. *S. aurantiacum* isolates (A) WM 06.482, (D) IHEM 15458 and (B, C) FMR 8630. (A) Colony growing on PDA after 14 days of incubation at 25°C. Light microscopy images from solid cultures of (B, C) a conidiogenous cell and conidia from solitary conidiophores, (D) an apical part of a synnema producing conidia. Scale bar = (B) 20 µm, (C, D) = 10 µm. Image has been modified from (Gilgado *et al*., 2005).

1.1.4.3. Molecular Identification Tools

Although the culture-based morphological approach is useful to identify *Scedosporium* spp. in clinical samples, it can fail to provide definitive species identification due to the presence of common morphological characteristics among them (Blyth *et al*., 2010a; Lackner *et al*., 2012). Recently, there has been an emphasis for the development of highly specific molecular diagnostic techniques that can enable fast and accurate identification of different members of *Scedosporium* spp. complex. Numerous molecular detection tools have also been introduced for genotyping *Scedosporium* spp. (Ramsperger *et al*., 2014; Harun *et al*., 2011; Harun *et al*., 2009; Lackner *et al*., 2012). In particular, polymerase chain reaction (PCR) based approaches including multiplex PCR, restriction fragment length polymorphism (RFLP), multilocus sequence typing (MLST) and nucleotide sequencing are most commonly used for rapid detection of different *Scedosporium* isolates due to their high resolving capacity (Bouchara *et al*., 2009; Castelli *et al*., 2008; Lau *et al*.,

2007; Lau *et al*., 2008a; Lau *et al*., 2008b; Zhou *et al*., 2008). Most of the PCR based techniques involve exponential amplification of a specific target region using short primers leading to rapid quantitative detection of amplified DNA. The target DNA is selected either based on specific sequence information from databases which allows designing of specific primers for conserved regions across different species, or cloning and sequencing of random parts of fungal genome (Atkins and Clark, 2004).

Gilgado *et al.* first demonstrated the use of a combined molecular and morphological approach to study around 141 isolates of *S. boydii* species complex to investigate the species level systematics (Gilgado *et al*., 2005). They analysed the nucleotide sequences of the housekeeping genes such as β-tubulin (*β-TUB* and *BT2*) and calmodulin (*CAL*) and ITS (internal transcribed spacer) loci and proposed two new clades (I and II) representing two new species called *S. aurantiacum* and *S. dehoogi* (Gilgado *et al*., 2008). The maximum amount of species was resolved with the help of BT2 and TUB regions whereas the non-coding ITS region was insufficient to distinguish between the species (Gilgado *et al*., 2005). Gilgado's work carved the way for future taxonomy based studies into *Scedosporium* spp.

Delhaès and colleagues first provided the epidemiological associations of the novel species S. aurantiacum. They used ITS based restriction fragment length polymorphism (ITS-RFLP) analysis to genotype a large number of *Scedosporium* isolates identified in Australia during the nation-wide investigation of scedosporiosis from 2003-2005 (Delhaès *et al*., 2008). The technique involved PCR amplification of the ITS1, 5.8S, ITS2 regions using primers SR6R (5-AAGTARAAGTCGTAACAAGG-3) and LR1 (5-GGTTGGTTTCTTTTCCT-3) followed by double digestion with *Sau*96I and *Hha*I restriction endonucleases and gel electrophoresis. Distinct patterns were observed in the ITS-RFLP sequence of Australian *S. apiospermum* isolates, which revealed the presence of a substantial number of *S. aurantiacum* (>15.8% of total isolates) in Australia. These results were confirmed by ITS sequencing which showed a sequence variation of 5-10% between *S. aurantiacum* and *S. apiospermum*. *S. aurantiacum* was thus classified as a sub-category of the previously identified *S. apiospermum* species (Heath *et al*., 2009; Delhaès *et al*., 2008; Gilgado *et al*., 2005). Although the RFLP technique is easy to use and produces reproducible results, its use is generally limited as it offers only species level identification (Harun *et al*., 2009).

In 2011, Harun and his co-workers developed a multiplex PCR technique which involved PCR amplification of the ITS regions (ITS1 and ITS2) and 28S ribosomal DNA using primers specific for *Scedosporium* species. The applicability of this approach was tested using sputum specimens from the adult CF patients in Australia. Using this approach, three major clinically important *Scedosporium* species including *S. aurantiacum*, *L. prolificans* and *S. boydii* complex were identified from the pure cultures with 100% specificity (Harun *et al*., 2011).

Harun and co-workers are currently working on the development of MLST (multilocus sequence typing) scheme for *Scedosporium* spp. (Meyer *et al*., unpublished data). MLST involves characterisation of the organisms based on the partial sequence analysis of a defined set of 7-10 housekeeping genes (Aanensen and Spratt, 2005) and has been successfully applied to fungal pathogens such as *C. albicans* (Bougnoux *et al*., 2002), *S. tropicalis* (Tavanti *et al*., 2005), *C. neoformans* (Litvintseva *et al*., 2006) and *F. oxysporum* (O'Donnell *et al*., 2004). A preliminary MSLT study was performed on the genetically closely related and phylogenetically characterised *S. aurantiacum* and *S. apiospermum* species using several gene loci. Based on the results, candidates such as *EF1α* (elongation factor 1- α), *SOD2* (superoxide dismutase), β-tubulin, *CHS* (chitin synthase) and *CAL* (calmodulin) were chosen for the MLST scheme development for *Scedosporium* spp. (Harun *et al*., 2009).

Traditionally the identification of fungi has been performed using methods based on morphology. However, techniques such as RFLP and MLST have also evolved as useful tools for this purpose. In addition to improving our ability to distinguish *S. aurantiacum* from other closely related species, the use of these sensitive laboratory diagnostic techniques may also be accounted for the reported emergence of this organism. In future, a combination of morphology and molecular based approaches can help to obtain a more detailed classification of *Scedosporium* species and facilitate the identification of yet new species.

1.1.5. Physiological Studies and Genome Sequencing in *S. aurantiacum*

In addition to the morphology, physiology-based studies including nutrient utilisation also hold importance for the identification of fungal communities (Buyer *et al*., 2001).

Filamentous fungi can utilise a wide variety of nutrient sources including carbon, nitrogen, phosphorous and sulphur and this property results in different fungal phenotypes which can be analysed using a nutrient utilisation assay (Bochner *et al*., 2001). Traditionally, nutrient utilisation experiments are performed using a defined minimal medium (solid or liquid) that can be supplemented with the nutrient of choice (*e. g.* carbon, nitrogen, sulphur, phosphorous and potassium). The nutrient utilisation profiles of different fungal species are obtained by cultivating them separately in a variety of nutrient compositions and observing or measuring the growth which can then be compared to identify the physiological similarities or differences among them (Rieger *et al*., 1997).

Guarro and colleagues were the first to analyse the assimilation of carbon sources such as sucrose, maltose, D-ribose, L-arabinitol and ribitol in different *Scedosporium* species. The inability to use sucrose (Table 1-3) distinguished *S. aurantiacum* from other members of the *Scedosporium* spp. complex (Gilgado *et al*., 2008).

Table 1-3. Carbon source utilisation patterns in different members of *Scedosporium* species. Table has been modified from (Guarro *et al*., 2006).

Species	**Conidiogenous cells**	**Sessile conidia**	**Colony reverse in orange**	**Assimilation of**				
				Ribitol	L-arabinitol	Sucrose	Maltose	D-ribose
P. boydii/ S. boydii	Cylindrical	Globose to sub-globose, thick walled	-	+	+	+	+	+
S. apiospermum	Cylindrical	Globose to sub-globose, thick walled	-	+	+	+	+	-
S. aurantiacum	Cylindrical or slightly flask shaped	Mostly obovoid, thick walled	+	+	+	-	+	+
S. dehoogi	Cylindrical or slightly flask shaped	Mostly obovoid, thick walled	-	+	+	+	+	-
S. prolificans	Flask shaped	Globose to sub-globose, thick walled	-	-	-	-	+	-

+ growth present, - growth absent

However, the conventional nutrient utilisation assays are often time consuming and laborious as it is impossible to screen the fungal response to more than one nutrient source in a single assay (Fong *et al*., 2005). These limitations can be overcome by the respiration-based phenotype microarray (PM) technology that provides a fast, sensitive and high-

throughput screening of cellular phenotypes in a 96 well microtitre plate containing a wide range of nutrient sources (Bochner, 2003). PM utilises a colourless tetrazolium dye that undergoes an irreversible reduction to a purple form during an increase in the cell respiration which indicates metabolism of a particular nutrient (Bochner *et al*., 2001). Bochner first demonstrated the use of microplate-based assays to study different bacterial phenotypes (Bochner *et al*., 2001). PM technology has also been employed to identify and characterise several species of filamentous fungi such as *Aspergillus*, *Neurospora*, *Fusarium*, *Hypocrea* and *Acremonium* (Caddick *et al*., 1994; Druzhinina *et al*., 2006; Hoyos-Carvajal *et al*., 2009; Tanzer *et al*., 2003). Phenotypic profiling of different *S. aurantiacum* strains using PM is described in the current study.

Growth and respiration in fungi are often regulated by signalling pathways that are conserved between related species (Harris *et al*., 2009; Rispail *et al*., 2009). Comparative genomics can provide comprehensive insights into the physiology of different species by predicting the presence of common signalling cascades and/or unique and unusual biochemical pathways. However, comparative genomic studies are limited by the availability of the sequenced genomes (Cornell *et al*., 2007; Soanes *et al*., 2007). The most common parallel sequencing platforms used for fungal genomes include 454 from Roche, the Applied Biosystems SOLiD and the Illumina GA2. These instruments allow cost-effective and efficient genome sequencing compared to the traditional Sanger sequencing methods. While Illumina sequencing produces shorter reads (up to 70 bp) that usually require comparison to a reference genome, 454 technology enables *de novo* assembly of unknown fungal genomes as it produces long and accurate reads of up to 500 bp. Currently a combination of Illumina and 454 sequencing technologies is used for generating rapid and affordable fungal genomes to provide high quality assemblies (Wilson and Talbot, 2009). So far, genomes of only two *Scedosporium* species have been sequenced including *S. apiospermum* strain IHEM 14462 (Vandeputte *et al*., 2014) and *S. aurantiacum* strain WM 09.24 (Perez-Bercoff *et al*., 2015). The genome of *S. aurantiacum* strain WM 09.24 (environmental, high virulence) was sequenced recently using Illumina HiSeq 2000 technology and 10,525 gene models and 11,661 transcripts were predicted in the draft genome assembly (Perez-Bercoff *et al*., 2015). It is expected that the availability of annotated *Scedosporium* genomes will be key to identify the diverse set of genes encoding enzymes involved in metabolic pathways, which are likely to vary in different members of the species.

1.1.6. *In vitro* Antifungal Susceptibility and Virulence of *S. aurantiacum*

Given the morphologic and molecular differences in *S. aurantiacum* observed in other studies, it becomes extremely important to determine whether *S. aurantiacum* also differs from other members of *Scedosporium* spp. in terms of antifungal susceptibilities and virulence level. A wide variety of antifungal compounds including azoles, polyenes (amphotericin B and nystatin), allylamines (terbinafine), pyrimidines (5-flucytosine) and exhinocandins have been employed to check the *in vitro* susceptibility of *Scedosporium* isolates (Cortez *et al*., 2008). Until now, voriconazole is the only drug that has been proved to be effective against all *Scedosporium* species including *S. aurantiacum* (Lau *et al*., 2008; Zhou *et al*., 2008). The antifungal susceptibility tests for Australian *Scedosporium* isolates were carried out by Heath and colleagues, which reported a similarity in the susceptibility patterns of *S. aurantiacum* and *S. apiospermum* (Gilgado *et al*., 2005). In a similar line of work, Lackner *et al.* tested a relatively higher number of *Scedosporium* isolates obtained from different geographical origins and found that *S. aurantiacum* was less susceptible to antifungal drugs compared to *S. apiospermum* (Lau *et al*., 2008; Atkins *et al*., 2004; Lackner *et al*., 2012 and 2014).

The presence of high intrinsic antifungal resistance in *S. aurantiacum* necessitates studies relating to the degree of virulence in these species to understand the molecular mechanisms underlying the different resistance profile. Significant efforts have been made recently to decipher the mechanism of *Scedosporium* virulence in cystic fibrosis using *in vivo* models of infection. The selection criteria for an *in vivo* model involves a model host organism which mimics the clinical disease states observed in humans and allows control over experimental variables such as fungal strain, inoculum size, route of administration and administration of pharmacotherapies (Capilla *et al*., 2007). A wide variety of statistically validated vertebrate and single cell invertebrate model organisms with tractable genetics and conserved immunity have been exploited to undertake research on fungal infections. These models can also facilitate studies relating to pathogenesis of various *Scedosporium* species and the host immune response (Lionakis, 2011). In addition to the frequently used mouse models, a number of invertebrate models have also gained popularity. Of the invertebrate models, the recently introduced *G. mellonella* is an excellent host as it offers a number of advantages such as similarity to the human system and susceptibility to a wide range of clinically relevant pathogens (Firacative *et al*., 2014). Distinct advantages and limitations associated with each model are briefly summarised in Table 1-4.

Table 1-4. Selected animal model systems used for studying the pathogenesis of various CF related bacteria and fungi. Table has been modified from Cassidy *et al*., 2014.

	Model organism	Genetic tractability	Breeding/ cultivation speed	Complementary information (genome/trans-criptome)	Susceptibility to clinically relevant pathogens	Similarity to human system	Model for	Reference
Invertebrate	*Caenorhabditis elegans*	+++	+++	+++	++	+	Intestinal development Microbial response Innate immunity Host metabolic activity	Cabreiro *et al.*, 2013; Marsh and May, 2012; Schrimpf and Hengartner, 2010; Tholey *et al.*, 2013
	Drosophila melanogaster	+++	+++	+++	++	+	Innate immunity Antimicrobial test system Pathogen response Innate immunity	Abuodeh *et al.*, 2000; Bier and Guichard, 2012; Engstrom *et al.*, 2004; Igboin *et al.*, 2012; Moule *et al.*, 2010
	Galleria mellonella	++	+++	++	+++	++	Antimicrobial test system Pathogen response Innate immunity	Champion *et al.*, 2010; Pustelny *et al.*, 2013; Wand *et al.*, 2013
Vertebrate	*Danio rerio*	+++	++	+++	+++	++	Innate and adaptive immunity Development Pathogen response	Kanther and Rawls, 2010; Medina and Royo, 2013; Rendueles *et al.*, 2012; Xiong *et al.*, 2011
	Mus musculus	+++	++	++	+++	++	Innate and adaptive immunity Intestinal development Host health Pathogen response	Kaiser *et al.*, 2012; Legrand *et al.* 2009; Lone *et al.*, 2013

+ low, ++ moderate, +++ high

1.1.6.1. Vertebrate Models

Amongst animal models, mice are the most common and effective subjects for the testing of microbial infections because of their ease of access and maintenance (Cano *et al*., 1992; Capilla and Guarro, 2004; Capilla *et al*., 2003a; Gonzalez *et al*., 2002). Mice share a striking similarity (about 95%) to the human genome and can be genetically engineered to mimic a number of disease conditions such as cancer and diabetes (Naglik *et al*., 2008; Capilla *et al*., 2007; Samaranayake and Samaranayake, 2001). In addition to this, mouse models are well characterised and well established in medical research and are used for a wide variety of fungi such as *Aspergillus* spp., *Candida* spp., *Scedosporium* spp., and *Cryptococcus* spp., and bacteria such as *Pseudomonas* spp. (Harun *et al*., 2010b; Rogers and Balish, 1976; Slesiona *et al*., 2012; Stotland *et al*., 2000). Mouse models are not usually affected by the mode of transmission of microbial infection into the animal (Cano *et al*., 1992; Capilla *et al*., 2004). Infection can be achieved in a variety of ways including intranasally, intraperitoneally, intracerebrally, intravenously, intratracheally and by inhalation (Krockenberger *et al*., 2010; Zaragoza *et al*., 2007). Among all these methods, intravenous injections *via* the lateral veins are most frequently used to establish infections in mouse models (Cano *et al*., 1992; Gilgado *et al*., 2009; Ortoneda *et al*., 2002). However, the widespread use of mouse models is debated due to the involvement of mouse specific immune response when exposed to certain infectious agents (Coers *et al*., 2009).

A variety of *in vivo* vertebrate infection models have been applied to check the virulence levels of *Scedosporium* spp., such as immunocompetent and immunocompromised mice, rabbits and guinea pigs (Cano *et al*., 1992; Capilla and Guarro, 2004; Capilla *et al*., 2003b; Gilgado *et al*., 2009; Gonzalez *et al*., 2002; Ortoneda *et al*., 2002). However, mice remain the most preferred choice amongst animal models due to their ease of handling and availability (Capilla *et al*., 2007). In view of the highly resistant nature of *S. aurantiacum* to various antifungal agents, the virulence of *S. aurantiacum* was first examined in a study conducted by Gilgado *et al.* using immunocompromised murine infection models. With an estimated mortality rate of around 80% in the immunocompetent mice, *S. aurantiacum* was described as the most significantly virulent member of the *Scedosporium* species (Gilgado *et al*., 2009). The virulence levels of *S. aurantiacum* were notably comparable to those of the formerly known most virulent species *Lomentospora prolificans* (Gilgado *et al*., 2009; Harun *et al*., 2010b; Ortoneda *et al*., 2002). Following this, a study performed by Harun and co-workers focussed on comparison between the virulence levels of genetically closely

related *S. aurantiacum* and *S. apiospermum* in immunocompromised mice. Their findings also suggested that *S. aurantiacum* was as virulent as *L. prolificans,* with the most virulent *S. aurantiacum* strain (WM 06.482) causing 100% mortality in mice (Harun *et al*., 2010b).

1.1.6.2. Invertebrate Models

Although mouse models offer valuable research tools for testing the virulence levels of fungal pathogens, their use is often limited by the high cost and ethical issues associated with them (Coers *et al*., 2009). During the past few years, focus has been shifted towards the development of insect models that can provide informative screening of the emerging opportunistic pathogens and provide insights into the host-pathogen interactions with a relatively economical method without strict ethical limitations. Invertebrates possess innate immunity against the infections and have shorter reproduction times than the mammalian counterparts, which makes them easy to maintain (Sabiiti *et al*., 2012). The major invertebrate infection hosts include *Caenorhabditis elegans, Drosophila melanogaster, Galleria mellonella* and Zebrafish (*Danio rerio*) (Lamaris *et al*., 2007; Mylonakis *et al*., 2002; Mylonakis *et al*., 2005; Sabiiti *et al*., 2012). Out of these hosts, *D. melanogaster* and *G. mellonella* have been used to study the virulence of *Scedosporium* spp.

Drosophila melanogaster: The fruit fly *Drosophila melanogaster* has been used as model system for testing infections by a wide range of bacterial and fungal pathogens such as *Burkholderia* cepacia, *Helicobacter pylori, Legionella pneumophila, P. aeruginosa* and *C. neoformans, C. albicans, A. fumigatus and Scedosporium* spp. (Panayidou *et al*., 2014; Apidianakis and Rahme, 2009; Chamilos *et al*., 2006; Sabiiti *et al*., 2012). *D. melanogaster* is an excellent model with a fully sequenced genome and provides advantages similar to *C. elegans* including a short generation time, small size and innate immune response to microbial infections. In addition to that, the immune pathways present in *D. melanogaster* are also similar to the human host immune pathways including Toll-like receptors (TLR) and interleukin-1 (IL-1) (Hoffmann, 2003; Sekiya *et al*., 2008). Both cellular and humoral components are involved in the innate immune system of *D. melanogaster* against foreign microbes (Lemaitre, 2004; Rizki and Rizki, 1984). Apart from this, several mutants of *D. melanogaster* have also been prepared to mimic the immunocompromised mammals that are susceptible to pathogens.

The mutant strain of *D. melanogaster* with a non-functional Toll receptor and a wild type *D. melanogaster* were used to test the infections by *S. apiospermum* and *L. prolificans.* The results lead to recognition of the role of host toll like pathway in the prevention of *Scedosporium* infection in case of wild type *Drosophila* flies which showed high resistance, compared to the mutant strains with high mortality (Lamaris *et al*., 2007). The only limitation with this method is the choice of the infection route, as pathogens injected directly into the haemolymph can escape the host immune response and cause killing of the fly (Jensen *et al*., 2007).

Galleria mellonella Larvae: The wax moth *Galleria mellonella* has been widely accepted as an excellent model for studying the pathogenesis of various bacterial and fungal human pathogens (Cook and McArthur, 2013). The ability to grow at 37°C is considered as an excellent advantage in investigating temperature sensitive virulence mechanisms in human pathogens (Cook and McArthur, 2013). The size of *G. mellonella* larvae is comparatively larger (12-20 mm) than insects such as *Drosophila* and can be easily manipulated to enable the collection of tissue samples for downstream analysis. The most common routes of infection in the moth is *via* the haemocoel and the microbes can be injected into the base of the proleg with a needle (Cook and McArthur, 2013). This model has been used to study host-pathogen interactions in many fungal pathogens such as *C. albicans*, *C. neoformans*, *A. fumigatus* and *S. aurantiacum* (Renwick *et al*., 2006; Firacative *et al*., 2014; Kaur *et al*., 2015; Mylonakis *et al*., 2005). Although *G. mellonella* lacks a sequenced genome, yet it has been widely accepted as a model organism to study the pathogenesis of a wide variety of human pathogens due to the structural and functional similarity to the human immune system (Mylonakis, 2008; Hoffmann, 1995; Lionakis, 2011).

Other Invertebrate Models for Studying the Virulence of S. aurantiacum

***C. elegans* and Zebrafish (*Danio rerio*):** The virulence studies of *Scedosporium* species could also be performed using other commonly used invertebrate model systems such as *C. elegans* and zebrafish. The nematode *C. elegans* has been used to study the virulence and host response to various human pathogenic prokaryotes and eukaryotes including *P. aeruginosa*, *C. albicans* and *C. neoformans* (Marsh and May, 2012; Anastassopoulou *et al*., 2011; Mylonakis *et al*., 2007). It serves as an amenable model to study the multispecies

dynamics due to its ease of handling, simplicity, short reproduction cycle and translucent body which also helps in visualisation of events under the microscope (Peleg *et al*., 2008).

Zebrafish, a tropical freshwater cyprinid is another emerging model system for a variety of human diseases (Rendueles *et al*., 2012; Renshaw *et al*., 2007). It has been used for assessing the host interactions with major fungal pathogens such as *C. albicans* (Brannon *et al*., 2009; Chao *et al*., 2010). Zebrafish possesses vertebrate like characteristics and induces the immune response through the release of pro-inflammatory cytokines and interferons through toll like receptors in a similar manner as humans (Cassidy and Tholey, 2014). During the early growth stages, zebrafish are transparent which helps in easy visualisation of fungi tagged with fluorescent proteins using confocal microscopy techniques (Medina and Royo, 2013; Meijer and Spaink, 2011). Apart from this, the availability of genetic information for zebrafish facilitates the molecular analysis of interaction between the host and pathogens (Rendueles *et al*., 2012; Renshaw *et al*., 2006).

While the use of *C. elegans* and zebrafish based infection models has not been demonstrated in *Scedosporium*, the presence of extensive genetic and molecular tools, real time imaging possibilities and conserved mammalian immune system features of *C. elegans* (Powell and Ausubel, 2008) and zebrafish (Cassidy and Tholey, 2014; Medina and Royo, 2013; Meijer and Spaink, 2011) may prove useful in understanding the host response against *Scedosporium* infections.

1.2. *S. aurantiacum* and Bacterial Inhabitants of CF Lung

In addition to fungi, the lungs of a cystic fibrosis patient are often colonised by bacterial species. Studies relating to the CF lung microbiome indicate the prevalence of different bacterial pathogens, *e.g. Streptococcus aureus, Burkholderia cepacia*, *Stenotrophomonas maltophila* and *P. aeruginosa* (Folkesson *et al*., 2012). *Pseudomonas aeruginosa* is the most predominant member of the Pseudomonadaceae family, which is known to cause recurrent pulmonary exacerbations in 80% of the cystic fibrosis patients (Harrison, 2007). It is a Gram-negative, biofilm forming, rod shaped bacterium capable of causing life threatening infections in CF patients which are often un-treatable owing to its continuously evolving drug resistance (Murray *et al*., 2007). The ubiquitous nature of *P. aeruginosa* results from the ability to withstand various environmental pressures (Suh *et al*., 2004).

While CF is primarily a genetic disease, these gene defects lead indirectly to a range of secondary changes within the human lungs that support the emergence of a complex polymicrobial microbiota thereby making it a polymicrobial disease (Sibley *et al*., 2008; Lipuma, 2010). For example, clinical reports published by Sibley *et al*, reveal the detection of fungi from lungs of the CF patients with a persistent bacterial infection (Chotirmall *et al*., 2010; Sibley *et al*., 2006). Similarly in 2003, Bakare *et al.* also reported co-isolation of bacteria and fungi from the sputum samples of CF patients (Bakare *et al*., 2003). Fig. 1-7 from Harrison *et al.* (2007) provides a brief summary of the microbial co-infections found within the CF lung.

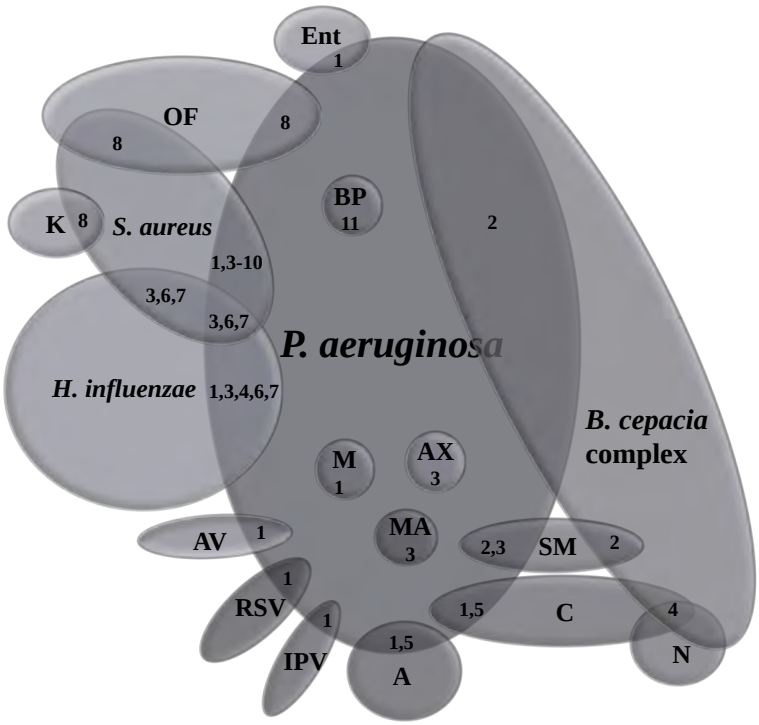


Fig. 1-7. Venn diagram showing the polymicrobial nature of the CF lung. Image has been modified from (Harrison, 2007). A: *Aspergillus* spp., AV: *adenovirus*, AX: *A. xylosoxidans*, BP: bacteriophage, C: *Candida* spp., ENT: *Enterobacteria*, IPV: Influenza and/or parainfluenza virus, K: *Klebsiella* spp., M: mycoplasma, MA: *Mycobacterium abscessus*, N: *Neisseria* spp., OF: Oropharyngeal flora, RSV: Respiratory syncytial virus, SM: *S. maltophilia*. The numbers refer to the references 1. Petersen *et al*., 1981; 2. Lambiase *et al*., 2006; 3. Wahab *et al*., 2004; 4. Moore *et al*., 2005; 5. Burns *et al*., 1999; 6. Hoiby, 1974; 7. Lording *et al*., 2006; 8. Santana *et al*., 2003; 9. Alvarez *et al*., 2004; 10. Anzaudo *et al*., 2005; 11. Ojeniyi *et al*., 1991.

Most of the studies have highlighted the isolation of other microorganisms from the lungs of the CF patients predominantly colonised by *P. aeruginosa*. Studies have suggested that mixed bacterial-fungal interactions have negative effects on the health of CF patients as compared to the patients infected with either bacteria or fungi (Chotirmall *et al*., 2010; Leclair and Hogan, 2010). The interactions between bacteria and fungi may also result in changes pertaining to the ability of the interacting organism to evade the host by changing its behaviour or the amount of virulence factors produced (Leclair and Hogan, 2010). The clinical importance of host response to bacterial-fungal interactions has been illustrated in a number of studies (Allard *et al*., 2009). Peleg *et al* plotted a universal survival curve for the host in case of polymicrobial infections as seen in Fig. 1-8. The model describes that interactions occurring during the polymicrobial infections including those between the pathogens and between pathogens and the host can affect the survival of the host (Peleg *et al*., 2010).

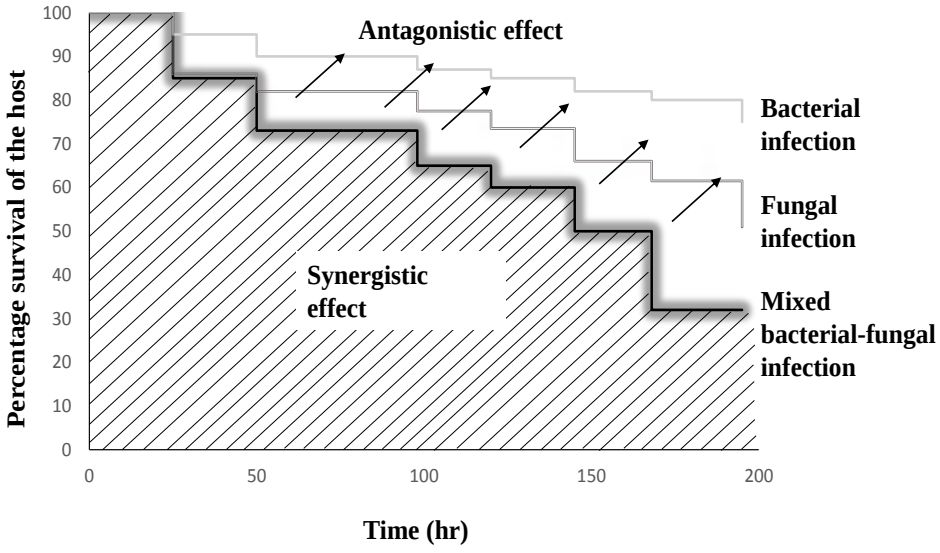


Fig. 1-8. Survival curve for polymicrobial infections. The killing of the host by each mixed infections (bacteria and fungi) is shown in the shaded region below the black line and the effect of individual pathogens is shown by pointed arrows (above the shaded region). Image has been modified from (Peleg *et al*., 2010).

If the sum of infection by each pathogen (Fig. 1-8, bacteria and fungi alone) equals the killing of the host by mixed bacterial-fungal infections (black line), it is termed as an additive effect. However, synergistic effect means greater host killing by mixed infections (shaded area below the black line) as compared to the individual pathogen alone. Antagonistic interactions between the pathogens happen when the rate of killing by an individual microbe is higher than that of mixed infections (area above the black line). In antagonistic interactions, one organism reduces the virulence of the other resulting in an efficient protective response by the host (Peleg *et al*., 2010).

1.2.1. Types of Interactions between Bacteria and Fungi

As the majority of studies have focussed on the interactions between known pathogens such as *Aspergillus*, *Candida* and *Pseudomonas*, there is a lack of understanding regarding the involvement of other fungi such as *Scedosporium* spp. in mixed interactions (Bandara *et al*., 2010; Cugini *et al*., 2010; Hogan and Kolter, 2002). Until now, there is only one report which indicates that *P. aeruginosa* was less likely to be isolated from the CF patients already colonised with *S. aurantiacum*, which may point towards the antagonistic interactions between the two opportunistic pathogens (Blyth *et al*., 2010b). The nature of interactions between *S. aurantiacum* and *P. aeruginosa* has not been explored at all.

