Proteases of

Scedosporium aurantiacum,

an opportunistic fungal pathogen

Zhiping Han

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Supervisors:

Prof. Helena Nevalainen

Prof. Paul Haynes

Dr. Liisa Kautto

MACQUARIE University
SYDNEY·AUSTRALIA

Department of Chemistry and Biomolecular Sciences
Macquarie University, Sydney, Australia

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Contents

Abstract

Scedosporium aurantiacum is an opportunistic filamentous fungal pathogen that is capable of causing a range of infections in immunocompromised people. In Australia, *S. aurantiacum* is the second most common fungus associated with cystic fibrosis after *Aspergillus fumigatus*. The majority of the work carried out with *S. aurantiacum* currently described in the literature features prevalence studies, animal studies including virulence and antifungal susceptibility studies. At the moment, very little is known about the infection mechanism of this fungus.

Secreted proteases have been shown to contribute to fungal virulence in several studies with other fungi. This work aimed at profiling and identifying proteases secreted by *S. aurantiacum* with a view to investigating a potential link with protease production and fungal pathogenicity. Clinical (WM 06.482) and environmental (WM 10.136) *S. aurantiacum* isolates were grown in a synthetic cystic fibrosis sputum medium supplemented with either casein supporting the synthesis and secretion of a wide range of proteases or mucin, which mimicked the cystic fibrosis lung sputum. Liquid cultivations were performed under both normoxic and hypoxic conditions considering the fact that fungi have to encounter oxygen level differences between the environment and human organs.

Secreted proteases in the culture supernatants were examined using enzymatic assays with class-specific substrates, inhibition assays, zymogram activity analysis and protein identification by mass spectrometry. Serine proteases from both strains were responsible for the majority of protease activity under normoxia; elastase- and trypsin-like proteases from the clinical isolate had significantly higher activities than those from the environmental isolate. Under hypoxia, secreted proteases from both isolates were predominated by the aspartic class; cysteine proteases were also detected. The clinical isolate had higher acidic protease activity including aspartic and cysteine protease activities, while the environmental isolate had higher alkaline protease activity featuring chymotrypsin-, subtilisin-, elastase- and trypsin-like protease activities.

Protein identification was conducted for both isolates grown under normoxia and hypoxia. Three protease homologs were identified with high sequence similarity to known fungal proteases under normoxia including subtilisin protease S8, putative leucine aminopeptidase and a PA-SaNapH-like protease. Aspartic protease homologs were identified under hypoxia. Cysteine proteases secreted by the clinical *S. aurantiacum* isolate under hypoxia were involved

in both detachment and death of the A549 human lung epithelial cells *in vitro*, causing rounding up of the cells and release of the cell contents into the environment. Chymotrypsin-like protease from hypoxic cultures was also involved in cell detachment by rounding cells up, yet the floating cells were still alive. Elastase-like protease from normoxic cultures was found to cause cell death by a yet unidentified mechanism.

This work expands the existing knowledge of the emerging fungal pathogen *S. aurantiacum* and provides insights into roles of secreted proteases in fungal invasion.

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 institute or university other than Macquarie University.

I also certify that the thesis is an original piece of research conducted by me and it contains no materials previously published or written by any other person except where due reference is made in the text. I consent that a copy of this thesis is available at Macquarie University Library for loan and photocopying forthwith.

Zhiping Han
March 2017

List of publications and manuscripts

This thesis includes published article 1, and manuscripts 1 and 2, presented as Chapter III, IV and V respectively.

Published article:

1. Han, Z., L. Kautto, and H. Nevalainen, Secretion of Proteases by an Opportunistic Fungal Pathogen *Scedosporium aurantiacum*. PLoS One. 2017;12(1):e0169403.

Manuscripts:

1. Han, Z., L. Kautto, and H. Nevalainen, Growth and protease secretion of *Scedosporium aurantiacum* under hypoxia. Manuscript prepared for submission to Fungal Biology.

2. Han, Z., L. Kautto, and H. Nevalainen, Effects of *Scedosporium aurantiacum* proteases on human lung epithelial cells. Manuscript prepared for submission to Experimental Cell Research.

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Abbreviations

CF	Cystic fibrosis
SCFM	Synthetic cystic fibrosis sputum medium
MS	Mass spectrometry
PDA	Potato dextrose agar
MIC	Minimum inhibitory concentration
NaH_2PO_4	Monosodium phosphate
Na_2HPO_4	Disodium phosphate
NH_4Cl	Ammonium chloride
KCl	Potassium chloride
KNO_3	Potassium nitrate
NaCl	Sodium chloride
MOPS	3-(N-morpholino) propanesulfonic acid
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
Tyr	Amino acid: tyrosine
Leu	Amino acid: leucine
Lys	Amino acid: lysine
Gly	Amino acid: glycine
Pro	Amino acid: proline
Glu	Amino acid: glutamic acid
His	Amino acid: histidine
Asp	Amino acid: aspartate
Arg	Amino acid: arginine
Val	Amino acid: valine
Ala	Amino acid: alanine
Phe	Amino acid: phenylalanine

2DE	Two-dimensional gel electrophoresis
1DE	One-dimensional gel electrophoresis
MW	Molecular weight
SDS	Sodium dodecyl sulphate
HCl	Hydrogen chloride
AEBSF	Protease inhibitor: 4-benzenesulfonyl fluoride hydrochloride
E-64	Protease inhibitor: N-[N-(L-3-trans-carboxyirane-2-carbonyl)-L-leucyl]-agmatine
PMSF	Protease inhibitor: Phenylmethylsulfonyl fluoride
ETDA	Ethylenediaminetetraacetic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
WST-1	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NAD+	Oxidized nicotinamide adenine dinucleotide
NSAF	Normalized spectral abundance factor

Chapter 1

Introduction

1.1 Fungi and disease

1.1.1 Fungi infecting humans

Fungi are eukaryotic microorganisms found widely distributed in soil and other organic matter and form associations with plants. They occur in two main morphological forms: single celled yeasts like *Candida* spp. with a typical diameter of 3-4 μm, and filamentous moulds like *Aspergillus* spp., which are complex multicellular organisms, composed of hyphae containing multiple nuclei. Many yeasts infecting humans are capable of shifting between yeast and filamentous forms depending on environmental conditions [1-4].

An estimated 5.1 million fungal species exist on earth and over 600 of them are able to cause infections in humans, and there are diverse diseases associated with fungal infection [5, 6]. Some fungal species are capable of colonizing the human skin tissue and nails causing superficial infections [2], whereas fungal infection occurring in the bloodstream or organs are called invasive fungal infection, usually associated with a mortality rate ranging from 30-60% [7, 8]. Globally, over 300 million people are afflicted with various fungal infections and 25 million are facing the high risk of dying from the infection (Table 1.1) [9, 10].

Fungal diseases are difficult to treat because fungi are eukaryotic organisms just like humans, making it hard to target fungi only without harming human cells [2]. For example, current pharmacy-available antifungal drugs, such as polyenes, azoles and allylamines, which work by disturbing membrane ergosterol in the fungal membranes, also harm human cell membranes that contain cholesterol [11]. Additionally, fungal diseases mainly occur in old, sick or immunosuppressed individuals, whose immune system is too weak to support the antimicrobial treatment [12].

Table 1.1 The top 10 significant invasive fungal infections and the associated fungal species (Reproduced from Brown et al., 2012 [9]).

Disease	Most common species	Estimated life-threatening infections
Aspergillosis	*Aspergillus fumigatus*	> 200,000
Candidiasis	*Candida albicans*	> 400,000
Cryptococcosis	*Cryptococcus neoformans*	> 1,000,000
Mucormycosis	*Rhizopus oryzae*	> 10,000
Pneumocystis	*Pneumocystis jirovecii*	> 400,000
Blastomycosis	*Blastomyces dermatitidis*	~ 3000
Coccidioidomycosis	*Coccidioides immitis*	~ 25,000
Histoplasmosis	*Histoplasma capsulatum*	~ 25,000
Paracoccidioidomycosis	*Paracoccidioides brasiliensis*	~ 4,000
Penicilliosis	*Penicillium marneffei*	> 8,000

Fungi that cause disease in humans or other organisms are called pathogenic fungi [13]. Fungal pathogens can be divided into two broad classes: primary pathogens and opportunistic pathogens. Primary fungal pathogens, such as *Coccidioides immitis* [14], *Histoplasma capsulatum* [15] and *Sporothrix schenckii* [16], are capable of infecting healthy hosts. On the other hand, opportunistic pathogens are not harmful for healthy individuals but only cause infections in immunocompromised hosts. This class comprises most fungi, including *Candida* spp., *Aspergillus* spp., *Penicillium* spp., *Mucormycetes* and *Fusarium* spp. [17].

Primary fungal infections usually result from inhalation of fungal spores, and mainly impact the respiratory tract [13]. Some of these types of infections have a characteristic geographic distribution, for example, coccidioidomycosis primarily occurs in the Southwestern States of America and Northern Mexico, while histoplasmosis primarily occurs in the Eastern and Midwestern states of America [18]. In contrast, opportunistic fungi gain access to the host by more diverse means, such as via the alimentary tract, or intravascular devices [13]. Opportunistic fungi are responsible for the majority of fungal infections [7].

Despite advances in medical mycology, the study and understanding of fungal infections is still limited. Some common fungi, such as *A. fumigatus* and *C. albicans* have drawn most attention in fungal research [18, 19], but less effort has been directed towards research into emerging fungal pathogens such as *S. aurantiacum*, studied here with a view to investigating a potential link between protease production and fungal pathogenicity.

1.1.2 *Scedosporium* species as fungal pathogens

Scedosporium spp. are opportunistic pathogenic fungi that have emerged as significant agents of disease in recent years owing to an increase in the frequency of reported *Scedosporium* spp. infections. They take up 25–29% of non-*Aspergillus* infections in organ transplant recipients in the USA and are associated with infections in patients receiving surgery [20-22]. Amongst cystic fibrosis patients they are the second most common fungi causing infection after *Aspergillus* spp. [23, 24]. Amongst *Scedosporium* spp., *S. aurantiacum* causes the highest number of infection incidences in cystic fibrosis patients (Fig 1.1).

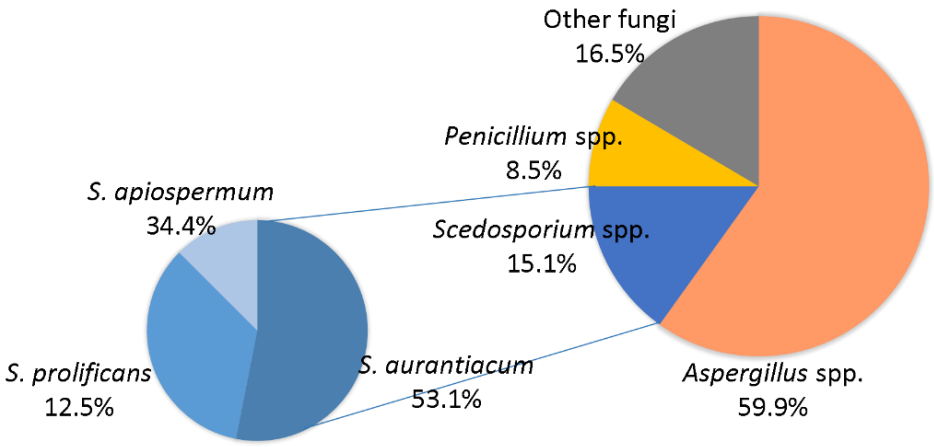

Fig 1.1 Major filamentous fungi isolated from 212 specimens sourced from cystic fibrosis patients (Reproduced from Blyth et al., 2010 [24]).

1.1.2.1 Taxonomy of genus *Scedosporium*

Scedosporium spp. were previously considered to contain two species of clinical interest: *Scedosporium prolificans* and *Scedosporium apiospermum* (the asexual form of *Pseudallescheria boydii*) [22]. *Scedosporium* was first discovered in 1889 as a causative agent for human otitis [22], and *P. boydii* was first described in 1911 as one of the causative agents of mycetoma [25]. Also in 1911, an anamorph stage was identified in the life cycle of *Scedosporium*, which demonstrated the connection between *Scedosporium* and *P. boydii*. Since then, an increasing number of clinical cases attributed to the fungus has been reported, and in 2000, the clinical significance of *Scedosporium* in the lungs of cystic fibrosis patients was described [26].

The taxonomy of this genus has been subject to change on the basis of emerging DNA sequence data. For example, *S. apiospermum*/*P. boydii* are recognised as separate species, whereas *S. prolificans*, previously considered as a member of the *Scedosporium* species complex, has been transferred to a related genus *Lomentospora* [27-30]. The updated classification of *Scedosporium* species is shown in Table 1.2.

Table 1.2. Classification of *Scedosporium* spp. based on ITS sequence analysis (Reproduced from Lackner et al., 2014 [30]).

Organism	Commonly used synonymous names
S. apiospermum	*Pseudallescheria apiosperma*
S. aurantiacum	
S. boydii	*Allescheria boydii, Petriellidium boydii, Pseudallescheria boydii, Polycytella hominis*
S. dehoogi	
S. minutisporum	*Pseudallescheria minutispora*
S. desertorum	*Pseudallescheria desertorum*

Amongst *Scedosporium* spp., *S. apiospermum*, *S. boydii* and *S. aurantiacum* are the principal human pathogens that mainly infect immunocompromised patients. Infection sites can be localized, expanded to the surrounding tissues, or the fungus can be disseminated to distant organs. However, species of the *Scedosporium* complex have also been found to cause disease in immunocompetent individuals [22, 31]. The range of disease caused by these fungi is broad but most infections caused by *Scedosporium* spp. are mycetomas. Other infections occur in the lungs, sinuses, eyes, bones, central nervous system and internal organs [22, 27, 32-34].

1.1.2.2 Morphology of *Scedosporium* spp.

Colonies of *Scedosporium* spp. can tolerate a wide range of stresses, such as a temperature between 25-42 °C, low oxygen tension and even strict anaerobism [22]. Microscopic features of *Scedosporium* conidiophores and sessile conidia (conidia borne individually along the sides of the hyphae) demonstrate differences in conidial shapes and colour between species, characteristics that also are important in distinguishing different species (Table 1.3).

Table 1.3 Morphological features of conidia among currently known species of *Scedosporium* (Reproduced from Gilgado et al., 2008 [28]).

Species	Conidiogenous cells	Sessile conidia	Colour of conidia
S. apiospermum	Cylindrical	Globose to sub-globose	Pale-brown
S. aurantiacum	Cylindrical or slightly flask-shaped	Mostly obovoid	Subhyaline
S. boydii	Cylindrical	Globose to sub-globose	Pale-brown
S. minutisporum	Cylindrical	Ellipsoidal to obovoid	_
S. dehoogi	Cylindrical or slightly flask-shaped	Mostly obovoid	_

Ramsperger et al. comprehensively studied the morphology of different *Scedosporium* species (Fig 1.2) [35]. Colonies of *S. aurantiacum* are dense and cottony with yellow or brown color.

The bottom of the colony mat has yellow diffusible pigment. *S. apiospermum* colonies are dark grey or smoky brown while *S. boydii* colonies are white to grey, and both have cottony texture with a pale reverse view. *S. dehoogii* colonies are grey with a colourless reverse. Under the microscope, *S. aurantiacum* has solitary conidiophores on aerial mycelium and conidia are smooth and thick-walled with obovoid or subcylindrical shape. Both *S. apiospermum* and *S. boydii* produce asexual hyaline septate hyphae, while *S. boydii* produces an ascocarp with brown wall and releases asci with ascospores at maturation [28, 35].