Generally bacteria and fungi can interact with each other in a variety of ways that involves either direct or indirect contact between them. Direct interactions represent the degree of intimacy/physical proximity shared between the organisms and indirect associations involve molecular communication/ cross talk that occur between them (Frey-Klett *et al*., 2011). Both direct physical and indirect chemical signalling interactions exist between bacteria and fungi and the combination of physical and molecular associations can result in a variety of outcomes for each interacting partner (Frey-Klett *et al*., 2011). The current knowledge of the interactions between the major bacterial and fungal CF pathogens is presented below.

1.2.1.1. Physical Association between Bacteria and Fungi

Physical interactions involve direct cell-to-cell contact between bacteria and fungi in a way that influences their survival and virulence. The mechanisms underlying direct physical interactions between bacteria and fungi were explored by Frey *et al.* as shown in Fig. 1-9. Bacteria can interact with the fungi in three different ways: by planktonic cells, mixed biofilm formation and interhyphal colonisation (Frey-Klett *et al.*, 2011). Direct physical interactions often prove advantageous to the bacteria for a number of reasons: a) the bacteria can use the fungus as a source of nutrients (*e.g.* fungal cell wall and secreted products); b) fungal hyphae can serve as an anchor to support bacterial growth as a biofilm or for movement within the lung environment to assist their growth; c) bacterial-fungal synergy can help in breakdown of complex substrates; d) bacteria can release antifungal compounds against the fungi to promote their own survival (Boer *et al.*, 2005; Hibbing *et al.*, 2010).

Fig. 1-9. Direct physical interactions between bacteria and fungi. Image has been modified from (Frey-Klett *et al.*, 2011).

Direct interactions have been reported between *P. aeruginosa* and *C. albicans* which showed that *P. aeruginosa* has a tendency to attach to the fungal hyphae by forming a dense population of cells surrounded by an extracellular matrix (biofilm) and cause fungal cell death (Hogan and Kolter, 2002). The level of virulence factors produced by bacteria may also be affected by mixed interactions. For *e.g.* phenazine toxins produced by *P. aeruginosa* have been known to cause damage to the host lungs and *C. albicans*, and the bacterial cells secrete higher level of these toxins in the presence of fungus (Hogan and

Kolter, 2002; Kerr, 1994; Lau *et al*., 2004; Wilson *et al*., 1988). The fact that the presence of *C. albicans* increases the production of virulence factors by *P. aeruginosa*, may propose a mechanism by which co-infection with bacteria and fungi causes more damage to the lungs of the CF patients (McAlester *et al*., 2008). Recent studies concerning the interactions between *A. fumigatus* and *P. aeruginosa* have suggested that *A. fumigatus* can convert the bacterial phenazines to highly toxic products such as 1-HP, 1-methoxyphenazine (1-MP), and phenazine-1-sulfate, which inhibit the fungal growth and induce iron starvation (Briard *et al*., 2015; Moree *et al*., 2012). Studies relating to direct physical interactions between *P. aeruginosa* and fungi other than *Candida* or *Aspergillus* have not been published in the current literature.

1.2.1.2. Molecular Communication between Bacteria and Fungi

The interactions between microorganisms in the lung microbiota can also be mediated by small signalling molecules (*e. g.* acylated homoserine lactones in bacteria and farnesol in fungi), which control the cell-cell signalling, also known as quorum sensing (Cugini *et al*., 2010; Laursen and Nielsen, 2004). The main function of quorum sensing (QS) in bacteria is to control processes such as biofilm formation, swarming motility and cell aggregation (Jarosz *et al*., 2011).

Bacterial QS system is complicated due to the involvement of several distinct QS sub-systems. One of the most well studied *P. aeruginosa* QS system is PQS, which secretes C4HSL, C12HSL molecules, has been shown to induce the change of *C. albicans* from yeast to hyphal form (Brand *et al*., 2008; Hogan and Kolter, 2002). The phenotypic switch of *C. albicans* from yeast to hyphal form makes it susceptible to killing by *P. aeruginosa* cells. The fungus responds to bacterial QS by secreting molecules such as farnesol, which can regulate the growth and virulence of the interacting bacterial partners by altering the production of toxic pigments such as pyocyanin (Hornby *et al*., 2001). The interactions between *P. aeruginosa* and *C. albicans* have been demonstrated in the Fig. 1-10 adopted from Mear *et al.* (2013).

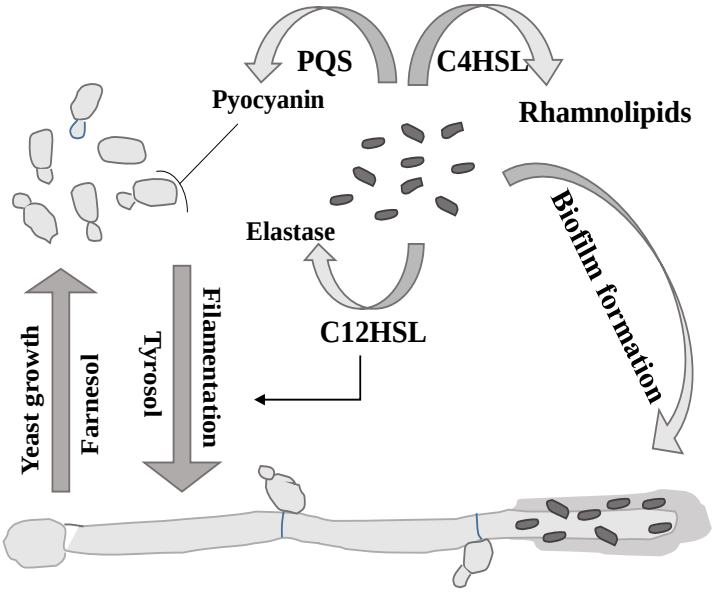


Fig. 1-10. QS Systems involved in the interactions between *P. aeruginosa* and *C. albicans*. Bacterial QS system (PQS, C12HSL) causes a morphological change in *C. albicans* whereas farnesol secreted by *C. albicans* causes a change in the virulence of the bacteria. Image has been modified from (Mear *et al*., 2013).

Another well-known bacterial QS system functional in *P. aeruginosa* is *LasI/LasR-RhlI/RhlR*, which is known to inhibit biofilm formation by *A. fumigatus*. In order to establish the role of the *Las* QS system in *P. aeruginosa*, Mowat and colleagues studied the response of the *LasI* and *LasR* knockout mutants of *P. aeruginosa* against *A. fumigatus* biofilms. Their results reported that none of the mutants could inhibit the biofilm growth of *A. fumigatus* to the same extent as the laboratory reference strain PAO1 (Mowat *et al*., 2010). These findings suggest that both the prokaryotic and eukaryotic microbial partners sense the presence of each other in their ecological niche through these secreted signalling molecules (Morales and Hogan, 2010).

In summary, the interactions whether direct or indirect, can either prove beneficial or harmful to the microbes involved as the compounds secreted by the bacteria or fungi can either enhance or negatively influence the growth and survival of the other (Sachs *et al*., 2011; Peleg *et al*., 2010). The majority of interactions reported in the literature have highlighted the antagonistic nature of *P. aeruginosa* against the fungal co-inhabitants, whereas synergistic interactions are rarely reported (Peleg *et al*., 2010).

1.2.2. Methods Used to Study Interactions

Yeast has been used as a genetically tractable model system to strengthen the understanding of interactions between different pathogens in polymicrobial infections (Wargo and Hogan, 2006). In particular, most of the research has been performed with the human pathogenic yeast *C. albicans* which has provided extended information of the role of complex interspecies signalling communication in the bacterial-fungal interactions (Hogan and Kolter, 2002; Hogan *et al*., 2004; McAlester *et al*., 2008). In general, a combination of an effective *in vitro* model system and innovative technologies such as proteomics, transcriptomics, metabolomics and imaging mass spectrometry is required to understand the interkingdom interactions. The role of an ideal *in vitro* model is to provide a stand-alone tool for the testing of new hypotheses while maintaining robustness and simplicity (Sabiiti *et al*., 2012). A system that includes parameters such as spatial proximity of the species, environmental influence, changes in the relationship over time and the host immune response would be ideal for studying the important aspects of the bacterial-fungal relationships (Peleg *et al*., 2010).

Numerous microbiology-based techniques are available for studying the interactions between bacteria and fungi. Traditional methods are based on confrontation assays where bacteria and fungi are incubated in the same solid or liquid medium for a period of time to examine their effects on each other (Souto *et al*., 2004; Toure *et al*., 2004). Most commonly used laboratory culture media involve either fungus-specific growth media such as SDA, PDA and/or minimal salts medium (Bandara *et al*., 2010; Hogan and Kolter, 2002; Kerr *et al*., 1999; Manavathu *et al*., 2014; McAlester *et al*., 2008; Stanley *et al*., 2014). These media can be made selective by addition of compounds, which are inhibitory to one species and allow the growth of the species resistant to the inhibitory agent (Kerr *et al*., 1999).

1.2.2.1. Solid Plate Assays

Most of the studies involving preliminary screening of the bacterial-fungal interactions are usually performed using solid plate assays. Kerr *et al.* demonstrated the use of a wide variety of plate assays such as disk diffusion assay, cross streak assay, dual culture plate assay and well plate method to detect the interactions between a wide variety of bacterial and fungal species (Kerr, 1999). These methods can also help in the prediction of

extracellular enzymes and molecules involved in the interaction (Kerr *et al*., 1999). The applicability of a cross streak assay has been tested with a large number of bacteria and fungi. For example, CF lung isolates of *P. aeruginosa* showed an inhibitory effect when cross streaked against *C. albicans* strains on Sabouraud dextrose agar (SDA) medium (Kerr, 1994).

Agar disk diffusion assay is generally used for cellular and sub-cellular fractions which are usually applied to the filter paper discs and applied to the solid medium seeded with the test microbial strain. The diameter of the clearing zone around the paper discs facilitates rough estimation of the inhibition capacity of a particular cellular fraction (Ventura *et al*., 2012). While growth inhibition studies provide useful preliminary information about the interactions present between bacterial and fungal species, the measurements do not yield information about the dynamic interactions existing between the fungal hyphae and bacteria at the cellular level (Stanley *et al*., 2014). Moreover, conventional plating techniques are labour-intensive and slow (Donlan and Costerton, 2002).

1.2.2.2. Liquid Cultures

Over the past few years, several liquid culture based assays have been used to study the bacterial-fungal interactions, which provide beneficial information at the microscopic level. Microbes are cultured together in a liquid medium and growth of each microbe is measured as viable cell count (Stanley *et al*., 2014). Viable count (CFU/ml) has been the preferred choice for calculating the rate of inhibition in the co-cultures involving yeast (*C. albicans*) and bacteria (*P. aeruginosa*) (Hogan and Kolter, 2002; Rella *et al*., 2012; Brand *et al*., 2008). However, it is rather difficult to apply these methods to filamentous fungi owing to their non-uniform growth patterns (hyphae) resulting in the formation of aggregates. The interspecies interaction between bacteria and filamentous fungi can be examined and characterised by microscopy based techniques that provide a visual representation of the morphological change and help in discrimination between the species (Frey-Klett *et al*., 2011). For studies involving bacteria and fungi isolated from the lungs of CF patients, Palmer *et al.* introduced the concept of synthetic cystic fibrosis medium (SCFM) that mimics the nutritional contents of human CF sputum. They analysed the growth of different strains of *P. aeruginosa* in SCFM and found that the growth phenotypes, carbon substrate preference and gene expression profiles of bacteria were

similar to those observed in CF sputum (Palmer *et al*., 2007). Due to the close resemblance of SCFM to the human CF sputum, it can provide an excellent means to study the interactions between the bacteria and fungi isolated from the CF patients and is also used in the current study.

1.2.2.3. Confocal Microscopy

Microscopy techniques, especially fluorescence imaging techniques, confocal microscopy and super-resolution microscopy such as electron microscopy have made enormous improvements in the visual interpretation of mixed cultures (Haagensen *et al*., 2011). Even though phase contrast and light microscopes have been used to study mixed species interactions to some extent (Hogan *et al*., 2004), confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) have become the preferred choice owing to the availability of detailed analysis. In a confocal microscope, the fluorescent dye used to stain the specimen is excited with a laser light beam to emit light at a longer wavelength which is then captured by the objective and transferred to the computer screen (Schmolze *et al*., 2011). Confocal microscopes are equipped with a considerable range of three to five laser systems, with argon laser (488 and 514 nm wavelength) and helium-neon laser (633 nm wavelength) as the most commonly used. The choice of the laser used for illumination depends on the dyes used for staining the microbes. Confocal microscopy offers a number of advantages over light microscopy such as three-dimensional view of location of the microorganisms in co-culture and quantitative estimation of cellular structures such as thickness, area and volume of the cell structures of the microbes involved (Schmolze *et al*., 2011).

In order to fully characterise the bacterial-fungal interactions through confocal microscopy, chemical stains are required to discriminate between each organism in a mixed microbe environment (Almeida *et al*., 2011). Usually dyes are selected on the basis of their non-toxic nature, pH sensitivity and stability to photobleaching. Specific cellular functions in target organisms can be probed with specific stains, which are designed to emit light at specific wavelengths (Fischer-Parton *et al*., 2000). For example, nucleic acid staining dyes such as DAPI (4’, 6-diamidino-2-phenylindole), acridine orange and Syto 9 can be used to stain the RNA or DNA present in the cells irrespective of their viability. Out of all these, Syto 9 is the most commonly used fluorogenic nucleic acid stain for quantification of

bacterial and yeast biomass (Boulos *et al*., 1999; Honraet *et al*., 2005; Honraet and Nelis, 2006; Strathmann *et al*., 2002). In order to discriminate between the viable and non-viable cells, techniques targeting the metabolic activity of the viable cells have also been employed. Viability stains such as propidium iodide and 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) can only be taken up by the cells with an active cytochrome system (Gabrielson *et al*., 2002; McCluskey *et al*., 2005). Similarly, a green fluorescent dye 2-chloro-4-(2,3-dihydro-3-methyl(benzo-1,3-thiozol-2-yl)methylidene)-1-phenylquinolinium iodide (FUN-1) has been used to discriminate between metabolically active and inactive fungal cells (Essary and Marshall, 2009). This dye diffuses into the plasma membrane of living fungal cells and is transported to the vacuoles in the metabolically active cells, which emit red fluorescence (Millard *et al*., 1997). Brand *et al.* (2008) used the FUN-1 stain to show the killing of *C. albicans* hyphae by *P. aeruginosa.* Chemical dyes such as $DiOC_6$ (3) (ER), BODIPY FL C5, MitoTacker FR and Syto 40 can be used to stain the sub-cellular structures including endoplasmic reticulum, Golgi bodies, mitochondria and nuclei. Bandara *et al.* (2010) showed the use of commercial Live and Dead stains (Syto 9 and propidium iodide) to study the interactions between *P. aeruginosa* and *C. albicans* in liquid culture. Confocal microscopy is immensely helpful in visualising monospecies communities, but mixed cultures cannot be demonstrated properly using CLSM owing to staining discrepancies/cross staining in the mixture of bacteria and fungi.

1.2.2.4. Electron Microscopy

Another microscopy-based approach that is employed in visualisation of bacterial-fungal co-cultures is electron microscopy, which provides the ability to view the ultrastructures of microbial cells (Smith and Oatley, 1955). Electron microscopes use a high beam of electrons to visualise and characterise the biological samples on a nanometer (nm) to micrometer (μm) scale (Klomparens, 1990). Electron microscopy is of two types: transmission electron microscopy (TEM) and scanning electron microscopy (SEM). TEM can reveal structures of fixed and sectioned specimens in flat two-dimensional images, which can then be combined computationally to obtain a detailed view of the target cell or organelle (Hohmann-Marriott *et al*., 2006). Cells are generally fixed using chemicals such as aldehydes, OsO4 (osmium tetroxide) and RuO4 (ruthenium tetroxide), which have toxic effects on the cells (Kaminskyj and Dahms, 2008). SEM on the other hand, provides a

three-dimensional surface view of cells (Jones, 2012) and provides the advantage of preserving the cells using freeze drying thus making it less harmful for organisms such as fungi, compared to chemical fixation methods used in TEM (Hess, 2007). Cells are fixed on the microscopy slides using aldehydes (4% glutaraldehyde) and dehydrated using a graded series of solutions of acetone or ethanol (from 30% to 100%). This is followed by replacement of dehydrating fluid with liquid carbon dioxide which is then dried to a critical point of carbon dioxide (31.1 °C at 1073 psi) in a critical point drier leaving the sample completely dry (Sabatini *et al*., 1963). The dried specimens are then mounted on the metallic holders (stubs) and coated with metal to aid in signal generation. Commonly used metal coatings are made from carbon, gold, gold-palladium, platinum-palladium, platinum and chromium (Jones, 2012).

Usually the interactions between bacteria and fungi are explored with a combination of different microscopy techniques. For example, Brand and colleagues used a range of microscopy techniques such as phase contrast, light microscopy, SEM and TEM to monitor the physical interactions between *P. aeruginosa* and *C. albicans* (Brand *et al*., 2008; Hogan and Kolter, 2002). Similarly, Bandara *et al.* used CLSM and SEM to explore the interactions between *P. aeruginosa* and six different species of *Candida* (Bandara *et al*., 2010). Mixed species biofilms between *P. aeruginosa* and *A. fumigatus* have been studied using light microscopy and SEM (Manavathu *et al*., 2014; Mowat *et al*., 2010). SEM has also been applied to study the physical interactions between *A. nidulans* and *S. hygroscopicus* (Schroeckh *et al*., 2009).

1.2.2.5. Molecular Tools: Using Bacterial and Fungal strains Tagged with Fluorescent Proteins

The non-specific nature of chemical stains such as crystal violet, Syto-9/propidium iodide and 4’,6-diamidino-2-phenylindole (DAPI) often makes it difficult to study the interactions between bacteria and fungi (Almeida *et al*., 2011). Another way to discriminate between bacteria and fungi in co-cultures is through development of bacterial and fungal transformants expressing genetically encoded fluorescent proteins (FP) (Almeida *et al*., 2011). Fluorescent proteins such as green fluorescent protein (GFP) serve as a non-invasive tool for biological imaging of mixed cultures as they do not require harsh fixation

and permeabilisation techniques which generally affect the cell integrity (Almeida *et al*., 2007; Hansen *et al*., 2001; Hansen *et al*., 2007; Rodrigues *et al*., 2003).

Green fluorescent protein that was discovered in 1960, is derived from a jellyfish *Aequorea victoria* and has been successfully used to label a number of microbes such as *P. aeruginosa*, *Staphylococcus aureus*, *C. albicans*, *Aspergillus* spp., *Trichoderma* spp., *Phytopthora* spp., *and Magnaporthe* spp. (Almeida *et al*., 2007; Chalfie, 2009; Peters *et al*., 2010; Shimomura *et al*., 1962). A red fluorescent protein (RFP) DsRed isolated from non-bioluminescent *Discosoma* spp. has also been used to label a wide range of filamentous fungi (Mikkelsen *et al*., 2003). The wide spectrum of DsRed ranging from 558 nm to 583 nm enables it to be used for multicolour experiments (Fradkov *et al*., 2002; Matz *et al*., 1999). However, the wild-type DsRed is tetrameric which leads to toxicity and slow maturation (Baird *et al*., 2000). Therefore, tetrameric wild type RFPs have been genetically manipulated to develop monomeric variants such as mCherry which have improved fluorescent properties, rapid maturation and reduced aggregation (Day and Davidson, 2009; Campbell *et al*., 2002; Shaner *et al*., 2004). Genetic modification has also led to the development of several other enhanced varieties of fluorescent proteins ranging in colour from cyan to far red such as ECFP (enhanced cyan fluorescent protein), EYFP (enhanced yellow fluorescent protein) and EGFP (enhanced green fluorescent protein) (Gurskaya *et al*., 2001; Heim *et al*., 1994; Lukyanov *et al*., 2000). The use of enhanced FPs was demonstrated by Hogan *et al.* (2009) who checked the colonisation of CFP labelled *Fusarium oxysporum*. f.sp. *radicis lycopersici* by *P. fluorescens* tagged with E-GFP.

Successful expression of fluorescent proteins in bacterial and fungal species requires the development of an efficient transformation system for introduction, integration and expression of the exogenous DNA. In case of the opportunistic bacterial pathogen *P. aeruginosa*, a wide array of genetic tools has been developed to facilitate genetic manipulation, especially expression of fusion proteins (Becher and Schweizer, 2000; Hoang *et al*., 2000; Schweizer, 1991; Schweizer and Chuanchuen, 2001; West *et al*., 1994). However, *S. aurantiacum* is a recently identified species with limited genetic information and almost non-existent molecular tools. Notwithstanding this, a genetic transformation system for *S. aurantiacum* can be developed using approaches devised for other filamentous fungi (Ruiz-Diez, 2002). Genetic transformation systems have been described for a wide variety of filamentous fungi and generally require identification and selection of a suitable gene promoter for gene expression, a reliable transformation marker

for selection of transformants and a delivery method for the introduction of DNA of interest into the fungal cells (Ruiz-Diez, 2002).

1.2.2.5.1. Expression Promoters

While efficient expression of homologous or heterologous genes in filamentous fungi depends on a strong promoter endogenous to the production host (Punt *et al.*, 1990; Daboussi *et al.*, 1989), the lack of an annotated genome precludes the use of a homologous promoter in *S. aurantiacum*. However, a large number of inducible and constitutive promoters are available for recombinant protein production in filamentous fungi. For example, *T. reesei cbh1* (cellobiohydrolase 1) is a strong inducible promoter that needs an inducer saccharide such as cellulose, sophorose or lactose to trigger the gene expression (Bergquist *et al*., 2002; Wang and Xia, 2011). The *cbh1* gene promoter has been used for the expression of a wide range of proteins in *T. reesei* and has been shown to function in yeasts such as *Kluyveromyces lactis* and *S. cerevisiae* (Aro *et al*., 2005; Madhavan and Sukumaran, 2014) and filamentous fungal species such as *Aspergillus* (Aro *et al*., 2005). Constitutive promoters such as *pki* (pyruvate kinase) do not need an inducer and are generally active *in vivo* at all the times (Li *et al*., 2012). Although the efficiency of constitutive promoters is lower than that of inducible promoters, they have higher chances of being active across species, as they do not require factors such as specific inducers for their function. For example, the constitutive promoter *gpdA* (glyceraldehyde-3-phosphate dehydrogenase) of *A. nidulans*, has been used to drive the protein expression in a wide variety of fungal species (Almeida *et al*., 2007; Ruiz-Diez and Martinez-Suarez, 1999). Punt *et al.* (1990) demonstrated the use of *gpdA* promoter to express the *hph* gene in fungal respiratory pathogen *Coccidoides immitis*. Other examples of constitutive promoters used across various fungal species include *trpC* and *Pna2/TPI* gene promoter from *A. nidulans* and *ToxA* promoter from the plant pathogenic fungus *Pyrenophora tritici-repentis* (Moore, 2007).

1.2.2.5.2. Selectable Marker

A wide variety of genes are available for use as selectable markers in different fungal transformation systems (Daboussi *et al*., 1989; d'Enfert, 1996). Genes that either complement the generation of nutritional deficiency in auxotrophic mutants or confer

dominant drug resistance are most commonly employed for fungal transformations. Auxotrophic markers such as *pyrG* and *pyr-4* are widely used to complement the mutants deficient in the metabolism of uracil (Ruiz-Diez, 2002). However the transformation strategies employing auxotrophic markers are limited by the lack of nutritional mutants in fungi without well-known genetic systems. A nutritional marker such as the *amdS* gene isolated from *A. nidulans* (Hynes *et al*., 1983) encodes an acetamidase enzyme that allows the fungus to utilise acetamide as a sole nitrogen source (Ruiz-Diez and Martinez-Suarez, 1999). The *amdS* gene has been widely used in the transformation of filamentous fungi such as *A. niger*, *Penicillium chrysogenum* and *T. reesei* (Debets *et al*., 1990; Kolar *et al*., 1988; Michielse *et al*., 2004; Saarelainen *et al*., 1997; Tilburn *et al*., 1983).

Dominant drug resistance markers have also proved to be of great utility in the transformation of filamentous fungal species and are known to provide resistance to the common antifungal drugs such as hygromycin B, phleomycin, bleomycin, oligomycin, G418 and nourseothricin, (Austin *et al*., 1990; Drocourt *et al*., 1990; Punt and van den Hondel, 1992; Ward *et al*., 1986). Hygromycin B (HygB) is an aminoglycosidic antibiotic that inhibits the protein synthesis in bacteria, fungi and higher eukaryotes (Gritz and Davies, 1983; Punt *et al*., 1987). *E. coli* hygromycin B phosphotransferase gene (*hph*) (Gritz and Davies, 1983) that confers resistance to hygromycin B has been extensively used for selection of transformants in filamentous fungi including *A. niger*, *T. reesei*, *Ophiostoma picaeae* and *O. ulmi* (Hoffman and Breuil, 2004; Mach *et al*., 1994; Punt *et al*., 1987; Royer *et al*., 1991). Ruiz and co-workers were the first to demonstrate the use of the *E. coli hph* gene as a selective marker for transformation of *L. prolificans* that showed susceptibility to hygromycin B at low concentrations (25 μg/ml) (Ruiz-Diez and Martinez-Suarez, 1999). The nourseothricin acetyltransferase gene from *Streptomyces noursei*, which provides resistance against the nourseothricin antibiotic, has been used as a selection marker in *Cryptococcus* spp. (Hua *et al*., 2000). The *kan* gene that confers resistance to antibiotic G418 has also been employed in transformation of *Neurospora crassa*, *Cephalosporium acremonium* and *Phycomyces blakesleanus* (Bull and Wootton, 1984; Revuelta and Jayaram, 1986). The advantages of using dominant drug resistance markers are that they do not require generation of auxotrophic mutants and can be used for a wide range of fungal species including those with little or no genetic information (Moore, 2007).

1.2.2.5.3. Methods of Transformation

Briefly, genetic transformation is the method of introduction of exogenous DNA into the recipient cells in case of filamentous fungi and the incoming DNA is integrated as a part of the fungal genome. Methods for transforming filamentous fungal species include PEG-mediated protoplast transformation, chemical treatment, electroporation, biolistic bombardment and *Agrobacterium tumefaciens*-mediated transformation (Olmedo-Monfil *et al*., 2004).

Protoplast Transformation: Protoplast transformation is the most common method used for transformation of filamentous fungi. The technique involves preparation of fungal protoplasts by removal of cell wall from the conidia, germinating conidia, young hyphae or yeast like cells using enzymatic methods (Buxton and Radford, 1983; Ruiz-Diez, 2002; Tanguay and Breuil, 2003). The successful preparation of protoplasts depends on the choice of suitable lysing enzyme (Fincham, 1989). Most popular commercial enzyme mixtures used for the production of protoplasts include a helicase and glusulase mixture from snail gut, Zymoylase T100 from *Arthrobacter luteus* and Novozyme 234 from *T. viride* (Olmedo-Monfil *et al*., 2004; Ruiz-Diez, 2002). Among them, Novozyme 234 is the most popular lysing enzyme for the preparation of fungal protoplasts for transformation. Due to the absence of cell walls, protoplasts are stabilised with an osmotic stabiliser such as sodium chloride, magnesium sulphate, mannitol or sorbitol (0.8-1.2 M) and can be stored at -70°C for later use. Internalisation of exogenous DNA by protoplasts is facilitated by calcium ions in the presence of high amounts of polyethylene glycol (PEG) (Fincham, 1989). PEG mediated transformation procedures are relatively simple and can be carried out in the laboratory without any specialised equipment. Protoplast transformation has been used as a routine method for filamentous fungi due to its simplicity, efficiency, reproducibility and ability to generate large amount of transformants (Liu and Vidali, 2011).

Chemical Treatment: Treatment with chemicals such as lithium acetate can be used as an alternative method to permeabilise the recipient cells for the incoming DNA without the need of forming protoplasts. Lithium acetate at a concentration of 0.1 M is used in

combination with PEG that causes agglomeration of the cells, which helps in trapping of the DNA. Lithium acetate mediated transformation has been successfully applied to *N. crassa*, *Coprinus lagopus*, *Humicola grisea* var. *thermoidea* and *Ustilago violacea* (Bej and Perlin, 1989; Binninger *et al*., 1987; Dantas-Barbosa *et al*., 1998; Dhawale *et al*., 1984).

Electroporation: Electroporation involves the use of high amplitude electric fields to change the cell permeability that helps in the DNA uptake. Electroporation can be performed using intact conidia, protoplasts (Olmedo-Monfil *et al*., 2004; Ruiz-Diez, 2002) or germinated conidia (Ward *et al*., 1986; Ozeki *et al*., 1994; Sanchez and Aguirre, 1996). When combined with the PEG treatment, electroporation can yield high transformation efficiencies. For example, protoplasts of *T. harzianum* with partially digested cell walls were transformed with PEG combined with electroporation resulting in high transformation efficiency of up to 400 transformants/µg of DNA (Goldman *et al*., 1990). Electroporation has been used to transform a wide range of fungi such as *N. crassa*, *P. urticae*, *A. oryzae*, *A. niger* and *T. harzianum* (Chakraborty *et al*., 1991; Goldman *et al*., 1990; Ozeki *et al*., 1994; Sanchez and Aguirre, 1996). The first report of the use of electroporation for transformation of *Scedosporium* spp. (*L. prolificans*) was in 1991 by Ruiz-Diez and Martinez-Suarez. Although they could not achieve high transformation efficiencies with this method, the work reported by them provides a good reference for the future studies.

Biolistic System or Microparticle Bombardment: Originally developed for plant tissue transformations (Sanford, 1990), this approach has been applied to a large number of bacteria, yeasts, filamentous fungi and eukaryotic cells over the years (Olmedo-Monfil *et al*., 2004). Biolistic transformation involves bombardment of the fungal cells with DNA coated on gold or tungsten microparticles. This method has found applications with many types of filamentous fungi such as *Cryptococcus neoformans*, *T. atroviride* and *A. nidulans* (Barcellos *et al*., 1998; Kingsbury *et al*., 2004; Rocha-Ramirez *et al*., 2002). Te'o *et al* showed the use of biolistic bombardment for the transformation of *T. reesei* using Bio-Rad™ seven barrels hepta adaptor system. Transformation efficiencies with this technique

are affected by a number of factors such as vacuum pressure in the bombardment chamber and the shooting distance of the microparticles (Te'o *et al*., 2002).

Agrobacterium-*mediated Transformation*: The Gram-negative soil bacterium *Agrobacterium tumefaciens* has been extensively used to transfer genes to the plants due to its ease of use, precision of integration into the plant genome and high transformation efficiencies (de Groot *et al*., 1998). The technique is based on the principle that *A. tumefaciens* causes tumor induction in plants and during tumor induction, it transfers part of its plasmid (T-DNA) into the plant cells. This T-DNA integrates into the plant genome at a random position and the genes present in the T-DNA are expressed (Abuodeh *et al*., 2000). The applicability of *Agrobacterium* mediated transformation has already been tested in yeasts such as *S. cerevisiae* and a number of filamentous fungi (Abuodeh *et al*., 2000; de Groot *et al*., 1998; Li *et al*., 2000; Mullins *et al*., 2001). The major advantages of using *Agrobacterium* mediated transformation involve the ability to use protoplasts, hyphae or spores as a target material and that it results in the integration of a single copy of the gene into the host (Mullins *et al*., 2001; Olmedo-Monfil *et al*., 2004).