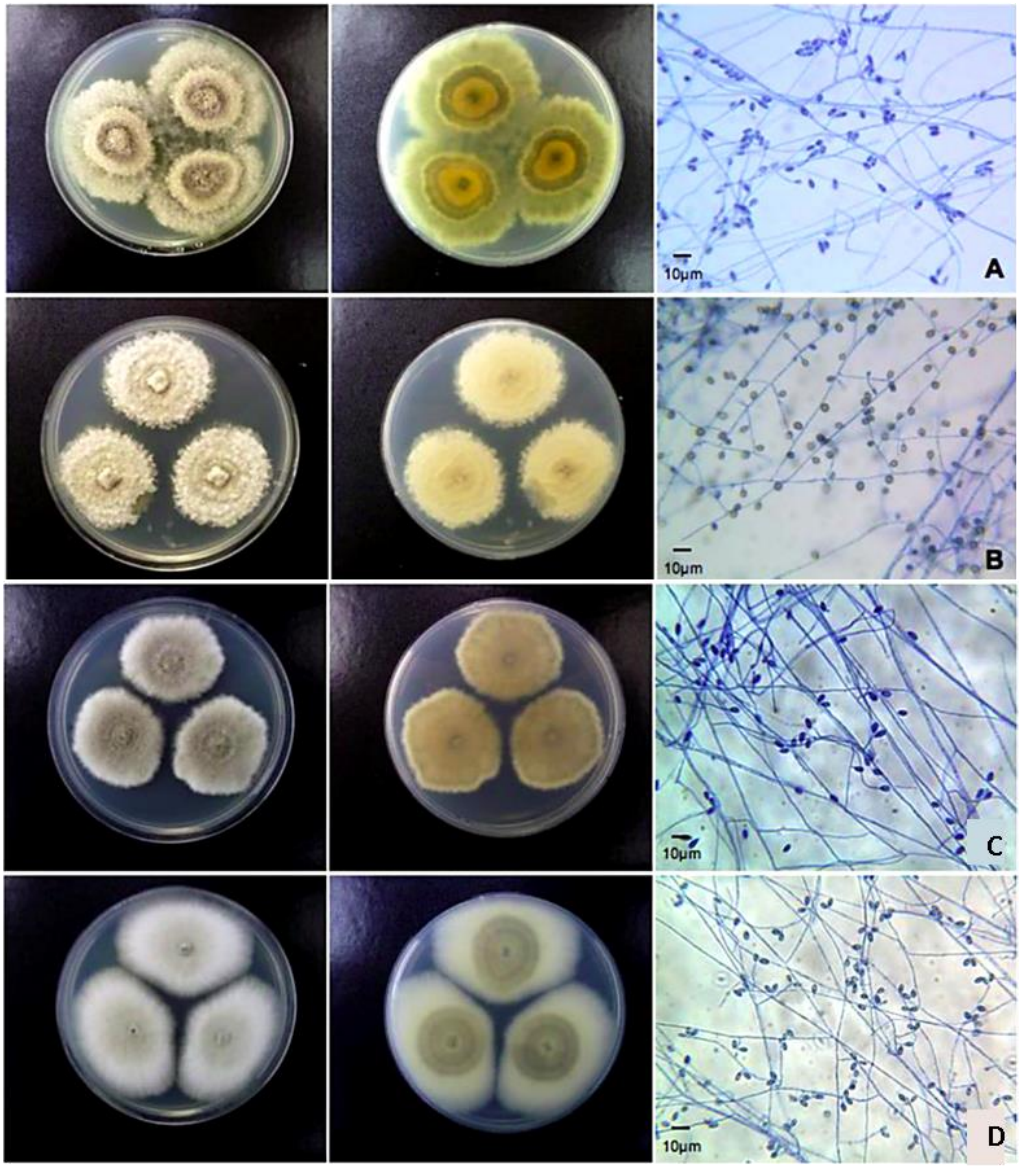

Fig 1.2 Colonies of different *Scedosporium* species on Potato dextrose agar (PDA) at 25 °C, comparing the plate obverse side (left panel) and reverse side (mid panel), and microscope morphology after staining with lacto-phenol cotton blue (magnification 400×, right panel). A: *S. aurantiacum*, B: *S. apiospermum*, C: *S. dehoogii* and D: *S. boydii* (Modified from Ramsperger et al., 2014 [35]).

The thickness of conidial cells and their germination pattern vary among *Scedosporium* species. Conidia of *S. boydii*, *S. apiospermum* and *S. aurantiacum* have thick walls, whereas conidia of *S. minutisporum* and *S. dehoogii* have thin walls [28]. Moreover, *S. apiospermum* germinates only from one site (pole), while *S. aurantiacum* germinates from one extremity or from both

conidial tips and *S. minutisporum* germinates from the central part of the conidial cell (Fig 1.3) [36].

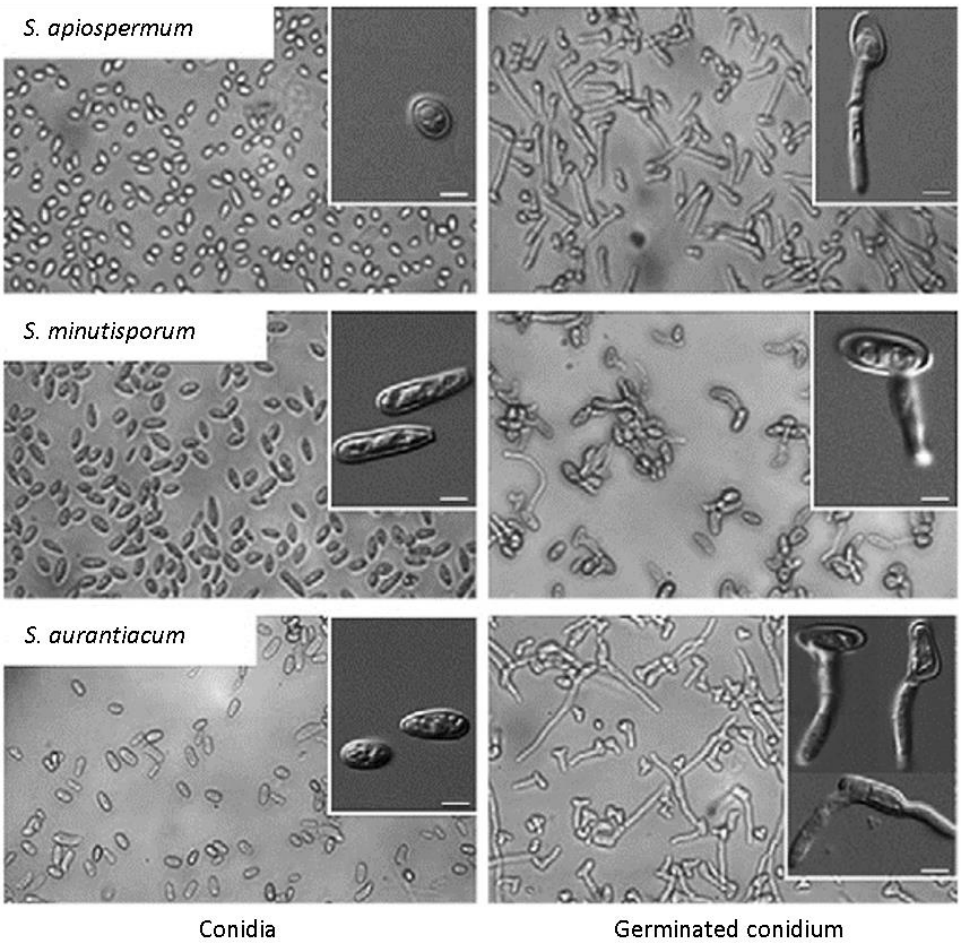

Fig 1.3 Dormant (0 h incubation) and germinated conidial cells (4 h incubation) of *S. apiospermum, S. minutisporum* and *S. aurantiacum* in Sabouraud medium at 37 °C in an atmosphere of 5% CO_2. Scale bar: 4 μm (Used with permission from Mello et al., 2016 [36]).

1.1.3 *Scedosporium aurantiacum*

S. aurantiacum is an opportunistic filamentous fungal pathogen in the genus *Scedosporium.* It was first identified as a new species in 2005 and was added to *Pseudallescheria boydii* complex as one of the clades [27, 28, 37, 38]. *S. aurantiacum* causes human infections mainly by inhalation, making the lungs the most common site of infection [39]. *S. aurantiacum* is prevalent in Australia and has a high isolation rate from cystic fibrosis patients [23, 40]. It can

also be isolated from the environment, especially in places where human activity is high [34, 41]. *In vivo* pathogenicity studies on mice showed that *S. aurantiacum* was highly virulent, displaying the same virulence as *Lomentospora prolificans* (formerly named *S. prolificans*), the current most virulent species in *Scedosporium* and related genera [20, 42-44]. In spite of the increasing prevalence of *S. aurantiacum* infections, very little is known about the virulence factors expressed by this fungal pathogen and the development of preventative strategies is currently limited.

1.1.3.1 Genotype and genome sequence

The traditional morphological approach has limitations when attempting to distinguish *Scedosporium* species due to the similar morphological characteristics [24]. Several molecular techniques, such as multiplex polymerase chain reaction (multiplex PCR), restriction fragment length polymorphism (RFLP) and multilocus sequence typing (MLST), have been introduced for genotyping, which enable accurate identification of *Scedosporium* spp. [35, 44-46]. So far, over twenty *S. aurantiacum* strains have been identified by a molecular approach and all these strains are phylogenetically close to *S. apiospermum* (Table 1.4) [38, 47]. Some of these species have been studied in terms of virulence, antifungal susceptibility and phenotypes, but their virulence factors and relevance in the clinical setting are still unknown [44, 46].

Genotypic analysis of *S. aurantiacum* still is not that easy due to the lack of annotated genomic data. So far, genomes of only two *Scedosporium* species have been sequenced, including *S. aurantiaucm* WM 09.24 [48, 49] and *S. apiospermum* strain IHEM 14462 [50]. Illumina HiSeq 2000 technology was used to sequence the genome of *S. aurantiaucm* WM 09.24 and 10,525 gene models and 11,661 transcripts were predicted in the draft genome assembly [49]. The availability of an annotated genome will greatly improve the characterization of different *S. aurantiacum* genotypes, similarly to work carried out with *Candida* spp. [51].

Table 1.4 Examples of *S. aurantiacum* strains and their location of isolation (Expanded from Harun et al., 2010 [44] and Delhaes et al., 2008 [38]).

Strain	CBS No.	Site of isolation
WM 06.482	136046	Broncho-alveolar lavage, Sydney, Australia
WM 06.390		Skin, Perth, Australia
WM 08.202	116910	Ankle ulcer, Spain
WM 08.269		Blood, USA
WM 06.385		Ear, Sydney, Australia
WM 06.555		Sputum, Sydney, Australia
FM 8842		Blood, USA
WM 09.13		Soil, Sydney, Australia
WM 09.24	136047	Soil, Sydney, Australia
WM 09.28		Soil, Sydney, Australia
WM 10.136	136049	Soil, Innsbruck, Austria
IHEM 15458		Soil, Austria

CBS No. was adopted from: http://www.westerdijkinstitute.nl/Collections/

In addition to assist in the characterization of different *Scedosporium* strains, an annotated genome will facilitate a comprehensive transcriptomic and proteomic analysis of *Scedosporium* species to enable global understanding of gene and protein expression in these fungi. Identification of protein function subsequently will be helpful in investigating the infection mechanism to further develop treatment strategies.

In this context, release of an annotated genome sequence of *Aspergillus* spp. has paved the way for analysis of their proteomes under various different growth and stress conditions. For example, the growth of *A. fumigatus* under hypoxia, which represents an important virulence attribute as fungi have to adapt to and survive in hypoxic conditions in diseased human tissues [52], has been studied at proteomic, transcriptional and metabolite levels. The results from the proteomic study demonstrated 117 proteins were implicated in fungal adaptation to hypoxia with various functions including glycolysis, the TCA-cycle, respiration, and amino acid

metabolism [53]. Contribution of these proteins to fungal virulence has been further studied by deleting individual genes coding for them, and subsequently investigating the virulence of the resulting mutants in a *Galleria mellonella* larval model and murine model [54]. From these studies, one specific protein (RbdA, a novel putative rhomboid family protease) was found to mediate fungal virulence and adaptation to hypoxia by potentially being involved in the cleavage of the sterol regulatory element binding protein SrbA. The *rbdA* deletion strain failed to grow under hypoxic conditions, was sensitive to phagocytic killing, showed low inflammatory response, and strongly reduced virulence in murine models of infection [54].

1.1.3.2 Geographic distribution

Scedosporium spp. cause infections worldwide, but more commonly in Western countries, such as France, Spain and Australia [21, 55]. In Australia, *S. aurantiacum* was the most common environmental *Scedosporium* species comprising 54.6% of the total isolates recovered from various sites in the Greater Sydney region [41]. The abundance of this fungus in the environment coincides with the relatively high frequency of isolation of the clinically important *S. aurantiacum* in Australia (with 17.4% prevalence in 69 adult cystic fibrosis patients); high prevalence may be linked with infection of human host, *e.g.* infection of lungs [23, 24, 40, 41].

Studies have showed that *S. aurantiacum* is prevalent in the environment with high human activity, such as urban areas in Sydney (Fig 1.4) [41]. It is believed that its prevalence is associated with organic contamination, with hydrocarbons and nitrogen originating from human, animal and industrial waste or crude oil [58]. For example, Darling Harbour in the central business district of Sydney with over 24 million international visitors yearly has over fifteen-fold colony units of *S. aurantiacum* compared to the Royal Botanic Garden with around 3.5 million annual visitations [41, 62, 63].

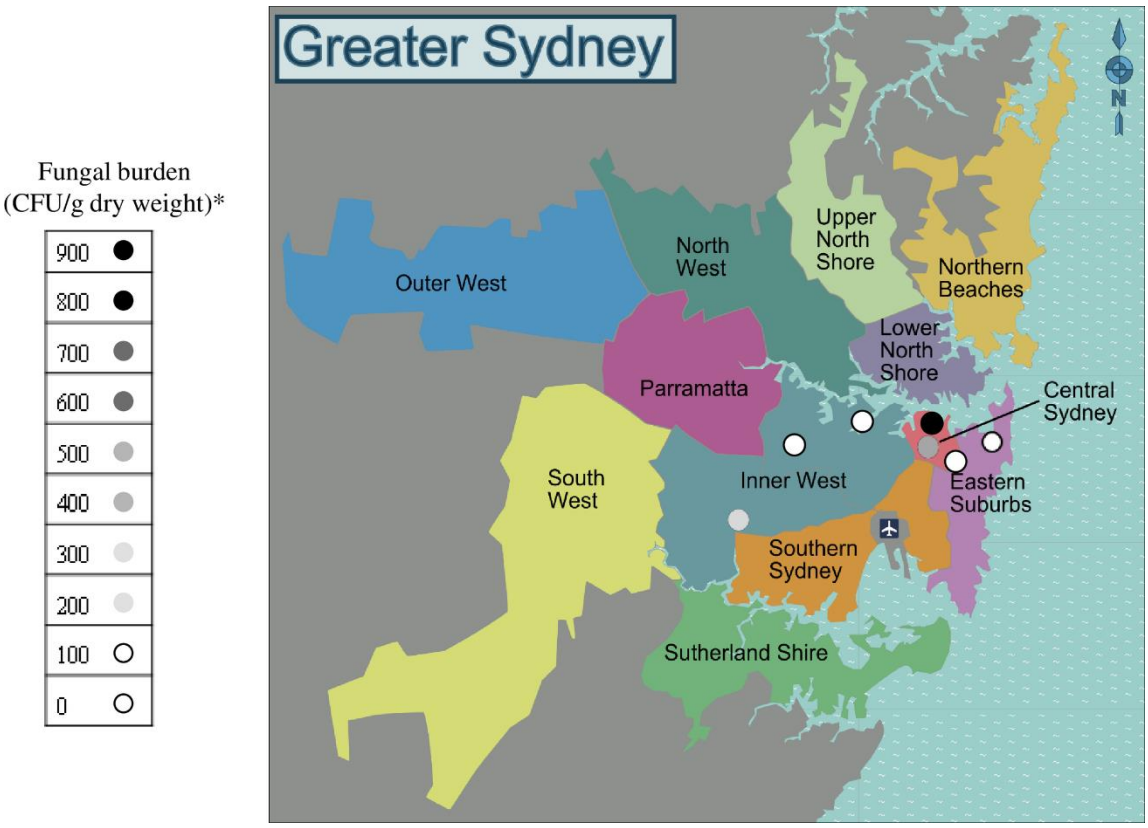

Fig 1.4 Sampling locations in the Greater Sydney Region. The white and black circles show locations from where *S. aurantiacum* has been isolated (Harun et al., 2010 [41]). The panel on the left indicates the fungal burden specifically featuring *S. aurantacum*. *CFU/g dry weight = Colony forming unit per gram of dry soil.

S. aurantiacum thrives best in an environment containing carbon sources such as glucose, sucrose, starch, cellulose and lactose and nitrogen sources such as soluble amino acids, peptides and proteins with a pH range of 6.1-7.5 [34, 58, 59]. It has been found to be able to utilize natural gas and/or aromatic compounds as carbon sources [25, 60]; this ability also has been reported in a study of other *Scedosporium* species and non-*Scedosporium* species such as *Cladosporium* spp. and *Fusarium* spp [61].

1.1.3.3 Macroscopic and microscopic features

The optimal growth temperature of *S. aurantiacum* is 37-40 °C, which allows it to adapt quickly and grow within the human host environment, although it is also capable of growing at lower temperatures [27]. In previous work carried out in this laboratory, Kaur et al. studied the macroscopic appearance of some strains of *S. aurantiacum* on Sabouraud's agar at 28 °C. The colour of the colonies varied among the isolates of different origin: greyish white in WM 06.482, white in WM 08.202, suede-like in WM 10.136 and brownish-white in WM 09.24 (Fig 1.5). All strains produced a light-yellow pigment on the reverse of the agar plates after 14 days of incubation [64].

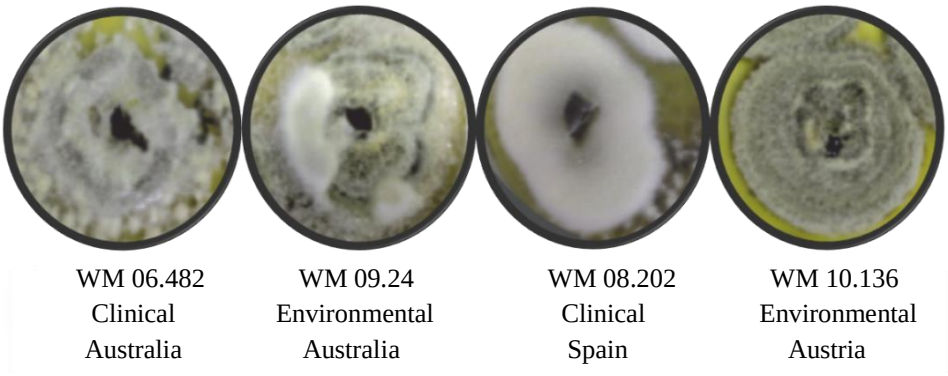

Fig 1.5 Obverse colony features of four different strains of *S. aurantiacum* growing on Sabouraud dextrose agar plates after incubation of 14 days at 28 °C (Modified from Kaur et al., 2015 [64]).

Microscopic features of *S. aurantiacum* were first described by Gilgado et al. in 2005 [27]. They reported that *S. aurantiacum* had two types of conidiophores: solitary on aerial mycelium and synnemata on the agar surface (Fig 1.6). Solitary conidiophores develop to a conidiogenous cell growing laterally on mycelium, and the conidia produced are subhyaline, smooth-walled, obovoid or subcylindrical. Synnemata are erect, smoke brown, smooth-walled, and terminated in a slimy head of conidia. The conidiogenous cells of synnemate conidiophores are subhyaline, smooth-walled and usually cylindrical. *S. aurantiacum* produces a third type of conidia from

the undifferentiated hyphae which are sessile, solitary, lateral, brown, smooth, and thick-walled [27].

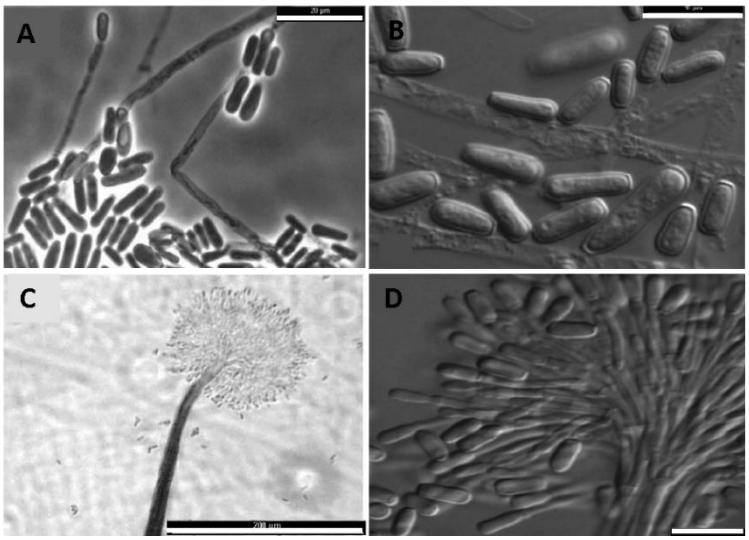

Fig 1.6 *S. aurantiacum* isolates (A, B) FMR 8630 and (C, D) IHEM 15458. (A, B) A conidiogenous cell and conidia from solitary conidiophores. (C) A synnema of the *Graphium* anamorph. (D) Apical part of a synnema producing conidia. Scale bar, A: 20 µm, B and D: 10 µm, C: 200 µm (Modified from Gilgado et al., 2005 [27]).

1.1.3.4 Virulence and *in vitro* antifungal susceptibility

Knowledge of the level of the virulence of *S. aurantiacum* and its response to the antifungal drugs will be very helpful for developing treatment for infections. *S. aurantiacum* has been found to have the same level of virulence as the most virulent species *S. prolificans* among *Scedosporium* and related genera [33, 44]. The association between the different *S. aurantiacum* strains and fungal virulence is yet to be explored. Nonetheless, all *S. aurantiacum* strains examined so far (clinical origin strains of WM 06.482, WM 06.390, WM 08.202, WM 08.269, WM 06.385, WM 06.555, and environmental origin strains of WM 09.13 and WM 09.28) demonstrated unique M13 PCR fingerprinting patterns, which may be a potential breakthrough for finding the association between genotype and virulence [44]. A further evaluation on the virulence of *S. aurantiacum* was conducted in a murine model, and results confirmed that *S.*

aurantiacum showed strain-specific virulence, and the specificity was linked to genotype of each strain [44].

Kaur et al. compared the virulence levels of four *S. aurantiacum* strains using a *Galleria mellonella* larval model by comparing the survival of infected larvae. They found that the *S. aurantiacum* WM 06.482 (clinical origin) caused 85% mortality within 8 days, followed by WM 09.24 (40%, environmental origin), WM 10.136 (25%, environmental origin) and WM 08.202 (20%, clinical origin) [64]. Virulence has also been tested in a mouse model by comparison of the mean survival time of mice infected by different *S. aurantiacum* strains. The results showed that WM 06.482 was the most virulent isolate among all examined *S. aurantiacum* strains. Its virulence level was significantly higher than that of the other examined isolates from clinic patients (WM 06.390, WM 08.202, WM 08.269, WM 06.385 and WM 06.555) and not significantly higher than that of the two environmental strains (WM 09.13 and WM 09.28) [44]. This indicates that the virulence of *S. aurantiacum* strains was independent of the origin of isolation. A high virulence level present in an environmental strain may consequently lead to serious infection.

The established high resistance *S. aurantiacum* to antifungal drugs also contributes to its virulence. The most virulent *Scedosporium* species, *S. aurantiacum* has also been found to be more resistant to amphotericin, itraconazole and voriconazole than *S. apiospermum* by comparing the amount of the antibiotic needed for inhibiting 50% of fungal colony growth [59]. Further antifungal susceptibility profiling with *S. aurantiacum* showed high resistance to amphotericin and low resistance to voriconazole *in vitro* (Table 1.5) [33]. The studies conducted by Lacker and Gilgado et al. also showed high *in vitro* resistance of *S. aurantiacum* to amphotericin and itraconazole, and other antifungal drugs showed poor activity as well [46, 65]. All these results suggest that voriconazole is the only active antifungal drug efficient against *S.*

aurantiacum (MIC_{90} 0.25 mg/L). A clinical trial of the efficacy of voriconazole on *S. aurantiacum* has been conducted on 107 patients with various infection sites. Results indicated that 57% of the patients showed a successful therapeutic response [66]. This drug has been proven to be able to lower mortality and help to cure *S. aurantiacum* osteomyelitis after surgery [33, 59, 67]. Also, voriconazole has been reported to be well tolerated by most patients regardless of age [68]. On the basis of these studies, the recommended treatment of *S. aurantiacum* infections is administration of voriconazole 6 mg/kg twice on day 1 then 4 mg/kg twice daily) [66, 69].

Table 1.5 *In vitro* antifungal susceptibility patterns of *S. aurantiacum* against four antifungal agents (Reproduced from Heath et al., 2009 [33]).

Antifungal drug (no of isolates tested)	MIC (mg/L)			
	Range	GM[a]	MIC_{50}[b]	MIC_{90}[c]
Amphotericin (27)	2-16	7.42	8	16
Itraconazole (27)	0.25-2	0.61	0.5	1
Voriconazole (27)	0.03-0.5	0.20	0.25	0.25
Posaconazole (26)	0.125-1	0.43	0.5	0.5

a: GM, geometric mean of the minimum inhibitory concentrations (MICs).

b: MIC at which 50% of the isolates were inhibited.

c: MIC at which 90% of the isolates were inhibited.

1.1.3.5 Nutrition and growth

Fungi are heterotrophic organisms that obtain carbon and nitrogen and other nutrition by absorption of simple molecules from their surrounding environment. These simple molecules are formed as a result of the fungi secreting various enzymes that cleave large organic molecules into simpler ones [2]. Carbon metabolic pathways in fungal life circle, such as gluconeogenesis and the TCA cycle, have been studied intensively and are thought to be involved in virulence [70]. Sugars are widely used as carbon source for fungal cultivation, ranging from simple

hexoses, such as glucose, to polysaccharides, such as starch, cellulose and aromatic hydrocarbons [70]. Kaur et al. studied the carbon utilization of different *S. aurantiacum* strains, and found that there was a potential link between the utilization of turanose and fungal virulence, as *S. aurantiacum* strains WM 06.482 and WM 09.24, showing higher virulence in the *Galleria* model, also displayed a good ability to grow on turanose [64]. In addition, carbon utilization can be used to distinguish different strains within *S. aurantiacum*, since each strain showed a different pattern in carbon utilization [64].

To obtain nutrition for growth, fungal mycelia secrete various enzymes into the environment to break down complex macromolecules into smaller molecules so that they can cross the hyphal cell wall to fulfil the nutritional needs [71]. Different fungal species secrete different enzymes due to their inherent properties and in response to substrates available [2]. Examples of enzymes secreted by fungi include cellulases, hemicellulases, pectinases, proteases, lipases, amylases chitinases and phospholipases (Table 1.6). These enzymes are synthesized and secreted selectively based on the substrates present in the environment and other conditions, so that cellular resources are allocated to production of the enzymes to result in valuable returns to the cell [72, 73].

Some classes of enzymes secreted by fungi have been found to be potential virulence factors in pathogenic fungi enabling them to breach and invade host tissues. In this respect, the most highly studied secreted enzymes include proteases, lipases and phospholipases since cell membranes are made up of lipids and proteins [74]. A correlation between secretion of proteases and fungal pathogenicity to humans has been established in studies of pathogenic *Aspergillus* spp. [75, 76], *Candida* spp. [77, 78] and Dermatophytes [79]. Proteolytic activities identified from these fungi are generally involved in permeating the lung epithelial barrier and digestion of cells and molecules of the host immune system [80, 81].

Table 1.6 Enzymes secreted by fungi for the degradation of polymeric compounds (Reproduced from Walker et al., 2005 [70]).

Substrates	Fungal secreted enzymes	Degradation products
Proteins	Proteases	Amino acids
Inulin	Inulinase	Fructose
Cellulose	Cellulases	Glucose
Lipids	Lipases	Fatty acids
Chitin	Chitinase	*N*-acetylglucosamine
Starch	Amylases, glucoamylase	Glucose
Hemicellulose	Hemicellulases, xylanase	Xylose, glucose
Pectin	Pectin lyase, polygalacturonase	Galacturonic acids
Lignin	Ligninase, manganase, peroxidase, laccase, glucose oxidase	Variety of largely phenolic products

There are numerous studies on the contribution of secreted fungal lipases to virulence. For example, lipases from the skin-associated fungal pathogen *Malassezia* spp. have been found to play roles in destruction of epidermal/epithelial tissues [82, 83]. Extracellular lipase activity of *C. albicans* has cytotoxic effects on the host macrophages and hepatocytes [84, 85]. As for phospholipases, phospholipase B, a common phospholipase secreted by different clinical fungi has been reported as a universal virulence factor, which has been found to damage host cell membranes and release various inflammatory mediators *in vivo* [86]. Additionally, there are many studies that have reported that phospholipases play a role in the physiology and virulence of *Cryptococcus neoformans*, which is a human pathogenic fungus causing pulmonary cryptococcosis in immunocompromised individuals [87, 88]. Targeting these virulence factors has been proposed as a means of infection control [89].

1.2 Proteases secreted by fungi

1.2.1 Classification of secreted fungal proteases

Proteases (proteases or proteolytic enzymes) catalyse the hydrolysis of peptide bonds in proteins, leading to degradation of protease substrates into their constituent amino acids [90]. Proteases can be divided into seven broad groups according to the nucleophilic amino acid or catalytic residue at the (enzyme's) active site: serine proteases, cysteine proteases, threonine proteases, aspartic proteases, glutamic protease, metalloproteases and asparagine peptide lyases. Furthermore, each class has a subfamily based on the structure, hydrolysis mechanism and catalytic residue order [91]. An up to date classification of proteases can be found in the MEROPS database (http://merops.sanger.ac.uk) [92]. Biological function, substrate specificity and other properties of proteases vary among classes and/or subclasses (some examples are shown in Table 1.7) [93, 94].

Although seven classes of proteases have been characterized, only four of them have been found to be secreted by fungal hyphae including serine, cysteine, aspartic and metalloproteases [95, 96]. Serine proteases are the most common proteases secreted by fungi and have been studied most intensively across different fungal species. These proteases have been further categorized based on their substrate specificity, and four sub-family members are frequently detected in fungal culture supernatant, namely chymotrypsin, subtilisin, trypsin and elastase [97-99].

Fungal secretion of proteases is subject to several regulatory mechanisms, including carbon, nitrogen, and sulphur metabolite repression and pH control [100]. Therefore, although all fungi secrete proteases they do not secrete the same ones. Table 1.7 lists proteases that have attracted attention in pathogenic fungi and their association with fungal pathogenicity.

The first report on the proteases secreted by *Scedosporium* species was by Larcher et al. in 1996, who purified and characterized a 33 kDa subtilisin-like protease from *S. apiospermum* culture supernatant [101]. This protease is able to degrade human fibrinogen, and thus has been considered to contribute to pulmonary damage in chronic bronchopulmonary inflammation from which cystic fibrosis patients suffer [101]. In 2006, Silva et al. detected two distinct extracellular metalloproteases in *S. apiospermum.* These proteases were capable of degrading several protease substrates, such as human serum albumin, casein, haemoglobin and non-immune IgG, to help the fungus to cross natural human barriers and defence [102]. To date, there have been no reports regarding proteases secreted by *S. aurantiacum*, which limits the understanding the pathogenicity of this fungus.

Table 1.7. Summary of the main proteases secreted by human fungal pathogens (Expanded from Wu, 2007 [103]).

Protease	pH range	Cleavage specificity(α)	Fungal species	Pathology in fungal secretion
Serine	Neutral-basic			
- Chymotrypsin		Tyr or Phe	*Aspergillus fumigatus*	Degradation of membrane protein laminin, resulting in disruption of membrane structure [104]
- Subtilisin		Tyr, Phe, Leu, Val or Ala	*Trichophyton rubrum*, *Microsporum canis*,	Invasion of keratinized tissues, arthroconidia adherence and tissue destruction [105-107]
- Trypsin		Lys or Arg	*Beauveria bassiana*, *Aspergillus clavatus*, *Aspergillus nidulans*	Pathogenicity marker; degradation of insect cuticles and breaking down gingival tissues of mice [108-110]
- Elastase		Gly, Ala or Val	*Aspergillus fumigatus*	Damage of lung tissue, deterioration in respiratory function, bronchiectasis and other inflammations in airways [98, 111-113]
Metallo	Acidic-neutral	Leu, Gly or Pro	*Aspergillus fumigatus*	Digestion of structural components of tissue and generation of inflammatory mediators [114, 115]
Aspartic	Acidic	Arg, Glu, Lys or Asp	*Candida albicans*	Degradation of many mouse proteins, digestion of cells and molecules of the host immune system and damaging to human oral tissue [80, 116]
Cysteine	Neutral-basic	His, Leu, Phe or Tyr	*Aspergillus fumigatus*	Breaching the alveolar epithelial cell barrier by disruption of the actin cytoskeleton and focal attachment in human lung cancer cells; also causing dissociation and migration of human cells [81, 117]

(α) Cleavage may occur at the carboxyl side of the amino acid in a peptide. Cleavage specificity of proteases was sourced from online protein database: http://merops.sanger.ac.uk/substrate_menu.shtml and https://prosper.erc.monash.edu.au.

1.2.2 Proteomic analysis of secreted proteins from filamentous fungi

Since 1995, when the term 'proteomics' was first coined, proteomic analysis has been used widely to obtain a global and integrated view of all the proteins of a cell. Today, proteomics is used to study protein-protein interaction, protein expression, function, identification and quantification [118]. Proteomic studies on secreted proteins have been conducted in a number of filamentous fungi, such as *A. flavus* [119, 120], *A. oryzae* [121], *A. fumigatus* [122, 123], *A. nidulans* [124], *Fusarium graminearum* [125] and *T. harzianum* [126]. Basically, the proteomic workflow consists of precipitation of secreted proteins from the fungal culture medium, separation of the proteins by electrophoresis, digestion and extraction of peptides from the separated proteins, and analysis of the resulting material using mass spectrometry (MS) [127].

1.2.2.1 Recovery of proteins from the culture medium for their analysis

Filamentous fungi secrete proteins mainly through the hyphal tip, although secretion also occurs in sub-apical regions at relatively lower amounts [128, 129]. In laboratory conditions, most filamentous fungi secrete proteins at concentrations of 5-50 μg/ml [130]. Most commonly, these secreted proteins are glycosylated, containing modifications such as oligomannose *N*- and *O*-glycans. Glycosylation helps secreted proteins to increase their stability and solubility in the culture medium, as well as enhance their resistance to environmental conditions [131]. In addition, fungi also secrete plenty of other compounds, such as polysaccharides, organic and fatty acids, mucilaginous material, phenols and other aromatic compounds. These compounds can interfere with protein quantification and MS analysis via spectrophotometric measurements and ionization [130, 132, 133]. These challenges to some extent limit research into proteins secreted by fungi. Therefore, it is advantageous to isolate and concentrate secreted proteins from fungal cultures to enable further studies.

When grown on a solid medium, secreted proteins are first extracted from the solid substrate by washing the surface of the agar plate with protein extraction buffer. The eluate is then centrifuged to remove pieces of fungal mycelia and agar and the supernatant containing secreted proteins is collected for further protein analysis [134]. When grown in a liquid medium, the medium containing secreted proteins is collected usually by centrifugation to allow separation and removal of fungal mycelia. Buffer exchange can be performed to remove the unused nutrients in the medium [135]. Proteins present in the clarified medium may be concentrated for further analysis.

Many strategies have been reported to enhance the recovery of proteins secreted by fungi including precipitation by trichloroacetic acid (TCA), methanol or acetone and concentration through freeze-drying or evaporation. Some combinatorial methods have been used to improve the recovery and purity of concentrated proteins. Fragner et al. used a combination of filtration (0.22 μm) and 10% TCA precipitation (at 4 °C), followed by ice-cold acetone washes, to clarify supernatants and extract secreted proteins from basidiomycetes [130]. Adav et al. and Salvachua et al. used a combination of filtration (0.22 μm) or dialysis in a tangential ultra-filtration system and freeze-drying to purify and isolate secreted proteins from *T. reesei* and *Irpex Lacteus* respectively [136, 137]. Arnold and Ulbrich-Hofmann modified a protocol for precipitating samples with a low concentration of proteins. They added sodium deoxycholate (DOC) to disrupt and dissociate protein interactions before preforming TCA precipitation and acetone washing [138].