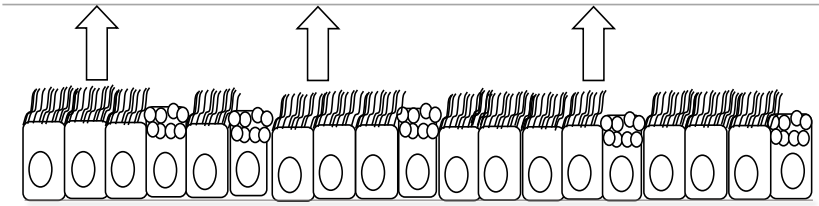
The development of a genetic transformation system for *S. aurantiacum* can present significant challenges owing to the lack of sufficient genetic information and the presence of high inherent resistance to antibiotics. However, it seems possible to develop a genetically tagged *S. aurantiacum* strain using a strategy based on the availability of extensive range of tools and approaches used for genetic transformation of other filamentous fungal species.

1.3. Mixed Infections and Host Response

Although *in vitro* methods of testing allow studying the interactions between the bacteria and fungi in a controlled environment, their authenticity is often limited by the absence of host immune response (Peleg *et al*., 2010). This disadvantage can be overcome to some extent using various cell-culture models that involve the use of disease specific mucosal epithelial cell lines derived from relevant organ systems (Duell *et al*., 2011). Human airway epithelium present in the respiratory tract is the initial point of contact with the

inhaled substances such as airborne microbes, allergens, smoke and environmental pollutants (Diamond *et al*., 2000). Two types of lung alveolar epithelial cells are known, namely type I cells that cover the inner alveolar surface and type II cells that secrete surfactant proteins and differentiate into type I cells (Herzog *et al*., 2008). Type II epithelial cells provide an interface between the external environment and lung milieu and mediate the host immune response against foreign microbes through the production of cytokines, chemokines, proteinase inhibitors, surfactant proteins and antimicrobial peptides (Fig. 1-11), which are known to regulate processes such as inflammation, immunity and wound repair (Diamond *et al*., 2000; Herzog *et al*., 2008; Holgate *et al*., 2000).

Inflammatory mediators	**Chemotactic mediators**	**Antimicrobial substances**	
Cytokines	LL-37/CAP-18	B-defensins	Phospholipase A2
Chemokines	B-defensins	Lysozyme	Lactoferrin
Leukotrienes	Chemokines	SLPI	LL-37/CAP-18
Calprotectin	Leukotrienes	Calprotectin	Anionic peptides



Airway epithelial cells

Fig. 1-11. Overview of the molecules secreted by the airway epithelial cells as a defense mechanism against infection. Chemokines are secreted primary to the basolateral side whereas antimicrobial peptides are secreted to the apical side. Image has been modified from (Bals and Hiemstra, 2004).

Failure of lung epithelial cells to display host defense properties results in microbial colonisation and airway infections (Bals and Hiemstra, 2004). Epithelial cells recognise the pathogens through toll-like receptors (TLR) that bind to pattern-recognition molecules displayed by the microorganisms (Bals and Hiemstra, 2004).

1.3.1. Cell Culture Models

Limited models of alveolar infection are available for studying the interaction between pathogens and the host (Osherov, 2012). Due to difficulties in growing type I and type II alveolar epithelial cells in culture (Osherov, 2012), almost all the studies analysing the interactions between host and pathogen have been undertaken in cell culture with lung epithelial cancer cell lines such as NCI-H292 and A549 and/or immortalised epithelial cell line such as HBE1 and 16HBE (Kreda *et al.*, 2012). Among these cell lines, A549 cells derived from lung carcinoma (adenocarcinoma) have been extensively used as an experimental model to study the interactions between CF pathogens and the host due to high degree of metabolic and morphological similarity between A549 cells and type II alveolar epithelial cells (Foster *et al.*, 1998). The majority of studies use microscopy techniques such as SEM or CLSM for the analysis of interactions between the host and the pathogens (Osherov, 2012). Transcriptome analysis of both the host cells and the pathogens is also increasingly carried out to understand the mechanisms involved in the infections (Gomez *et al.*, 2010; Ichikawa *et al.*, 2000).

The host and pathogen transcriptomes can be analysed simultaneously by a dual RNA-sequencing approach that helps to provide a deeper understanding of the infection process by allowing monitoring of the gene expression changes in both the pathogen and the host. Dual RNA-sequencing allows simultaneous and unbiased sequencing of mixed species RNA samples (rRNA depleted) that are obtained from the host infected with the pathogen. Individual RNA samples from the host and the pathogen are also isolated in parallel for comparison (Westermann *et al.*, 2012). RNA molecules are reverse transcribed into cDNA libraries, which can be sequenced using either amplification-based sequencing methods or non-amplification based single-molecule sequencing (SMS). A wide variety of RNA sequencing platforms such as Illumina (Solexa), Life Technologies (SOLiD) and Roche (454) are available for genome-wide transcriptomic studies (Liu *et al.*, 2012). The RNA expression profiles for the host and the pathogen are generated by differentiating the sequenced reads *in silico* (bioinformatics) and subsequent mapping onto respective reference genomes (Westermann *et al.*, 2012).

1.3.2. Fungal interactions with Epithelial Cells

The majority of fungal pathogens associated with CF including *A. fumigatus, C. albicans* and *C. neoformans* invade the host epithelial cells during mucosal and respiratory infections and induce their own endocytosis. However, there is a paucity of knowledge concerning the mechanism used by the fungal pathogens to invade the epithelial cells (Filler and Sheppard, 2006). Since *A. fumigatus* is the most predominant fungal CF pathogen, the majority of host-pathogen interaction studies have been performed using a co-culture of *A. fumigatus* conidia or culture filtrate (CF) with the lung epithelial cells (Osherov, 2012). Co-culture studies of *A. fumigatus* and A549 cells have shown that *A. fumigatus* conidia can penetrate and internalise within the epithelial cells (Botterel *et al*., 2008; DeHart *et al*., 1997; Han *et al*., 2011; Paris *et al*., 1997; Wasylnka and Moore, 2002). Epithelial cells with internalised conidia show a depolymerised F-actin cytoskeleton and loss of focal adhesions (Kogan *et al*., 2004; Sharon *et al*., 2011). Transcriptomic and proteomic studies have reported that internalisation of fungal conidia into the epithelial cells results in the activation of different signalling pathways in the infected host including MyD88 dependent NF-kB, PI3K (phosphatidylinositol 3-kinase) and p38 MAPK (mitogen activated protein kinase) signalling, which ultimately lead to the synthesis of chemokines and cytokines (Osherov, 2012). The cytokines produced by the infected A549 cells include IL-6, IL-8, TNFα, GM-CSF and MCP1 (Bellanger *et al*., 2009; Sharon *et al*., 2011; Zhang *et al*., 2005). Similarly, co-culture of *A. fumigatus* with bronchial and nasal epithelial cells has also been studied by several groups which showed that around 50% of the conidia were internalised into the phagosomes where they can survive up to 20 hours whereas the remaining conidia adhered to the external cell surface, penetrated after germination and caused cell death (Amitani *et al*., 1995).

The presence of *A. fumigatus* conidia is recognised by the human bronchial epithelial (HBE) cells through dectin-1 receptors that binds to the glucans present on the fungal surface (Cunha *et al*., 2010; Sun *et al*., 2012). Transcriptomic analysis of the cells with internalised conidia showed an up-regulation of genes associated with repair and inflammatory response such as matrix metalloproteinase, chemokines and glutathione S-transferase (Cortez *et al*., 2006). The infected cells suffered a decrease in the rate of proliferation as suggested by the down-regulation of the genes associated with cell cycle phase, mitosis and intracellular organelles (Oosthuizen *et al*., 2011). Fungal protease was thought to be responsible for the pathogenesis of the epithelial cells as no cytokine release,

cell peeling and mucin secretion was observed in the co-cultures treated with serine protease inhibitors (Kauffman *et al*., 2000; Oguma *et al*., 2011; Tomee *et al*., 1997).

The host immune response to infections caused by *Scedosporium* spp. has been studied to a lesser extent compared to *A. fumigatus* (Santos *et al*., 2009). Most of the insight into the pathogenesis of *Scedosporium* spp. has been gained through the *in vitro* co-culture of host phagocytic cells and monocytes with *S. apiospermum* (Cortez *et al*., 2008). These studies indicated that *Scedosporium* infection leads to an enhanced immune response in the host monocytes, which release more tumor necrosis factor alpha and interleukin-6 (IL-6) when compared to *Aspergillus* spp. (Warris *et al*., 2005). Host cells such as macrophages and dendritic cells infected with *S. boydii* are also known to release high amounts of cytokines through toll like receptors, CD14 and MyD88 pathways (Gersuk *et al*., 2006; Steele *et al*., 2005). Toll like receptors are present on a variety of human cells and are known to activate the host immune response through recognition of pathogen-associated molecular patterns (PAMPs) usually displayed by the fungal pathogens (Carpenter and O'Neill, 2007). Some researchers have evaluated the role of (1-3)-β-glucan as a ligand for toll like receptors and induction of host immune response (Hohl *et al*., 2005; Kedzierska *et al*., 2007; Miyazaki *et al*., 1995). So far, there is only one report by Pinto *et al* (2004) who assessed the interaction between *S. boydii* conidia and human epithelial cell lines including HEp2 and A549 through light microscopy (Fig. 1-12). They found that *S. boydii* conidia attached to the epithelial cells soon penetrated into the epithelial cell membrane after germ tube formation and were ingested by the epithelial cells. They also indicated that binding of *S. boydii* conidia to HEp2 cells is mediated by an antigen called peptidorhamnomannan (PRM), which is present on the fungal conidial surface.

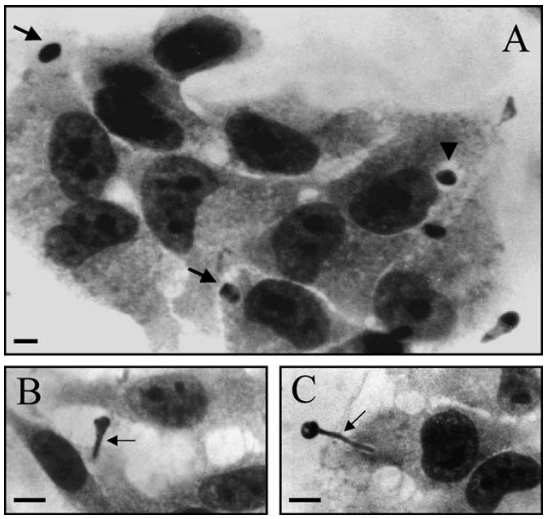


Fig. 1-12. Light microscopy images of different phases of interaction between *S. boydii* conidia and HEp2 cells after a) 1h, b) 2h and c) 4 h. (Note: Arrows show internalisation of *S. boydii* conidia by HEp2 cells in (a) and projection of germ tubes in (b) and (c). Scale bar = 10 μm. Image has been used with permission from (Pinto *et al*., 2004)

1.3.3. Bacterial Interactions with Epithelial Cells

Similar to the fungi, other CF associated pathogens such as bacteria are also capable of attaching to the respiratory mucus and respiratory epithelial cells (Cervin *et al*., 1994; Chi *et al*., 1991). The interaction between bacteria and host cells is mediated by the bacterial surface or secreted products that are normally recognised by the host resulting the activation of the host immune response (Ichikawa *et al*., 2000). The disease state of CF stems from the accumulation of viscous mucus in response to the presence of bacteria (Li *et al*., 1997). Several studies have been carried out to assess the host epithelial cellular response to one of the most dominant opportunistic CF bacterial pathogens, *P. aeruginosa* (Hawdon *et al*., 2010; Ichikawa *et al*., 2000). Studies focussing on interaction between different genotypically and phenotypically characterised *P. aeruginosa* isolates and A549 cells have revealed that the *P. aeruginosa* cells adhered to A549 cells are rapidly internalised and lead to the production of reactive oxygen species (ROS), apoptosis of infected cells and induction of innate and adaptive immune responses (Hawdon *et al*., 2010). The adherence of bacteria to the epithelial cells is mediated by a number of factors such as pili, flagella, alginate, toll-like receptors and lipopolysaccharides (LPS) (Adamo *et al*., 2004; Feldman *et al*., 1998; Gupta *et al*., 1994). The mechanism of bacteria-induced host cell apoptosis involves a number of secreted bacterial products such as toxins (ExoS,

ExoT, ExoU and ExoA) and pigments (pyocyanin) (Jia *et al*., 2006; Shafikhani *et al*., 2008). However, recent studies have indicated that bacterial flagellin can also induce apoptosis of the epithelial cells through activation of various extrinsic and intrinsic caspase pathways (Zeng *et al*., 2006). The hallmark of the disease is the production of pro-inflammatory molecules *i. e.* cytokines TNF-α, IL-1, IL-6, IL-8 and neutrophils by the host cells infected by bacteria leading to severe tissue damage and lung failure (Bjarnsholt *et al*., 2009; Sadikot *et al*., 2005).

1.3.4. Mixed Bacterial-Fungal Interactions with Epithelial Cells

Multispecies interaction models with the lung epithelial cells are far more limited than the monospecies models. Kerr *et al.* (2013) were the first to examine the effect of combined cultures of the CF pathogens *A. fumigatus* and *P. aeruginosa* on the A549 cell line. They found that *P. aeruginosa* strain PA14 alone was able to cause a higher mortality in the A549 epithelial cells compared to the cells treated with both bacteria and fungi. Elastase was considered as a major bacterial virulence factor that was involved in the blebbing and detachment of A549 cells. Until now, there are no further reports of the use of A549 cells as a model to assess the interactions between CF pathogens such as *S. aurantiacum* and *P. aeruginosa*. Therefore, the successful application of A549 cell models to study pathogen-host interactions can be extended to include mixed *S. aurantiacum-P. aeruginosa* mixed communities for a better understanding of the pathobiology of the CF lung with mixed infections.

1.4. Aims of Thesis

The broad aims of the thesis were 1) to biochemically characterise selected strains of *S. aurantiacum*, a recently identified fungal pathogen causing frequent infections in Australian CF patients (Delhaès *et al.*, 2008; Heath *et al.*, 2009); and 2) advance the understanding of biological mechanisms involved in its interactions with the human alveolar epithelial cells and prokaryotic bacterial lung inhabitants especially *P. aeruginosa*.

The specific aims of the thesis were:

A. To undertake morphological, physiological and metabolic assessment of four different strains of *S. aurantiacum* displaying different levels of virulence using the phenotype microarray (PM) technology.

B. To assess the effect of two clinical strains PASS1 and PASS2 and one reference laboratory strain PAO1 of the major bacterial lung pathogen *P. aeruginosa* on the growth of two different *S. aurantiacum* isolates WM 06.482 (high virulence) and WM 08.202 (low virulence).

   This question was pursued using solid plate assays and liquid cultures carried out in a synthetic cystic fibrosis medium (SCFM) that mimics the nutritional contents of the human sputum. Visualisation of the liquid cultures was facilitated by confocal microscopy of *S. aurantiacum* and *P. aeruginosa* strains tagged with chemical fluorescent stains. A genetic transformation system was also devised in order to achieve an mCherry labelled *S. aurantiacum* strain suitable for confocal microscopy.

C. To explore the interactions of *S. aurantiacum* with a carcinogenic lung epithelial cell line A549 to improve the understanding of the mechanism of infection of *S. aurantiacum* in the lungs.

   This involved infecting the A549 cells with *S. aurantiacum* strain WM 06.482 and observing the interactions using microscopy techniques such as CLSM and SEM. Specific response of the epithelial cells was noted by carrying out the transcriptome based analysis of the infected and non-infected cells.

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2

MATERIALS AND METHODS

Materials and Methods

The materials and methods used in the present work are described in the table below. Detailed description has been outlined in the corresponding chapter.

Table 2-1. A list of materials and methods as described in chapter 3, 4 and 5 respectively.

Materials and Methods	**Chapter**
Cultivation and growth of *S. aurantiacum* strains	3, 4, 5
Cultivation and maintenance of *P. aeruginosa* strains	4
Cultivation and maintenance of the A549 cell line	5
Construction of *S. aurantiacum* and *P. aeruginosa* strains tagged with fluorescent proteins	4
Pathogenicity testing of *S. aurantiacum* using the *Galleria mellonella* larvae model	3
Phenotype microarray (PM) analysis of different *S. aurantiacum* strains	3
Correlation of respiration (PM assays) with the growth of *S. aurantiacum* strains	3
Metabolic reconstructions of the PM data	3
Measurement of the inhibitory effect of *P. aeruginosa* on the growth of *S. aurantiacum* in both solid and liquid synthetic cystic fibrosis medium	4
Extraction of phenazines from *P. aeruginosa* strains	4
Measurement of adherence of *S. aurantiacum* to A549 cell monolayers	5
Analysis of co-culture of *S. aurantiacum* conidia and A549 cells using microscopy techniques	5
Extraction of RNA from A549 cells cultured with *S. aurantiacum*	5
Transcriptome profiling of A549 cells exposed to *S. aurantiacum*	5
Functional annotation and pathway analysis of the transcriptome of A549 cells infected with *S. aurantiacum*	5
qRT-PCR (quantitative reverse transcriptase polymerase chain reaction) validation of the transcriptomics results	5

3

PHENOTYPIC PROFILING OF *SCEDOSPORIUM AURANTIACUM*, AN OPPORTUNISTIC PATHOGEN COLONIZING HUMAN LUNGS

3.1. Introduction

S. aurantiacum is a recently identified pathogen in Australia and the majority of published studies concerned with *S. aurantiacum* either comprise clinical case reports or epidemiological studies, with no knowledge of the biochemistry and physiology of the organism. In Chapter 3, I describe the use of morphological, physiological and metabolic assessment to characterise four different strains of *S. aurantiacum* including WM 06.482, WM 09.24, WM 08.202 and WM 10.136 exhibiting different virulence levels. A difference was observed between the high and low virulence *S. aurantiacum* strains on the basis of their substrate utilisation patterns.

The results of this research were reported in a peer-reviewed paper that was published in *PLosOne* (Publication 1).

3.2. Contribution to Publication 1

This publication was developed with the co-operation of my mentors at Macquarie University (Helena Nevalainen, Anahit Penesyan and Ian Paulsen), and collaborators at Westmead Hospital (Wieland Meyer and his team Lea Vaas and Shu Yua Duan). All experiments related to the phenotypic profiling of *S. aurantiacum* strains were performed by me and the data were analysed with the assistance of Lea Vaas and Anahit Penesyan. The virulence testing of *S. aurantiacum* strains was carried out at Westmead Hospital by Shu Yao Duan. The manuscript was written by me and reviewed by Anahit Penesyan, Wieland Meyer, Ian Paulsen and Helena Nevalainen.

Table 3-1. Author contribution for Publication 1.

	JK	SD	LV	AP	WM	IP	HN
Experimental design	•				•	•	•
Data collection	•	•					
Data analysis	•		•	•			
Manuscript	•			•	•	•	•

*JK= Jashanpreet Kaur, SD= Shua Yau Duan, LV= Lea Vaas, AP= Anahit Penesyan, WM= Wieland Meyer, IP= Ian Paulsen, HN= Helena Nevalainen

3.3. Publication 1

Kaur, J., Duan, S. Y., Vaas, L. A., Penesyan, A., Meyer, W., Paulsen, I. T. & Nevalainen, H. 2015. Phenotypic Profiling of *Scedosporium aurantiacum*, an Opportunistic Pathogen Colonising Human Lungs. PLoS One, 10, e0122354.

PLOS | ONE

Phenotypic Profiling of *Scedosporium aurantiacum*, an Opportunistic Pathogen Colonizing Human Lungs

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Abstract

Genotyping studies of Australian *Scedosporium* isolates have revealed the strong prevalence of a recently described species: *Scedosporium aurantiacum*. In addition to occurring in the environment, this fungus is also known to colonise the respiratory tracts of cystic fibrosis (CF) patients. A high throughput Phenotype Microarray (PM) analysis using 94 assorted substrates (sugars, amino acids, hexose-acids and carboxylic acids) was carried out for four isolates exhibiting different levels of virulence, determined using a *Galleria mellonella* infection model. A significant difference was observed in the substrate utilisation patterns of strains displaying differential virulence. For example, certain sugars such as sucrose (saccharose) were utilised only by low virulence strains whereas some sugar derivatives such as D-turanose promoted respiration only in the more virulent strains. Strains with a higher level of virulence also displayed flexibility and metabolic adaptability at two different temperature conditions tested (28 and 37°C). Phenotype microarray data were integrated with the whole-genome sequence data of *S. aurantiacum* to reconstruct a pathway map for the metabolism of selected substrates to further elucidate differences between the strains.

Citation: Kaur J, Duan SY, Vaas LAI, Penesyan A, Meyer W, Paulsen IT, *et al.* (2015) Phenotypic Profiling of *Scedosporium aurantiacum*, an Opportunistic Pathogen Colonizing Human Lungs. PLoS ONE 10(3): e0122354. doi:10.1371/journal.pone.0122354

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Data Availability: All relevant data are within the paper and its Supporting Information files.

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Competing interests: The authors have declared that no competing interests exist.

Introduction

S. aurantiacum is a ubiquitous ascomycetous fungus found in diverse ecological niches including soil, sewage and polluted waters [1]. It has been recently added to the *S. boydii* species complex as a subset of isolates previously identified as *Scedosporium apiospermum* [2–5]. This emerging pathogen has been reported to be less susceptible to antifungals than other members of the *S. boydii* complex, such as *S. apiospermum* [6–8]. *S. aurantiacum* is an opportunistic pathogen capable of causing a wide variety of localized and superficial infections, such as malignant otitis externa, osteomyelitis, invasive sinusitis, keratitis and pneumonia [9, 10]. While *S. aurantiacum* has been associated with airway colonization in Europe, *S. aurantiacum* related infections have been reported mainly in Australia [9, 11]. Recent population-based surveys have indicated that 17.4% of sputum specimens of Australian cystic fibrosis (CF) patients contain *S. aurantiacum*. This makes *S. aurantiacum* the second most common filamentous fungus associated with CF in Australia after *Aspergillus fumigatus* [12, 13]. CF, a genetically inherited disease, is characterized by defective mucociliary clearance, which provides an ideal environment for the growth of airborne fungal conidia in the lung [14]. The colonization of the respiratory tracts of Australian CF patients by *S. aurantiacum* can possibly be attributed to its relative high abundance in the Australian environment [2].

Considering the increasing incidences of *Scedosporium* infections, and high mortality rate associated with CF, there is a need to develop treatment strategies for these fungal infections [11]. The successful development of preventative strategies is limited by the similarity between the mammalian and fungal cell structures and metabolic pathways. The majority of the work reported on *S. aurantiacum* features clinical case studies and epidemiology research, with no published literature on the physiology and biochemistry of the organism [15]. Therefore, studies relating to cell growth, viability and general metabolism can provide a good starting point to facilitate the identification of novel targets to inhibit fungal growth without affecting the human host [15, 16].

As individual cell-based growth assays are relatively slow and can be used to test only a few phenotypes at a time [17], high throughput systems have been devised for the profiling of nutrient utilization in microorganisms [16]. One such method is a phenotype microarray (PM) carried out in 96-well microtitre plates, containing a variety of nutrients (e.g. sugars), where cell viability and respiration is automatically recorded [18]. Carbon utilization profiles in some filamentous fungi including *Aspergillus*, *Neurospora*, *Hypocrea* and *Acremonium* have been studied with this approach [16, 19–21].

In this study, we have evaluated the phenotypes of four *S. aurantiacum* strains isolated from clinical and environmental sources by recording their respiration rates on 94 substrates in microtitre plate assays. The results generated from respiration-based PM assays were validated by shake flask cultivation of the strains on selected carbon sources. Data obtained from the PM assays were compared against the *S. aurantiacum* draft genome for the presence or absence of particular metabolism related genes. The *Galleria mellonella* larvae models were used to assess the virulence levels of the four *S. aurantiacum* strains studied in this work.

Materials and Methods

Scedosporium aurantiacum strains

Scedosporium strains selected for the studies were obtained from the culture collection of the Medical Mycology Research Laboratory, Centre for Infectious Diseases and Microbiology, Westmead Hospital, Sydney, Australia and included: (1) WM 06.482 isolated from the broncho-alveolar lavage of a cystic fibrosis patient in Australia; (2) WM 09.24 isolated from Sydney Circular Quay [7, 22]; (3) WM 08.202 (FMR8630; CBS116910) a type strain of *S. aurantiacum* isolated from a wound exudate of a patient in Santiago de Compostela, Spain and originally sourced from CBS culture collection (CBS-

KNAW Fungal Biodiversity Centre, Netherlands), and (4) WM 10.136 (INS1120) isolated from a valley near Innsbruck, Austria. All the strains are a part of the Australian and global MLST (Multilocus Sequence Typing) network. Potential virulence of two of the *S. aurantiacum* strains addressed in this work (WM 06.482 and WM 08.202) has previously been assessed in an immunocompetent mouse model by Harun *et al* [7].

Growth measurements

All *S. aurantiacum* strains were cultured on Sabouraud dextrose agar plates (BD, Difco™, Australia) for 5 days at 37°C to achieve sufficient growth and conidiation. Three independent plate cultures were maintained for each strain in order to establish the differences in the appearance of colonies. Conidia were harvested from the cultures using 5 ml of sterile saline (0.9% w/v NaCl and 0.01% v/v Tween 80). The suspension was then filtered through sterile glass wool to separate conidia from the hyphae. The concentration was adjusted to $1x10^6$ conidia/ml for inoculation of liquid cultures each containing 50 ml of Sabouraud's broth (Sigma Aldrich, Australia) in a 250 ml conical flask. Different flasks were maintained for each time point and all the cultures were incubated at both 28 and 37°C on an orbital shaker at 250 rpm for a total of seven days. The mycelia were collected from the growing cultures by taking out one flask after every 12 hours, filtering the content through pre-weighed Whatman filter paper number 1 and drying in a vacuum oven at 70°C to a constant weight. Dry weight was calculated as the difference between the weight of the filter paper with and without the mycelia.

Pathogenicity testing using a *Galleria mellonella* larval model

Pathogenicity of the four *S. aurantiacum* strains was assessed using the invertebrate *G. mellonella* infection model [23]. Conidia were washed twice and diluted in PBS (phosphate-buffered saline) to a final concentration of 10^6 conidia/ml in an inoculum. *G. mellonella* larvae were obtained after the oviposition of the adult moths reared and maintained at 26°C and 60% relative humidity in the insectarium of the Westmead Hospital Animal Care Facility, Sydney, Australia. Ten similar sized larvae were weighed (about 3000 mg each) and placed in a 90 mm plastic Petri dish. Fungal inoculum (10 μl) was then injected into the last left pro-leg of the hemocoel of each larva using a 50 U syringe with a 29-gauge needle. Two different controls were also included in the assay: a group of 10 larvae inoculated with PBS to monitor potential effects on survival due to physical injury, and 10 untreated larvae. After injection, the larvae were incubated in Petri dishes at 35°C for 10 days and checked daily for survival. Larvae were considered dead when they were dark coloured and failed to respond to physical stimuli applied with a forceps. Survival of the larvae against each fungal strain was plotted after performing statistical analysis using Graph Pad Prism 6 (La Jolla, CA, USA).

Phenotype microarray

Biolog Phenotype analysis was carried out for all four *S. aurantiacum* strains using GEN III MicroPlate™ (Biolog Inc, USA) containing 94 assorted substrates (sugars, amino acids, hexose acids and carboxylic acids) and a positive and negative control (S1 Fig.). Fungal conidial suspensions ($1x\ 10^5$/ml) were prepared in the inoculating fluid (IF, Biolog, USA) and 100 μl of the inoculum was dispensed in three replicates into each well of the plate using a multichannel pipette (Biolog). After inoculation, the plates were incubated in the OmniLog incubator/reader (Biolog) for 72 hours at 28°C and 37°C. Cell respiration was recorded every 15 minutes by a charge-coupled device camera and plotted as a kinetic curve depicting reduction of the colorless tetrazolium blue dye to violet (formazan) by cell respiration. Raw values were imported from the OmniLog reader and analyzed using R package software opm [24]. This resulted in two datasets which comprised four strains x three replicates x two measurement temperatures x 94 substrates giving rise to 2256 individual cell respiration curves. Classification of phenotypes was performed based on the maximal curve height calculated as Omni Log units; an Omni Log value greater than 100 was considered as a positive phenotype. Comparison of substrate utilization in different strains was carried out using heat maps, which classified the strains based on estimation of the maximum height of the cell respiration curve [24].

Correlation of growth and respiration rates

Growth as biomass formation was determined for the four *S. aurantiacum* strains in shake flask cultivations on selected carbon sources also used in the Biolog respiration assay. Fungal conidia (1 x 10^5/ml) were inoculated into 50 ml of M9 minimal medium (Sigma), supplemented with a selected carbon source (1 mM) in a 250 ml shake flask and incubated at 37°C and 200 rpm for 60 hours. Carbon sources tested included maltose, D-trehalose, sucrose, D-turanose, D-salicin, D-glucose and D- fructose. All compounds were obtained from Sigma-Aldrich, dissolved in sterile water and filter sterilized. Cell dry weight was measured at the end of the incubation period as described above, and plotted against the OmniLog units obtained from the respiration curves. Each compound was tested in biological triplicate.

Metabolic pathway analysis and genome correlation

Sequencing of the genomes of the four *S. aurantiacum* strains used in this study has been completed recently with pending annotation [25]. Therefore, information on the putative pathways and enzymatic steps involved in the metabolism of the selected sugars was extracted from KEGG and MetaCyc and additional literature searches [26]. Amino acid translations of the gene sequences involved in the metabolic pathways of interest were identified from closely related reference organisms including *Trichoderma reesei*, *Aspergillus fumigatus*, *Penicillium chrysogenum* and *Neurospora crassa* and mapped back into the *S. aurantiacum* genome data using the tblastn program of BLAST algorithm [27]. Biological function was assigned for each gene encoding a particular enzyme in the predicted pathway based on the homology between the genome sequences for each *S. aurantiacum* strain and reference gene sequences [28, 29].

Results and Discussion

Growth pattern of *Scedosporium aurantiacum*

The first phenotypic feature that separated the four *S. aurantiacum* strains was the appearance of colonies on Sabouraud's agar. The color of the colonies varied from greyish white in WM 06.482, white in WM 08.202, suede-like in WM 10.136 and brownish-white in case of WM 09.24 (Fig. 1). All strains were slow-growing and produced a light yellow pigment on the reverse of the agar plates after 14 days of incubation.

Fig 1. Colony morphology of the four different strains of *S. aurantiacum* growing on Sabouraud dextrose agar plates after 14 days of incubation.

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Submerged cultures were grown in triplicate to establish a growth pattern for each strain. Growth of *S. aurantiacum* can be divided into four phases: lag, 0 to 36 hr; log, 36 to 48 hr; stationary, 48 to 72–84 hr and death after 84 hr (Fig. 2). A shorter lag phase (0–24 hours) was observed for the environmental strains (WM 09.24 and WM 10.136) whereas the clinical strains (WM 06.482 and WM 08.202) showed a longer lag phase, with the first significant change in the dry weight after 48 hours of incubation. As a general trend, the average mycelial dry weight reached its maximum in the log phase and decreased thereafter. WM 08.202 (type strain) was the slowest growing *S. aurantiacum* strain and also produced least biomass as compared to the others (Fig. 2).

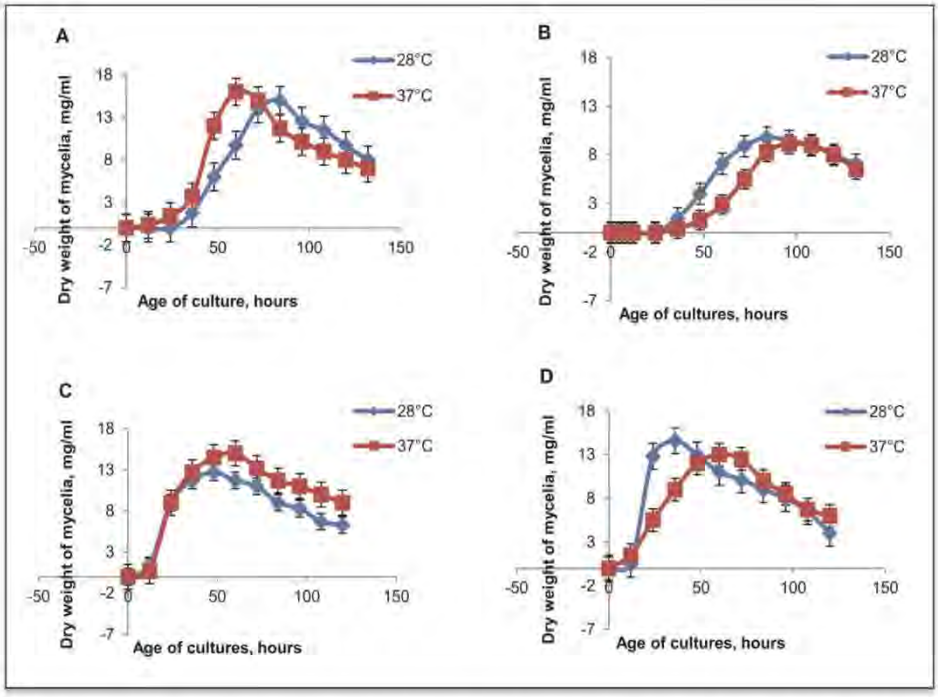

Fig 2. Growth of the *S. aurantiacum* strains in liquid culture in Sabouraud's broth, measured as change in the mycelial dry weight (mg/ml) over time. A) WM 06.482, B) WM 08.202, C) WM 09.24 and D) WM 10.136. Each experiment was repeated in triplicate, with bars representing ±1.25 standard errors, and 95% confidence interval.

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Temperature had an impact on the growth of *S. aurantiacum* strains. WM 06.482 (clinical isolate) and WM 09.24 (environmental isolate) exhibited better growth at 37°C (as seen in Fig. 2), but were able to adapt to the lower temperature (28°C). The slowest growing strain WM 08.202 (clinical isolate) showed equal mycelial dry weights at 28°C and 37°C and strain WM 10.136 (environmental isolate) preferred 28°C, at which temperature the growth was fast and efficient. All four strains tested were able to adapt to the mammalian body temperature *i.e.* 37°C. Adaptation to various culture conditions, especially tolerance of mammalian temperatures (37°C) is a well-established phenomenon in other opportunistic fungal pathogens such as *Cryptococcus neoformans* [30].

Ranking the *S. aurantiacum* strains according to virulence

Virulence levels of the four *S. aurantiacum* isolates were explored using the *G. mellonella* larvae invertebrate model that has been previously used to assess the virulence of different strains of the human fungal pathogen *Candida albicans* [31]. Survival of larvae infected with the different strains is shown in Fig. 3. No larval death was observed in any of the control groups i.e. non-treated larvae and larvae inoculated with PBS.

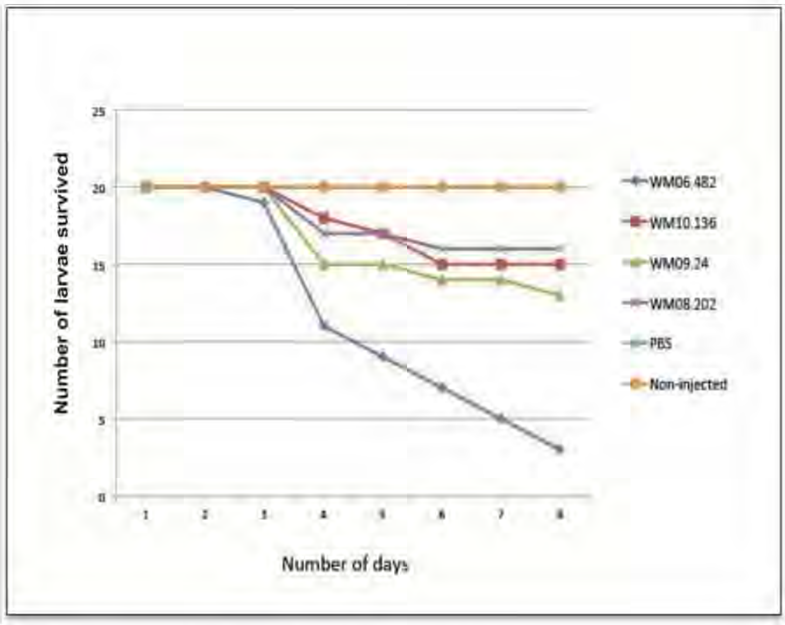

Fig 3. Survival of *G. mellonella* larvae infected with different strains of *S. aurantiacum*.

doi:10.1371/journal.pone.0122354.g003

As seen in Fig. 3, all *S. aurantiacum* strains were able to kill larvae; WM 06.482 (clinical isolate) was the most virulent among the strains tested, as the majority (85%) of the larvae failed to survive after eight days of infection and WM 08.202 (type strain isolated from a wound exudate) had the least effect on larval mortality (20%) The environmental strains, namely WM 09.24 and WM 10.136, killed approximately 40% and 25% of the larvae, respectively, within eight days.

While the *G. mellonella* model did clearly separate the strains with highest (WM 06.482) and lowest virulence (WM 08.202 and WM 10.136), the nature of WM 09.24 is less clear. Nevertheless, from this analysis, WM 09.24 was the second most virulent strain of the four *S. aurantiacum* strains tested. Similar to previous studies [11, 32] the virulence levels observed for different *S. aurantiacum* strains used in this study were independent of the origin of the strain as the environmental isolate WM 10.136 had similar virulence properties as the clinical strain WM 08.202.

Carbon utilization by *S. aurantiacum* strains

The virulence levels of *S. aurantiacum* strains can be attributed to the physiological differences [33], analysis at the phenotypic level can help to further understand the mechanisms of pathogenicity in this organism [34, 35]. Thus, utilization

of a variety of nutrients (especially carbon sources) by the four *S. aurantiacum* strains was tested using an automated high throughput Biolog assay. The opm package for R was used for data analysis as it provides a range of benefits such as visualization and curve parameter estimation, metadata management, customizable plots and automated generation of tabular reports [36].

All four strains were able to respire on 54 out of the 94 substrates displayed on the GenIII microplates, including a range of sugars, a few amino acids and hexose acids at either 28 or 37°C. Temperature-based differences were observed specifically, when comparing the utilization of the most common substrate groups such as carbohydrates, carboxylic and amino acids. For example, maltose, D-salicin, D-fructose and lactose were utilized by two *S. aurantiacum* strains namely WM 08.202 and WM 10.136 only at 37°C. This is different from both WM 06.482 and WM 09.24, which could respire on these substrates at both 28 and 37°C thereby showing a similar metabolic response to the cultivation temperature (Fig. 4). Adaptation to the imposed temperature conditions has been described as an essential attribute of many highly virulent pathogens [37, 38].

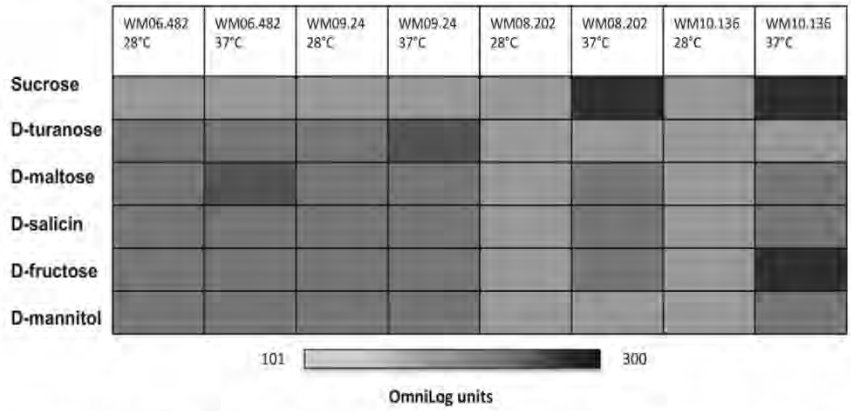

Fig 4. Different sugar utilization patterns of low and high virulent strains. OmniLog units with a minimum value of 100 depict a positive phenotype.

doi:10.1371/journal.pone.0122354.g004

Carbon acquisition and metabolism is central to the virulence and persistence of many lung pathogens and can be used to distinguish different strains within the species [34]. For example, difference in assimilation of five different carbon sources (ribitol, L-arabinitol, sucrose, maltose and ribose) was used to discriminate between the species. *S. aurantiacum* was distinguished from other members of the complex based on its inability to use sucrose as a substrate for growth [4]. In this study, two strains of *S. aurantiacum* (WM 06.482 and WM 09.24) were unable to metabolize sucrose but instead showed higher cellular respiration on its isomeric form turanose. While the outcome may seem surprising at the first glance, however there are previous studies proposing utilization of turanose as a potential indicator for virulence [39]. Turanose is known to promote high level of mycelial growth in the plant pathogen *Fusarium virguliforme*, and activates defense responses in higher plants suggesting a possible association between turanose assimilation and pathogenic properties [40–42]. High respiration rates on sucrose in WM 08.202 and WM 10.136 point towards the presence of a functional sucrose utilization pathway.

Correlation of growth and respiration assays

Cell respiration generally correlates with cell growth. In order to confirm the cellular respiration results obtained in the Biolog assay, growth of *S. aurantiacum* strains was determined in liquid cultures on a minimal medium supplemented with a panel of selected carbon compounds, incubated at 28°C (Fig. 5A) and 37°C (Fig. 5B). In accordance with the PM data, WM 06.482 and WM 09.24 showed growth on maltose, D-trehalose, D-salicin and D-fructose at both temperatures (28 and 37°C), whereas WM 08.202 and WM 10.136 were able to grow on these substrates only at high temperature (37°C).

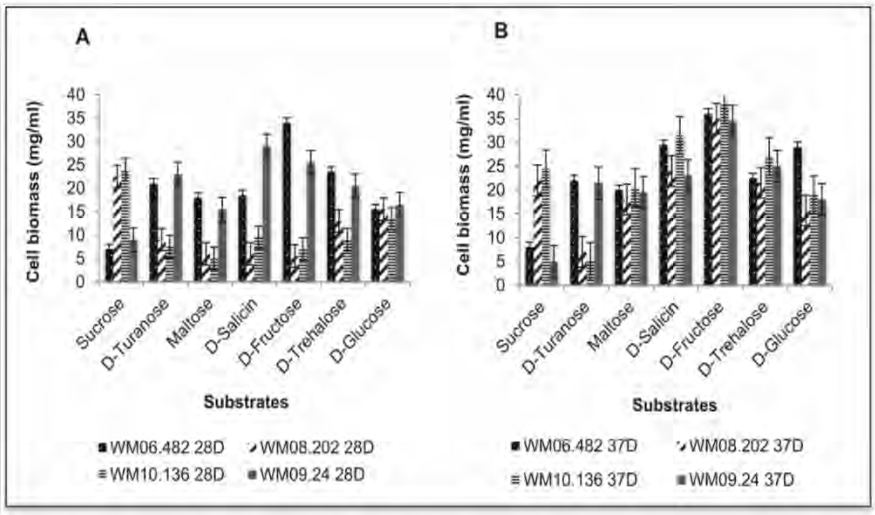

Fig 5. Relative growth of *S. aurantiacum* in minimal medium with different carbon sources, measured as average dry weight of mycelia at a) 28°C and b) 37°C. A different pattern is assigned to each strain as shown in the key below. The graphs clearly distinguish between the growth patterns of high and low virulent strains on selected carbon sources at both temperatures. (Weight of the inoculum was 10 mg/ml. Therefore biomass value above this limit was considered as significant growth). Each experiment was repeated in triplicate, with bars representing ±1.25 standard errors, and 95% confidence interval.

doi:10.1371/journal.pone.0122354.g005

Growth in the shake flasks was consistent with cell respiration on all six tested substrates as shown by plotting the cell biomass against the OmniLog units. Examples of this correlation at 37°C are shown in Fig. 6A for sucrose and Fig. 6B for turanose. *S. aurantiacum* strains WM 06.482 and WM 09.24 that showed no respiration on sucrose in the Biolog assay also did not show any growth in the minimal medium supplemented with sucrose. Instead, these strains grew well on turanose which is in accordance with efficient respiration measured in the PM assay on this substrate at 37°C. In contrast, the established low virulence strains WM 08.202 and WM 10.136 exhibited good respiration and growth on sucrose. Overall, the growth assays in liquid media were concordant with the Biolog phenotype microarray data, thus validating the respiration assay and illustrating its usability as a high-throughput phenotype assay for *Scedosporium*. These findings suggest that common characteristics were shared between two groups of *S. aurantiacum* strains WM 06.482 and WM 09.24; and WM 08.202 and WM 10.136 as reflected in their substrate utilization patterns.

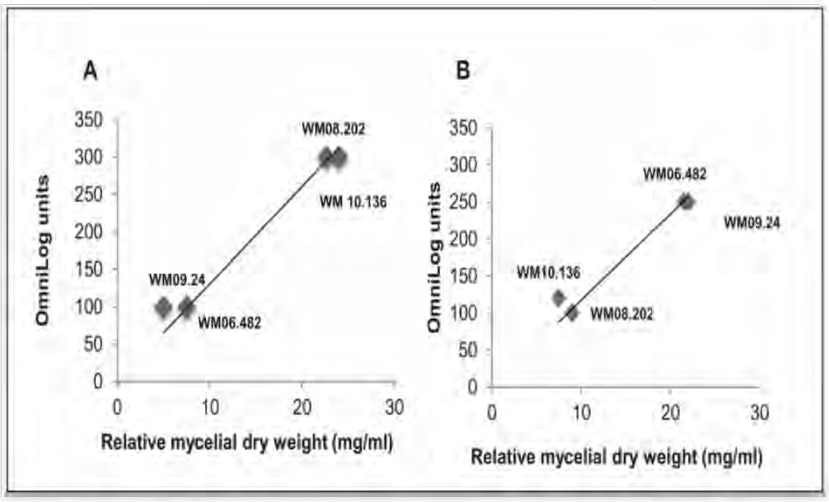

Fig 6. Correlation between cell growth and cell respiration for sucrose and turanose for *S. aurantiacum* at 37°C. Cell biomass was calculated for all the strains grown in sucrose and turanose supplemented minimal media and plotted against the respiration rates (OmniLog units). a) 1mM sucrose; b) 1mM turanose.

doi:10.1371/journal.pone.0122354.g006

Resistance to selected chemicals

PM analysis revealed that all four strains of *S. aurantiacum* were highly halotolerant as high respiration rates were observed on high sodium chloride concentrations (4% and 8%). The salt tolerance capacity of *S. aurantiacum* is close to the optimum salt concentration required for the growth of the halophilic black yeast *Hortaea werneckii*, the most salt tolerant eukaryotic organisms reported to date [43]. Saline resistance could be one of the reasons for the persistence of *S. aurantiacum* in the salt-rich airway fluid of CF patients [44]. CF patients have abnormal salt transport across the airway epithelium, which causes defective mucociliary clearance and reduced clearance of the infectious agents [45]. Similarly, resistance of *S. aurantiacum* to other chemical treatments such as nalidixic acid and low pH under different temperature conditions can explain their pervasive nature and ability to survive under extreme environmental conditions [46].

The effect of amino acids on growth

The phenotype assays revealed some hexose acids, carboxylic acids, esters and fatty acids that resulted in slow or no growth. Examples of such compounds are aspartic acid, D-serine, L-histidine, L-pyroglutamic acid, D-galacturonic acid, L-galactonic acid-g-lactose, D-gluconic acid, D-glucuronic acid, mucic acid, D-saccharic acid, D-lactic acid methyl ester, a-keto-glutaric acid, D-malic acid and sodium formate. Some of these compounds such as D-lactic methyl ester that caused slow growth or no growth at all have been considered as inhibitors of the spore germination process *e.g.* in *Hypocrea jecorina* [21]. Given the high resistance of *S. aurantiacum* to many antifungal agents, similar combined strategies can be devised for screening the potential inhibitors against this pathogenic fungus.

Metabolic reconstructions using the *S. aurantiacum* draft genomes

Metabolic reconstructions were carried out for all the four *S. aurantiacum* strains by ascribing the observed phenotypic differences to the presence or absence of certain enzyme encoding genes in a particular carbohydrate utilization pathway. The main metabolic pathways found in the analysis were then used for the generation of biochemical maps (Fig. 7) that show the network of genome-encoded enzymes catalyzing the metabolism of different carbohydrates in the four different *S. aurantiacum* strains studied in this work. Five major carbohydrate metabolism pathways were revealed in the genome-wide analysis of *S. aurantiacum*, namely sucrose, fructose, mannose and galactose metabolism and glycolysis (Fig. 7).

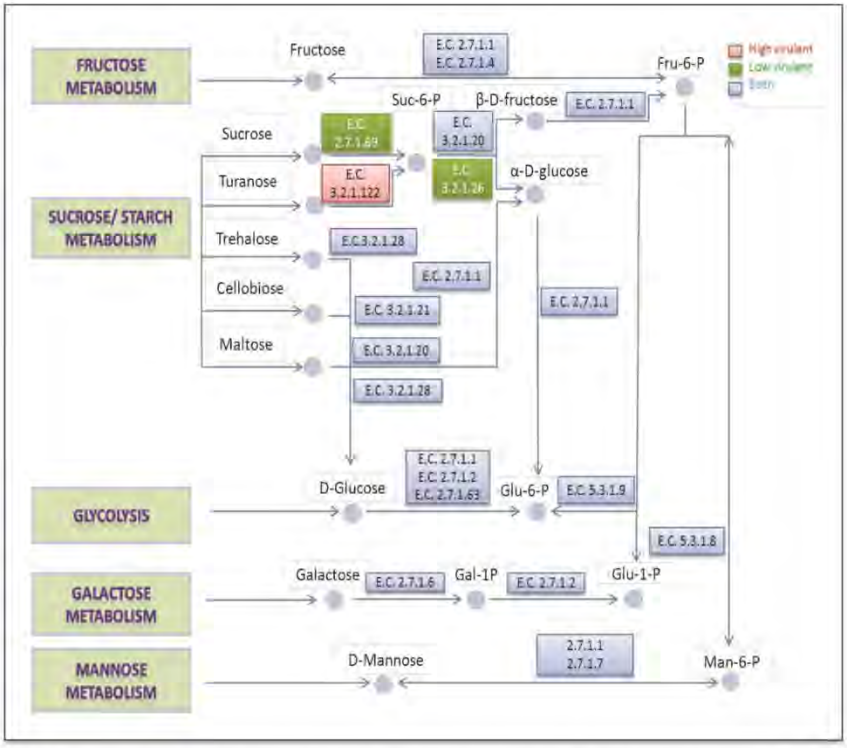

Fig 7. Schematic overview of various metabolic pathways present in *S. aurantiacum* obtained after superimposition of PM data with the assembled genome.

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Sucrose metabolism can involve either one-step or a two-step reaction depending on the enzymes involved in the overall process. In a two-step reaction common in many filamentous fungi, sucrose is converted to sucrose-6-phosphate by the phosphotransferase enzyme and ultimately to the end product glucose in a reaction catalyzed by invertase [47, 48]. Alternatively, sucrose can be directly hydrolyzed to glucose solely by invertase. Based on the metabolic pathways reconstructed from genome data, only the low virulence strains WM 08.202 and WM 10.136 seemed to possess both the phosphotransferase (E.C. 2.7.1.69) and invertase (E.C. 3.2.1.26) enzymes which allowed them to utilize sucrose for growth and respiration.

On the other hand, inability of WM 06.482 and WM 09.24 to grow/respire on sucrose (as seen in Fig. 4 and 6) can be explained by the absence of sucrose-hydrolysing enzymes as revealed by the genome correlation. The assimilation of turanose in these strains can be attributed to the presence of phospho-alpha-glucosidase (E.C. 3.2.1.122) which was not found in the low virulence strains. The other three metabolic pathways studied including fructose, galactose and mannose metabolism were similar in all four *S. aurantiacum* strains. Thus phenotypic testing allowed us to specifically search for the genetic factors underpinning the phenotypes of different strains of *S. aurantiacum*.

In principle, the presence or absence of respective enzymes in different *S. aurantiacum* strains can be verified by amplification of the gene sequences from the genomic DNA. However, considering the non-availability of a fully annotated *S. aurantiacum* genome and the limited overall sequence homology (~40%) for the identified gene sequences between *S. aurantiacum* and the reference organisms, this methodology can lead to inconclusive results. Nevertheless, the ability of the strains to metabolize the discussed carbohydrates is a strong, yet indirect indication for the presence of genes encoding the required enzymes [49].

Conclusions

We have used morphological, physiological and metabolic assessment to characterize four different *S. aurantiacum* strains exhibiting different virulence levels. The analysis helped to identify metabolic differences between two groups of *S. aurantiacum* strains, WM 06.482 and WM 09.24; and WM 08.202 and WM 10.136. Correlation of the genome information with the metabolic assessment assisted in exposing five putative carbohydrate metabolism pathways of which sucrose and D-turanose utilization different between the above *S. aurantiacum* groups. While classification of the environmental strain WM 09.24 as a high or low virulence strain was not straightforward from the *Galleria mellonella* assay, it behaved similar to the high virulent strain WM 06.482 for which the virulence has also been established in a mouse model. Therefore we group WM 09.24 together with WM 06.482, which leads us to speculate on metabolic differences between high and low virulence strains, such as ability to utilize D-turanose. The differences can be investigated further with fully annotated genomes available in the near future.

Supporting Information

S1 Fig. Layout of biolog GenIII plate. Layout of the Biolog GenIII plate depicting various conditions/substrates used to detect substrate utilization of *S. aurantiacum* strains WM06.482, WM08.202, WM10.136 and WM09.24. The various substrates listed can be categorized as follows: 1) Control: A1. 2) Sugars: A2-A9, B1-B9 and C1-C9. 3) Hexose phosphates: from D06 and D07. 4) Amino acids: from E1-E9. 5) Hexose acids: from F1-F9. 6) Carboxylic acids, esters and fatty acids: G1-G9 and H1-H9. 7) Acidic pH: A11 and A12. 8) NaCl: B10-B12. 9) Lactic acids: C10. 10) Reducing agents: F11 and F12. 11) Gram negative/gram positive: F10 and G10.
(TIFF)

Author Contributions

Conceived and designed the experiments: JK SD LV AP WM IP HN. Performed the experiments: JK SD. Analyzed the data: JK LV AP. Wrote the paper: JK HN.

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4

PSEUDOMONAS AERUGINOSA INHIBITS GROWTH OF *SCEDOSPORIUM AURANTIACUM*, AN OPPORTUNISTIC FUNGAL PATHOGEN ISOLATED FROM LUNGS OF CYSTIC FIBROSIS PATIENTS

4.1. Introduction

The lungs of a CF patient serve as a niche for a wide variety of microorganisms including bacteria and fungi. Bacteria such as *P. aeruginosa* are the most predominating species commonly isolated from the sputum samples of CF patients. Numerous reports can be found concerning interactions between *P. aeruginosa* and major fungal lung pathogens such as *A. fumigatus*, *C. neoformans* and *C. albicans*. However until now, interactions between *P. aeruginosa* and *S. aurantiacum* have not been studied. In Chapter 4, I aimed to explore the effect of *P. aeruginosa* on the growth of *S. aurantiacum* in synthetic cystic fibrosis medium (SCFM) that mimics the nutritional contents of human sputum.

The results from the work are reported in the form a manuscript that has been published in the journal *Frontiers in Microbiology*.

4.2. Contribution to Publication 2

The work presented in this chapter was the product of collaboration between the two different groups working with a) *S. aurantiacum* (Helena Nevalainen, Anwar Sunna, Junior Te'o, Liisa Kautto, Minkyoung Kim, Bhavin Pethani and Jashanpreet Kaur) and b) *P. aeruginosa* (Ian Paulsen, Anahit Penesyan and Sheemal Kumar) at Macquarie University. I worked very closely with all the researchers mentioned above in building the conceptual design of this research. All experiments related to the co-culture of *P. aeruginosa* and *S. aurantiacum* in the solid and liquid media were conducted by me. The effect of *P. aeruginosa* cell fractions including cell supernatant, cell lysate and heat inactivated cells against *S. aurantiacum* was checked by Bhavin Pethani. The vector for genetic transformation of *S. aurantiacum* was developed by Minkyoung Kim and myself under the guidance of Junior Te'o and the transformation procedure and further confirmation of transformants through confocal microscopy was carried out by me. The genetic transformation of *P. aeruginosa* was undertaken by Sheemal Kumar. The phenazine extraction from all *P. aeruginosa* strains was conducted by me. All the data was analysed by me with the assistance of Liisa Kautto and Anahit Penesyan. The manuscript was prepared by me and reviewed by Anwar Sunna, Liisa Kautto, Anahit Penesyan, Ian Paulsen and Helena Nevalainen.

Table 4-1. Author contribution summary for chapter 4.

	JK	BP	SK	MK	AS	LK	AP	JT	IP	HN
Experimental design	•	•	•	•	•	•	•	•	•	•
Data collection	•	•	•	•						
Data analysis	•					•	•			•
Manuscript	•				•	•	•		•	•

*JK= Jashanpreet Kaur, BP= Bhavin Pethani, SK= Sheemal Kumar, MK= Minkyoung Kim, AS= Anwar Sunna, LK= Liisa Kautto, AP= Anahit Penesyan, JT= Junior Te'o, IP= Ian Paulsen, HN= Helena Nevalainen

4.3. Publication 2 (Manuscript)

Kaur, J., Pethani, B. P., Kumar, S., Kim, M., Sunna, A., Kautto, L., Penesyan, A., Paulsen, I. & Nevalainen, H. 2015. *Pseudomonas aeruginosa* inhibits the growth of *Scedosporium aurantiacum*, an opportunistic fungal pathogen isolated from the lungs of cystic fibrosis patients. *Frontiers in Microbiology,* 6**,** 866.

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Pseudomonas aeruginosa inhibits the growth of *Scedosporium aurantiacum*, an opportunistic fungal pathogen isolated from the lungs of cystic fibrosis patients

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The filamentous fungus *Scedosporium aurantiacum* and the bacterium *Pseudomonas aeruginosa* are opportunistic pathogens isolated from lungs of the cystic fibrosis (CF) patients. *P. aeruginosa* has been known to suppress the growth of a number of CF related fungi such as *Aspergillus fumigatus*, *Candida albicans*, and *Cryptococcus neoformans*. However, the interactions between *P. aeruginosa* and *S. aurantiacum* have not been investigated in depth. Hence we assessed the effect of *P. aeruginosa* reference strain PAO1 and two clinical isolates PASS1 and PASS2 on the growth of two clinical *S. aurantiacum* isolates WM 06.482 and WM 08.202 using solid plate assays and liquid cultures, in a synthetic medium mimicking the nutrient condition in the CF sputum. Solid plate assays showed a clear inhibition of growth of both *S. aurantiacum* strains when cultured with *P. aeruginosa* strains PASS1 and PAO1. The inhibitory effect was confirmed by confocal microscopy. In addition to using chemical fluorescent stains, strains tagged with yfp (*P. aeruginosa* PASS1) and mCherry (*S. aurantiacum* WM 06.482) were created to facilitate detailed microscopic observations on strain interaction. To our knowledge, this is the first study describing successful genetic transformation of *S. aurantiacum*. Inhibition of growth was observed only in co-cultures of *P. aeruginosa* and *S. aurantiacum*; the cell fractions obtained from independent bacterial monocultures failed to initiate a response against the fungus. In the liquid co-cultures, biofilm forming *P. aeruginosa* strains PASS1 and PAO1 displayed higher inhibition of fungal growth when compared to PASS2. No change was observed in the inhibition pattern when direct cell contact between the bacterial and fungal strains was prevented using a separation membrane suggesting the involvement of extracellular metabolites in the fungal inhibition. However, one of the most commonly described bacterial virulence factors, pyocyanin, had no effect against either of the *S. aurantiacum* strains. This study shows that *P. aeruginosa* has a substantial inhibitory effect on the growth of the recently described CF fungal pathogen *S. aurantiacum*. The findings also highlighted that *P. aeruginosa* biofilm formation is important but not crucial for inhibiting the growth of *S. aurantiacum* in a lung- mimicking environment.

Keywords: co-culture, *S. aurantiacum*, *P. aeruginosa*, interactions, growth inhibition, phenazines, SCFM, biofilms

Introduction

Cystic fibrosis (CF) is one of the most common, potentially lethal, genetically inherited disorders affecting mainly the European Caucasian population (O'Sullivan and Freedman, 2009). Although the disease affects a number of organs and systems in the human body, lungs remain the main site of infection in CF patients (Quinton, 1999). The inherited condition stems from the mutation of the CF transmembrane conductance regulator (CFTR) gene, which regulates the transport of chloride ions across the plasma membrane of the epithelial cells (Boucher, 2007). Impaired ion exchange reduces the mucociliary clearance, which leads to accumulation of hyper-viscous mucus in the airway surfaces, thus providing ideal conditions for the growth of microorganisms (Delhaes et al., 2012). Various molecular and microbiology based approaches have revealed the polymicrobial nature of the infections in CF with the identification of complex microbiota including bacteria, fungi, and viruses (Lynch and Bruce, 2013). Most of these microorganisms are either acquired from the environment or through contact with other infected patients (Lipuma, 2010).

Bacteria constitute the major portion of the microorganisms associated with CF. The most common bacterial inhabitants of the CF airways include *Haemophilus influenzae*, *Staphylococcus aureus*, *Pseudomonas Aeruginosa*, and *Burkholderia cepacia* complex (BCC) (Harrison, 2007; Lipuma, 2010). Among them, *P. aeruginosa* is the most dominant bacterial species known to cause chronic respiratory infections in more than 50% of adult CF patients (Coutinho et al., 2008). *P. aeruginosa* is a ubiquitous Gram-negative bacterium possessing a wide variety of pathogenicity factors to evade the host defense system (Davies, 2002). During the early stages of infection, the bacterium attaches itself to lung epithelial cell surface receptors through specific adhesins and secretes extracellular products to prolong its survival in the CF airways (Tang et al., 1995). The extracellular products secreted by *P. aeruginosa* include enzymes such as elastase and alkaline protease, exotoxins, siderophores, and phenazines such as pyocyanin with a known role in virulence (Haas et al., 1991). Moreover, *P. aeruginosa* cells form biofilms in order to proliferate inside the lungs and protect themselves from antibiotic agents (Singh et al., 2000).

In addition to bacteria, some fungal species are also known to colonize the respiratory tracts of CF patients (Cimon et al., 2000; Pihet et al., 2009). Mycological examination of the specimens obtained from CF patients have shown that *Aspergillus fumigatus* is the most predominant fungal colonizer of the CF lungs as it has been recovered from 6 to 71% of CF patients (Bakare et al., 2003; Horre et al., 2010). However, the presence of non-*Aspergillus* fungal species often remains unnoticed owing to the lack of sensitive culture techniques to examine the sputum specimens from CF patients (Delhaes et al., 2012). Recently, a more targeted approach has been developed by combining molecular techniques with laboratory culture methods, which can now identify a wide range of fungal pathogens in the expectorated sputa (Middleton et al., 2013). Studies conducted on CF patients in Australia and certain parts of Europe have confirmed the emergence of a new fungal genus *Scedosporium* (originally called *Pseudallescheria*) that causes infections in the lungs of immunocompromised hosts (Blyth et al., 2010a; Paugam et al., 2010; Lackner et al., 2014). *Scedosporium* sp. have been isolated from the sputum specimens of 14.7–17.4% of Australian CF patients which makes it the second most common fungal respiratory pathogen associated with CF (Blyth et al., 2010a,b). *Scedosporium aurantiacum* is a recently identified, highly virulent member of the *Scedosporium* sp. complex recovered from one in six CF patients in Sydney (Heath et al., 2009; Blyth et al., 2010b; Harun et al., 2010). The clinical consequences of the *S. aurantiacum* colonization or infections in the CF patients remain to be explored (Harun et al., 2010).