Each of these techniques has its own limitations and may not work for enrichment of all secreted proteins. The composition and concentrations of secreted proteins vary among fungi and these variations may have different influences during the process of protein recovery [130, 132, 133]. Therefore, when a new filamentous fungus is studied, it is necessary to compare and optimize

the protein enrichment protocol to enable high protein recovery. For example, different enrichment methods resulted in different recovery rates and profiles of proteins secreted by *Coprinopsis cinerea* at day 3 post inoculation in a glucose plus yeast extract medium at 37 °C. TCA/acetone protocols recovered 60%, chloroform/methanol 54%, acetone 30%, ethanol 8% of the proteins; also, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of the protein samples from different enrichment methods were not identical [130].

1.2.2.2 Sample preparation for mass spectrometry based analysis

Depending on the protein enrichment method used, the extraction product may be a complex protein mixture. This presents significant problems for subsequent MS analysis because enzymatic digestion of a big protein gives a large number of peptide products, which overwhelm or suppress peak capacity of mass spectra from other proteins, leading to inaccurate interpretation of the protein complex [139]. Thus, proteins are usually pre-separated by one-dimensional gel electrophoresis (1DE) or two-dimensional gel electrophoresis (2DE) before subsquent analysis [140].

1DE is commonly used in many proteomics applications for protein separation. It is easily reproducible and facilitates identification of higher numbers of peptides compared to other protein separation techniques [141]. Protein(s) contained in a gel band are digested into smaller peptides using trypsin or other suitable proteases, such as GluC and LysC, for analysis by mass spectrometry [142].

1.2.2.3 Protein identification by mass spectrometry

Mass spectrometry (MS) is currently the most widely accepted and utilized method of protein identification [142]. The peptides from protein digestion are usually separated by a chromatographic method directly coupled to the MS, and thus only a few peptides elute at the

same time, which allows a greater coverage of protein to be achieved. The eluted peptides are usually ionised by knocking one or more electrons off to give a positive ion. The ions are then accelerated to get the same kinetic energy and transferred to the mass analyzer where they are separated according to their mass-to-charge ratio (m/z). A mass spectrum is generated based on the ion intensity at different m/z values. The most abundant ionized ions can be further fragmented, possibly by collision, to form MS/MS spectra. For each MS/MS spectrum, software (search engines) can be used to determine which peptide sequence in a database of protein sequences gives the best match. After individual peptide sequences are identified, the set of peptide sequences is used to infer which proteins may have been present (Fig 1.7) [143, 144].

Ionization and mass analysis are two important steps in mass spectrometry. The most commonly used ionization methods in proteomics are electrospray ionization (ESI) and matrix-assisted laser desorption and ionization (MALDI) [145]. When the peptide mixture is analyzed by ESI-MS, the peptide mixture is usually pre-fractionated by high-performance liquid chromatography (HPLC) on different columns (usually reverse phase HPLC or LC), generally named LC-MS/MS. Alternatively, MALDI-MS analysis of protein is achieved by peptide mass fingerprinting. The mass analyser utilizes either electric or magnetic fields to separate and detect ions according to their mass-to-charge ratio, which is the key of MS technology. Basically, there are four types of mass analysers commonly used in proteomics research including orbitrap, time-of-flight (TOF), quadrupole and Fourier transform ion cyclotron (FT-MS) analysers. These analysers usually work alone, but in some cases are put together in tandem to improve the process [143].

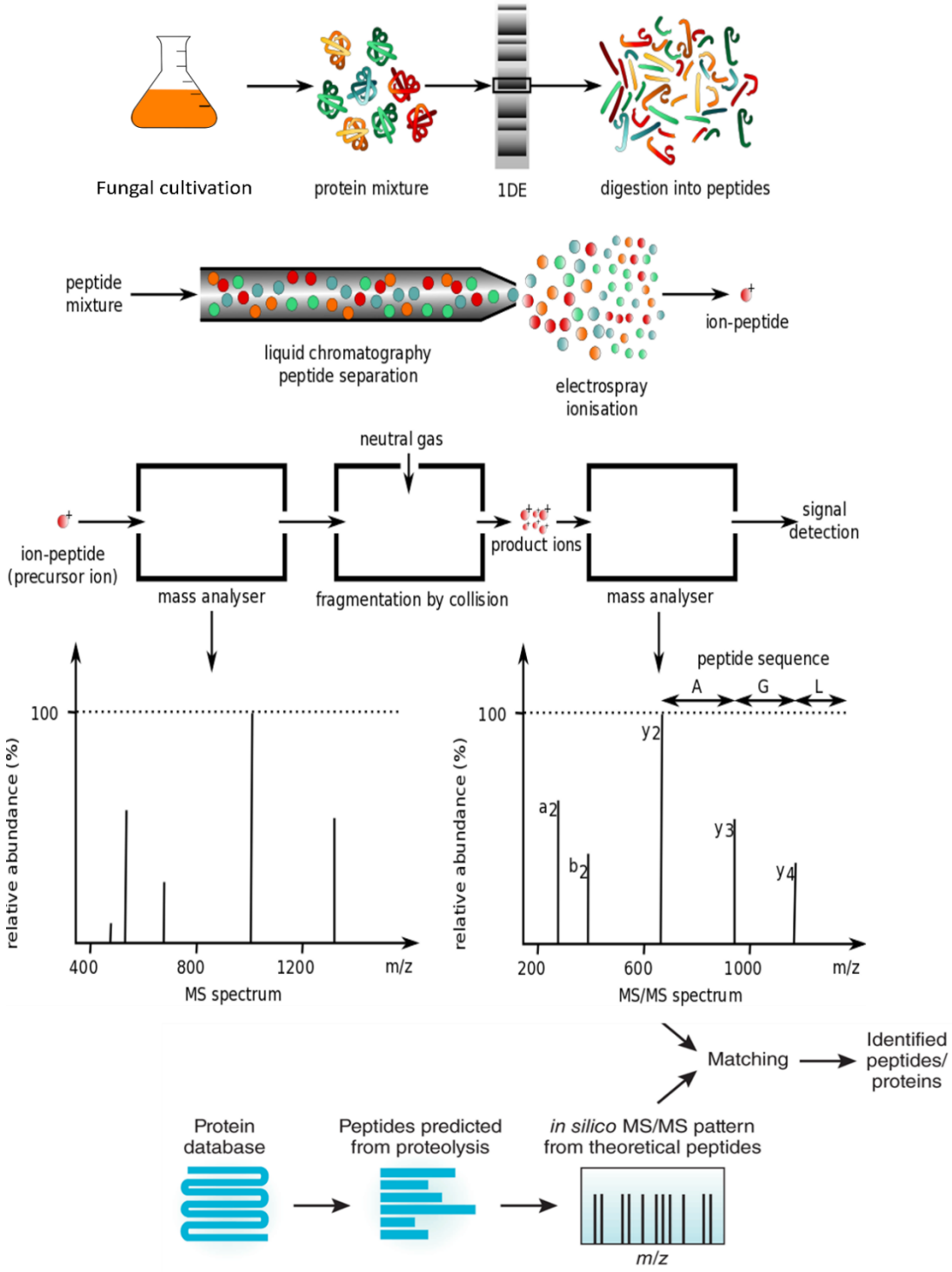

Fig 1.7 Workflow chart of an MS-based proteomic experiment for protein identification (Reproduced from Barillot et al., 2012 [146] and Duncan et al., 2010 [147]).

Currently, there are several search engines available for performing searches of MS/MS data (Table 1.8). As to protein sequence source databases, there are primarily two databases: NCBInr (www.ncbi.nlm.nih.gov) and SwissProt (www.expasy.org/sprot/). When the organism source of the protein is known and the annotated genome sequence has been made available, matches with database proteins and subsequent identification can be relatively straightforward. However, proteins from organisms whose genomes have not been sequenced or annotated have to been searched against other organisms with high sequence similarities [144, 148]. Organisms with close phylogeny exhibit similarities in their genome sequences and thereby a high degree of similarity between the proteins encoded by the genome is also likely. Therefore, protein identification based on existing protein sequences of closely-related phylogenetic species can still be achieved [149].

Table 1.8 Database search engines for MS/MS data (Reproduced from Cottrell, 2011 [148]).

Online tools	
X!Tandem (The GPM)	http://thegpm.org/TANDEM/index.html
PepProbe	http://bart.scripps.edu/public/search/pep_probe/search.jsp
InsPect	http://proteomics.ucsd.edu/LiveSearch/
Mascot	http://www.matrixscience.com/search_form_select.html
OMSSA	http://pubchem.ncbi.nlm.nih.gov/omssa/index.htm
Phenyx	http://phenyx.vital-it.ch/pwi/login/login.jsp
Popitam	http://www.expasy.org/tools/popitam/
Sonar	http://hs2.proteome.ca/prowl/knexus.html
Off-line tools	
X!Tandem (The GPM), MassMatrix, MyriMatch, Paragon, PepSplice, ProLuCID, ProbID	

Analysis of a protein through MS provides not only information about protein identity, but also sequence information for the whole protein complement in a cell or organism from a dynamic perspective [143, 145, 150]. This is useful to understand the biological functions of particular

proteins in the development of organisms or cells, so that a targeted inhibition or activation can be studied or performed. For example, MS analysis at different time points during germination and early development of *A. fumigatus* revealed proteins required for fungal aerobic respiration and ATP generation and the results impacted antifungal drug discovery and biomarker assessment [151].

Proteomic analysis of secreted proteins from *S. boydii* (also known as *P. boydii*) mycelial cells was conducted in 2012 by da Silva et al [48]. They found that most of the proteins were focused at the pH ranging from 4 to 7 with molecular masses from 14 to >117 kDa on two-dimensional gels. The subsequent MS analysis suggested that about 60% of the separated proteins were consistently identified, while approximately 40% of them showed no resemblance to any known protein from other fungi [48]. The identified proteins were involved, for example, in energy/carbohydrate metabolic pathways, protein degradation/nutrition and drug elimination and/or detoxification [48]. Their identification provides information for understanding immune modulation of *S. boydii* and the roles of these proteins may have in pathogenesis or pathogen survival.

1.2.3 Characterization of proteases

1.2.3.1 Protease specificity and activity assays

The classes and activities of secreted fungal proteases can be assessed using liquid protease assays. Despite of similar catalytic mechanisms, the amino acid residues and structures of the active sites are different for different proteases, and each class of protease can only cleave specific amino acids (Table 1.7) with a particular structure [103]. Usually the active site of a protease is a catalytic triad consisting of three amino acid residues [121]. For example, both chymotrypsin- and subtilisin-like proteases are able to cleave the carboxyl side of amino acids

of Tyr or Phe in a peptide/protein, but the catalytic triad of chymotrypsin-like proteases consist of amino acid residues His57, Ser95 and Asp102, while that of subtilisin-like proteases consist of Asp32, His64 and Ser221 [128]. The sequence difference leads to a different structure of the catalytic triad, which makes the two proteases bind to specific substrates to initiate catalysis [128].

Sequence-specific peptides synthesized artificially have been widely used as substrates to measure the class and activity of a protease. In the assay process, proteases hydrolyze a substrate to induce quantitative changes in light absorbance (colorimetric assay) or fluorescence (fluorimetric assay) in the assay medium [118]. These changes are used to calculate the amount of product released from a substrate or the amount of a substrate hydrolyzed by proteases. Protease activity can be defined as the amount of product increased or substrates decreased within a time range under described conditions. As catalytic efficiency of a protease is highly dependent on the reaction conditions, such as temperature, pH and ions, the reaction conditions must be specified when reporting a protease activity [119].

Proteolytic specificity is relative to the amino acid sequence of a substrate, substrate conformation and accessibility [120]. In Table 1.8 (Section 1.2.1), the amino acid cleavage specificity of some proteases is listed, and proteolytic cleavage happens when the amino acids are in a proper site. For example, papain (a cysteine protease) cleaves amino acids Phe and Leu when they are the second rather than the first amino acid residue to the bond, while the first residue may vary widely [121]. Some servers such as PROSPER have been developed to predict novel protease substrates and cleavage sites. PROSPER is capable of predicting cleavage sites of 24 different protease families from the primary sequences and structural characteristics

(https://prosper.erc.monash.edu.au). Based on the specificity of proteases, artificial peptide-form substrates have been synthesized to profile proteases [122].

1.2.3.2 Protease activity zymography

Zymography is an electrophoretic technique with proteinaceous substrates copolymerized in agar gels for the detection of hydrolytic enzymes. [123]. This technology was first reported by Gross and Lapiere in 1962 when they detected collagen degradation in tadpole tissue and found a matrix of metalloproteases [124]. Now it is widely used to detect the molecular weight, activity and quantity of enzymes in mixed samples [123]. Zymogram gels usually have the same appearance as regular protein gels (SDS-PAGE). The difference between them is that in a zymogram gel, a protein or another substrate (*e.g.* gelatin, casein, chondroitin sulfate, fibrin, hyaluronan and xylan) is co-polymerized with the acrylamide during casting [123, 125]. Thus, in zymography, enzymes are also separated by their MW. During electrophoresis, enzymes are inactivated by SDS, which binds to the surface of the enzymes without influencing the enzyme core. After electrophoresis, the zymogram gel is washed in renaturing buffer to remove SDS and reactivate the enzymes. Then the gel is transferred into an appropriate incubation buffer to enable hydrolysis of the substrate in the gel by the localised enzymes. Digestion of the substrate in the gel is usually visualized by staining (Fig 1.8 a) [118]. Casein- and gelatin-substrate zymography have been frequently used to visualize proteases secreted by fungi, as casein is the most common substrate for proteases while gelatin is useful to assess some classes of metalloproteases, such as MMP-2 and MMP-9 [112]. For example, Peterson et al. used gelatin and casein zymogram gels to test the proteases present in the culture supernatants of *T. reesei* RUT-C30. The number and intensity of proteolytic bands on the zymogram gels correlated well with the activity levels measured by protease assays, although the casein gel resulted in clearer bands of protease activity [126].

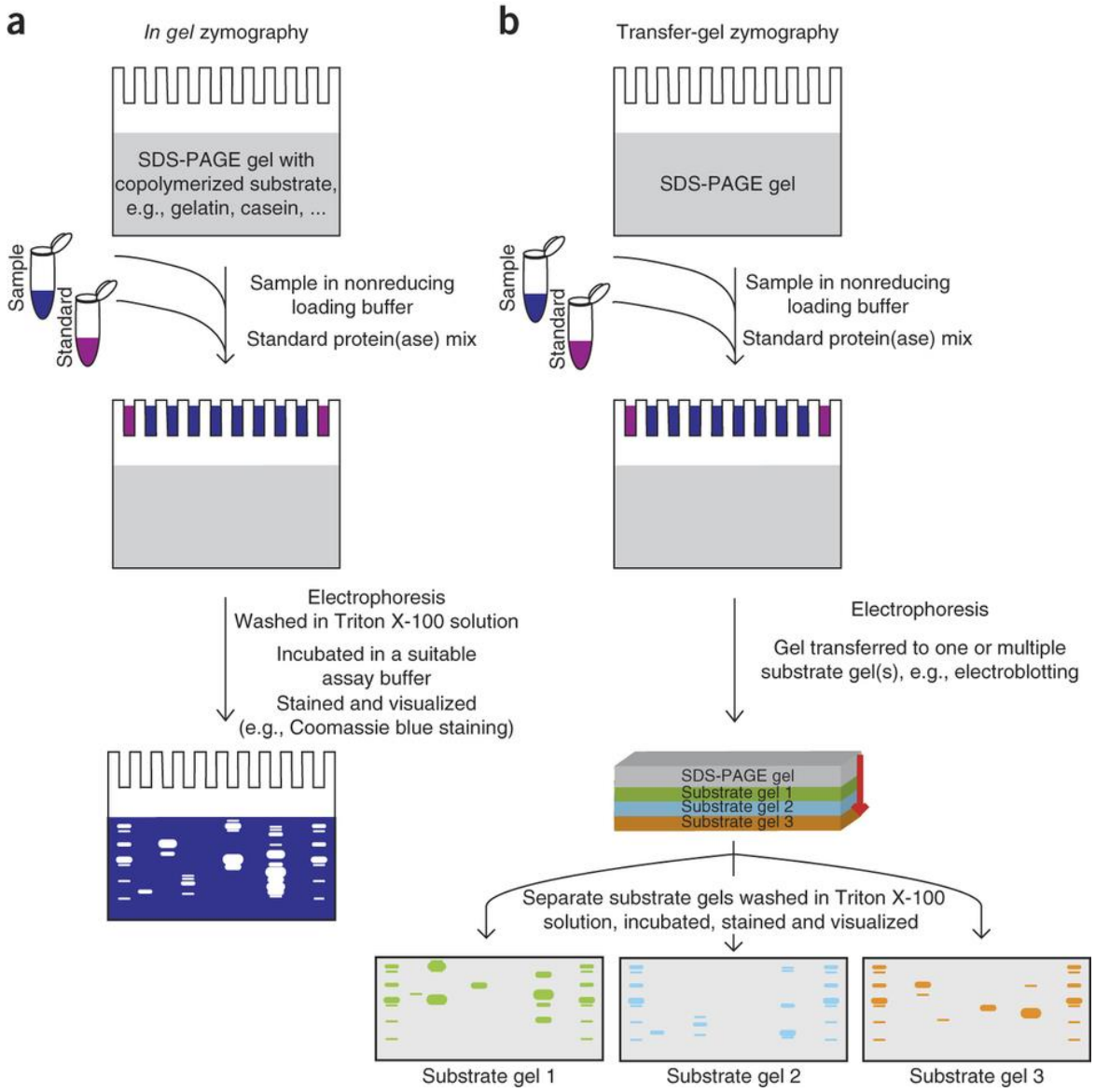

Fig 1.8 Two types of zymography. a) In gel zymography. Enzymes are separated by electrophoresis according to MW on an SDS-PAGE gel with co-polymerized substrate. The resulting gel is incubated in an appropriate buffer after removal of SDS, and then stained. b) Transfer-gel zymography. Enzymes are first separated by MW on a standard SDS-PAGE gel. The resulting gel is stacked on one or more substrate-containing gels to transfer the enzymes to the substrate gels electrophoretically. The substrate gels are then incubated in an appropriate buffer for each gel and then stained (used with permission from Vandooren et al., 2013 [123]).