According to the clinical reports, the prevalence of fungi in the respiratory tracts of CF patients is mainly affected by the bacteria present, and the interactions between the bacteria and fungi potentially impact the disease outcome (Sibley et al., 2006; Chotirmall et al., 2010; Leclair and Hogan, 2010). Several *in vitro* studies have reported an inhibitory effect of *P. aeruginosa* against the common lung co-inhabitants such as *A. fumigatus* or the yeasts *Candida albicans*, and *Cryptococcus neoformans* (Hogan and Kolter, 2002; Bandara et al., 2010; Cugini et al., 2010). Similar data for *S. aurantiacum* are lacking. Reflecting the increasing importance of *S. aurantiacum* in CF, we examined the effect of clinical *P. aeruginosa* CF isolates PASS1 and PASS2 and laboratory reference strain PAO1 on the growth of two clinical *S. aurantiacum* isolates WM 06.482 and WM 08.202 using solid plate assays and liquid co-cultures containing medium that mimics the nutritional content of human CF sputum (Palmer et al., 2007).

Materials and Methods

Growth and Maintenance of Strains

Strains used in the study are listed in **Table 1**. *P. aeruginosa* PASS1 and PASS2 were isolated from the sputum samples of CF patients (Penesyan et al., under review). A common laboratory 'reference' strain PAO1 (Lewenza et al., 2014) was also included in the study. *S. aurantiacum* strains WM 06.482 and WM 08.202 were obtained from the culture collection of the Medical Mycology Research Laboratory, Centre for Infectious Diseases and Microbiology, Westmead Hospital, Sydney, NSW, Australia (Kaur et al., 2015). Virulence levels of all *P. aeruginosa* strains used in this study have been tested previously using *Caenorhabditis elegans* based infection model (Lewenza et al., 2014; Penesyan et al., under review). Virulence studies of *S. aurantiacum* have been performed using *Galleria mellonella* larvae model (Kaur et al., 2015).

Pseudomonas aeruginosa strains were revived from frozen stocks stored at −80°C by streaking on LB (Luria Bertani, Sigma) plates and incubation overnight at 37°C. Bacterial colonies were inoculated into LB broth and incubated at 37°C on an orbital shaker (200 rpm) overnight. Following fractions were prepared from overnight cultures of the *P. aeruginosa* strains: (1) Heat killed cells were obtained by incubating 1 ml of an overnight cell culture at 80°C for 60 min. Absence of any viable cells was confirmed by plating on LB agar medium; (2)

TABLE 1 | *Pseudomonas aeruginosa* and *Scedosporium aurantiacum* strains used in the study.

Strain	Strain name	Source	Virulence level	Reference
PASS1	*P. aeruginosa*	Sputum sample of a cystic fibrosis (CF) patient Sydney, NSW, Australia	High	Penesyan et al. (under review)
PASS2	*P. aeruginosa*	Sputum sample of a CF patient Sydney, NSW, Australia	Low	Penesyan et al. (under review)
PAO1 (ATCC 15692)	*P. aeruginosa*	Wound exudate Melbourne, VIC, Australia	High	Holloway (1955)
WM 06.482	*S. aurantiacum*	Invasive clinical isolate from CF patient Sydney, NSW, Australia	High	Kaur et al. (2015)
WM 08.202	*S. aurantiacum*	Type strain from a wound exudate Santiago de Compostela (Spain)	Low	Kaur et al. (2015)

Cell lysates were obtained after sonicating the cells (50 ml) on ice for 10 min in an ultrasonic processor followed by collection of the supernatant after centrifugation at 10,000 × *g* for 30 min; (3) Cell culture supernatants were collected by centrifuging 50 ml of overnight cultures of *P. aeruginosa* strains at 10,000 × *g* for 30 min. Supernatants were then freeze dried and resuspended in 100 µl of 1x PBS and stored at 4°C until use.

Fungal strains were maintained on PDA (potato dextrose agar, BD, Difco™) plates at 37°C. After 5 days of growth, the conidia were scraped into sterile saline solution (0.9% w/v NaCl and 0.01% v/v Tween 80) and the suspension was filtered through a sterile cotton wool to separate the conidia from the hyphal debris. Conidia were washed with 1x PBS to remove traces of saline and the inoculum was adjusted to a McFarland standard concentration of 2.5×10^5 conidia/ml. Concentration of conidia was confirmed using Neubauer counting chamber and additional plate counting.

Construction of Strains Tagged with Fluorescent Proteins

Pseudomonas aeruginosa Strain Expressing Yellow Fluorescent Protein (YFP)

Plasmid pUCP*yfp* (Gloag et al., 2013) encoding yellow fluorescent protein (YFP) was used to transform the *P. aeruginosa* PASS1 strain. In order to make electrocompetent cells, PASS1 was cultured in 5 ml of LB broth overnight at 42°C and 200 rpm. Cells were harvested by centrifugation (14,000 *g* for 15 min at 4°C) and the ionic strength of the suspension was reduced by rigorous washing with 1x M9 minimal salts medium (Sigma) followed by two washes with ice-cold sterile milliQ water. Bacterial cells were transformed by electroporation as described by Dower et al. (1988) by adding 1 µg of the plasmid DNA to 20 µl of the washed cell aliquots. At the end of the procedure, cells were streaked on LB plates containing 8 mg/ml ampicillin and incubated for up to 48 h at 37°C to select for the transformants.

Construction of the *S. aurantiacum* Strain Expressing mCherry

The *mCherry* gene was PCR amplified from the pmcherry-c1 vector (Clontech Laboratories, USA) using *mCherry.fwd* and *mCherry.rev* primers (**Table 2**) and was expressed under the *Trichoderma reesei* pyruvate kinase (*pki*) promoter, which was amplified from the pCBH1corlin vector (Te'o et al., 2000) using *pki.fwd* and *pki.rev* primers. In addition,

TABLE 2 | Sequence of primers used for the construction of transformation cassettes.

Primer name	Sequence (5′–3′)
mCherry. fwd	**GAA GAACCT CTT AAC CTC TAG (*pki* sequence)** ATG GTG AGC AAG GGC GAG G
mCherry. rev	CAT GCG GGT ACC (*Kpn*I) CTA TTA CTT GTA CAG CTC GTC CAT GC
pki. fwd	TGC TGC GAT ATC (*EcoR*V) CTT AAG TTA G TA ACT AGT GGA TC
pki.rev	**CTC GCC CTT GCT CAC CAT (*mCherry* sequence)** CTA GAG GTT AAG AGG TTC TTC
pki-hph. fwd	TAC GCG GCG CGC C CT TAA G (*Afl*II) TT AG T AAC TAG TGG ATC
pki-hph.rev	CAT GCT AAG CTT (*Hind*III) CTA TTC CTT TGC CCG CGG AC

The primers contain engineered restriction sites shown in shading and the overlapping sequences are shown in bold.

a DNA fragment featuring the *pki* promoter together with the hygromycin B resistance gene (*pki-hph*) was PCR amplified using primer *pkihph.fwd* and *pki-hph.rev* to allow selection of transformants. The fragments were engineered to contain restriction sites as needed (**Table 2**).

The primers *pki.fwd* and *mCherry.rev* were used to fuse the separately amplified *pki* and *mCherry* fragments in an overlap extension PCR as described by Thornton (2015). The fragment *pki-hph* was digested with restriction enzymes *Hind*III and *Afl*II (Fermentas, Thermo Scientific, USA) and fragment *pki-mcherry* was digested with *EcoR*V and *Kpn*I. The digested products *pki-hph* and *pkimcherry* were gel purified using QIAquick gel extraction kit (Qiagen, USA) and inserted into MCS-1 (multiple cloning site) and MCS-2 of the pETDuet-1 plasmid, respectively (Supplementary Figure S1). Finally, the purified vectors and inserts were ligated using T4 ligase (Fermentas, USA) at a 1:3 molar ratio for 2 h at room temperature. The final ligated vector (pETDuet-phpm) was introduced into *Escherichia coli* DH5α competent cells as described by Inoue et al. (1990). Selection of transformants was performed on LB agar plates containing ampicillin (100 µg/ml) and incubating at 37°C. Selected transformants were grown in 3 ml of LB and plasmid DNA was isolated using QIAprep Spin Miniprep kit (Qiagen, USA). The plasmid pETDuet-phpm was sequenced by AGRF, Sydney, NSW, USA to check sequence alignment of the inserted gene cassettes.

The pETDuet-phpm DNA was introduced into highly virulent *S. aurantiacum* WM 06.482 using protoplast-mediated transformation based on the method adopted from Penttilä et al. (1987) with modifications. The young hyphae obtained from an overnight culture of WM 06.482 on PDA plates with cellophane at 28°C were digested with 10 mg/ml of lysing enzyme from *T. harzianum* (Sigma–Aldrich, Australia) to obtain protoplasts which were then filtered through a sterile sintered glass filter (porosity 1). Osmotically stabilized protoplasts were transformed with 5 μg of plasmid DNA as described by Penttilä et al. (1987). Transformed protoplasts were mixed with 10 ml of molten agar (1.5% w/v KH_2PO_4, 0.5% w/v NH_4SO_4, 2% w/v glucose, 1 M sorbitol, pH 5.5) containing hygromycin B (410 U/ml) and overlayed onto PDA plates which were incubated at 28°C for 3–5 days. Hygromycin resistant colonies were restreaked onto fresh PDA plates containing hygromycin B (410 U/ml) for a second round of selection. Transformation efficiency was calculated as number of transformants per μg of plasmid DNA. Expression of the mCherry protein in selected transformants was confirmed using Fluoview FV1000 inverted confocal microscope (Olympus) with an excitation and emission wavelength 488/633 nm (HeNe).

Growth Inhibition Assays

The effect of *P. aeruginosa* on the growth *of S. aurantiacum* was tested in different combinations on both solid and liquid growth media. Combinations of bacterial and fungal strains for the testing are presented in **Table 3**.

Cross Streak Assay using Live Cells

The effect of bacteria on fungal growth was assessed using an agar plate method described by Kerr (1999), with slight modifications adopted from Chen et al. (2013). *P. aeruginosa strains* PASS1, PASS2, and PAO1; and *S. aurantiacum* strains WM 06.482 and WM 08.202, were cultured together on a synthetic cystic fibrosis medium (SCFM) that mimics the nutritional content of human CF sputum. SCFM contains average concentrations of ions, free amino acids, glucose, and lactate present in the CF sputum samples (Palmer et al., 2007). Solid SCFM agar plates were made with an addition of 2% w/v agar to liquid SCFM medium. A sterile cotton swab was used to draw a straight vertical line of *P. aeruginosa* cells (1×10^8 CFU/ml=0.5 McFarland standard concentration) across the plate. At the same time, *S. aurantiacum* conidia (2.5×10^5 conidia/ml = 0.5 McFarland standard concentration) were inoculated with a cotton swab horizontally across the upper part of the plate preventing any direct contact between fungi and bacteria. The plates were dried at room temperature for 15 min and incubated at 37°C. Digital photography was performed after 24 h to visualize the growth of both bacterial and the fungal strains tested on the plate.

Disk Inhibition Method using Live Cells and Cell Fractions

Sterile filter paper disks (Whatman no. 1; Sigma–Aldrich), 7 mm in diameter, were impregnated with 20 μl of the *P. aeruginosa* PASS1, PASS2, and PAO1 cell fractions, i.e., cell lysates, cell culture supernatant and heat inactivated cells (see preparation in section 1.1) and placed on an SCFM plate that was freshly surface seeded with 100 μl (2.5×10^5 conidia/ml) of *S. aurantiacum* conidia (WM 06.482 or WM 08.202). A suspension of live *P. aeruginosa* cells was included for comparison. The plates were incubated at 37°C for up to 3 days and observed at regular intervals for the appearance of any clear inhibition zones around the disks. Assays were repeated in three biological replicates. A relative inhibition index was calculated for each *P. aeruginosa* isolate by dividing the area of activity (difference between the area of the inhibition zone and area of the colony) by the area of the colony.

Effect of Bacteria on the Fungal Growth in Liquid Co-cultures

Interactions between *P. aeruginosa* and *S. aurantiacum* were observed in liquid medium using both chemical fluorescent stains and genetically labeled strains of bacteria and fungi in a direct contact with each other. In case of fluorescently labeled co-cultures, 1×10^8 CFU/ml of *P. aeruginosa* PASS1, PASS2, and PAO1 and 2.5×10^5 conidia/ml of *S. aurantiacum* WM 06.482 and WM 08.202 were inoculated in 20 ml SCFM medium in 100 ml shake flasks and incubated for 24 h at 37°C on an orbital shaker at 150 rpm. Aliquots were taken on a sterile glass slide from the co-cultures after every 4 h, washed with 1x PBS and fixed using 2% v/v paraformaldehyde (Sigma–Aldrich). The co-cultures were stained with DNA specific Syto9 (0.6 μM) and mitochondria specific Mito-Tracker^R Red FM (25 nM) for 15 min in the dark as per the manufacturer's protocol (Molecular Probes, Life Technologies). Bacterial cells were expected to stain with Syto9 whereas fungal cells would stain with Mito-Tracker^R Red FM. Fixed specimens were imaged using Fluoview FV1000 inverted confocal microscope (Olympus) with an excitation and emission wavelength of 488 nm (Ar) and 633 nm (HeNe).

The genetically tagged *P. aeruginosa* PASS1yfp strain and *S. aurantiacum* WM 06.482mCherry strain were also cultured together in 20 ml of SCFM for 24 h at 37°C, shaking at 150 rpm. At the end of the incubation period, cells were washed and fixed on sterile glass slides as above. Imaging was performed with a confocal microscope using an excitation and emission

TABLE 3 | Types of cultures used to investigate the effect of different *P. aeruginosa* strains on *S. aurantiacum*.

Type of co-culture	*P. aeruginosa* strains	*S. aurantiacum* strains
Solid plate (cross streak, disk inhibition assay)	PASS1, PASS2, PAO1	WM 06.482, WM 08.202
Liquid cultures (chemical fluorescent dyes)	PASS1, PASS2, PAO1(stained with Syto9)	WM 06.482, WM 08.202 (stained with Mito tracker FR)
Liquid culture (genetically tagged strains)	PASS1 (*yfp*-labeled)	WM 06.482 (*mCherry*-labeled)
Liquid culture (addition of an antibiotic)	PASS1 (*yfp*-labeled)	WM 06.482 (*mCherry*-labeled)

wavelength of 488 nm (blue laser diode for yfp) and 561 nm (yellow-green laser for mCherry) respectively. Liquid co-cultures with genetically labeled PASS1 and WM 06.482 strains were also repeated by adding different concentrations of gentamicin (2.5–10 mg/ml), which is a commonly used antibiotic against bacteria (Döring et al., 2000; Lin et al., 2011). Image analysis for both types of co-cultures was performed using IMARIS imaging software.

Transwell Assay with Polycarbonate Membranes

In order to explore the role of secreted bacterial metabolites on the fungi, *P. aeruginosa* strains PASS1, PASS2, and PAO1 and *S. aurantiacum* strains WM 06.482 and WM 08.202 were co-cultured in SCFM in sterile six-well Transwell plates (Corning) with polycarbonate cell culture inserts (0.4 μm, Sigma–Aldrich) in order to prevent direct contact between the fungal and bacterial strains. *P. aeruginosa* (1×10^8 CFU/ml) and *S. aurantiacum* (2.5×10^5 conidia/ml) were inoculated in the bottom and top of the membrane insert, respectively. The plates were incubated at 37°C for 24 h and any inhibition of the growth of *S. aurantiacum* was measured as a difference in the dry weight of the *S. aurantiacum* cultured with or without *P. aeruginosa*. The method of calculating dry weight was adopted from Kaur et al. (2015).

Effect of Phenazines

Phenazines were extracted from all *P. aeruginosa* strains (PASS1, PASS2, and PAO1) cultured in 5 ml of LB (in three biological replicates) for 2 days at 37°C using chloroform according to a method described by Mavrodi et al. (2001). Crude phenazine extracts were dried under reduced pressure to remove the solvent, resuspended in 80% acetonitrile (ACN) and applied to the filter paper disks (Whatman paper no. 1). The presence of pyocyanin in the crude phenazine extracts was confirmed using Ultra High Performance Liquid Chromatography (UHPLC) as described by Penesyan et al. (under review). While the absolute concentration of pyocyanin in the crude phenazine extracts from the three *P. aeruginosa* strains was not known, major experimental discrepancy was minimized by using same amount (20 μl) of crude extracts for the testing. The effect of these crude extracts on the fungal growth was determined using a disk inhibition assay (as described in section Disk Inhibition Method using Live Cells and Cell Fractions) where disks containing 20 μl of phenazine extracts from different *P. aeruginosa* isolates were air-dried and placed on SCFM agar plates freshly spread with 2.5×10^5 conidia/ml of *S. aurantiacum* strains (WM 06.482 and WM 08.202). The activity of blank 80% ACN, blank LB medium extract and the solution of commercial pyocyanin (10 mM, Sigma–Aldrich) were also tested against *S. aurantiacum* for comparison. Plates were incubated for 48 h at 37°C and observed for the presence of clearing zones around the filter paper disks as an indication of inhibitory activity of the extracts on fungal growth.

Statistical Analysis

Statistical significance between the means of different experimental datasets was analyzed using two-tailed Student's *t*-test. SD with p-value less than 0.05 was considered significant. All experiments were performed in biological triplicates.

Results

Inhibition of *S. aurantiacum* Growth by Live *P. aeruginosa* Cells

When *S. aurantiacum* strains WM 06.482 (high virulence) and WM 08.202 (low virulence) were cross streaked against three different *P. aeruginosa* isolates PAO1, PASS1, and PASS2 on the SCFM agar medium, an area of inhibition was observed in the growth of both *S. aurantiacum* strains after 24 h (**Figures 1A–F**). It was evident from the size of the inhibition area that the bacterial strains had lesser impact on the highly virulent *S. aurantiacum* strain WM 06.482 (**Figures 1A,B**) compared to the less virulent WM 08.202 strain (**Figures 1D,E**). Out of the three bacterial strains studied, *P. aeruginosa* PASS2 had the weakest inhibitory effect on the *S. aurantiacum* strains in the plate test as seen in **Figures 1C,F**.

Effect of *P. aeruginosa* Cell Extracts on *S. aurantiacum*

The effect of different cell fractions, i.e., the culture supernatant and cell lysate, and heat inactivated cells of *P. aeruginosa* strains PASS1, PASS2, and PAO1 was further tested on the growth of *S. aurantiacum* WM 06.482 and WM 08.202 using the disk inhibition method. Following 48 h incubation, clear inhibition zones were observed on plates inoculated with living cells of *P. aeruginosa* PASS1 and the reference strain PAO1 and their respective cell lysates. The inhibitory effect of *P. aeruginosa* was expressed as a relative inhibition index (**Figure 2**).

Living cells of both PAO1 and PASS1 and their corresponding cell lysates displayed a higher inhibitory activity against the less virulent *S. aurantiacum* strain WM 08.202 compared to the high virulence strain WM 06.482. Cell supernatants and heat killed *P. aeruginosa* cells failed to elicit a response against either of the fungal strains. In a separate experiment, the effect of *S. aurantiacum* was also tested against *P. aeruginosa* by incubating filter disks impregnated with *S. aurantiacum* conidia and cell fractions on the plates freshly seeded with *P. aeruginosa* cells. As *S. aurantiacum* failed to display any inhibition against *P. aeruginosa*, these interactions were not studied further (data not shown).

Effect of *P. aeruginosa* on Fungal Physiology

Pseudomonas–Scedosporium interactions were also studied using confocal microscopy by imaging cellular aggregates from liquid co-cultures labeled with fluorescent stains. Confocal images demonstrated an inhibitory effect of the *P. aeruginosa* PASS1 (isolated from sputum of a CF patient) and the reference strain PAO1 on the growth and development of both *S. aurantiacum* strains tested (**Figures 3A–F**). In the course of 24 h, the bacteria had attached to the surface of fungal hyphae and formed biofilm-like structures containing a high density of bacterial cells but very few fungal hyphae. The tested bacterial strains had a weaker impact on the more virulent WM 06.482 compared

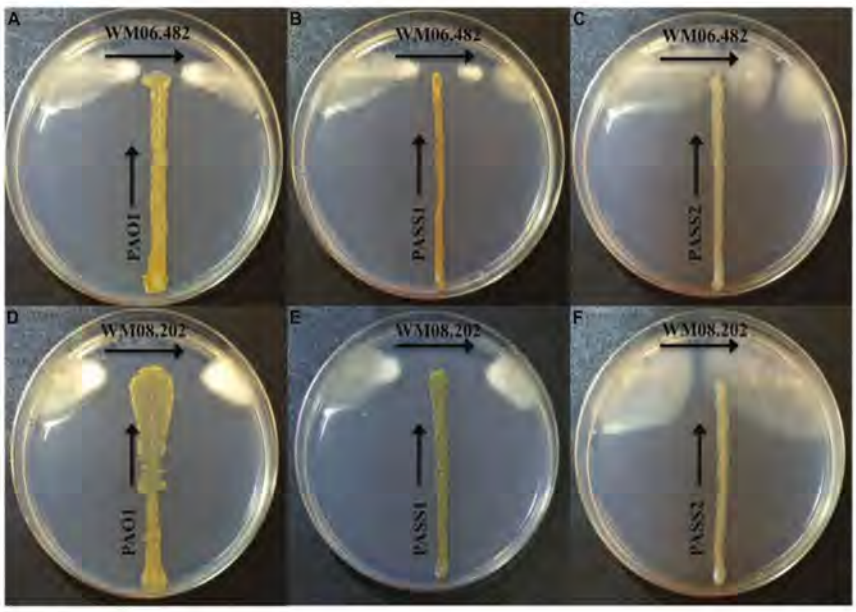

FIGURE 1 | Cross-streak plate assay between different strains of *Pseudomonas aeruginosa* and *Scedosporium aurantiacum* on synthetic cystic fibrosis medium (SCFM) agar plates. All bacterial strains were inoculated vertically whereas the fungal strains were streaked horizontally across the upper part of the SCFM agar plate. The plates were incubated at 37°C for 24–48 h. **(A–C)** Inhibition of *S. aurantiacum* strain WM 06.482 by PAO1, PASS1, and PASS2 strains of *P. aeruginosa*. **(D–F)** Inhibition of *S. aurantiacum* strain WM 08.202 by PAO1, PASS1, and PASS2 strains of *P. aeruginosa*.

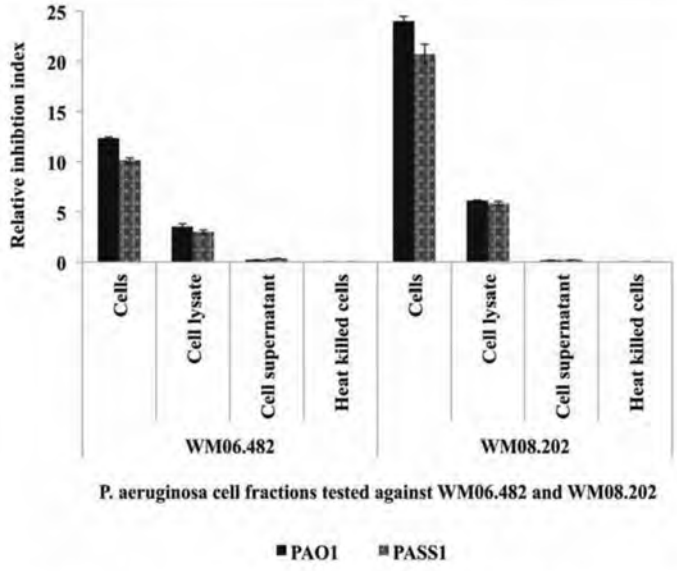

FIGURE 2 | Susceptibility of *S. aurantiacum* (WM 06.482 and WM 08.202) to *P. aeruginosa* (PAO1, PASS1, and PASS2) and their cell lysate fractions. Relative inhibition index was calculated as the average value of three replicates ($n = 3$) with a p-value <0.05 considered as significant.

to the less virulent WM 08.202, showing the resistant nature of the more virulent strain also highlighted in the plate tests. Although different fluorescent stains were used to distinguish between *P. aeruginosa* and *S. aurantiacum* in liquid cultures, it was difficult to visualize the detailed effect of bacteria on the fungal hyphae due to permeabilisation of the Syto9 dye by both

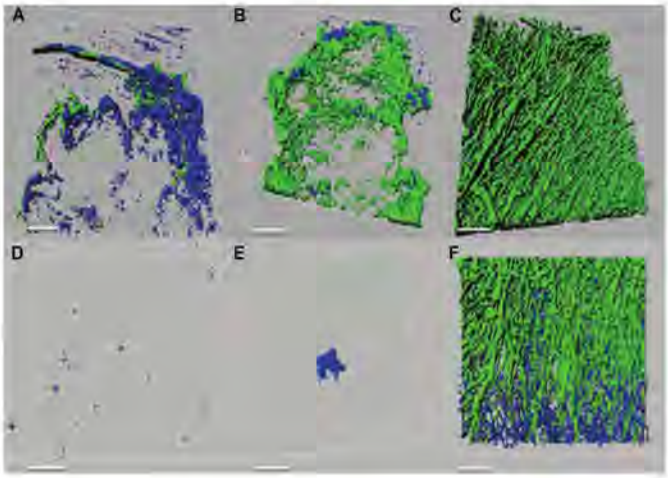

FIGURE 3 | Confocal laser scanning microscope (CLSM) images of interactions between *P. aeruginosa* (PAO1, PASS1, and PASS2) and *S. aurantiacum* (WM 06.482 and WM 08.202) as observed after co-incubating both the organisms in SCFM liquid medium at 37°C for 24 h. *P. aeruginosa* cells are stained with Syto9 (shown in green) and *S. aurantiacum* with Mito-tracker deep red FM (shown in blue). 3D re-construction of CLSM datasets was performed using IMARIS software package (Bitplane). Scale bar = 50 μm. **(A–C)** CLSM images of co-culture of WM 06.482 with PAO1, PASS1, and PASS2, respectively. **(D–F)** CLSM images of co-culture of WM 08.202 with PAO1, PASS1, and PASS2, respectively.

P. aeruginosa and *S. aurantiacum*. No growth inhibiting effect was observed when the fungal strains were co-cultured with PASS2 as indicated by dense growth of fungi in **Figures 3C,F**. These observations were also consistent with the results seen in the assays carried on plates.

Interactions between Genetically Tagged *P. aeruginosa* and *S. aurantiacum* Strains

To circumvent the difficulty in differentiating between bacteria and fungi in liquid co-cultures, genetically tagged *P. aeruginosa* strain PASS1 expressing yfp and *S. aurantiacum* strain WM 06.482 expressing mCherry were developed. With this arrangement, it was observed that the bacteria started colonizing the fungal conidia soon after incubating them together in the SCFM (**Figure 4A**). Thus, within 8 h, bacteria began aligning themselves along the length of fungal hyphae as seen in **Figures 4B,C**. After 24 h, large clumps of *P. aeruginosa* cells were observed on *S. aurantiacum* hyphal filaments (**Figure 4D**), and the amount of hyphae was also reduced in number compared to the *S. aurantiacum* control without the bacteria (**Figure 4E**).

Effect of Antibiotics used in Clinical Practice on Co-cultures

Analysis of the plate cultures and confocal images confirmed that *P. aeruginosa* had an inhibitory effect on the growth of *S. aurantiacum*. Therefore, in order to further validate this finding and to reveal the possible effect of antibiotic therapy on *S. aurantiacum* and *P. aeruginosa* mixed populations present in CF lungs, co-culturing was repeated with an addition of varying amounts of gentamicin (2.5–10 mg/ml) to selectively inhibit the growth of *P. aeruginosa*. *S. aurantiacum*-*P. aeruginosa* co-cultures were also maintained without the addition of gentamicin for comparison (**Figure 5A**). All bacteria were killed at a concentration of 8 mg/ml of gentamicin. As seen from **Figure 5B**, *S. aurantiacum* strain WM 06.482 was growing actively in the absence of *P. aeruginosa* strain PASS1 indicating the reversal of the inhibitory effect caused by live bacteria against the fungus.

Indirect (non-physical) Interactions between *P. aeruginosa* and *S. aurantiacum*

To investigate whether physical contact between *P. aeruginosa* and *S. aurantiacum* was important to trigger growth inhibition, co-cultures were performed in six-well plates fitted with polycarbonate membranes to prevent direct contact between *P. aeruginosa* and *S. aurantiacum* cells while allowing free exchange of nutrients and extracellular molecules between the organisms. Growth of the less virulent *S. aurantiacum* strain WM 08.202 was inhibited when co-cultured with PAO1 and PASS1, evident from the substantial decrease in the fungal biomass (**Figure 6**) when compared to the culture of WM 08.202 maintained for the same amount of time.

Pseudomonas aeruginosa isolate PASS1 and the reference strain PAO1 showed a milder inhibitory effect against the high virulence *S. aurantiacum* strain WM 06.482. The PASS2 strain had little or almost no effect on growth of either of the *S. aurantiacum* strains. The results suggested that cell–cell contact was in fact not necessary to bring about inhibition of the growth of *S. aurantiacum* by *P. aeruginosa* and that the inhibition might involve bacterial metabolites and/or extracellular signaling molecules. In addition, *S. aurantiacum* strains WM 06.482 and WM 08.202 produced a red colored pigment when co-cultured with clinical *P. aeruginosa* strain PASS1 and reference strain PAO1. No such pigment was observed in the co-cultures

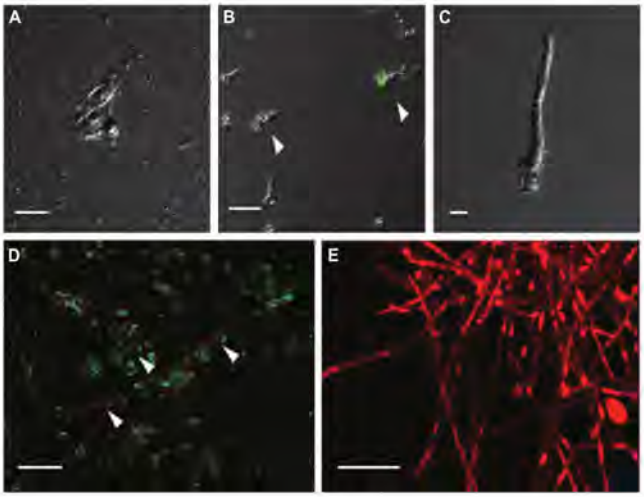

FIGURE 4 | Adhesion and colonization of mCherry-tagged *S. aurantiacum* strain WM 06.482 (shown in red) by *P. aeruginosa* strain PASS1 tagged with yfp (shown in green) during coculturing in SCFM for 24 h at 37°C. Scale bar = 20 μm. **(A)** *P. aeruginosa* cells adhered to germinating *S. aurantiacum* conidia after 2 h of incubation as viewed by DIC. **(B,C)** after 8 h, some young hyphae were surrounded by bacterial cells. **(D)** Bacteria can be seen attached to the hyphal filaments after incubation for 24 h. **(E)** Healthy growing culture of WM 06.482 expressing *mCherry* in the absence of bacteria. *White arrows indicate fungal filaments that are being colonized by the bacteria.

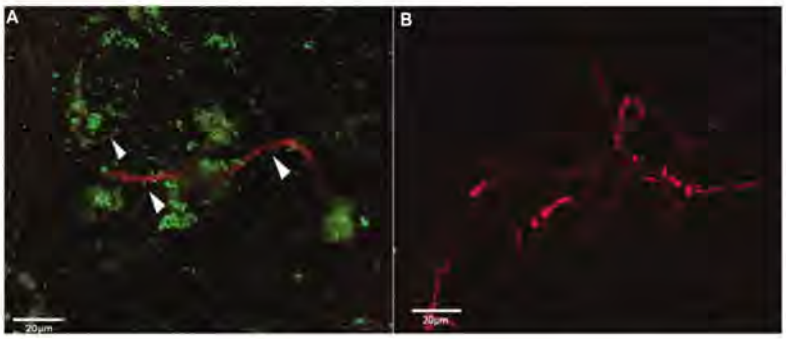

FIGURE 5 | The effect of gentamicin on *P. aeruginosa* (PASS01) and *S. aurantiacum* (WM 06.482) co-cultures growing in SCFM at 37°C for 24 h. Scale = 20 μm. **(A)** Co-culture of WM 06.482 and PASS01 without the antibiotic. **(B)** Active growth of *S. aurantiacum* in a co-culture treated with 8 mg/ml of gentamicin to eradicate the bacterial growth.

involving *S. aurantiacum* and PASS2 strain (Supplementary Figure S2).

Effect of Phenazines on the Growth of *S. aurantiacum*

To test whether known virulence factors such as phenazines secreted by *P. aeruginosa* were involved in the inhibition of *S. aurantiacum* growth, the effect of crude phenazine extracts from *P. aeruginosa* strains PAO1, PASS1, and PASS2 were tested on the two *S. aurantiacum* strains using a disk inhibition assay. No inhibition was observed with disks saturated with the crude extracts as seen in **Figure 7**. All *S. aurantiacum* strains also showed resistance to a high concentration (10 mM) of commercial phenazine pyocyanin. Phenazines are known to have an inhibitory effect against a wide range of fungal species (Kerr et al., 1999).

Discussion

Most of the studies targeting bacterial-fungal interactions *in vitro* have been performed with bacterial laboratory reference strains using either fungus-specific culture media (PDA, SABD) and/or minimal salts medium (Kerr et al., 1999; Hogan and Kolter, 2002; McAlester et al., 2008; Bandara et al., 2010; Manavathu et al., 2014). Differently to previous studies and to provide a better focus, we used a CF sputum-mimicking medium, i.e., SCFM to

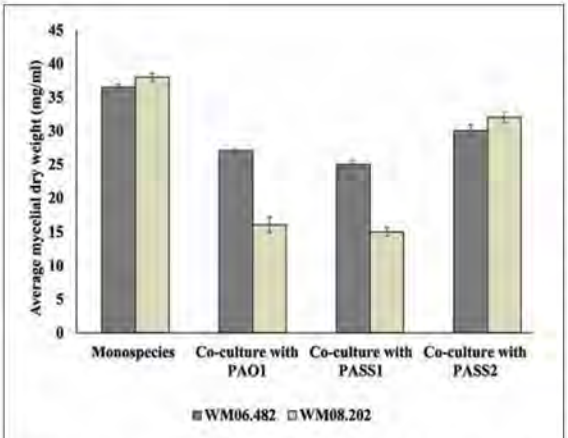

FIGURE 6 | The inhibitory effect of *P. aeruginosa* on *S. aurantiacum* during co-culture in SCFM, determined by the change in dry weight of *S. aurantiacum* hyphae. A sterile polycarbonate membrane was used to separate *P. aeruginosa* from *S. aurantiacum* in six well culture plates. Error bars on each data point represent SEM of three independent experiments. *P*-values were calculated by student's *t*-test, where $p < 0.05$ was considered significant.

explore the possible effect of *P. aeruginosa* on *S. aurantiacum* in the CF lung environment. We also used two recently isolated clinical CF strains of *P. aeruginosa*, PASS1 and PASS2, together with PAO1, a commonly used reference strain, and a clinical *S. aurantiacum* isolate (WM 06.482) with a high established virulence and a less-virulent type strain WM 08.202 to add to the clinical relevance of the findings.

Our results demonstrated that *P. aeruginosa* strains exhibit an inhibitory effect against *S. aurantiacum*. Consistent with the co-culture studies involving *P. aeruginosa* and other fungi, initial screening using plate assays suggested that presence of metabolically active (live) bacteria was necessary to inhibit the growth of the fungus as heat killed cells had no effect on *S. aurantiacum* growth (Mowat et al., 2010). Further on, extracts obtained from the bacterial monocultures failed to show any inhibitory effect. Thus it is possible that inhibition pathways might involve genes that are expressed only in bacterial-fungal co-cultures. In this respect our findings are similar to those of Rella et al. (2012) who showed that the growth of *C. neoformans* was not affected by the cell extracts obtained from *P. aeruginosa* strains PAO1 and PA14 cultured separately. The inhibition of *S. aurantiacum* by cell lysates of *P. aeruginosa* may be explained by the presence of bacterial exotoxins that are released during the cell lysis.

Confocal microscopy has been used to study interactions between chemically stained *P. aeruginosa* and major fungal lung pathogens such as *C. albicans* and *A. fumigatus* in liquid co-cultures (Bandara et al., 2010; Manavathu et al., 2014). However, the use of chemical stains was limited by the cross staining of bacteria and fungi thereby making it impossible to differentiate between them under a confocal microscope (Bandara et al., 2010). One of the key features of the current study is the use of *P. aeruginosa* and *S. aurantiacum* strains that were genetically tagged with fluorescent proteins in order to characterize the interactions in detail. To the best of our knowledge, this is the first report on successful genetic transformation of the newly described *S. aurantiacum* species. As no homologous promoters are available for this fungal species as yet, the fluorescent marker mCherry and the *E. coli hph* gene encoding hygromycin B phosphotransferase were expressed under a heterologous *pki* (pyruvate kinase) promoter derived from another ascomycetous fungus, *T. reesei* (Te'o et al., 2002; Boon et al., 2008; Klix et al., 2010). In previous studies, heterologous promoters such as *pki* and *gpdA* have been successfully used for gene expression across various phylogenetically close species (Punt et al., 1990; Jieh-Juen Yu, 1998; Ruiz-Diez and Martinez-Suarez, 1999; Almeida et al., 2007). The amount of hygromycin B required to inhibit the growth of *S. aurantiacum* was relatively high (410 U/ml) compared to some other fungi, which shows the highly resistant nature of *S. aurantiacum* also observed in antifungal susceptibility tests described in other studies (Lackner

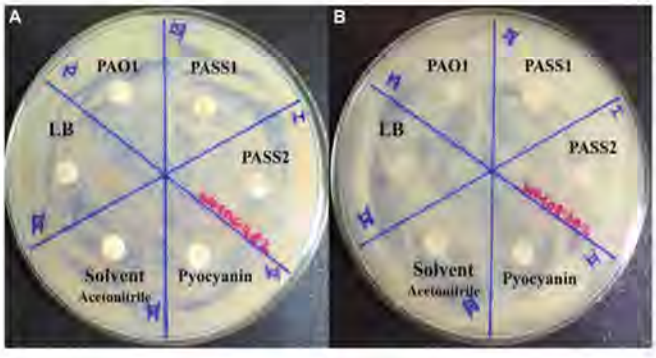

FIGURE 7 | Effect of *P. aeruginosa* phenazines on two *S. aurantiacum* isolates (A) WM 06.482 and (B) WM 08.202. Phenazines were extracted from different *P. aeruginosa* strains (PAO1, PASS1, and PASS2) and redissolved in acetonitrile (ACN). LB medium extract, ACN solvent and commercial phenazine (pyocyanin) were also tested against *S. aurantiacum*.

et al., 2012). Although the transformation efficiency was low (2.2 μg of plasmid DNA), transformant strains expressing the mCherry protein were obtained.

Confocal microscopy of the bacterial-fungal co-cultures revealed that bacteria elicit a specific inhibitory response by establishing a physical contact with the fungal hyphae. Similar types of interactions have also been observed in yeasts such as *C. albicans* and ascomycetous fungi such as *A. nidulans* and *Alternaria alternata* (Hogan and Kolter, 2002; Jarosz et al., 2011). This association might be directed toward utilization of the fungus by bacteria as an additional source of nutrients, or as an additional matrix support to form biofilms (Hibbing et al., 2010), or it may be a strategy to promote their own survival by inhibiting the fungal growth owing to nutrient limiting conditions in the medium (Brand et al., 2008).

Under nutrient limiting conditions, biofilm formation has been described as an important characteristic for *P. aeruginosa* mediated killing of other fungi such as *C. albicans* and *A. fumigatus* (Hogan and Kolter, 2002; Manavathu et al., 2014). Similarly, an inhibitory effect was also displayed by the biofilm forming strains of *P. aeruginosa* (PASS1 and PAO1) against the two *S. aurantiacum* strains in this study. PASS1 and PAO1 are high virulence strains, which share many similarities in their respective genomes. In contrast, the least virulent bacterial strain PASS2 (Penesyan et al., under review) that failed to show an effect against the fungi lacks several virulence related genes such as those encoding phenazines and the *psl* (polysaccharide synthesis locus) gene cluster which is required for biofilm formation (Ma et al., 2009; Penesyan et al., under review). The effect of bacteria on the growth of the less virulent *S. aurantiacum* strain WM 08.202 was much higher compared to the more virulent WM 06.482 both in the plate assays and in liquid co-cultures. This difference probably results from their different physiology as shown by Kaur et al. (2015) and possibly higher resistance to antifungals of the more virulent *S. aurantiacum* strain WM 06.482. These factors will be studied further when annotated *S. aurantiacum* genomes are available.

While biofilm formation and colonization of fungal hyphae in the nutrient limited SCFM liquid medium clearly contributed to the inhibition of *S. aurantiacum* by *P. aeruginosa*, it was not absolutely essential for the inhibitory effect as the cross streak assay with cultures not touching each other and disk inhibition experiments using cell lysates also resulted in inhibition of fungal growth. These indicated the possible involvement of secreted diffusible bacterial exoproducts/metabolites in fungal growth inhibition. One of these metabolites pyocyanin, a phenazine, is an extracellular redox-active virulence factor which is widely known to affect the growth of a large number of fungal species such as *A. fumigatus*, *C. albicans*, and *C. neoformans* (Kerr et al., 1999; Laursen and Nielsen, 2004; Gibson et al., 2009). Corroborating the highly resistant nature of *S. aurantiacum*, the amount of commercial pyocyanin (i.e., 10 mM) included in the test for comparison, was much higher than the MIC (minimum inhibitory concentration) of pyocyanin used for *C. albicans* and *A. fumigatus* (>0.3 mM; Kerr et al., 1999). These amounts are significantly higher than the amount of pyocyanin normally detected in the lungs of CF patients (100 μM; Wilson et al., 1988). However, neither crude phenazines nor pyocyanin showed an inhibitory effect against *S. aurantiacum* in our assays. A similar phenomenon has been observed in some ascomycetous fungi such as *A. sclerotiorum* (Hill and Johnson, 1969). Although it is not yet known if the phenazines are modified or sequestered by *S. aurantiacum*, the production of a red colored pigment in co-cultures could be due to a detoxification mechanism used by the fungus against bacterial phenazines. However, further studies into the chemical structure and UV and visible absorption spectra are required in order to ascertain if the red pigment indicates a modified phenazine.

In addition to phenazines, *P. aeruginosa* has also been reported to produce a wide variety of other exoproducts/metabolites such as proteases, elastases, haemolysin, and rhamnolipids that contribute to bacterial virulence (McAlester et al., 2008; Ben Haj Khalifa et al., 2011; Heeb et al., 2011; Rella et al., 2012; Mear et al., 2013). Their possible activity against *S. aurantiacum* will be worthy of a further study.

Most of the CF associated filamentous fungal species have been isolated from the lungs of patients with prolonged antibiotic therapies (Bakare et al., 2003). Previous clinical reports by Blyth et al. (2010b) have also showed an increased prevalence of *S. aurantiacum* in CF patients administered with antibacterial drugs indicating that the presence of bacteria has an effect on the susceptibility of the lungs to fungal infection. In support of this view, an increase in the growth of the fungus was observed upon a decline in the bacterial growth through addition of gentamicin to the co-culture medium in the present study. Therefore, it seems that the *P. aeruginosa* strains prevalent in CF patients during early stages of CF hinder fungal infection of lungs by inhibiting their growth.

Conclusion

We have assessed the effect of clinically relevant strains of *P. aeruginosa* on a newly discovered fungal lung pathogen *S. aurantiacum* in a synthetic lung-mimicking medium (SCFM) that closely resembles the chemistry of CF sputum. An inhibitory effect of *P. aeruginosa* was observed on the growth of *S. aurantiacum*, which can be mediated by the production of biologically active metabolites. Biofilm formation and colonization of fungal hyphae by bacteria were also important for *S. aurantiacum* growth inhibition. Surprisingly, the toxic *P. aeruginosa* phenazine pigments, such as pyocyanin, known to have an inhibitory effect against other fungal species including *A. fumigatus* and *C. albicans*, proved to be ineffective against *S. aurantiacum*. This suggests involvement of other virulence determinants and emphasizes the resilient nature of *S. aurantiacum* compared to other fungi present in lung infections. Further research may include transcriptomic studies of *P. aeruginosa* – *S. aurantiacum* co-cultures in order to reveal detailed molecular mechanisms underlying these interactions; these studies will be facilitated by the upcoming annotated *S. aurantiacum* genome.

Author Contributions

Conceived and designed the experiments: JK, LK, AP, AS, IP, HN. Performed the experiments: JK, SK, BP, MK. Analysed the data: JK, AP, HN. Wrote the paper: JK, HN.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00866

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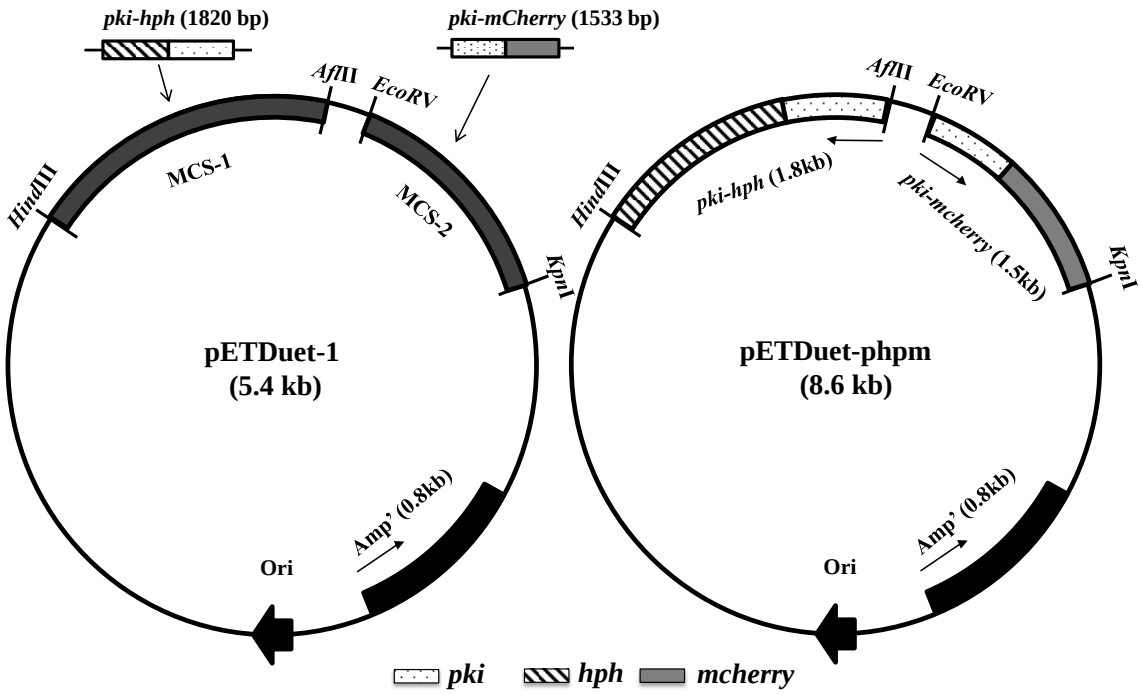
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1. **Supplementary Figure**



Supplementary Figure 1. Schematic representation of the construction of plasmid pETDuet-phpm containing *pki-hph* (1.8 kb) with *Hind*III and *Afl*II restriction sites and *pki-mcherry* (1.5 kb) with *Eco*RV and *Kpn*I restriction sites. The plasmid is 8.6 kb long and was made by insertion of *pki-hph* and *pki-mcherry* fragments in two different multiple cloning sites of the pETDuet-1 vector. The patterns used in the image are described in the key below.

5

INTERACTIONS OF AN EMERGING FUNGAL PATHOGEN *SCEDOSPORIUM AURANTIACUM* WITH HUMAN LUNG EPITHELIAL CELLS

5.1. Introduction

Most of the fungal lung invaders first come in contact with the epithelial cell membrane lining the lung alveoli and interaction between the fungi and epithelial cells is the most important aspect of the fungal pathogenesis. In chapter 5, I investigated the interactions between the human epithelial lung cell line and a high virulence *S. aurantiacum* strain with an aim to understand the overall pathophysiology of the disease. The interactions were studied using techniques such as confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) followed by functional analysis of the host transcriptome. Due to the lack of an annotated *S. aurantiacum* genome, the pathogen transcriptome could not be analysed at this time. Submission of this chapter as an extended manuscript including the analysis of *S. aurantiacum* transcriptome is planned after the annotated genome is made available.

5.2. Contribution to Publication 3

The concept of this publication was developed in partnership with my mentors including Liisa Kautto and Anahit Penesyan, supervisors Ian Paulsen and Helena Nevalainen at Macquarie University and my co-supervisor Wieland Meyer at Westmead Hospital. All the experiments were performed by me under the guidance of Liisa Kautto. The transcriptome data was assembled by Anahit Penesyan and functional annotation was performed by me. The manuscript was prepared by me and critical comments were provided by Anahit Penesyan, Liisa Kautto, Ian Paulsen and Helena Nevalainen.

Table 5-1. Author contribution for Publication 3.

	JK	LK	AP	WM	IP	HN
Experimental design	•	•	•	•	•	•
Data collection	•					
Data analysis	•		•			
Manuscript	•	•	•	•	•	•

*JK= Jashanpreet Kaur, LK= Liisa Kautto, AP= Anahit Penesyan, WM= Wieland Meyer, IP= Ian Paulsen, HN= Helena Nevalainen

5.3. Publication 3 (Written as Manuscript)

Kaur, J., Kautto, L., Penesyan, A., Paulsen, I. T., Nevalainen, H. Interactions of an Emerging Fungal Pathogen *Scedosporium aurantiacum* with the Human Lung Epithelial Cells. Manuscript prepared for submission.

Interactions of an Emerging Fungal Pathogen *Scedosporium aurantiacum* with Human Lung Epithelial Cells

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Abstract

S. aurantiacum is an opportunistic environmental pathogen that can cause infections in immunocompromised people. These infections may result from the inhalation of airborne *S. aurantiacum* conidia into the human respiratory system where they come into contact with a monolayer of epithelial cells present at the interface of the fungus and the host lungs. In order to elucidate the pathobiology of *S. aurantiacum* infections in the human host, it is important to understand the interactions between the fungal conidia and the respiratory epithelial cells. We used an *in vitro* model system to represent the carriage of *S. aurantiacum* to the human lungs. The experimental system involved co-culture of a highly virulent *S. aurantiacum* strain WM 06.482 with human alveolar epithelial cell line (A549). The interactions between A549 cells and *S. aurantiacum* (WM 06.482) were assessed using confocal microscopy (CLSM) and scanning electron microscopy (SEM). Transcriptional changes were also studied for A549 cells in response to interaction with WM 06.482 conidia. The experimental findings suggest that 75-80% of fungal conidia were able to bind to the A549 cells within 4 hr of incubation and the germinated conidia invaded the cells by penetrating through the epithelial cell layer. Analysis of the transcriptomes of A549 cells infected with WM 06.482 conidia and the non-infected control cells revealed 3950 genes that were differentially expressed. The up-regulated

genes were mainly associated with cell repair and inflammatory processes. These results suggest that A549 epithelial cells exhibited a protective response against *S. aurantiacum* infection. Differentially expressed genes were also used for network analysis that showed the activation of innate immune system (NF-kB pathway) leading to the release of pro-inflammatory cytokines such as IL8. The differential expression of genes associated with the inflammatory pathway was confirmed by a quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). This study provides substantial information for further analysis of the genes and pathways that are activated in pneumocytes following *S. aurantiacum* infection.

Introduction

The filamentous fungus *Scedosporium aurantiacum* is an emerging pathogen in Australia (Harun *et al*., 2010). *S. aurantiacum* is ubiquitous as it has been isolated from a wide variety of environmental sources such as agricultural and garden soil, sewers, polluted ponds and sediments (Kaltseis *et al*., 2009). According to an environmental survey carried out by Harun *et al.* in the greater Sydney area, the amount of *S. aurantiacum* found was roughly estimated to range from 60 to 905 colony forming units (CFU) per gram dry weight of soil samples. While sampling spanned both urban and suburban areas, *S. aurantiacum* was mostly isolated from the areas with high human impact (Harun *et al*., 2010), which increases the likelihood of acquiring *S. aurantiacum* infection by humans.

Scedosporium spp. are capable of causing numerous serious infections ranging from localised mycetomas to disseminated tissue infections in immunocompromised hosts (Cortez *et al*., 2008). The infections caused by *S. aurantiacum* have been studied to a much lesser extent than those caused by other major lung pathogens such as *Aspergillus fumigatus*. Although the episodes of *S. aurantiacum* infections have been described in various sites of the human body including blood, skin, ear, brain and sinuses, lungs have been highlighted as the main target of infection (Heath *et al*., 2009). Recently, *S. aurantiacum* has also gained particular attention due to its occurrence in the airways of the Australian cystic fibrosis (CF) patients. The small size of *S. aurantiacum* conidia (2-5 μm) can easily allow them to enter the respiratory tract *via* inhalation and traverse to the innermost areas of the lungs such as alveoli (Gilgado *et al*., 2005). It is believed that *S. aurantiacum* infection/colonisation is present in every one in six CF patients in Sydney

(Blyth *et al*., 2010). Furthermore, the treatment of *S. aurantiacum* infections is challenging as the fungus is highly resistant to most of the currently used antifungal agents (Heath *et al*., 2009).

The most likely mechanism of fungal invasion of epithelial cells involves attachment of the fungal conidia to the lung tissue followed by penetration into the intact alveolar lining which results in a tissue injury (DeHart *et al*., 1997). Invasion of the host cells and subsequent defense mechanisms used by the host against the pathogen are central to the pathogenesis of the disease (Filler and Sheppard, 2006). Human alveolar epithelial cells lining the lung alveoli are the first point of contact between the inhaled external particulates including dust, allergens and microorganisms (bacteria and fungi) and the inner lung milieu (Diamond *et al*., 2000). Therefore it is important to understand the response of alveolar epithelial cells to the invading pathogens as it may help to expose the pathogenic properties of the invading microbe as well as understand the host immune response (Filler and Sheppard, 2006). Type-II pneumocytes such as A549 cells have been widely used to study the infection process and host immune response to a large number of CF pathogens such as *Pseudomonas aeruginosa*, *Aspergillus fumigatus*, *Candida albicans* and *Cryptococcus neoformans* (Gomez *et al*., 2010; Santos *et al*., 2009; Hawdon *et al*., 2010; Foster *et al*., 1998). Traditionally the interactions between host epithelial cells and fungi have been assessed by confocal microscopy (CLSM) and scanning electron microscopy (SEM) (Botterel *et al*., 2008; DeHart *et al*., 1997; Han *et al*., 2011; Moore *et al*., 2005; Paris *et al*., 1997; Wasylnka and Moore, 2002). Recently, transcriptome and proteome based analyses have also gained importance as they can help to identify novel responses and pathways activated in the infected cells (Gomez *et al*., 2010; Oosthuizen *et al*., 2011; Sharon *et al*., 2011).

Despite the increasing importance of *S. aurantiacum* as a pathogen, the mechanism used by the fungus to infect the lungs has not been explored at all. Hence the main aim of the current study was to assess the interactions between *S. aurantiacum* and human alveolar epithelial cells *in vitro*. To investigate the conidial attachment and infection process, A549 pneumocytes derived from a human lung carcinoma were used and the co-culture of A549 cells and *S. aurantiacum* conidia was analysed using both CLSM and SEM. RNA sequencing was performed to evaluate the transcriptional differences between the infected and non-infected A549 cells.

Materials and Methods

S. aurantiacum strain

All experiments were performed using a high virulence strain of *S. aurantiacum* WM 06.482 (Kaur *et al*., 2015). The strain was obtained from the culture collection of the Medical Mycology Research Laboratory, Centre for Infectious Diseases and Microbiology, Westmead Hospital, Sydney, Australia (Kaur *et al*., 2015). Conidia were prepared and stored as described previously (Kaur *et al*., 2015).

A549 Cell Line

The A549 alveolar epithelial cell line was obtained from the American Type Culture Collection (ATCC® CCL-185™). The A549 cells were maintained in RPMI 1640 medium (Life technologies, Australia) supplemented with 10% v/v FBS (Fetal bovine serum, Life technologies, Australia), 1mM glutamine (Life technologies, Australia), 100 U/ml penicillin and 100 µg/ml streptomycin in the small cell culture flasks (Sigma-Aldrich, Australia) at 37°C in a humidified 5% CO_2 atmosphere. Cells were grown to confluence, washed three times in 1x PBS to remove excess medium. Cell monolayers were detached from the culture flask by adding 0.25% v/v trypsin (Life technologies, Australia) and 0.02% w/v EDTA in PBS and incubating at 37°C for 1-5 min. Cell count and viability were calculated with TC20™ Automated Cell Counter (Biorad, Australia) using 10 µl of cells mixed with 0.4% v/v Trypan blue (1:1). Cells were then seeded in RPMI 1640 medium in Falcon 8-chamber tissue culture slides (BD Falcon™ CultureSlides) approximately 1 x 10^5 cells/well at 37°C for 12 hr for confocal microscopy.

Measurement of Adherence of *S. aurantiacum* Conidia to Epithelial Cells

The confluent A549 cell monolayers seeded in Falcon 8-chamber tissue culture slides were incubated with 0.1:1, 1:1 and 10:1 multiplicity of infection (MOI = number of conidia/cell) of *S. aurantiacum* conidia in 500 µl of RPMI 1640 medium for 2 hr and 4 hr respectively at 37°C. The co-culture of epithelial cells with each MOI of *S. aurantiacum* conidia was maintained in three independent replicate experiments. After incubation, unbound conidia were removed by washing three times with 1 ml of PBS and epithelial cell monolayers were detached from the plate using trypsin (as described earlier). *S. aurantiacum* conidia were lysed using 400 µl of 0.5% v/v Triton X-100 and serial dilutions (10-fold) of the

released conidia were plated on solid potato dextrose medium (three replicates/well) for 24 hours at 37°C to determine the number of adhered conidia per well.

Analysis of the Co-culture of *S. aurantiacum* Conidia and A549 Cells by Microscopy Techniques

Confocal Microscopy

The co-cultures of *S. aurantiacum* (MOI = 1 conidia/cell) and A549 cell monolayers (1 x 10^5 cells/well) were maintained (in triplicate) in Falcon 8-chamber tissue culture slides at 37°C for 4 hr and 24 hr respectively. After indicated time intervals, the co-cultures were washed with 1ml PBS to remove unbound *S. aurantiacum* conidia and fixed with 2% v/v paraformaldehyde (Sigma-Aldrich) in PBS for 1 hr at room temperature. Cell co-cultures were then washed three times with 1 ml PBS and stained with Hoechst 33342 (1:100 of 10 mg/ml stock in PBS, Life technologies) for 15 min at room temperature to label the cell nuclei. After staining, the co-cultures were again washed with 1 ml PBS and covered with a coverslip. Images were acquired using a Fluoview FV1000 IX81 Inverted Confocal Microscope (Olympus) with an excitation and emission wavelength of 350/461 nm. Three-dimensional reconstructions of the cell monolayers and associated conidia were prepared by taking a series of images of the co-culture specimens in the Z-plane.

Scanning Electron Microscopy (SEM)

For SEM, the co-cultures were prepared as above and an additional time point of 8 hr was included to assess the interactions during germination of fungal conidia. At the end of each incubation period, the co-cultures were washed with PBS and fixed in 3% glutaraldehyde (50% stock, ProSciTech, Australia) in PBS. The samples were dehydrated using a series of ethanol concentrations (30-100%) and dried to the critical point using a K850 critical point dryer (Emitech). Dried specimens were mounted on the specimen stubs and coated with gold particles using K550 gold splutter coater unit (Emitech) and visualised using JSM-7100F Field Emission Scanning Electron Microscope (Jeol) at 10 kV working voltage.

RNA Extraction

Confluent monolayers of A549 cells were cultured (in triplicate) in the Greiner CELLSTAR^{R} dishes (Sigma-Aldrich, Australia) with and without *S. aurantiacum* conidia (MOI=1 conidium/cell) for 8 hours at 37°C. Upon completion of the incubation, the co-cultures were detached from the culture Petri dish using trypsin (as described previously) and centrifuged for 5 min at 1000 rpm and 4°C to harvest the cells. The cells were then washed three times with PBS to remove traces of medium and stored at -80°C. RNA extraction was performed using the TRizol method adopted from Schumann *et al*. (2013), with slight modifications. The harvested cells were ground to a fine powder using liquid N_2, and incubated with 1 ml TRizol reagent per 50-100 mg of sample (Sigma-Aldrich) for 5 min at room temperature. Organic extraction steps were carried out twice and involved addition of 200 µl chloroform to the samples followed by vigorous shaking and centrifugation at 12,000 rpm for 15 min at 4°C. The supernatant from the final extraction step was precipitated with 500 µl of isopropanol and RNA was collected after centrifugation at 12,000 rpm for 10 min at 4°C. The RNA pellet was washed with 75% v/v ethanol (cold), purified using RNeasy MinElute Cleanup kit (Qiagen) according to manufacturer's instructions, dried and resuspended in 20 µl of DEPC-treated water (diethyl-pyrocarbonate, Sigma-Aldrich). The concentration of the RNA was measured using a NanoDrop Spectrophotometer. The quality of RNA was assessed by running 1-1.5 µg of the RNA samples on 1% w/v agarose gel stained with GelRed (Biotium, Australia). The RNA samples were sequenced at the ACRF Biomolecular Resource Facility, John Curtin School of Medical Research, Canberra, Australia.

Transcriptome Profiling

The forward and reverse RNA-Seq Illumina paired-end sequence reads for A549 cells infected by WM 06.482 and non-infected control A549 RNA samples (in biological triplicates) were trimmed to remove adapter sequences and low-quality sequences using Trimmomatic tool (Bolger *et al.*, 2014). Trimmed high-quality sequence reads were mapped against human genome (*Homo sapiens*, UCSC release hg19) using Tophat2 software (Kim *et al*., 2013). Transcript assembly was performed with Cufflinks software tool; differential expression levels and statistical significance for each gene were calculated with Cuffdiff (Trapnell *et al*., 2010).

Functional Annotation and Pathway Analysis

Statistically significant ($p < 0.05$) differentially expressed genes were analysed using DAVID (The **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery) functional annotation tool, version 6.7 (http://david.abcc.ncifcrf.gov/). The gene dataset was uploaded to DAVID in a tab-delimited format and mapped against the *Homo sapiens* reference database to extract information including gene ontology terms, molecular function, biological processes and important pathways. The DAVID functional analysis tool was used with a threshold count of 1.0 and EASE threshold of 0.5. Network analysis for the corresponding gene sets was performed using IPA (**I**ngenuity **P**athway **A**nalysis), version 21249400, released in March 2015 (www.qiagen.com/ingenuity). The list of significantly differentially expressed genes was uploaded to the IPA software and mapped against a reference knowledge base that contains curated information from the published scientific literature. Genes that successfully mapped to the knowledge base were then used to generate the biological pathways and their associated networks.