Variations of the conventional zymography technique have been developed, such as transfer-gel zymography, 2D zymography and mixed-substrate zymography. In transfer-gel zymography, proteins are first separated in a non-reducing SDS-PAGE, and then electrophoretically transferred to one or more substrate gels which is cast like the conventional zymogram gel described above (Fig 1.8 b) [123, 127].

1.2.3.3 Protease inhibition assays

The catalytic efficiency of an enzyme (particularly a proteolytic enzyme) can be decreased or eliminated by binding of various enzyme inhibitors at its active site [128]. Enzyme inhibitors are usually short peptide-like molecules. They are able to bind to the enzyme active site so that the enzyme cannot bind to the substrate to finalise the catalysis. According to the mode of binding, inhibitors are divided into two categories: reversible and irreversible [129]. Reversible inhibitors can be removed from the enzyme to which they bind rapidly so that the enzyme can function normally again. In contrast, an irreversible inhibitor binds to the target enzyme over a time that is longer than the functional lifetime of the enzyme itself, so that the enzyme will lose its function.

Protease inhibitors come in three major forms: proteins, peptides and small molecules. Many different protease inhibitors are commercially available for both *in vitro* and *in vivo* assays with varying working concentrations (Table 1.9) [130, 131]. Protease inhibitors are classified according to the proteases they inhibit, and exist individually or as a cocktail containing several protease inhibitors. Individual protease inhibitors are ideal for assays of purified proteins or monitoring the action of a protease of interest, while a protease inhibitor cocktail results in a comprehensive inhibition of many proteases in a sample [130]. Each inhibitor has its advantages and disadvantages that may affect the effectiveness, thus, a pre-test is necessary to achieve the ideal inhibition.

Table 1.9 Examples of protease inhibitors (Compiled from Thermo Scientific [132] and Santa Cruz [133]).

Inhibitor	Target Class	Type	Solubility (solvent)	Typical Working Concentration
AEBSF	Serine proteases	Irreversible	200 mg·ml^{-1} (water)	0.2-1 mM
Aprotinin	Trypsin proteases	Reversible	10 mg·ml^{-1} (water)	0.1-0.2 µM
Bestatin	Amino-proteases	Reversible	5 mg·ml^{-1} (methanol)	1-10 µM
EDTA	Metalloproteases	Reversible	1 g·ml^{-1} (water)	2-10 mM
PMSF	Serine proteases	Reversible	18 mg·ml^{-1} (methanol)	0.1-1 mM
Leupeptin	Serine and cysteine proteases	Reversible	1 mg·ml^{-1} (water)	10-100 µM
Pepstatin A	Aspartic acid proteases	Reversible	1 mg·ml^{-1} (methanol)	1-20 µM
6-aminohexianoic	Chymotrypsin proteases	Reversible	20 µg·ml^{-1} (0.001 M HCl)	5 mM
Boc-VF-NHO -Bz-pCL	Subtilisin proteases	Irreversible	Any concentration in water	10-100 µM
Elastinal	Elastase proteases	Reversible	20 µg·ml^{-1} (0.08 M Tris buffer, pH 8.6)	10-100 µM
E-64	Cysteine proteases	Irreversible	20 mg·ml^{-1} (50% ethanol)	1-20 µM

1.2.4 Effect of secreted fungal proteases on lung epithelial cells

1.2.4.1 *In vitro* cell models

The human lung epithelium forms an interface between fungi and the host and is the initial point to be affected by fungal growth [168, 169]. Two types of lung alveolar epithelial cells have been revealed, namely type I and type II [170]. Type I cells comprise 95% of the alveolar epithelium, making them a major component of the physical barrier to respiratory pathogens and constitute the gas exchange area of the alveoli [170, 171]. Type II cells cover only 5% of surface area, and they are responsible for the secretion of various substances, such as cytokines,

protease inhibitors, surfactant proteins and antimicrobial peptides, to mediate the host immune response [170-172].

It is difficult to grow both type I and type II epithelial cells in co-culture [173], therefore most *in vitro* studies use lung epithelial cancer cell lines as models to analyze the effects of fungal infection on human lung epithelial cells. Commonly used cell lines include NCl-H292, A549, and immortalized epithelial cell lines HBE1 and 16HBE [174]. The A549 cell line (ATCC CCL-185) in particular, has been widely used to study the effects of secreted fungal proteases on human lung epithelial cells due to a high degree of metabolic and morphological similarity between A549 cells and type II alveolar epithelial cells [175]. For instance, serine proteases secreted by *A. fumigatus* have been shown to induce the production of cytokines by A549 epithelial cells [176]. Another study showed that serine proteases and cysteine proteases secreted by *A. fumigatus* were able to breach the alveolar epithelial cell barrier by disruption of the actin cytoskeleton and sites of focal attachment in human lung cancer cells A549 [81]. Aspartic proteases secreted by *C. albicans* induce apoptosis of epithelial cells A549 [177].

The A549 cell line grows adherently as a monolayer and individual cells have a characteristic pebble-like shape (Fig 1.9) [178, 179]. The basic medium for this cell line is F-12K Medium which contains various inorganic salts, amino acids, vitamins, D-glucose, sodium pyruvate and lipoic acid. For optimum growth, the basic medium needs to be supplemented with fetal bovine serum to a final concentration of 10% [178]. Like other human cell lines, A549 cells undergo cell division by which a parent cell divides into two genetically identical daughter cells, and the cell population doubles over about 22 h [178, 180].

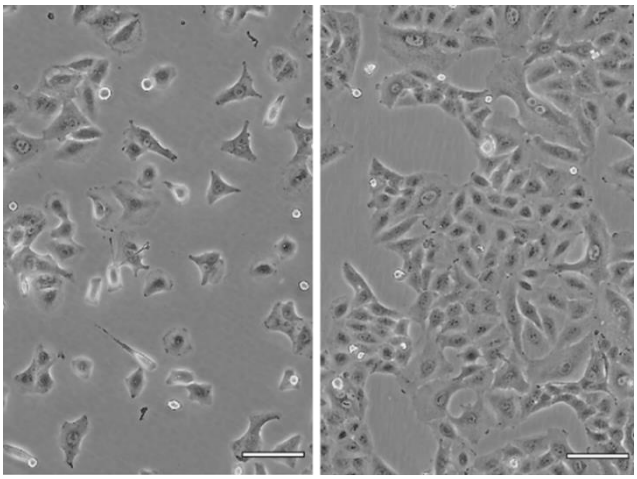

Fig 1.9 Standard microscopy images of A549 cell lines (adopted from ATCC [178]). Left image: A549 with low density in culture plate, right image: A549 with high density in culture plate. Scale bar = 100 μm.

1.2.4.2 Cell viability

Cell viability is a parameter of living or dead cells, based on a total cell sample [180]. Cell viability can be regulated by both internal and external factors. External factors feature foreign proteins, toxicants, temperature and other ambient conditions [181]. These external factors alter cell signalling processes or cause a switch in signalling proteins so that growth of the cells is disrupted [182, 183]. In addition, cell mitogens, which usually are extracellular proteins capable of stimulating cell viability by directly controlling the growth and division of cells, can be deactivated due to proteolysis, overheating and other external factors. As a result, cell viability decreases [184, 185].

Cell viability assays are generally used to assess the effects of external factors on cell growth and division, in which the total number of cells or the change in the proportion of cells is measured [186]. There are primarily four methods to assay cell viability: measuring the rate of DNA replication, analysis of metabolic activity, cell surface antigen recognition and measuring ATP concentration [187, 188]. Among them, analysis of the metabolic activity is most

commonly used in a multi-well plate format. This method is based on the fact that living cells produce a lactate dehydrogenase enzyme which can reduce tetrazolium salts forming a colorimetric or fluorescent readable formazan dye. The absorbance or fluorescence of the released formazan dye can be recorded and converted to a calculation of the number of the living cells [188].

The most commonly used tetrazolium salts include MTS, MTT, XTT and WST-1; consequently, the methods for testing cell viability based on these salts are named MTS assay, MTT assay, XTT assay and WST-1 respectively [188]. All these methods are routinely used for cell viability assays and have been shown to be reliable [186].

The released formazan dye from MTS salts has an advantage of being soluble in the cell culture medium and directly readable via colorimetric change. In addition, the absorbance measurements can be recorded from assay plates periodically within 1-4 h of addition of the MTS solution to cell culture medium; the plates can be returned back to the incubator between the readings to maintain a constant environment, so that the time-depending effect of external factors can be assessed continuously within 4 h [187, 188].

The effects of secreted fungal proteins on cell viability have been previously studied with some fungal strains, and the results showed that some proteins had a cytotoxic effect on human cells. For example, a *P. boydii* culture supernatant could cause death of around 60% of the A549 cells in 24 h at 1 $\mu g \cdot ml^{-1}$ final concentration of proteins [48]. Studies of *A. fumigatus* showed that serine and cysteine proteases secreted by this fungus caused around 60% loss in A549 viability within 24 h of exposure [81]. This study also demonstrated that the cytotoxic effect of *A. fumigatus* culture supernatant could be significantly reduced by addition of serine and cysteine protease inhibitors [81]. However, another study showed that *A. fumigatus* culture supernatant

had no effect on A549 cell viability although around 5% of the cell population became rounded in appearance and detached from the plastic surface of the culture flasks; these cells were still viable [189]. In the current work, the effect of *S. aurantiacum* culture supernatant on the viability of A549 cells was studied.

1.2.4.3 Cell attachment

Cell attachment is a primary feature of the architecture of many tissues. In this process cells interact and attach to a solid surface or other cells through interactions between transmembrane glycoproteins of the cell surface [190, 191]. These glycoproteins can mediate the cell attachment sites and allow cells to attach [190]. It has been found that cell attachment is an important factor in cell life. It is a necessary step in cell growth and cell cycle progression [192, 193], essential in cell communication and regulation, and of fundamental importance in the development and maintenance of tissues [194, 195].

Changes in cell attachment can be a defining event in many diseases, such as arthritis [194, 195], cancer [196, 197], osteoporosis [198, 199], and atherosclerosis [200, 201]. Decrease in cell attachment is generally observed in human cancers, which can result from the action of proteolytic enzymes, such as trypsin, which disrupts the protein-protein interactions that hold cells together [190]. Secreted fungal proteins have also been found to cause dissociation of cells. In the study of *A. fumigatus* it was shown that its secreted serine and cysteine proteases caused over a 50% loss in A549 cell adherence 24 h post-incubation [81].

There are mainly two methods to assess cell attachment. In a wash assay, cells are cultured in multi-well plates for cell attachment to occur. Cell washing is performed to remove the non-attached cells after which the cells adhered to the substrate are quantified by cell count, quantification of DNA content, protein count, or antibody binding [81, 202]. The second

method option is resonance frequency, which uses a biosensor to measure cell attachment. The sensor is coated with a substrate which can be digested by enzymes secreted by the cells and the products will lead to changes in the resonant frequency of the piezoelectric resonators. The biosensor collects these signals and converts them to a measure of cell attachment rate [203-205]. Wash assay is easy to perform and has been widely used to assess the disrupting effect of secreted fungal proteases on cell attachment. For example, Kogan et al. found that a cysteine protease induced 50% detachment of A549 cells from the culture plates using wash assay method [81].

1.3 Hypoxia and fungal pathogenesis

1.3.1 Occurrence of hypoxia in infected sites

The oxygen level in the air is approximately 21% (v/v). In healthy human lungs, alveolar air contains about 14% (v/v) oxygen (105 mmHg) and the arterial blood has an oxygen tension of 97 mmHg [206]. However, the oxygen concentration may decrease in unhealthy human tissues due to inflammation, thrombosis and necrosis, which reduce gas perfusion and consequently cause a decrease in available oxygen [52, 207, 208]. Oxygen concentration less than 1% (v/v), specifically hypoxia, has been well described in tumors, wounds and sites of necrotic tissue, and is typically a feature of cystic fibrosis (CF) [200, 209].

Unhealthy, sick or people with a weakened immune system, are at a higher risk of fungal infection [210]. For instance, CF is characterized by chronic sinopulmonary infection and 80-95% of CF patients suffer from infection caused by various fungi and bacteria [211]. As a result of infection, the degree of hypoxia in diseased tissues will be enhanced due to tissue damage and oxygen consumption of fungal growth [212, 213]. For example, development of hypoxia

was observed *in vivo* in murine models of invasive pulmonary aspergillosis, and the intensity of hypoxia increased with the growth of *A. fumigatus* (Fig 1.10) [212].

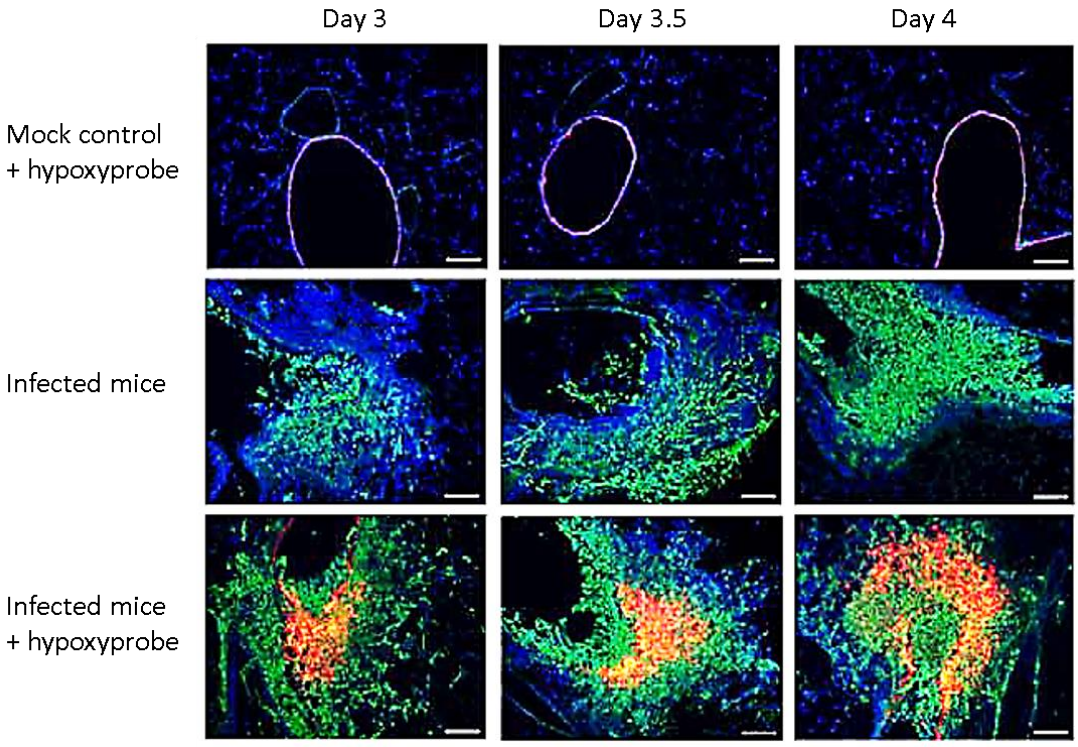

Fig 1.10 Development of hypoxia at the site of *A. fumigatus* infection in murine lungs. Mock control = 0.01% Tween inoculated; Infected mice = inoculated with *A. fumigatus* strain; Infected mice + hypoxyprobe = hypoxyprobe-1 injected into indicated mice, detected by specific antibodies. Red colour = hypoxyprobe-1; Green colour = *A. fumigatus* hyphae; Blue colour = host immune cells. Bar = 100 μm (Reproduced from Grahl et al., 2011 [212]).

The mechanism for fungal infection contributing to hypoxia expansion has been studied, and the results showed that fungi are able to modulate host angiogenesis by secretion of secondary metabolites, such as gliotoxin. These secondary metabolites may further compromise tissue perfusion and consequently aggravate coagulative necrosis, and ultimately reduce the oxygen delivery to infected sites [214]. This study also found that levels of hypoxia gradually increased during the time course of *A. fumigatus* infection in mice, while was almost not detected at the early stages of infection. The findings suggest that fungi need some time to adapt to hypoxic environments before penetrating the lung parenchyma and invasion into the vasculature [212].

1.3.2 Growth of fungi under hypoxia

Filamentous fungi, such as *A. fumigatus, P. boydii and Fusarium oxysporum,* are able to grow in hypoxic conditions, [58, 215, 216]. An electron acceptor is a critical factor for fungal cell growth. Under normoxia, molecular oxygen plays an irreplaceable role in growth. It participates in the process of generating chemical energy via oxidization of metabolic ethanol to carbon dioxide (CO_2) [217]. In low oxygen containing environments, fungi have been found to produce energy by multi-energy metabolic mechanisms. In oxygen-limited environments, nitrate (NO_3^-) is used as an alternative electron acceptor for respiration through denitrification, and metabolic ethanol is also oxidized to CO_2 [215]. When the oxygen concentration is even lower (hypoxic and anoxic conditions), ammonia fermentation is performed through which metabolic ethanol is converted to acetate (CH_3COOH) to generate energy. However, in the presence of glucose, lactic acid fermentation will initially occur and ammonia fermentation starts only after the glucose present in the medium is utilized. Also, metabolic ethanol is converted to lactate (Fig 1.11) [215, 217, 218].