Quantitative (q) RT-PCR

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis was performed on a selection of differentially expressed genes to validate the transcriptomics results. Total RNA was extracted from A549 cell lines cultured with and without *S. aurantiacum* conidia as described above. All RNA samples were subjected to DNase digestion using DNA-free Turbo DNase Digestion Kit (Ambion) and reverse transcribed to cDNA using QuantiTect® Reverse Transcription Kit (Qiagen) following the manufacturer's guidelines. A standard 10 µl real time-PCR reaction mix comprised 2 µl of cDNA (2.5 ng/µl) template, 3 µl forward and reverse primers (2 µM each) and 5 µl of Superscript III Platinum SYBR Green One master mix (Invitrogen). All reactions were carried in triplicate in a qTOWER 2.2 (Analytik, Jena) with an initial denaturation of 95°C for 10 min followed by 40 cycles of 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. The primers for each gene (Table S1) were synthesised using Primer3 (Rozen and Skaletsky, 2000) and checked for their amplification efficiencies. PCR reactions were also set for RNA only samples to check gDNA contamination in the RNA samples and confirm the quality of cDNA. Housekeeping gene GAPDH was included as an internal reference. Cycle threshold (C_T) values were exported from the qPCRsoft 2.0

software as an excel file and relative abundance of RNA was calculated in each sample using $\Delta\Delta C_T$ method (Livak *et al.*, 2001).

Results

Adherence of *S. aurantiacum* Conidia to the A549 Pneumocytes

Human lung infection with *S. aurantiacum* conidia was mimicked using the human lung adenocarcinoma epithelial cell line (A549 cells), co-cultured with a highly virulent *S. aurantiacum* strain WM 06.482.

Cell Adherence Assay

The adherence of *S. aurantiacum* to the A549 cell line was determined after co-incubating the A549 cell monolayers with WM 06.482 conidia (MOI = 10, 1 and 0.1 per human cell) at 37°C for 2 and 4 hours (hr) respectively. The relative extent of adhesion of WM 06.482 conidia to A549 cells was directly proportional to the amount of conidia added at each time point *i. e.* 2 and 4 hr (Fig. 5-1), with maximum adherence observed for a conidia to pneumocyte ratio of 10:1.

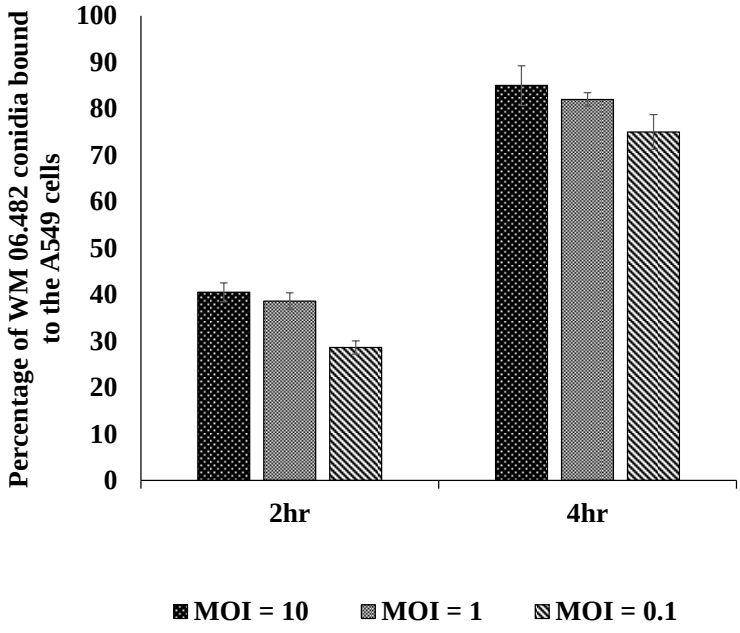

Fig. 5-1. The extent of adhesion of *S. aurantiacum* WM 06.482 conidia to the A549 epithelial cells as measured after 2 hr and 4 hr respectively. Error bars represent standard error (±SE) of the mean of three biological replicates for each time point.

Only about 40% of the fungal conidia were attached to the epithelial cells over a period of 2 hr whereas at the end of 4 hr, the adhesion percentage increased to about 80% in the A549 cells infected with different doses of WM 06.482 conidia.

Confocal Microscopy of the WM 06.482 and A549 Cell Co-cultures

The attachment of WM 06.482 conidia to the cultured epithelial cells was visualised using confocal microscopy of the co-cultures stained with a nucleic acid stain (Hoechst). *S. aurantiacum* conidia became attached to the A549 cell monolayers within 4 hr of co-incubation as seen in Fig. 5-2 A-C. Fungal attachment to the A549 cells appeared to be specific as no attachment was observed on the glass coverslips without the epithelial cells (data not shown).

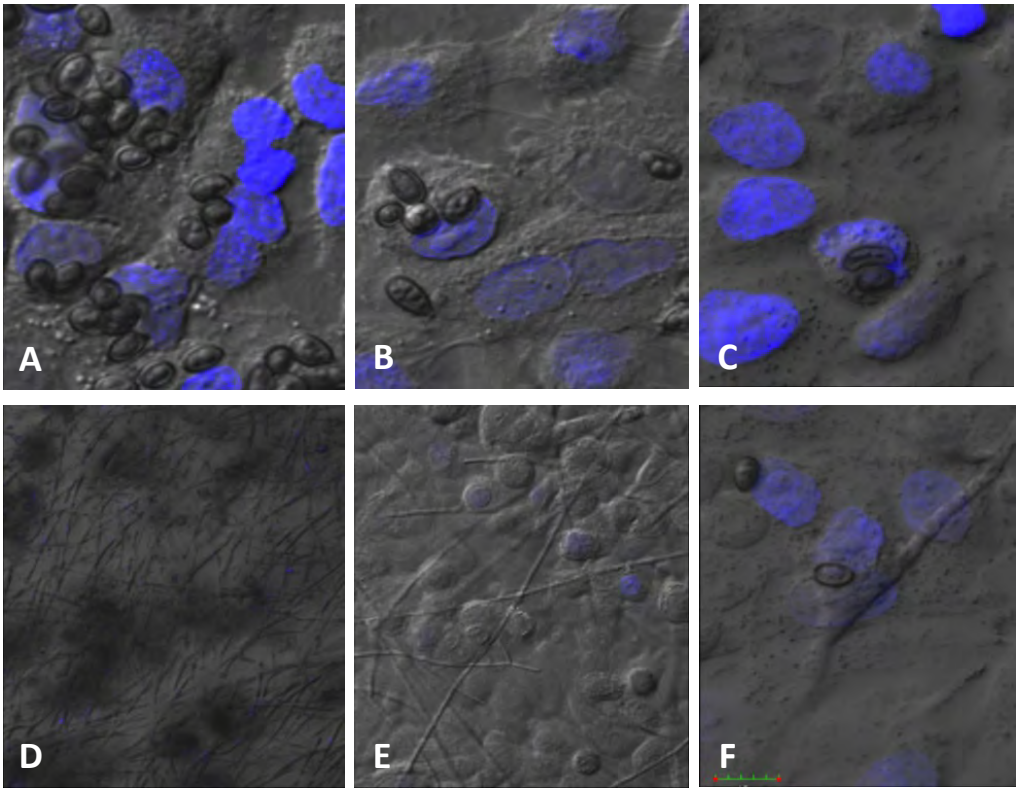

Fig. 5-2. Confocal laser scanning microscopy (CLSM) images of the co-culture of *S. aurantiacum* strain WM 06.482 and A549 pneumocyte monolayers after 4 hr and 24 hr of incubation at 37°C. The epithelial cells were stained with nucleic acid specific stain Hoechst 33342 (shown in blue). Scale bars (A) = 5 µm, (B, C and F) = 10 µm and (D and E) = 20 µm. A549 cells after incubation with WM 06.482 conidia **(A, D)** MOI = 10:1, **(B, E)** MOI = 1:1 and **(C, F)** MOI = 0.1:1 for 4 hr (A-C) and 24 hr (D-F), respectively.

CLSM images (Fig. 5-2 A-C) show that pneumocytes carried more than one *S. aurantiacum* conidium that were mainly attached to the A549 cell surface. Following 24 hr of co-incubation (Fig. 5-2 D-F), most of the fungal conidia germinated and formed a network of hyphae on the A549 cells. Conidia to pneumocyte ratio of 1:1 was considered optimum for the binding of WM 06.482 conidia to the A549 cells (Fig. 5-2 B and E) as a higher fungal burden (10:1, Fig. 5-2 A and D) resulted in greater pneumocyte damage, and a lower fungal load (0.1:1, Fig. 5-2 C and F) was not sufficient to cause any effect.

Study of Attachment of Fungal Conidia to A549 Cells using SEM

Interactions between *S. aurantiacum* and human pneumocytes were further characterised using SEM. The cultures were constituted with a conidia to pneumocyte ratio of 1:1 and maintained for 4, 8 and 24 hr respectively to assess the interactions between *S. aurantiacum* strain WM 06.482 and A549 epithelial cells at different stages of fungal growth *i. e.* before and after conidial germination.

SEM analysis of the co-cultures showed that after 4 hr of incubation, most of the *S. aurantiacum* conidia were attached to the pneumocyte cell surface in small groups (Fig. 5-3 A). Small germ tube projections were observed for many fungal conidia that were mainly present in the interstitial regions between the pneumocytes (Fig. 5-3 B). WM 06.482 infected the A549 cells by penetrating the epithelial cell membrane with the help of the germ tubes (Fig. 5-3 B). At a later time point, *i. e.* 8 hr, WM 06.482 conidia penetrated many A549 cells as seen in Fig. 5-3 C, while some pneumocytes still appeared morphologically intact. At the end of 24 hr (Fig. 5-3 D), the pneumocytes were completely covered by *S. aurantiacum* and damaged cells showed distinct signs of membrane blebbing and apoptosis which was visualised by the appearance of a large number of small sphere-like apoptotic bodies as seen in Fig. 5-3 E. After 24 hr, the A549 cells were completely detached from the culture plate whereas conidia free control pneumocytes remained adhered (Fig. 5-3 F).

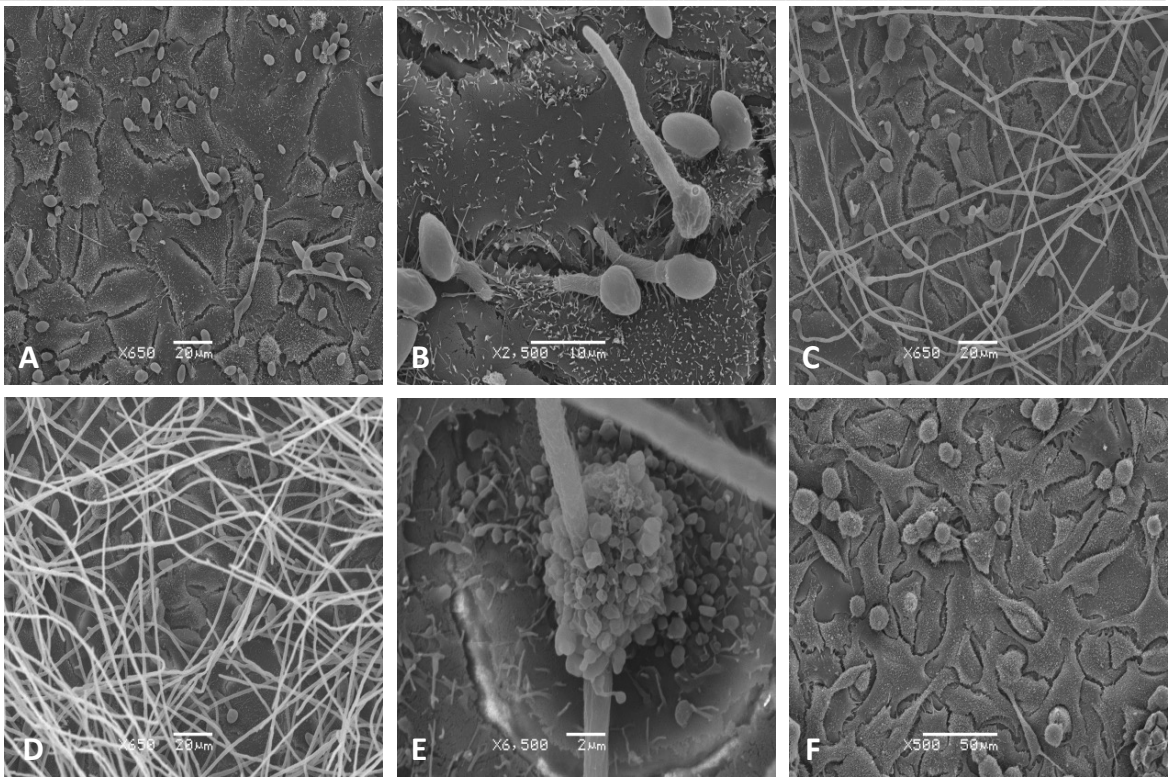

Fig. 5-3. The invasion of A549 epithelial cells by *S. aurantiacum* WM 06.482 as observed by scanning electron microscopy (SEM). Scale bars (A, C and D) = 20 µm, (B) = 10 µm, (E) = 2 µm and (F) = 50 µm. (A) WM 06.482 conidia adhered to A549 cells after 4 hr of incubation (x 650). (B) Germ tube projections from the WM 06.482 conidia attached to the A549 interstitial sites (x 2500). (C) A549 cells covered with WM 06.482 hyphae after 8 hr of co-incubation (x 650). (D) WM 06.482 hyphae completely covering the A549 cells after 24 hr (x 650). (E) Rounding of A549 cells after 8 hr (x 6500). (F) Conidia free A549 control cells maintained for 24 hr (x 500).

Transcriptome Analysis of Mixed *S. aurantiacum* and Pneumocyte Cultures

RNA sequencing was carried out for A549 epithelial cells infected with live WM 06.482 conidia in order to investigate the gene expression changes in pneumocytes in response to *S. aurantiacum* infection. RNA was extracted from the A549 cells infected with WM 06.482 conidia for 8 hr and the A549 cells maintained for the same amount of time. The statistical analysis of the RNA seq paired end reads revealed around 3950 differentially expressed genes ($p \leq 0.05$). Out of this statistically significant list of genes, 2008 genes were found to be up-regulated and 1941 were down-regulated. The complete list of genes is provided as a supplementary file (5-S2). Top 25 differentially expressed genes are shown in Table 5-1.

Table 5-1: A list of top 25 differentially expressed genes in the A549 cells in response to infection with WM 06.482. The genes were sorted with a log2 (fold change) cutoff of 1.0.

Gene Symbol	Gene name	Function	Log2 (Fold change)
EGR1	early growth response 1	Cell growth and differentiation, apoptosis	4.07
NR4A1	nuclear receptor subfamily 4, group A, member 1	Induction of apoptosis	3.09
FOS	FBJ murine osteosarcoma viral oncogene homolog	Regulation of apoptotic cell death	2.431
ANGPTL4	angiopoietin like 4	Regulation of glucose metabolism	2.292
RASD1	RAS, dexamethasone induced 1	Regulation of cell morphology	2.238
CXCL8	chemokine (CXC motif) ligand 8	Inflammatory response	2.168
ATF3	activating transcription factor 3	Cellular stress response	1.927
MUC5B	mucin 5B, oligomeric mucus/gel forming	Airway defense response	1.867
TNFAIP3	tumor necrosis factor, alpha induced protein 3	Involved in immune and inflammatory response	1.52
IL11	interleukin 11	Activation of T-cell development	1.27
GPR157	G protein coupled receptor 157	Signal transduction	1.21
IER3	immediate early response 3	Resistance against cell stress and starvation	1.19
MAPK8IP3	mitogen activated protein kinase 8 interacting protein 3	Wound healing	1.13
NFkB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells	Inflammation and immune response	1.10
NFKBID	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta	Inflammatory response	1.07
CCL2	chemokine (CC motif) ligand 2	Signalling, Inflammatory process	1.01
TXNIP	thioredoxin interacting protein	Regulation of glucose homeostasis	-1.080
TLR3	Toll like receptor 3	Activation of NF-kB	-0.874
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	Induction of apoptosis	-0.639
CEP70	centrosomal protein 70 kDa	Organisation of microtubules	-0.599
KNSTRN	Kinetochore localized astrin/SPAG5 binding protein	Chromosome segregation and progression into anaphase	-0.597
MAP9	microtubule associated protein 9	Bipolar spindle organisation, mitosis and cytokinesis	-0.594
CENPL	centromere protein L	Kinetochore assembly and mitosis progression	-0.581
GSPT2	G1 to S phase transition 2	G1 to S phase transition	-0.55
CKAP2	cytoskeleton associated protein 2	Regulation of cell division	-0.43

Functional annotation of the differentially expressed genes

Functional annotation and gene ontology (GO) classification of the list of genes was performed using DAVID online tool. In DAVID annotation system, EASE Score, a modified Fisher Exact P-Value is used to perform the gene set enrichment in annotation terms. The GO categories significantly over represented in the up-regulated genes are shown in Table 5-2A and down-regulated genes are shown in Table 5-2B, with *p* values less than 10^{-7}. The up-regulated genes showed enrichment of GO terms associated with cell death, inflammation and signalling molecules such as cytokines whereas GO terms categories for down-regulated genes included processes related to cell cycle progression such as cell division, mitosis and chromosome segregation.

Table 5-2. Gene ontology (GO) term annotation for the list of differentially expressed genes.

A. List of gene ontology terms associated with up-regulated genes

GO ID	Function	Ontology	*p*-value
GO:0009611	response to wounding	BP	1.63E-07
GO:0008219	cell death	BP	8.09E-06
GO:0007155	cell adhesion	BP	6.41E-06
GO:0006955	immune response	BP	1.07E-06
GO:0006954	inflammatory response	BP	5.3E-05
GO:0005125	cytokine activity	MF	2.32E-05
GO:0009991	response to external stimulus	BP	1.01E-04
GO:0030036	actin cytoskeleton organisation	BP	7.34E-04
GO:0006915	apoptosis	BP	9.48E-04

B. List of gene ontology terms associated with down-regulated genes			
GO ID	**Function**	**Ontology**	***p*-value**
GO:0000279	M-phase	BP	9.82E-07
GO:0022402	cell cycle process	BP	7.63E-07
GO:0051301	cell division	BP	1.05E-06
GO:0000278	mitotic cell cycle	BP	1.63E-06
GO:0007067	mitosis	BP	1.17E-06
GO:0048285	organelle fission	BP	7.85E-05
GO:0007098	centrosome cycle	BP	1.43E-05
GO:0043229	intracellular organelle	CC	4.40E-04
GO:0007059	chromosome segregation	CC	1.74E-03
GO:0000280	nuclear division	BP	1.17E-02

* BP= biological process, MF= molecular function and CC= cellular component

Pathway detection and gene network analysis using Ingenuity Pathway System (IPA)

The statistically significant list of genes (Supplementary data) was further analysed using Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com) for the detection of biological pathways and gene networks. IPA software classified the differentially expressed genes according to the biological processes associated with them (Fig. 5-4). Biological processes for the corresponding gene sets were mainly related to cell death and survival, cell to cell signalling, immunological processes or inflammation.

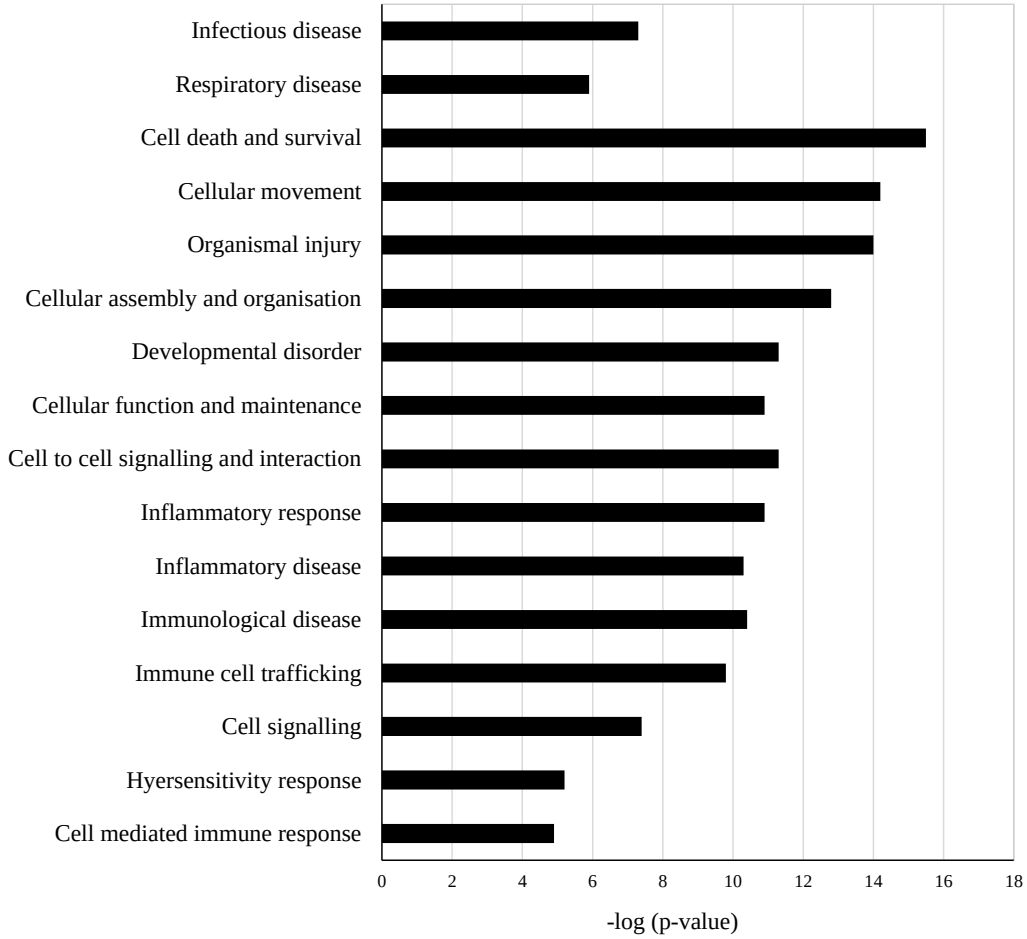

Fig. 5-4. Biological processes active in the A549 pneumocytes infected with *S. aurantiacum* WM 06.482 conidia. y-axis represents the biological processes and x-axis is the –log (p-value) as obtained after IPA analysis.

The differentially expressed genes involved in these biological processes were then mapped onto several pathways using IPA to study the gene networks and their interaction patterns. For example, a pathway map for inflammation (Fig. 5-5) revealed interactions between genes that pertain to NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) complex and/or NF-kB family such as *NFKB2* and *RELB*. In the same cluster, transcription regulators such as NFKBID (inhibitor, delta), JUNB, AP1 (activator protein) and ATF3 (activating transcription factor 3) were also unveiled. Interleukins such as IL11 and CXCL8 (interleukin 8) were also present in the same group. The only gene that was downregulated in the network was *TXNIP* (thioredoxin-interacting protein).

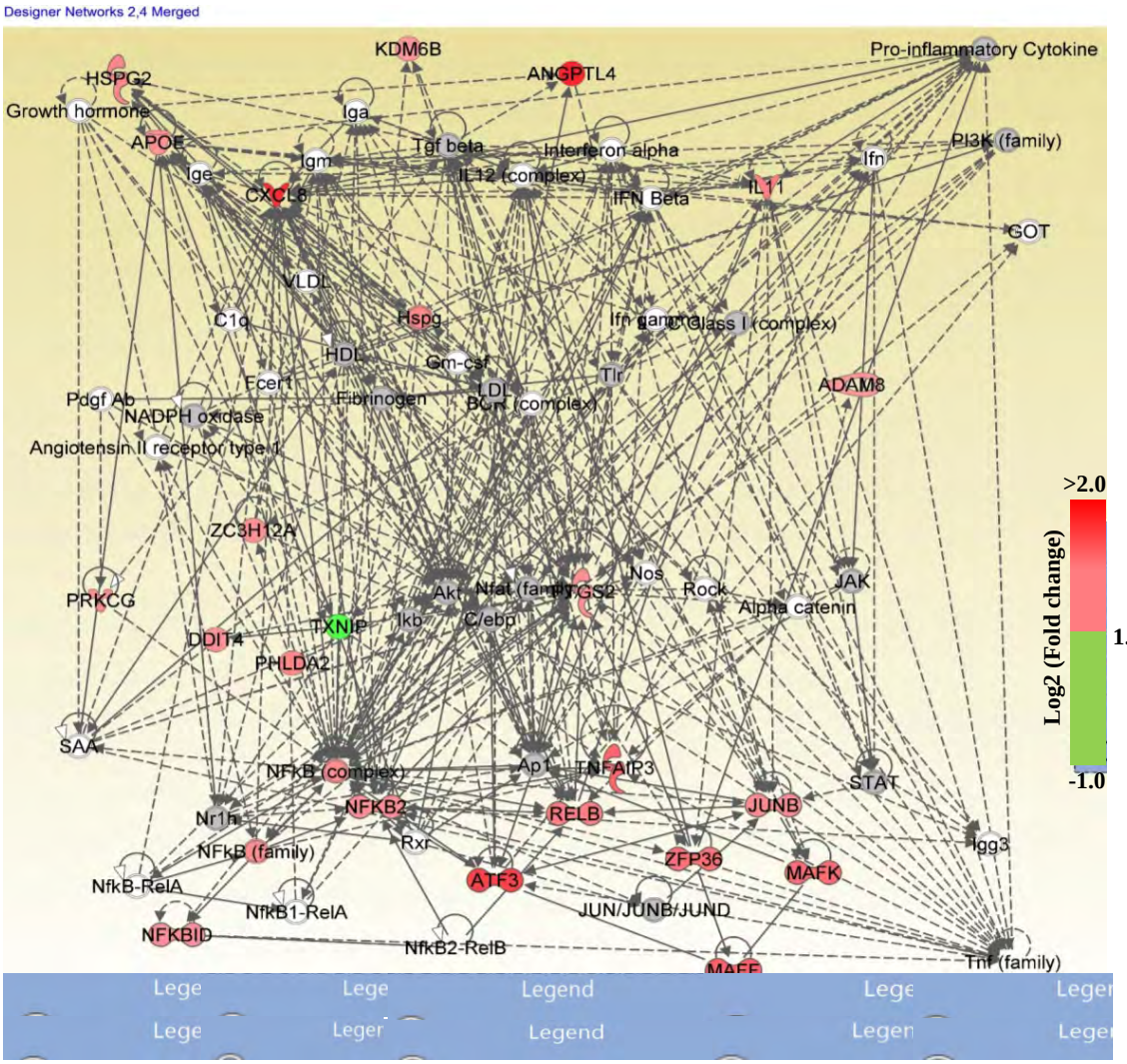

Fig. 5-5. Innate signalling pathway obtained after IPA analysis of the genes that were differentially expressed in the A549 human alveolar epithelial cells exposed to *S. aurantiacum* WM 06.482 conidia. The colour red or green in the network map symbolises significantly up and down regulated genes, respectively. The intensity of the red colour indicates the level of up-regulation of the gene expressed as log2 (fold change). Grey colour indicates the genes that did not show any changes in expression after 8 hr of incubation with WM 06.482.

Analysis of the subnetwork of immunological cluster (Fig. 5-6) revealed that integrated signaling between transcription factors such as JUNB, Ap1 and TNFAIP3, and members of NF-kB family including *RELB* and *NFKB2* might be involved in the up-regulation of proinflammatory cytokines such as IL11 and CXCL8.

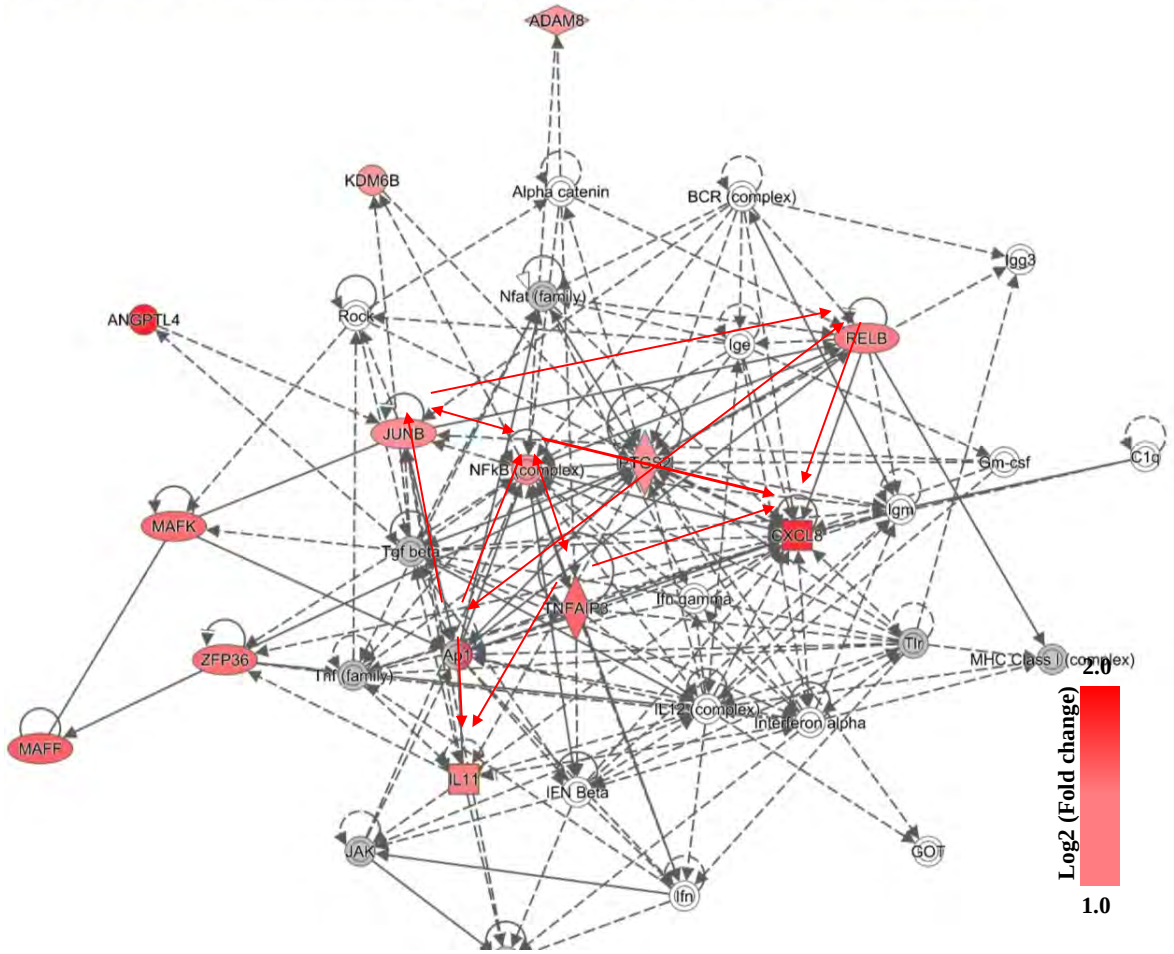

Fig. 5-6. Sub-network of the innate signaling pathway obtained after IPA analysis of the differentially expressed genes in A549 epithelial cells infected with WM 06.482 conidia. Significantly up-regulated genes are shown in red and the intensity of red color shows the level of up-regulation of the gene expressed as log2 (fold change). The genes that did not show any changes in expression after 8 hr of incubation with WM 06.482 are shown in grey.

Validation of Selected Differentially Expressed Genes using qRT-PCR

Five different genes were selected to validate the transcriptomics data using qRT-PCR analysis. These genes were selected based on the immunological relevance and included *RELB*, *ATF3*, *TNFAIP3*, *MUC5B* and *CXCL8*. *CXCL8* showed highest up-regulation (8 fold) in the A549 cells infected with *S. aurantiacum* compared to the A549 cells alone (Fig. 5-7). Other genes including *ATF3*, *TNFAIP3* and *MUC5B* were up-regulated 5.34 fold, 4.2 fold and 4.6 fold respectively. A low level but significant up-regulation was also observed for *RELB* (1.77 fold). No amplification was observed for RNA samples, which confirmed the purity of cDNA.

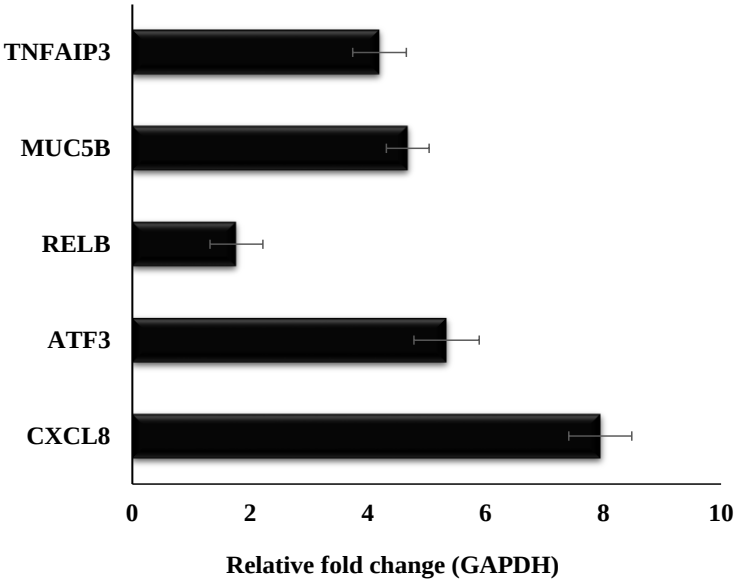

Fig. 5-7. Relative expression of selected genes in the A549 pneumocytes exposed to *S. aurantiacum* WM 06.482 conidia for 8 hr. Error bars represent standard error (±SE) of the mean of three biological replicates for each time point.

Discussion

The airway epithelial cells lining the respiratory tract regulate the development of chronic respiratory diseases by acting as a physical barrier for the inhaled microbes including bacteria and fungi (Holtzman *et al*., 2014). The importance of lung epithelial cells in clearance of inhaled microbes has been a motivation for numerous studies involved in assessing the interaction of bacteria and/or fungi with these cells (Claudia *et al*., 2002; Gafa *et al*., 2006; Gafa *et al*., 2007). These studies have primarily focussed on exploring the mechanism of epithelial cell invasion by major bacterial and fungal pathogens such as *A. fumigatus, C. neoformans, C. albicans, P. aeruginosa* and *Mycobacterium tuberculosis* (Filler and Sheppard, 2006). To date there is only one report, which has shown the process by which *S. boydii*, a member of *Scedosporium* spp., invades the mammalian epithelial cells including A549 and HEp2 cells (Pinto *et al*., 2004). However, there is no published literature concerning the interaction between *S. aurantiacum* and human alveolar epithelium. Hence the main aim of our study was to assess the adherence and subsequent effects of a highly virulent *S. aurantiacum* strain WM 06.482 on the cultured A549 cell line during the initial stages of infection (up to 24 hr). A549 cell line derived from a human

lung carcinoma was used in the study as it shares metabolic and morphological features with the human type II alveolar epithelial cells (Foster *et al*., 1998) and has been used previously to represent the carriage of a large number of bacterial and fungal pathogens to the human lungs (Daly *et al*., 1999; DeHart *et al*., 1997; Hawdon *et al*., 2010; Lieber *et al*., 1976; Paris *et al*., 1997; Tomee *et al*., 1997).

The most crucial step in the pathogenesis of an infectious microbe is the adherence to the host alveolar epithelium (DeHart *et al*., 1997). Our results demonstrated, for the first time, the ability of *S. aurantiacum* (WM 06.482) conidia to adhere to the pneumocytes (A549 cells). The percentage of adhesion of WM 06.482 conidia to the A549 cells surface was monitored using the conidia to pneumocyte ratios (MOI) of 10:1, 1:1 and 0.1:1 that were maintained for 4 hr and 24 hr respectively. During the first 2 hr of infection, only 40% of WM 06.482 conidia were adhered to the A549 pneumocytes in all the co-cultures but the rate of adherence doubled (80%) at the end of 4 hr (Fig. 5-1). These results indicate that a minimum of 4 hr is needed for the attachment of the majority of WM 06.482 conidia to the A549 cells. This is different to *A. fumigatus* conidia that bind to the A549 cells within 40 min of incubation as shown by DeHart *et al.* (1997). Most of the studies involving interaction between filamentous fungi and lung epithelial cells have been performed using a high MOI *i. e.* conidia to pneumocyte ratio of 10:1 (Gomez *et al*., 2010; Pinto *et al*., 2004; DeHart *et al*., 1997). However, in our study the confocal microscopy of the A549 pneumocyte and WM 06.482 co-cultures revealed that high conidial input *i. e.* MOI = 10:1 resulted in greater cell damage and injury and cells were difficult to image after 24 hr (Fig. 5-2 A and D), whereas low conidial input (MOI = 0.1:1) did not affect the cells even after 24 hr (Fig. 5-2 C and F). Therefore, a MOI of 1:1 was chosen to carry out further quantitative studies on interaction of *S. aurantiacum* conidia with the pneumocytes.

The electron microscopy studies demonstrated that WM 06.482 infection in A549 cells was initiated by the attachment of the conidia to the cells within 4 hr followed by formation of conidial germ tubes. This is different to *A. fumigatus* conidia, which show high attachment to the pneumocytes within initial 30 min of co-incubation but germinate only after 8 hr (Bellanger *et al*., 2009). Almost all previous studies on the interaction of filamentous fungi and yeast with the lung epithelial cells have shown the endocytosis of fungal conidia/yeast cells within the cells after an initial adhesion step (Gomez *et al*., 2010; Park *et al*., 2005; Pinto *et al*., 2004). However, the confocal and SEM images (Fig. 5-2 and 5-3) obtained in our study showed that most of the *S. aurantiacum* conidia were present on

the cell surface, with no signs of conidial internalisation. Therefore, we focused our studies primarily on the externally adherent conidia that germinated outside the cells.

It has been observed that the differentiation of conidia is important for the infection of a host by opportunistic fungi (Osherov and May, 2001). The appearance of germ tubes within the early 4 hr of interaction of *S. aurantiacum* conidia with the pneumocytes suggests an active participation of the fungus in the interaction process. Furthermore, the SEM images (Fig. 5-3B) showed that germ tubes were able to penetrate the epithelial cells through the intercellular spaces. Previous studies by DeHart and others showed that the majority of *A. fumigatus* conidia also invade the alveolar airway epithelium by adhering to the cell surface, germinating externally and penetrating the cells (Bellanger *et al*., 2009; Wasylnka and Moore, 2002; DeHart *et al*., 1997). After 8 hr of incubation, WM 06.482 penetrated many of the A549 cells by elongating hyphae. Almost all pneumocytes were invaded by the fungal hyphae within 24 hr and the infected cells underwent loss of membrane integrity *i. e.* membrane blebbing and cell rounding (Fig. 5-3 E) whereas the morphological integrity of few non-infected pneumocytes appeared to be preserved. Membrane blebbing is considered as an important phenomenon that represents the onset of apoptosis in the cells as a response to stress factors and allergens (Barros *et al*., 2003). It was not possible to assess the *S. aurantiacum* infection in A549 cells beyond 24 hr as profuse hyphal growth caused cell death and detachment from the surface.

Transcriptional profiling was then applied to analyse the response of A549 cells that were incubated with WM 06.482 conidia for 8 hr. Transcriptome studies have been used to assess the interactions between mammalian hosts and a large number of microbes (Cummings and Relman, 2000; Huang *et al*., 2002; Liu *et al*., 2006; Waddell *et al*., 2007); however to the best of our knowledge, this is the first report on the transcriptome of human alveolar epithelial cells exposed to *S. aurantiacum* conidia. A list of 3950 genes were found to be differentially expressed in the A549 cells infected with WM 06.482 conidia compared to non-infected A549 cells maintained for the same length of time (8 hr). In particular, the cells showed increased levels of transcripts from genes associated with repair and inflammatory processes (*e. g.* chemokines) (Table 5-2 A). Genes involved in mucin production were also found to be up-regulated which indicates that the cells might initiate a mucosal response against *S. aurantiacum* infection. The down-regulated genes were mainly involved in cell cycle progression, which suggests a reduction in the

proliferation of the A549 cells in response to *S. aurantiacum* infection (Table 5-2 B). Reduction in the cell cycle progression has also been reported in case of human lung epithelial cells infected with *A. fumigatus* (Gomez *et al*., 2010). Further analysis of the most significantly differentially expressed genes using DAVID and IPA strengthened the relevance of the identified genes.

Network analysis of the corresponding gene sets revealed the up-regulation of inflammation pathway in the A549 pneumocytes in response to the interaction with *S. aurantiacum* (Fig. 5-6). Genes that showed the highest degree of up-regulation in the inflammation pathway were two chemokines (CXCL8/IL8 and IL11) and members of NF-kB family involving *RELB* and their transcriptional regulators such as TNFAIP3 and ATF3. These genes were not only up-regulated in the transcriptomics data set but were also confirmed by qRT-PCR (Fig. 5-7). NF-kB is the most predominant transcription factor in human respiratory epithelial cells that is involved in cell responses to allergen activity. It is also known to control a wide variety of physiological processes including cell differentiation, cytokine expression, cell survival and proliferation (Rahman and MacNee, 1998). Under normal conditions, NF-kB is present in the cell cytosol in an inactive state. However, under stress conditions, such as invasion of cells by pathogenic microorganisms, NF-kB enters the nucleus and activates the expression of pro-inflammatory genes encoding proteins such as CXCL8, that are responsible for activating T-cells and recruiting neutrophils and eosinophils at the site of injury (Lawrence, 2009; Pahl, 1999; Wright and Christman, 2003; de Oliveira *et al*., 2013). Previous studies have reported NF-kB mediated increase in the level of IL8 production in the human respiratory epithelial cells exposed to other fungi including *A. fumigatus* and *A. alternata* and bacteria such as *P. aeruginosa* (Mukaida, 2000; Pease and Sabroe, 2002; Craig *et al*., 2009; Babiceanu *et al*., 2013; Bellanger *et al*., 2009; Kauffman *et al*., 2000; Tomee *et al*., 1997). Our dataset also revealed the presence of a metalloprotease ADAM8 that is known to protect the lungs against allergic pulmonary disease, and can be induced by allergens and Th2 cytokines (Matsuno *et al*., 2008). Together these findings suggest that human alveolar cells could recognise the *S. aurantiacum* conidia that were attached to them and initiate a host defense response by secreting inflammatory cytokines *via* NF-kB pathway.

While the adhesion of fungal conidia to the host pneumocytes and their subsequent immune reaction has been explored widely, the mechanism by which the fungus penetrates

the epithelial cell layer is largely unknown (Filler and Sheppard, 2006). Fungal penetration into the pneumocytes can be accomplished *via* a variety of mechanisms that have been studied in some fungi and involve fungal enzymes (proteases such as elastases) and metabolites (Holden *et al*., 1994; Kothary *et al*., 1984; Miyaji and Nishimura, 1977). The mechanism of epithelial cell invasion by *S. boydii*, a member of *Scedosporium* spp., was first studied by Pinto *et al* (2004). They reported the involvement of a fungal antigen, peptidorhamnomannan (PRM), in the initiation of epithelial cell invasion (Pinto *et al*., 2004). *S. aurantiacum* has been recently acknowledged as an emerging pathogen in the Australian sub-continent because of an increase in the incidences of *S. aurantiacum* infections in the lungs of Australian cystic fibrosis (CF) patients. Therefore there is limited genetic information concerning *S. aurantiacum* (Blyth *et al*., 2010; Harun *et al*., 2011; Pihet *et al*., 2009). In this context, genomes of four different *S. aurantiacum* strains (including WM 06.482) were sequenced recently (Perez-Bercoff *et al*., 2015) and the annotation is ongoing. In addition to providing an important reference for all further studies into *S. aurantiacum*, an annotated genome will also help to determine the mechanism used by the fungus for lung invasion.

Conclusions

We report for the first time, interactions between a highly virulent *S. aurantiacum* strain (WM 06.482) and human alveolar epithelial cells (A549 cell line) *in vitro* using microscopy techniques and transcriptome analysis of the infected A549 cells. The invasion process of WM 06.482 including initial attachment of fungal conidia to the A549 pneumocytes followed by conidial germination and penetration into the cells, follows that of other fungal pathogens, which have been previously described in the literature, but appears faster. Whole genome transcriptional profiling revealed that A549 pneumocytes showed a protective response against *S. aurantiacum* infection by activating their defense system. This response was characterised by the activation of immunological pathways leading to the production of chemokines and cytokines against the fungus. This study represents a critical step in advancement of the understanding of the interactions between *S. aurantiacum* and human respiratory epithelial cells and paves the way for more complex studies in the future aimed at assessing the mechanisms of hyphal invasion into the pneumocytes.

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Supplementary table 5S-1.

Table 5S-1. Primer sequences for selected differentially expressed genes validated by qRT-PCR.

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	CTCTGACTTCAACAGCGAC	TGGTCCAGGGGTCTTACT
CXCL8	TCCTGATTTCTGCAGCTCTG	GTCCACTCTCAATCACTCTCAG
ATF3	CCTCGGAAG TGAGTGCTTCT	ATGGCAAACCTCAGCTCTTC
MUC5B	CACATCCACCCTTCCAAC	GGCTCATTGTCGTCTCTG
TNFAIP3	AAAGCCCTCATCGACAGAAA	CAGTTGCCAGCGGAATTTA
RELB	CATCCTGGACCACTTCCTGCC	GAACATGTTGCTGCCCACAAG

Supplementary table 5S-2.

Table 5S-2. A list of differentially expressed genes in human lung epithelial cells exposed to *S. aurantiacum* strain WM 06.482.

*Table 2 is provided as an excel file in a CD.

6

CONCLUSIVE SUMMARY AND FUTURE PROSPECTS

Conclusive summary and future prospects

The work described here is the first attempt to understand the overall physiology of *S. aurantiacum* as well as mechanisms underlying its interactions with the host lungs and prokaryotic lung inhabitants such as *P. aeruginosa*. This is a step forward as most of the previous studies have focussed on the clinical and epidemiological aspects of *S. aurantiacum*.

Chapter-3 gives an overview of the phenotypic profiling of four different strains of *S. aurantiacum* (WM 06.482, WM 08.202, WM 10.136 and WM 09.24) displaying different levels of virulence in the *Galleria mellonella* larvae assays. Three of the four *S. aurantiacum* strains were classified as high virulence (WM 06.482, high rate of killing = 85%) or low virulence (WM 08.202 and WM 10.136, low rate of killing =20%) based on the larval assay. However, approximately 40% of larvae were killed when exposed to WM 09.24, thereby making it difficult to initially classify WM 09.24 either as a high or a low virulence strain. Phenotype Microarray (PM) technology was then used for profiling carbon source utilisation in *S. aurantiacum* with an aim to identify metabolic differences between the high and low virulence strains. Comparison of the phenotypic profiles of the strains at two different temperatures (28 and 37 °C) suggested that WM 06.482 and WM 09.24 were able to adapt the metabolic pathways to best utilise the available nutrients under both the temperature conditions tested. On the other hand, WM 08.202 and WM 10.136, both classified as low virulence strains, could utilise the available nutrients only at a higher temperature (37 °C). The two above-mentioned groups of *S. aurantiacum* strains also differed from each other in their ability to utilise turanose (WM 06.482 and WM 09.24) and sucrose (WM 08.202 and WM 10.136). Therefore, considering the similarity in the behavior of WM 09.24 and the high virulence strain WM 06.482 in the nutrient utilisation assays, these strains were grouped together in the high virulence group. These results were further validated by performing growth assays and correlating the metabolic data with the assembled genome data available for *S. aurantiacum* to identify the presence of corresponding carbohydrate utilisation pathways. The biggest challenge in the validation of metabolic data was the absence of an annotated *S. aurantiacum* genome. Therefore, the sequences of the genes involved in the metabolic pathways of interest were identified from other closely related filamentous fungal species and compared against the draft genome assemblies of the four *S. aurantiacum* strains to carry out metabolic reconstructions.

The ability of PM assays to unveil the metabolic differences between two groups of *S.*

aurantiacum strains proves the applicability of this technique for identifying intra-specific variations in members of the *Scedosporium* species as well as providing a base for the future metabolic studies. While the present work was mainly focussed on assessing the utilisation of carbon sources in different *S. aurantiacum* strains, the application of PM nutrient utilisation assays can be extended to include a wide variety of nitrogen, sulphur, phosphorous and potassium sources (Bochner *et al*., 2001, Singh, 2009). Another potential approach for future studies into *Scedosporium* spp. may involve the identification of fungal secondary metabolites using either functional analysis or discovery by integration of high performance analytical methodology (Smedsgaard *et al*., 2005). Metabolite profiling or metabolomic studies of *Scedosporium* spp. may prove as a powerful tool for taxonomy and identification and classification of different members of *Scedosporium* spp.

In **Chapter 4**, the nature of interactions occurring between *S. aurantiacum* and a major bacterial pathogen *P. aeruginosa* are discussed. The bacterium *P. aeruginosa* and the fungus *S. aurantiacum* are opportunistic pathogens that have been isolated from the lungs of CF patients. The interactions between *P. aeruginosa* and leading fungal pathogens such as *A. fumigatus* and *C. albicans* have been investigated extensively (Abuodeh *et al*., 2000, Bandara *et al*., 2010, Cugini *et al*., 2010, Hogan and Kolter, 2002). However, there is limited information on the nature of physiochemical, molecular and biological mechanisms involved in the interactions between *P. aeruginosa* and *S. aurantiacum*. In our study, interactions between three strains of *P. aeruginosa* (PAO1, PASS01 and PASS02) and two clinical *S. aurantiacum* isolates (WM 06.482 and WM 08.202) were assessed using solid plate assays and liquid co-cultures in a medium mimicking the contents of sputum of CF patients. Apart from using chemical fluorescent stains, microbial strains genetically labelled with yfp (*P. aeruginosa* PASS1) and mCherry (*S. aurantiacum* WM 06.482) were developed in order to visualise the interactions under Confocal Laser Scanning Microscope (CLSM), which indicated that bacteria inhibited fungal growth. The development of an expression vector for expression of the mCherry gene in *S. aurantiacum* was the biggest challenge as there was not enough genome information to source for suitable homologous promoters and a transcription terminators required for the vector construction. Due to absence of a homologous promoter, the *mCherry* gene was successfully expressed under a heterologous promoter *pki* derived from *Trichoderma reesei*, a related ascomycetous species. Considering the high antifungal and antibiotic resistance of *S. aurantiacum*, it was also difficult to identify a suitable selection marker. Eventually, the commonly used *E. coli*

hph gene that confers resistance to the hygromycin antibiotic was chosen as a selective marker and was also expressed under the *pki* promoter. The expression cassette featuring the *mCherry* gene and a hygromycin resistance marker was introduced into the *S. aurantiacum* strain WM 06.482 using protoplast transformation. Although the transformation efficiency obtained was low, successful expression of mCherry was observed in a number of transformant strains. Development of a more efficient gene expression system for *S. aurantiacum* will be possible with an upcoming annotated genome in hand.

In general, *P. aeruginosa* strains displayed an inhibitory effect against *S. aurantiacum* in both solid plate and liquid assays, but differed from each other in the levels of inhibition. For example, biofilm forming high virulence clinical strains PAO1 and PASS01 demonstrated a higher inhibitory effect than the non-biofilm forming low virulence strain PASS02. The addition of a commonly used antibacterial agent gentamycin to the co-culture medium resulted in an increase in the growth of *S. aurantiacum* upon decline in the bacterial load. It has been observed that anti-pseudomonal antibiotics are increasingly administered in the CF patient cohort nowadays (Blyth *et al*., 2010b). These results may therefore provide direct evidence on the potential impact of antibiotic exposure either systemically and/or locally (*via* nebuliser) on the rates of recovery of *Scedosporium* spp in the CF patients. Therefore the use of optimal antimicrobial drug regimes along with the use of more aggressive laboratory diagnostics approaches such as multiplex viral PCR assays should be tested to evaluate the significance of *S. aurantiacum* in CF.

Examination of the liquid co-cultures showed that *P. aeruginosa* formed biofilms on the surface of *S. aurantiacum* hyphae that had an inhibitory effect against fungal growth. Hence it was proposed that biofilm formation by *P. aeruginosa* might be an important attribute for inhibition of the growth of *S. aurantiacum* in the medium mimicking CF sputum as the non-biofilm forming PASS2 did not an affect the growth of either of the two *S. aurantiacum* strains tested.

It is also worth mentioning that a direct cellular contact between *P. aeruginosa* and *S. aurantiacum* was not needed for the inhibition of fungal growth as a similar inhibitory trend was observed in the co-cultures involving both organisms separated with a membrane. This led us to speculate the involvement of extracellular bacterial metabolites and or exoproducts in the inhibition of fungal growth. Therefore, to explore this possibility, the effect of one of the most predominant bacterial virulence factors, pyocyanin (PCN^{-}), a

blue redox-active secondary metabolite (Lau *et al*., 2004) was evaluated against the *S. aurantiacum* strains. Somewhat surprisingly, pyocyanin did not inhibit the fungal growth. This might reflect the resilient nature of *S. aurantiacum* as compared to other opportunistic fungal pathogens or indicate the involvement of bacterial metabolites other than pyocyanins in the inhibition of fungal growth (Ben Haj Khalifa *et al*., 2011; Rella *et al*., 2012).

One aspect for the future studies is to identify the activity of other commonly described bacterial virulence factors including enzymes such as proteases/elastases, haemolysin and rhamnolipids (Ben Haj Khalifa *et al*., 2011; Rella *et al*., 2012) against *S. aurantiacum* using a similar approach as described in the thesis. These studies would help to provide more information about the mechanisms involved in the interaction between *S. aurantiacum* and *P. aeruginosa* and their subsequent impact on the progression of CF disease. In addition to that, with the forthcoming annotation of the *S. aurantiacum* genome, it will be possible to evaluate the interactions between *S. aurantiacum* and *P. aeruginosa* in more detail through transcriptomic analysis of the co-cultures.

As the *P. aeruginosa* strains used in the present study are fresh clinical isolates, it is not known whether they share similar genetic characteristics with the two dominant Australian *P. aeruginosa* shared strain clusters AUST-01 and AUST-02 that have been reported in various cross-sectional studies carried across different Australian CF centres (Kidd *et al*., 2013). Therefore further studies are also required to examine the genotypic relationships between the *P. aeruginosa* isolates PASS01 and PASS02 and the shared *P. aeruginosa* strains common in Australian CF patients.

Apart from the interactions of *S. aurantiacum* with prokaryotic lung inhabitants, the existing knowledge on *S. aurantiacum* also lacks basic information of its interactions with the human lung epithelial cells. A better knowledge of the mechanisms used by *S. aurantiacum* to interact with the host lung epithelial cells is crucial for understanding the pathogenesis of the fungus. For this reason, the interactions between *S. aurantiacum* and human alveolar type-II like A549 cell line were evaluated and are described in **Chapter 5**. The time-course of the interaction showed that the adhesion of *S. aurantiacum* conidia to the A549 pneumocytes occurred within 2 hr of incubation and the extent of adhesion also escalated with an increase in the incubation period. The interactions between *S. aurantiacum* conidia and the alveolar epithelial cells were assessed using microscopy techniques involving a confocal laser scanning microscope and a scanning electron

microscope. These studies showed that *S. aurantiacum* used a mechanism similar to that used by other pathogenic fungi (Bellanger *et al*., 2009; DeHart *et al*., 1997; Wasylnka and Moore, 2002) which involves an initial attachment of the fungal conidia to the epithelial cell surface, followed by germination of the conidia and subsequent penetration of the germ tubes into the cells through the intercellular junctions. Finally, the biologically relevant response of epithelial cells to *S. aurantiacum* infection was assessed using a transcriptomic approach. Analysis of the transcriptomic data using online software tools including IPA and DAVID showed that genes associated with inflammatory processes were upregulated in the A549 epithelial cells exposed to *S. aurantiacum*. These genes were involved in the NFkB pathway and included *NFkB*, *RELB* and their transcriptional regulators such as JUNB, NFKBID, AP1 and ATF3. Network analysis showed a direct link between activation of the NFkB pathway and up-regulation of inflammatory chemokines such as CXCL8 (also known as IL 8) and IL 11. Further analysis using qRT-PCR also confirmed the differential expression of these genes in the epithelial cells exposed to *S. aurantiacum*. These results suggest that the alveolar epithelial cells could recognise the presence of *S. aurantiacum* and protect themselves against the damage caused by the fungus by initiating an inflammatory response.

There is a growing interest in the development of a dual organism transcriptomic approach to assess the complex nature of interactions between pathogenic fungi and human lung epithelial cells. The application of the technique was first shown by Oosthuizen *et al.,* who examined the interactions between *Aspergillus fumigatus* conidia and the airway epithelial cells by studying the early transcriptomic responses of both interacting partners. They reported that interaction between *A. fumigatus* conidia and primary human airway epithelial cells lead to an up-regulation of cytokine IL-6 in the epithelial cells and pathways such as iron acquisition, vacuolar acidification and formate dehydrogenase activity in the fungus (Oosthuizen *et al*., 2011). Due to lack of an annotated *S. aurantiacum* genome, the transcriptomic response of *S. aurantiacum* conidia could not be studied in this work. The anticipated availability of an annotated *S. aurantiacum* genome will provide an opportunity to analyse the fungal transcriptomics data which we have collected and will help to gain insight into the response of the fungus to the host cell environment. In future, a transcriptomic approach could also be devised for the analysis of interactions between *S. aurantiacum, P. aeruginosa* and the host lung epithelial cells which would ultimately help

to validate the implications of *in vitro* findings of bacterial-fungal interactions in a clinically relevant system.

In the past few years, a new concept of human lung microbiome has gained special attention as it provides a deeper understanding of the microorganisms contributing to the chronic respiratory diseases. This emerging field allows the identification of candidate respiratory microbes during both homeostasis and infection conditions with the use of culture independent methods such as next generation sequencing. Clinical studies in respiratory microbiome suggest that fungi (mycobiome) present in the chronic respiratory diseases such as asthma, chronic obstructive pulmonary diseases, cystic fibrosis and bronchiectasis contribute to the decline of the lung function and disease progression by interacting with the bacteriome and/or virome (Nguyen *et al*., 2015). Similarly lung-mapping project is another recent initiative, which utilises techniques such as deep genetic sequencing of both DNA and RNA, and mass spectrometry based chromatography to remodel various parts of the lungs. In addition to providing a three-dimensional view of different types of microbes and their metabolites present within the lung, this three-dimensional model also helps in the identification of various microbial interaction patterns that lead to lung inflammation (Chakradhar, 2015). These studies involving human lung microbiome and human lung mapping project have lead to an increased understanding of the complexity of human CF lungs. Therefore the application of these techniques along with *in vitro* and *in vivo* methods described in the present study can revolutionise the identification and characterisation of bacterial-fungal interactions by comparing the cross-kingdom relationships in healthy and disease conditions.

To summarise, the research presented in the thesis aims to provide a better understanding of the physiological aspects of *S. aurantiacum* and mechanisms underlying its interactions with the most predominant bacterial lung inhabitant *P. aeruginosa* and the human alveolar epithelial cells.

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Appendix: Biosafety Approval

Macquarie University Mail - NLRD Ref 5201200092- Annual Report

MACQUARIE University

Jashanpreet Kaur <jashanpreet.kaur@mq.edu.au>

NLRD Ref 5201200092- Annual Report

Bio Safety <biosafety@mq.edu.au> Mon, Feb 16, 2015 at 11:08 AM
To: Jashanpreet Kaur <jashanpreet.kaur@mq.edu.au>

Dear Jashanpreet,

Thank you for sending that through. Your progress report has been approved. Please note the next progress report is due 1st April 2016. If the project is discontinued before this time, please submit a Final Report.

Many thanks,

Kate

On 16 February 2015 at 09:44, Jashanpreet Kaur <jashanpreet.kaur@mq.edu.au> wrote:

> Dear Kate,
>
> Please find attached a scanned copy of the annual report for the project entitled,'Re: "Molecular toolbox for the studies into the filamentous fungus Scedosporium aurantiacum" (Ref: 5201200092'
>
> My apologies for sending it late.
>
> Many thanks and regards
>
> Jashanpreet Kaur
>
> PhD Candidate
> Room 305, E8A
> Department of Chemistry and Bimolecular Sciences
> Macquarie University
> NSW 2109

--
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MACQUARIE University

Fwd: Biosafety Application Ref 5201200092- Final Approval

Helena Nevalainen < helena.nevalainen@mq.edu.au> Mon, Apr 9, 2012 at 8:42 AM
To: Jashanpreet Kaur <jashanpreet.kaur@mq.edu.au>

---------- Forwarded message ----------
From: Bio Safety <biosafety@mq.edu.au>
Date: Thu, Apr 5, 2012 at 1:31 PM
Subject: Re: Biosafety Application Ref 5201200092- Final Approval
To: Helena Nevalainen <helena.nevalainen@mq.edu.au>

Dear Prof Nevalainen,

Re: “Molecular toolbox for the studies into the filamentous fungus Scedosporium aurantiacum” (Ref: 5201200092)

Thank you for your recent correspondence. Your responses have been reviewed by the institutional Biosafety Committee and Final Approval of the above application is granted, effective 4 April 2012.

Approval has been granted subject to your compliance with the Office of the Gene Technology Regulator’s standard conditions for exempt work listed below:

1. The project must be conducted in accordance with the OGTR Guidance Notes for the Containment of Exempt Dealings (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/exemptdealclass-2).

2. You must inform the Institutional Biosafety Committee if you complete or abandon the exempt dealings with GMOs.

The following personnel are authorised to conduct this research:

Prof Helena Nevalainen- Chief Investigator
Dr Junior Te’o – Associate Investigator
Dr Anwar Sunna - Associate Investigator
Prof Ian Paulsen - Associate Investigator
Dr Anahit Penesyan - Associate Investigator
Jashanpreet Kaur - Associate Investigator

3. Face masks are to be worn when handling the organism as it is not well characterized in addition to the use of ‘fungal cabinets’ to safeguard any personnel against toxicity.

Please note the following standard requirements of approval:

Approval will be for a period of 3 years subject to the provision of annual reports. If, at the end of this period the project has been completed, abandoned, discontinued or not commenced for any reason, you are required to submit a Final Report. If you complete the work earlier than you had planned you must submit a Final Report as soon as the work is completed. These reports are located at the following address:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/biosafety_research_ethics/forms
A progress/Final report for this project will be due on: 4 April 2013

2. Please remember to notify the Committee of any alteration to the project by completing a 'Request for Amendment' form and submitting it to Biosafety@mq.edu.au The 'Request for Amendment' form is located at the following address:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/biosafety_research_ethics/forms

3. If you will be applying for or have applied for internal or external funding for the above project it is your responsibility to provide the Macquarie University's Research Grants Management Assistant with a copy of this email as soon as possible. Internal and External funding agencies will not be informed that you have final approval for your project and funds will not be released until the Research Grants Management Assistant has received a copy of this email. Is you need to provide a hard copy letter of Final Approval to an organisation as evidence that you have Final Approval, please do not hesitate to contact the Committee Secretary at the address below.

Please retain a copy of this email as this is your formal notification of final Biosafety approval.

Yours Sincerely,

A/Prof Subramanyam Vemulpad
Chair, Macquarie University Institutional Biosafety Committee
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Office of the Deputy Vice Chancellor (Research)

Macquarie University Biosafety Secretariat

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