Fungi grown under hypoxia show slower growth and less production of biomass compared to growth under normoxia, as shown in studies on many fungi, such as *A. nidulans, C. albicans* and *P. boydii* [58, 124, 219]. The main reasons for this phenomenon is that the fungal energy metabolic pathway produces less energy under hypoxia. Under hypoxia, fungal metabolic ethanol is oxidised to acetate in glucose-free medium and lactate in presence of glucose. These reactions released up to 33-fold less energy than the reaction performed by fungi under normoxia which converts ethanol to CO_2 [217]. Also, lactic acid fermentation causes a decrease in medium pH and many enzymes cannot function normally in acidic conditions. It has been found that fungi may maintain an optimal pH level within the hyphae by pumping in or out ions. When the extracellular pH deviates too much from the optimal range, fungi lose their ability to keep the optimal inner cell pH, enzymes will be inactivated and consequently less energy is

produced [217, 220-222]. Another possibility is that ammonia fermentation is insufficient to re-oxidize NADH to NAD^+, which results in a higher NADH/NAD^+ ratio, which breaks the redox balance and limits fungal growth [222].

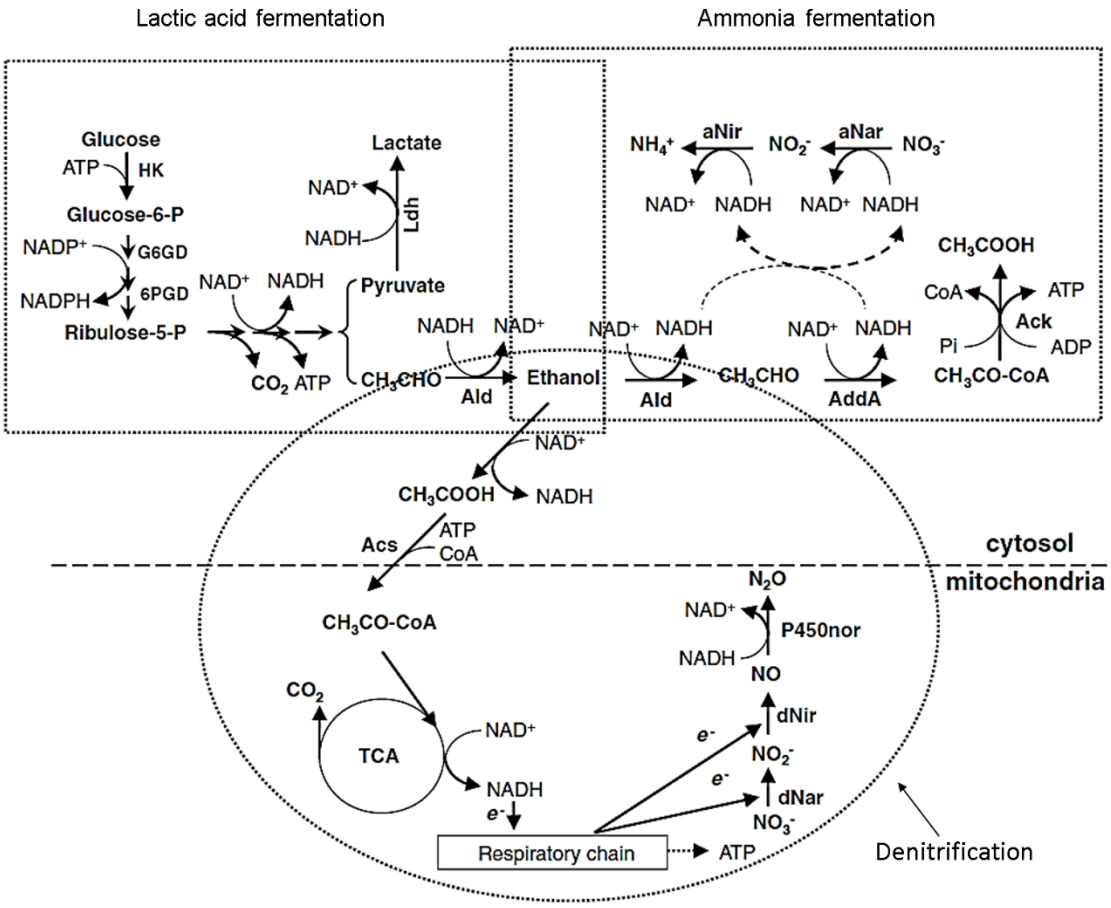

Fig 1.11 Three types of metabolic pathways of fungal energy metabolism under low oxygen environments. HK, hexokinase; G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phoshpogluconate dehydrogenase; Ldh, lactate dehydrogenase; Ald, alcohol dehydrogenase; AddA, acetaldehyde dehydrogenase (acylating); Ack, acetate kinase; Acs, acetyl-CoA synthetase; aNar, assimilatory nitrate reductase; aNir, assimilatory nitrite reductase; dNar, dissimilatory nitrate reductase; dNir, dissimilatory nitrite reductase; P450nor,cytochrome P450nor (Modified from Zhou et al., 2010 [215].)

Microscopic and morphological features of fungal hyphae in hypoxic growth are also different from those in normoxic growth. Studies on *A. fumigatus* showed that hypoxia caused an increase in the cell wall width and a decrease in overall hyphal diameter. An increase in the

electron dense layer on the periphery of the cell wall was also observed in hypoxic grown hyphae [216]. In addition, solid culture of this fungus demonstrated that there were more aerial hyphae, less production of conidia, and consequently a 'fluffy' appearance of colony morphology in hypoxic growth [223].

The dynamics of fungal cell morphology and growth rate under hypoxia may affect the pathogenesis of invasive pulmonary aspergillosis. The low growth rate enables fungi to save energy for the synthesis of essential amino acids and other matters, and the thicker cell wall protects the fungal cell from the acidified environment, induced by fungal growth under hypoxia. In addition, thickening of the fungal cell wall driven by hypoxia may augment phagocyte responsiveness beyond optimal levels required for fungal eradication. This scenario would contribute to the development of immunopathology and subsequent poor treatment outcomes.. Therefore, it is worthy to conduct further research on the interaction between fungi and the host they infect. For instance, if the fungal growth is reduced under hypoxia, introduction of hypoxic conditions may be a potential new therapeutic strategy for the treatment of fungal infection.

1.3.3 Effect of hypoxia on protein secretion in fungi

Hypoxia alters the fungal energy metabolic pathway and growth rates; accordingly, proteins involved in energy-costly processes have been found to be downregulated during oxygen deprivation, indicating an energy-saving effort [222]. Hypoxia-dependent regulation of inner-cell proteins has been observed in *A. nidulans*, *A. fumigatus*, *T. reesei* and *Saccharomyces cerevisiae* [124, 224, 225]. For example, one hundred and seventeen proteins from *A. fumigatus* mycelia were found to significantly change in their abundance under hypoxia in comparison to normoxic growth conditions, including many proteins involved in carbon metabolism, amino acid metabolism, cell wall construction, glycolysis and vitamin and cofactor synthesis. Proteins

involved in respiration and electron transport increased in abundance to increase the respiratory capacity [53].

Response of secreted fungal enzymes to hypoxia is poorly studied. It has been proposed that extracellular fungal enzymes would be affected by hypoxic condition, since fungi change their metabolic pathways under hypoxia [215, 217, 218]. There are a few reports about the effect of hypoxia on secreted fungal non-enzyme proteins. For example, studies on *S. cerevisiae* showed that secretion of proteins, which were supposed to be involved in cell wall remodelling at different oxygen levels was decreased under hypoxia [226-228]. It is therefore important to acquire this knowledge in order to understand how fungi regulate the secretion of enzymes to obtain nutrition for growth under hypoxia and then affect host cells.

1.4 Aims of the present study

The overall aim of the present study was to profile and identify proteases secreted by *S. aurantiacum* with a view of investigating a potential link between protease production and fungal pathogenicity.

The more specific aims were:

1) To examine the growth and protease secretion of clinical (WM 06.482) and environmental (WM 10.136) *S. aurantiacum* isolates in a medium mimicking the cystic fibrosis lung sputum.
2) To explore the growth and protease secretion of *S. aurantiacum* grown under hypoxic conditions characterized by low O_2 levels.
3) To compare proteases secreted by the clinical and environmental *S. aurantiacum* isolates under normoxic and hypoxic conditions.

4) To study the effects of each class-specific protease secreted by *S. aurantiacum* on viability and attachment of human lung cells *in vitro*.

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Chapter 2

Materials and Methods

2.1 Preparation of *S. aurantiacum* growth medium

In this work, *S. aurantiacum* was grown in synthetic cystic fibrosis sputum medium (SCFM) supplemented with casein or mucin (SCFM+C or SCFM+M). SCFM has been developed to mimic the nutrition composition of CF sputum and contains the average concentrations of ions, free amino acids, glucose, and lactate in CF sputum samples (Table 2.1) [1]. To prepare SCFM, NaH_2PO_4, Na_2HPO_4, KNO_3, NH_4Cl, KCl and NaCl were dissolved in MilliQ-water; MOPS was also added to buffer the pH changes due to fungal growth. After the addition of various components to 70% (v/v) of the final medium, pH of solution was adjusted to 5.7 with HCl to support fungal growth, and the medium was filter sterilized through a 0.22 μm membrane [1]. All chemicals used in this work were from Sigma-Aldrich (Australia), unless otherwise stated.

Casein or mucin (see publication 1) were dissolved in MilliQ-water to 30% (v/v) of the final medium, after which the pH of solution was adjusted to 5.7, autoclaved and mixed with SCFM just before use. During preparation, a small amount of sodium hydroxide (NaOH) was added to the casein solution to help the casein to dissolve. Hydrochloric acid (HCl) was added to the casein or mucin solution slowly by stirring to titrate the pH to 5.7 before autoclaving. The casein solution was autoclaved once at 121 °C for 20 min, while mucin solution was autoclaved twice with a 24 h break between the two cycles to achieve a contaminant-free medium.

Table 2.1 Composition of SCFM (Reproduced from Kelli et al., 2007 [1]).

Components	Concentration (mM)
Ions	
NaH_2PO_4	1.3
Na_2HPO_4	1.25
KNO_3	0.035
NH_4Cl	2.36
KCl	14.94
NaCl	51.85
$CaCl_2$	1.75
$MgCl_2$	0.61
FeSO4	0.0036
Amino acids	
Serine	1.4
Threonine	1.0
Alanine	1.8
Glycine	1.2
Proline	1.7
Isoleucine	1.1
Leucine	1.6
Valine	1.1
Aspartate	0.8
Glutamate	1.5
Phenylalanine	0.5
Tyrosine	0.8
Tryptophan	0.01
Lysine	2.1
Histidine	0.5
Arginine	0.3
Ornithine	0.7
Cysteine	0.2
Methionine	0.6
Others	
Glucose	3.2
MOPS	7.79

2.2 Other materials and methods

General materials and methods used during the present study are listed in the table below (Table 2.2). Details of the specific methods are outlined in the corresponding experimental chapters as indicated.

Table 2.2 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 and 4
Construction of hypoxic culture device	4
Cultivation and maintenance of the A549 cell line	5
Measurement of fungal growth rates	3 and 4
Measurement of general protease activity	3 and 4
Class and activity profiling of proteases	3, 4 and 5
Protease inhibition assays	3 and 4
SDS-PAGE and protease activity zymograms	3 and 4
Isolation and enrichment of secreted proteins	3, 4 and 5
Protein identification by mass spectrometry	3 and 4
Protein separation by size exclusion chromatography	5
Cell viability assay	5
Cell attachment assay	5

Reference

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Chapter 3

Secretion of proteases by an opportunistic fungal pathogen *Scedosporium aurantiacum*

3.1 Introduction

S. aurantiacum is a recently identified pathogen and is an important cause of infection in cystic fibrosis patients in Australia [1, 2]. This fungus shows high virulence to human hosts and resistance to many types of antifungal agents [3-5]. To date, little is known about the virulence factors of this fungal pathogen. In this chapter, production and secretion of proteases in a synthetic cystic fibrosis sputum medium supplemented with casein or mucin by a clinical (WM 06.482) and an environmental (WM 10.136) *S. aurantiacum* isolate were investigated and compared. Casein supports the synthesis and secretion of a range of proteases, while excess mucin is representative of the lung environment of cystic fibrosis patients [6]. Secreted proteases are considered putative virulence factors in a number of other pathogenic fungi [7-9]. The mucin-containing medium, which has similar nutritional components as lung sputum of cystic fibrosis patients, was found to support the growth and protease secretion of the clinical strain the best and generate the greatest difference in fungal growth and protease secretion between the clinical and environmental strains. Protease produced in mucin-containing medium was further analysed by LC−MS/MS and homologs of some proteases were identified with a view of exploring potential differnces between proteases secreted by the clinical and environmental isolates.

The results of this research were reported in a peer-reviewd paper published in PLoS One (Publication 1).

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3.2 Contribution to publication 1

This publication was developed with supervision of my mentors at Macquarie University (Helena Nevalainen and Liisa Kautto). All experiments were performed by me and data were analysed with the assistance of Helena Nevalainen and Liisa Kautto. The manuscript was written by me and Helena Nevalainen and reviewed by Helena Nevalainen and Liisa Kautto.

3.1 Author contribution for publication 1.

	ZH	HN	LK
Experimental design	•	•	•
Data collection	•		
Data analysis	•	•	•
Manuscript	•	•	

* ZH = Zhiping Han, HN = Helena Nevalainen, LK = Liisa Kautto

3.3 Publication 1

Han, Z., L. Kautto, and H. Nevalainen, Secretion of Proteases by an Opportunistic Fungal Pathogen *Scedosporium aurantiacum*. PLoS One. 2017;12(1):e0169403.

PLOS ONE

Secretion of Proteases by an Opportunistic Fungal Pathogen *Scedosporium aurantiacum*

Zhiping Han [1, 2*], Liisa Kautto[1, 2] and Helena Nevalainen[1, 2]
[1] Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, Australia
[2] Biomolecular Frontiers Research Centre, Macquarie University, Sydney, Australia
* zhiping.han@students.mq.edu.au

Abstract

Scedosporium aurantiacum is an opportunistic filamentous fungus increasingly isolated from the sputum of cystic fibrosis patients, and is especially prevalent in Australia. At the moment, very little is known about the infection mechanism of this fungus. Secreted proteases have been shown to contribute to fungal virulence in several studies with other fungi. Here we have compared the profiles of proteases secreted by a clinical isolate *Scedosporium aurantiacum* (WM 06.482) and an environmental strain (WM 10.136) grown on a synthetic cystic fibrosis sputum medium supplemented with casein or mucin. Protease activity was assessed using class-specific substrates and inhibitors. Subtilisin-like and trypsin-like serine protease activity was detected in all cultures. The greatest difference in the secretion of proteases between the two strains occurred in mucin-supplemented medium, where the activities of the elastase-like, trypsin-like and aspartic proteases were, overall, 2.5-75 fold higher in the clinical strain compared to the environmental strain. Proteases secreted by the two strains in the mucin-supplemented medium were further analyzed by mass spectrometry. Six homologs of fungal proteases were identified from the clinical strain and five from the environmental strain. Of these, three were common for both strains including a subtilisin peptidase, a putative leucine aminopeptidase and a PA-SaNapH-like protease. Trypsin-like protease was identified by mass spectrometry only in the clinical isolate even though trypsin-like activity was present in all cultures. In contrast, high elastase-like activity was measured in the culture supernatant of the clinical strain but could not be identified by mass spectrometry searching against other fungi in the NCBI database. Future availability of an annotated genome will help finalise identification of the *S. aurantiacum* proteases.

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Introduction

Members of *Scedosporium* spp. are ubiquitous in nature and can be isolated from a wide range of human-impacted environments [1, 2]. Some species of the *Scedosporium* spp. complex such as the recently identified *S. aurantiacum* are opportunistic pathogens that affect people with diabetes, solid tumours, chronic lung diseases and stem cell transplants [3]. In Australia, *S. aurantiacum* is the second most common filamentous fungus isolated from the sputum of cystic fibrosis (CF) patients after *Aspergillus fumigatus* [4, 5].

The majority of the work carried out with *S. aurantiacum*, currently described in the literature, features prevalence studies [4, 6, 7], animal studies including virulence [8-10] and antifungal susceptibility studies [1, 11, 12]. Recently, we have reported phenotypic profiling of this fungus that revealed differences in the carbon substrate utilisation patterns between clinical and environmental *S. aurantiacum* isolates [13].

A correlation between production of proteases and fungal pathogenicity to humans has been established in the studies with pathogenic *Aspergillus* spp. [14-16], *Candida* spp. [17, 18] and Dermatophytes [19]. Proteolytic activities identified from these fungi feature the families/subfamilies of elastase-like, chymotrypsin-like, subtilisin-like, and trypsin-like serine proteases, aspartic proteases, metalloproteases and cysteine proteases [16, 20, 21]. Amongst the proteases, elastase activity has emerged as the main indication for virulence and has been linked to germination and penetration into mice lungs [22], deterioration of respiratory function [14], and lung injury [23]. Secreted serine proteases and cysteine proteases from *A. fumigatus* strain AF293 were able to breach the alveolar epithelial cell barrier by disruption of the actin cytoskeleton and sites of focal adhesion in human lung cancer cells [16]. Extracellular aspartic proteases have been implicated in the virulence of *C. albicans* in a mouse model; these proteases were found to have broad substrate specificity, degrade many mouse proteins and digest cells and molecules of the host immune system [24].

Secreted proteases may also contribute to the virulence of the fungal species of the *Pseudoallescheria boydii* complex including *S. aurantiacum* [6, 25, 26] since research has demonstrated that infections caused by *P. boydii* highly resemble those of *A. fumigatus* [27, 28], whose secreted proteases have been reported as putative virulence factors [16, 23, 29, 30]. The first report on the proteases secreted by *P. boydii* was by Larcher et al. in 1996, who purified and characterized a 33 kDa subtilisin-like protease from the fungal culture supernatant [31]. Recently, a zinc-metalloprotease active in an acidic pH was detected in *P. boydii* mycelia [32]. In addition, *Scedosporium apiospermum*, a member of the *P. boydii* complex, was found to produce six distinct mycelial metalloproteases ranging from 28 to 90 kDa in size [33]. To date, there are no reports relating these proteases to virulence.

In this study, we have explored, for the first time, proteases secreted by *S. aurantiacum*, as a prelude to studies into potential roles of these proteases in fungal virulence. Protease profiles were compared between a clinical isolate and an environmental strain. The amount, type, and activity of major proteases secreted by these two strains were determined in response to cultivation on synthetic cystic fibrosis sputum medium supplemented with mucin or casein.

Materials and Methods

Liquid cultivation media

Synthetic cystic fibrosis sputum medium (SCFM) containing mineral salts, amino acids and 1% (w/v) glucose was used as a base for all liquid media and prepared as previously described [34]. Additions to SCFM featured 1% (w/v) casein from bovine milk (Sigma-Aldrich, Australia; SCFM+C) or 1% (w/v) mucin from porcine stomach (type III; Sigma-Aldrich, Australia; SCFM+M). Mucin and casein were suspended in Milli-Q H2O; sodium hydroxide was added to dissolve casein. These substrates were autoclaved separately and mixed with SCFM just before use. SCFM was sterilized by filtering through a 0.22 μm membrane (Millipore) and final pH of media was adjusted to 5.7 to support fungal growth [35].

Fungal strains and cultivation conditions

The *Scedosporium aurantiacum* strains studied were WM 06.482 (clinical isolate) isolated from broncho-alveolar lavage of a CF patient in Australia and WM 10.136 (INS1120; environmental strain) originating from a valley near Innsbruck, Austria [8]. The strains were obtained from the culture collection of the Medical Mycology Laboratory, Centre for Infectious Diseases and Microbiology, Westmead Hospital, Sydney, Australia. Virulence status of WM 06.482 has been established using immunocompromised mice [8]. In this paper, no significant virulence differences were found between WM 06.482 and environmental strains examined. The virulence of both strains used in the current study has been assessed using the invertebrate wax moth *Galleria mellonella* larval model, and results showed that the clinical isolate WM 06.482 was about three times more virulent than the environmental strain WM 10.136 [13]. However, there are no published reports regarding comparison of these strains in a mammalian model to establish their relative virulence.

S. aurantiacum strains were cultured on Malt Extract agar (Oxoid, Australia) at 37 °C for 7 to 10 days to attain sufficient conidiation. Conidia were harvested by gentle agitation into a solution containing 0.9% (w/v) sodium chloride and 0.01% (v/v) Tween 80, filtered through an autoclaved 5 mL tip packed with cotton wool to remove hyphal fragments and counted using a Neubauer haemocytometer. Liquid cultures were performed in 250 ml conical flasks containing 50 ml of growth medium, inoculated with 2×106 conidia/ml and incubated for up to seven days at 37 °C on an orbital shaker at 200 rpm with three individual flasks dedicated for each time point. Culture supernatants were collected at every 24 h from flasks dedicated to each time point. Contents were centrifuged at 4500 g for 30 min, and the supernatant filtered through a 0.22 μm membrane (Millipore, Australia) at 4 °C. Cleared supernatants were then aliquoted in 1.5 ml Eppendorf tubes and stored at -80 °C. Fungal protease inhibitor cocktail (0.05% v/v, Sigma-Aldrich, Australia) was added in the culture supernatant samples used for proteomic analysis [36].

Measurement of growth and pH

Measurements were performed every 24 hours (three replicates). After removal of the culture supernatant, mycelia left in the centrifuge tubes were washed three times with 50 ml Milli-Q water by inverting. The washed mycelia were frozen at -30 °C for at least 4 h, and then freeze dried. Biomass was calculated as the difference between the weight of the centrifuge tubes with and without the freeze dried mycelia. The starting biomass was recorded as the weight of the same amount of freeze dried conidia used to inoculate each flask. The pH of each culture supernatant was measured at room temperature.

Protease activity assays

All protease activity assays were performed under sub-saturating conditions. General protease activity in thawed supernatant samples was assayed using azocasein (Sigma-Aldrich, Australia) as a substrate following the method of Rauscher [37] with some adjustments. Briefly, the substrate (10 mg/ml) was dissolved in citrate-phosphate buffer pH 7.5 which was deemed optimal for the assay of general protease activity (data not shown). A 75 μl aliquot of the supernatant was mixed with 50 μl substrate solution in a 96-well plate and incubated for 60 min at 37 °C. The reaction was terminated by adding 125 μl 5% (w/v) trichloroacetic acid (TCA). After centrifugation at 4000 g for 5 min, the supernatant was collected and absorbance of the released azo-dye read at 366 nm and activity calculated according to the method of Coelho et al [38]. Protease activity is calculated as μg of azocasein digested in one minute, normalized against dry fungal biomass. Reaction blanks were prepared by adding TCA to the culture supernatant to denature proteases before addition of azocasein. General protease activity was measured every 24 hours over seven days.

The types of proteases in the culture supernatant were studied using the following class-specific inhibitors: 1 mM PMSF (serine protease inhibitor; Sigma, Australia), 5 mM EDTA (metalloprotease inhibitor; Sigma, Australia), 100 μM pepstatin A (aspartic protease inhibitor; Merck, Nottingham, UK), and 5 μM E-64 (cysteine protease inhibitor; Sigma, Australia). The day-4 culture supernatants were pre-incubated with a protease inhibitor for 30 min at room temperature prior to performing a general protease activity assay using azocasein as substrate. Culture supernatants with no inhibitor added were also included in the assay for comparison.

Table 1. Class-specific substrates used to detect the protease class and activity in fungal culture supernatants

Specificity	Substrate	Positive control	Buffer and assay pH	References
Chymotrypsin-like	N-Benzoyl-L-Tyrosine-ρ-NA	Bovine pancreas alpha-chymotrypsin-like	0.1 mM Tris-HCl buffer, pH 8.0	[39]
Subtilisin-like	Z-Ala-Ala-Leu-ρ-NA	Sublitisin A from *Bacillus* sp	0.1 mM Tris-HCl buffer, pH 8.0	[40]
Elastase-like	N-Sue-Ala-Ala-Ala-MCA	Elastase-like type I from porcine pancreas	0.1 mM Tris-HCl buffer, pH 8.0	[41]
Trypsin-like	Boc-Phe-Ser-Arg-MCA	Trypsin-like from bovine pancreas	67 mM sodium phosphate buffer, pH 7.6	[42]
Cysteine	Z-Arg-Arg-MCA	Papain from *Papaya latex*	100 mM sodium acetate buffer containing 10 mM DTT, pH 5.5	[43]
Collagenase	Sue-Gly-Pro-Leu-Gly-Pro-MCA	Collagenase from *Clostridium histolyticum*	50 mM tricine buffer pH 7.5 with 10 mM $CaCl_2$ and 400 mM NaCl, pH 7.5	[44]
Aspartic	Boc-Leu-Ser-Thr-Arg-MCA	Pepsin from porcine gastric mucosa	0.2 M acetate buffer, pH 3.5	[45]

Based on the inhibitor studies, serine proteases (chymotrypsin, subtilisin-like, elastase-like and trypsin-like proteases), metallo and aspartyl proteases were studied in more detail using subclass specific substrates. These substrates were synthetic peptides coupled with either 7-Amino-4-methylcoumarin (MCA) or ρ-nitroanilide (ρ-NA) (Peptide Institute Inc, Japan), and protease activities were calculated based on fluorometric or colorimetric detection. Cysteine protease activity was also included in the assay. Substrates, buffers and proteins used as a positive control are listed in Table 1. Chymotrypsin-like, elastase-like, subtilisin-like, trypsin-like, cysteine, collagenase and aspartic protease activities were measured in their optimal pH described in the literature following the manufacturer's instructions with some modifications. The reaction mixture contained 50 μl of culture supernatant and 0.5 mM substrate in 200 μl of appropriate buffer. After incubation at 37 °C for 4 min, the absorbance of released ρ-NA was read at 410 nm, or fluorescence of the released MCA was measured at λex =380 nm and λem = 460 nm at 37 °C. The amount of released ρ-NA or MCA was calculated using a standard curve prepared with appropriate dilutions of ρ-NA or MCA. Activities are given as nM of MCA or μM of ρ-NA released per minute per mg of dry biomass. All assays were performed in triplicate, and statistical analysis of the biological replicates was conducted using Excel or OriginPro 8.5 (www.originlab.com).

Protein electrophoresis and enzyme activity zymograms

Culture supernatants were analysed by protein electrophoresis according Laemmli [46]. An aliquot of 21 μl of supernatant from each culture was mixed with 4× NuPAGE LDS sample buffer (Thermo Fisher Scientific), heated at 70 °C for 10 min, and subjected to electrophoresis at 120 V for approximately 1 h on a 12.5% (w/v) tris-glycine gel using a running buffer containing 1.4% (w/v) glycine, 0.1% (w/v) SDS and 24 mM Tris. The protein gel was stained overnight in Coomassie Brilliant Blue G-250 (Bio-Rad, CA, USA) and destained in 1% (v/v) acetic acid. To detect protease activity of the secreted proteins, another protease activity of the secreted proteins, another set of supernatants (not heated) was applied to zymogram gels made of 12.5% (w/v) Tris-glycine gels containing 0.1% (w/v) casein (from bovine milk, Sigma, Australia). Following electrophoresis, zymogram gels were soaked in 2.5% (v/v) Triton-X 100 to for 30 min twice and washed 3 × 10 min in MilliQ water to renaturate proteins and restore enzyme activity. The gels were then incubated for 16 h in 0.03 mol l-1 Tris-HCl buffer pH 7.5 at 37 °C. After incubation, the zymogram gels were subjected to the same staining and destaining procedures as the SDS-PAGE gels [47].

Sample preparation for proteomic analysis

Proteins in the day-4 culture supernatant were precipitated following the method of Fragner [48] with some modifications. Briefly, culture supernatants with protease inhibitors added were frozen and stored at -80 °C for 12 h. After thawing at 4 °C the samples were centrifuged at 10000 g for 1 h at 4 °C and the supernatant was removed into a new tube. Na-deoxycholate (2% w/v in dd H2O) was added into the supernatant to the final concentration of 0.02% (w/v) and the mixture was incubated on ice for 30 min after vortex briefly [49]. Na-deoxycholate was used to improve protein recovery [50]. Then, 10% (w/v) TCA was added to the solution and the content was incubated on ice for at least 12 h. After centrifugation at 4000 g and 4 °C for 5 min, the supernatant was discarded carefully and the pellet was washed twice using cold acetone (-30 °C). Protein pellets were kept at -80 °C until use.

Identification of proteases

Protein pellets from above were resuspended in 50 μl Tris-buffer (50 mM, pH 8.0) and protein concentration was measured according to Bradford [51]. Twenty micrograms of protein from each sample was heated for 10 min at 70 °C and subjected to electrophoresis, as described above. Each lane on the protein gel was cut across horizontally into 10 slices. Each slice was chopped finely, destained with 50% acetonitrile (ACN) in 100 mM ammonium bicarbonate, reduced with 10 mM dithiothreitol, alkylated with 55 mM iodoacetamide, and then washed twice with 25 mM ammonium bicarbonate. Gel slices were dehydrated with ACN before drying by vacuum centrifugation. The dry samples were kept on ice for 10 min and then digested with trypsin (Sequencing grade modified, Promega, Australia) using a trypsin: protein ratio of 1:30 (w/w) as described by Grinyer [52]. Biological triplicates were analyzed in parallel. In addition, GluC digestion (Sequencing Grade, Promega, Australia) was carried out in a similar fashion with a GluC: protein ratio of 1:50 (w/w) separately to the trypsin digestion. Peptides were extracted with 65% (v/v) ACN containing 1.65% (v/v) formic acid. Extracted peptides were desalted and concentrated using C18 zip-tips (Millipore, Australia), and 10 μl of the resulting peptides was subjected to reversed-phase nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC−MS/MS) using an LTQ-XL ion-trap mass spectrometer (Thermo, San Jose, CA). In brief, peptides were injected onto an in-house packed column with Halo C18 (Advanced Materials Techology, USA) and washed with buffer A (5% (v/v) ACN containing 0.1% (v/v) formic acid). The bound peptides were then eluted from the column using a linear solvent gradient buffer B (99.9% (v/v) ACN, 0.1% (v/v) formic acid), with the following steps: 1-50% of buffer B for 58 min, 50–85% of buffer B for 2 min, held at 85% for 8 min with a flow rate of 500 nl min^{-1} across the gradient. The resolved peptides were directed into the nanospray ionization source of the mass spectrometer and scanned in the spectral range 350−2000 amu. Automated peak recognition, dynamic exclusion window set to 90s and tandem MS of the top ten most intense precursor ions at 35% normalization collision energy was performed using Xcalibur software (version 2.06, Thermo, San Jose, CA) [53].

Protein identification was carried out using the Global Proteome Machine software of the X! Tandem algorithm (GPM, http://www.thegpm.org). For each experiment, the 10 fractions were processed sequentially with output files for each individual fraction and also a merged, non-redundant output file was generated for protein with log (e) values less than -1. An in-house database was created from NCBI protein entries comprising protease sequences of fungal species including *Saccharomyces* spp., *Schizosaccharomyces* spp., *Trichoderma* spp., *Neurospora* spp., *Aspergillus* spp., *Candida* spp. and *Pseudallescheria* spp. GPM search parameters included MS tolerance of ±2 Da, MS/MS tolerance of ±0.2 Da, carbamidomethylation of cysteine as fixed modifications, oxidation of methionine as variable modifications, and tolerance of *Aspergillus* spp., *Candida* spp. and *Pseudallescheria* spp. GPM search parameters included MS tolerance of ±2 Da, MS/MS tolerance of ±0.2 Da, carbamidomethylation of cysteine as fixed modifications, oxidation of methionine as variable modifications, and tolerance of two missed tryptic cleavages and K/R-P cleavages. Only the proteins that were identified in three biological replicates and had a total spectral count of at least 5 were considered as a valid protein identity [54].

Results

Growth and secretion of proteases in different media

The clinical isolate *S. aurantiacum* WM 06.482 and the environmental strain WM 10.136 were cultured in the SCFM medium with addition of casein or mucin to boost growth and induce protease production. The

growth rates were investigated by measuring the dry weight of the biomass from day zero to day seven. As shown in Fig 1 (A, B), the clinical and environmental strains showed a similar overall growth pattern in all media with some exceptions. The clinical isolate seemed to grow faster on SCFM supplemented with mucin peaking at day 2 and also produced the highest amount of biomass (9.94 mg/ml). The sharp peak on day one in the SCFM+C cultures was caused by the precipitated casein in the growth medium although the biomass was washed three times using Milli-Q water before freeze drying. SCFM without supplement was the least supportive of growth.

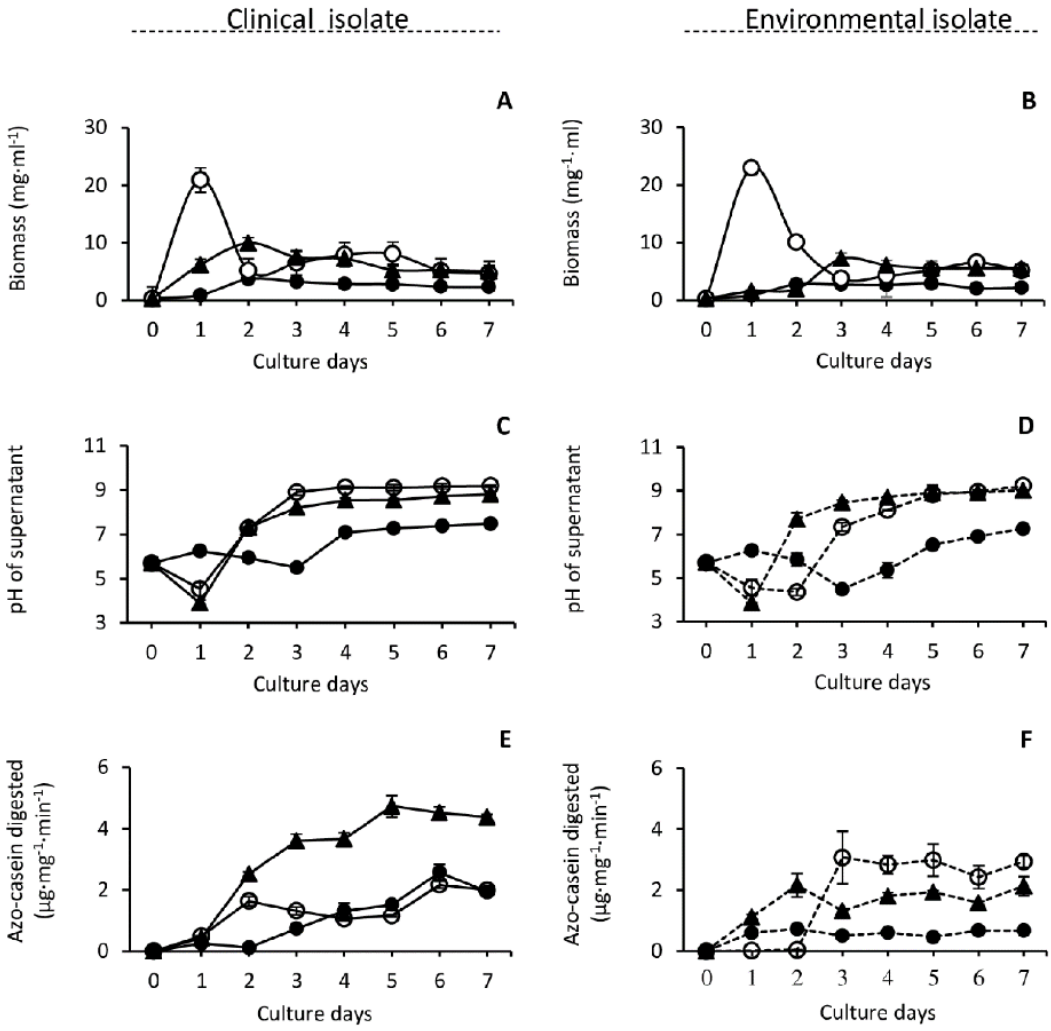

Fig 1. Growth and protease activity of *S. aurantiacum* WM 06.482 (clinical isolate) and WM 10.136 (environmental strain) in different media. (A, B) Change in the dry weight of mycelia over time in 50 ml cultures. The data of day 0 represent the dry weight of the same amount of freeze dried conidia used to inoculate each flask. Due to the precipitating casein on day one in the casein supplemented medium, the dry weight measured encompasses both dry mycelia and casein; (C, D) pH of the culture supernatant; (E, F) general secreted protease activity, normalized against dry biomass. Data represent mean ± SD (3 biological replicates). ● SCFM: Synthetic CF sputum medium, ○ SCFM+C (casein added); ▲ SCFM+M (mucin added).

All cultures experienced a decrease in the medium pH at the beginning of culture (Fig 1 C, D). The pH then increased to 9 in mucin and casein supplemented SCFM media and 7.5 in SCFM only. The decrease in pH was probably due to lactic acid fermentation in the presence of glucose [55]; after glucose had been exhausted, the pH started to increase due to ammonia fermentation [56]. In the SCFM mediu m without supplements, the decrease of pH in the culture supernatant was slight and occurred later. This might relate to the poor growth of *S. aurantiacum* in SCFM (Fig 1 A, B).

To assess the total protease activity in each culture supernatant, azocasein was used as a substrate and comparison was made between the two strains and culture time. The clinical and environmental strains exhibited a great difference in the protease activity in each cultivation medium. The highest protease activity (4.73 μg azocasein digested per minute per mg of dry biomass) was found in the culture supernatant of the clinical strain grown in the mucin supplemented medium (Fig 1 E); this was over two times higher than the activity produced by the environmental strain (Fig 1 F), supporting the hypothesis that the clinical strain was thriving in the presence of mucin, which is abundant in the CF lungs [57] by growing well and producing proteases (Fig 1 A, E). The dominant form of mucin in the porcine mucin used in this study is similar to the human mucin [58, 59].

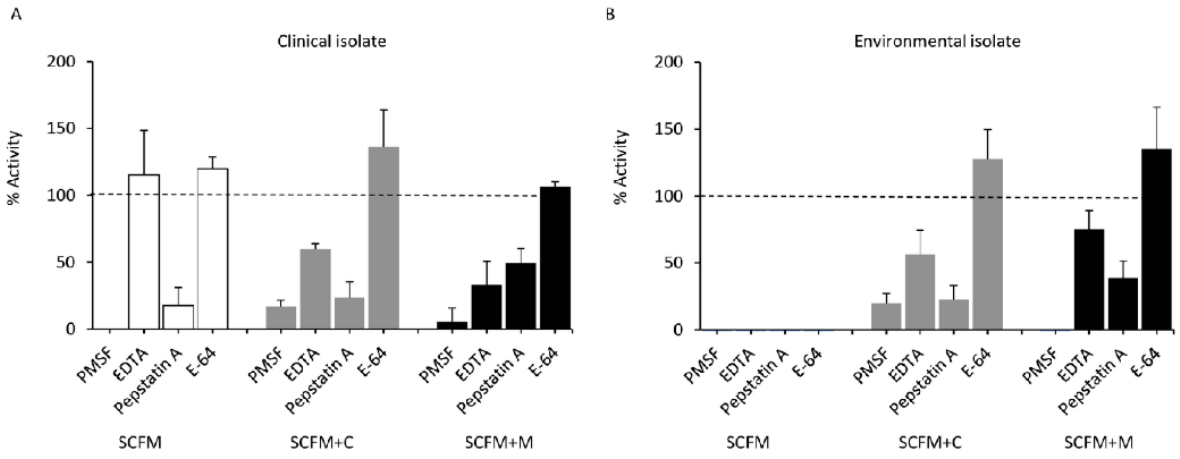

Fig 2. Inhibition of protease activity in day-4 culture supernatant of *S. aurantiacum* in three media. Percentage changes in the protease activity in the supernatant with an inhibitor relative to the non-inhibited control supernatant, set as 100% for each culture (dotted line). Data represent mean ± SD (3 biological repeats). A. Clinical strain; B. Environmental strain. SCFM: Synthetic CF sputum medium; SCFM+C (casein added); SCFM+M (mucin added).

Protease inhibitors were used to inhibit specific classes of proteases secreted by *S. aurantiacum* in the different day-4 culture media in order to explore the protease profile. The inhibitor concentrations used were chosen based on literature and previous testing in the laboratory (data not shown). The serine protease inhibitor PMSF showed the greatest inhibition in all cultures overall (Fig 2 A, B) indicating that serine proteases were responsible for the majority of proteolytic activity. Pepstatin A (aspartic protease inhibitor)

was the second strongest inhibitor followed by EDTA (metalloprotease inhibitor) across all media, suggesting secretion of metallo and aspartic proteases by both *S. aurantiacum* WM 06.482 (clinical isolate) and WM 10.136 (environmental isolate; Fig 2 A, B). E-64 (cysteine protease inhibitor) had no effect on protease activity in any of the cultures, suggesting that *S. aurantiacum* did not secrete cysteine proteases under the culture conditions used. The 'zero activity' in the supernatants of the environmental strain grown on SCFM may reflect the fact that the overall protease activity was low to start with (Fig 1F; Fig 2 B).

Protease assays using specific substrates

Specific substrates were then used to measure the activity of each type of protease secreted by the two *S. aurantiacum* strains cultured in different media at their optimal pH (Table 1). Samples taken at days 2, 4 and 6 were examined. Both strains were found to secrete different proteases in response to different supplements (casein or mucin) added in the SCFM medium. Subtilisin-like serine protease activity was found to be common in all cultures although the amount varied greatly (Fig 3). Trypsin-like protease activity was also found across the cultures but it was almost close to zero in the SCFM medium. Strong presence of serine proteases is in accordance with the inhibitor studies (Fig 2).

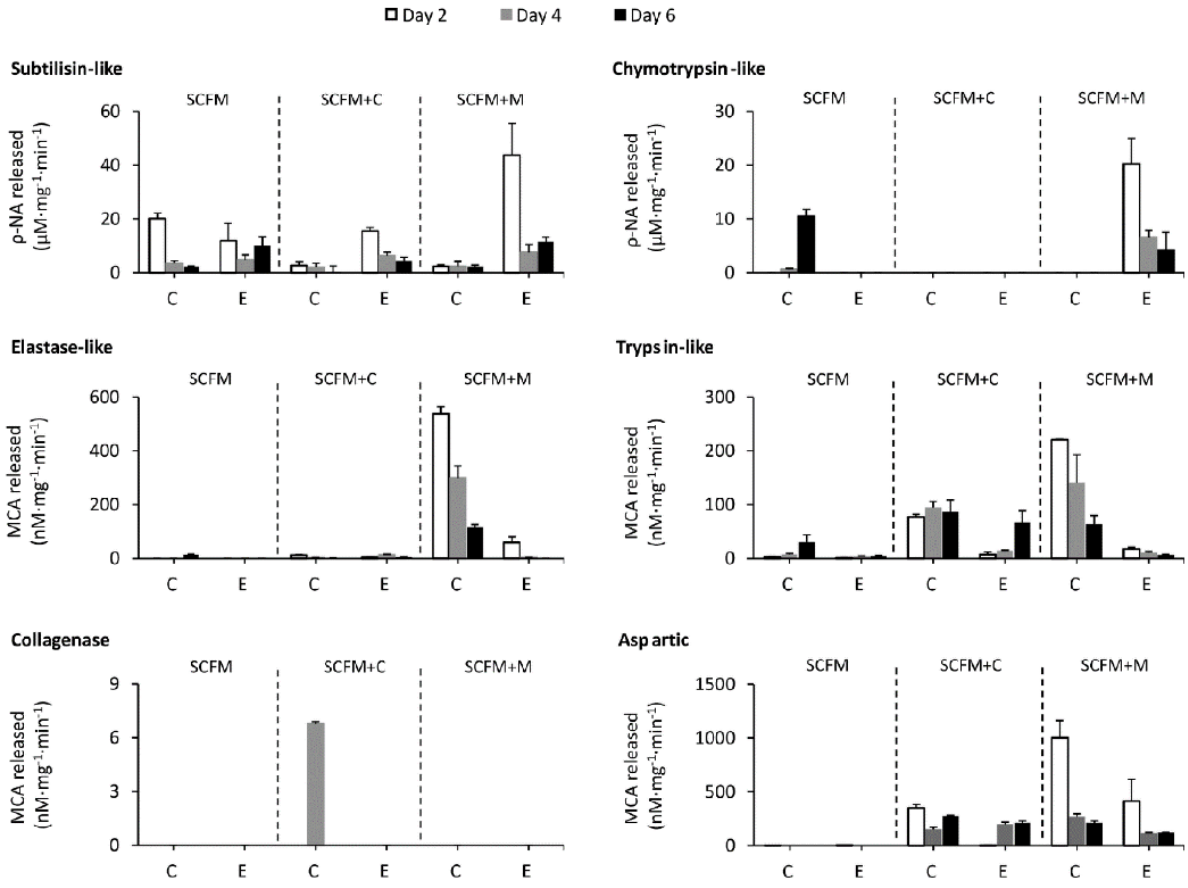

Fig 3. Classes and activities of specific secreted proteases in three media. Activities of specific proteases were expressed as the amount of ρ-NA or MCA released in one minute per mg of dry biomass. Substrates

and test conditions are given in Table 1. Data represent mean ± SD (3 biological repeats). SCFM: Synthetic CF sputum medium; SCFM+C (casein added); SCFM+M (mucin added). C: Clinical strain, E: environmental strain.

The highest subtilisin-like activity was measured on day 2 in the environmental strain grown on the mucin supplemented SCFM medium (Fig 3). The highest trypsin-like activity as well as the highest elastase-like and aspartic protease activity was recorded for the clinical isolate on day 2 on mucin-supplemented medium, (Fig 3). The highest chymotrypsin-like activity was detected in the environmental strain on day 2 in the mucin supplemented SCFM whereas chymotrypsin activity produced by the clinical strain was very low under the same circumstances. Cysteine protease activity was not detected in any of the cultures (data not shown). Overall, the casein supplemented SCFM medium (SCFM+C) did not induce production of proteases very well except for collagenase activity in the clinical strain measured on day 4 (Fig 3).

The mucin-containing SCFM medium (SCFM+M) supported production of a broad range of proteases and the activity of each class was also, in most cases, higher compared to the other two media. Subtilisin-like, chymotrypsin-like, elastase-like, trypsin-like serine proteases and aspartic proteases were all detected. Elastase-like, trypsin-like and aspartic protease activities of the clinical strain WM 06.482 were significantly higher (about 2.5-75 fold, $p < 0.05$) than those of the environmental strain WM 10.136 (Fig 3). In particular, the elastase-like protease activity in the clinical isolate was high in the mucin-supplemented medium but almost undetectable in all other cultures.

To conclude, the nature of the growth medium (SCFM supplemented with casein or mucin) affected the levels and types of proteases secreted into the cultivation medium by the *S. aurantiacum* strains. Of particular interest in the context of proteases being described as a virulence factor, higher protease activity in the mucin supplemented SCFM provided an indication towards this role as the medium mimics the lung environment of CF patients. In this medium, serine proteases formed up 95% activity of the secreted proteases in the clinical isolate (Fig 2). Furthermore, amongst serine proteases, elastase-like and trypsin-like activities were significantly higher in the clinical isolate compared to the environmental isolate (Fig 3, $p < 0.05$). These two protease-types warrant further investigation as the major contributors to secreted protease activity by the clinical strain.

Enzyme activity zymograms

The samples from day 2, day 4 and day 6 culture supernatants of the clinical (WM 06.482) and environmental (WM 10.136) isolates were further analyzed by protein electrophoresis and zymogram gels containing 0.1% (w/v) casein as substrate. The pH of zymogram gels was adjusted to 7.5 for visualization of the protease activity as this is close to the pH of the upper and lower airways of the human lungs in both CF and non-CF subjects [60]. In spite of the fact that only faint protein bands showed up on the protein gels from the culture supernatants due to the low concentration of secreted proteins (Fig 4 A), clear proteolytic bands could be seen on the zymograms (Fig 4 B). A proteolytic band with a molecular weight (MW) of approximately 30 kDa was common for most of the cultures, appearing in the three media at different time points in both isolates (Fig 4 B).

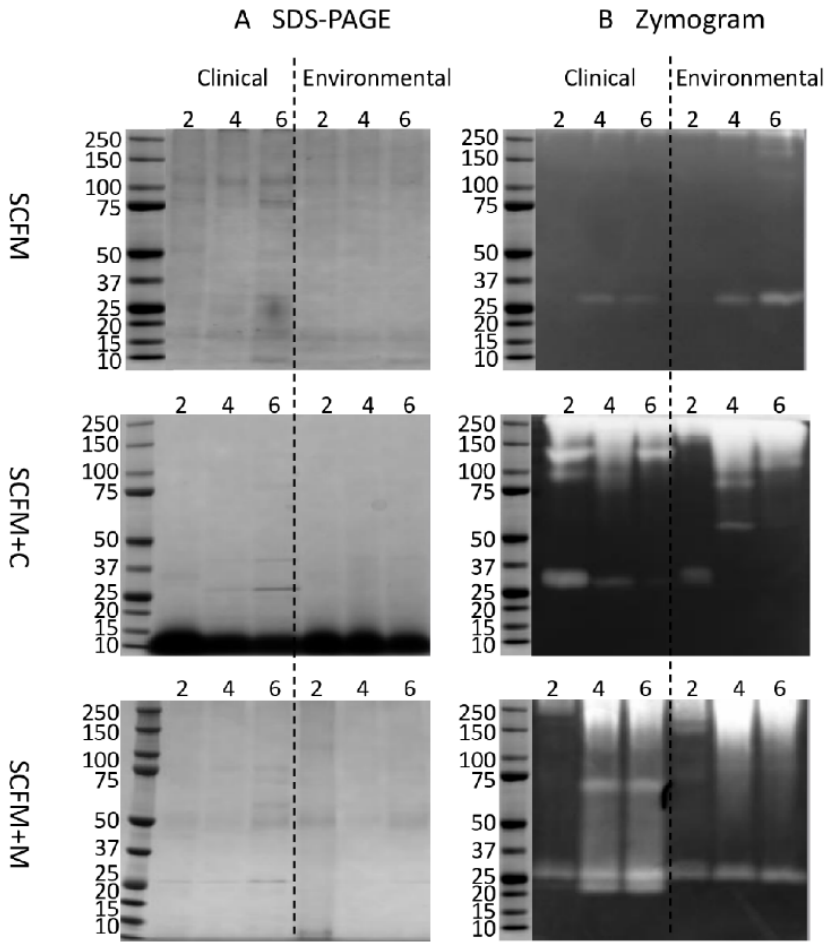

Fig 4. SDS-PAGE and zymogram analysis of *S. aurantiacum* culture supernatants. A. Proteins from the non-concentrated culture supernatant separated by protein electrophoresis. B. Enzyme activity zymograms containing 0.1% (w/v) casein as substrate. For protein gels without substrate, the supernatant was heated at 70 °C for 10 min and the electrophoresis was run at room temperature. For the zymogram analysis, the supernatant was not heated, the electrophoresis was run at 4 °C and incubation was conducted in Tris-HCl buffer pH 7.5 at 37 °C for 16 hours. All gels were stained by Coomassie Brilliant Blue G-250. SCFM: Synthetic CF sputum medium; SCFM+C (casein added); SCFM+M (mucin added). d2, d4, d6: culture days.

In addition to this 30 kDa band, the clinical isolate cultured in SCFM plus casein (SFCM+C) displayed two proteolytic bands with molecular weights of approximately 100 kDa and 135 kDa in the day 2 supernatant, and also a 135 kDa band in days 4 and 6. In terms of the environmental isolate cultured in casein supplemented SCFM, two proteolytic bands with molecular weights of about 60 kDa and 80 kDa were visible on the zymogram gels on day 4 (Fig 4 B). Apart from these well separated bands, all culture supernatants obtained from the casein-supplemented medium displayed a diffuse digested region of 150-250 kDa (Fig 4 B).

Zymogram gels of proteins from the mucin-supplemented SCFM (SCFM+M; Fig 4) showed that in addition to the bands common for both strains, the clinical strain produced two additional proteolytic bands with molecular weights of 22 kDa and 70 kDa respectively (Fig 4 B). Also, both isolates exhibited a digested area of about 150-250 kDa at the top of the zymogram as well as a distinct proteolytic band of about 30 kDa in all cultures (Fig 4 B).

Drawing a parallel to the protease diversity assays (Fig 3), subtilisin-like and trypsin-like activities were detected in all cultures using specific substrates. As subtilisin typically have molecular weights of about 20 - 45 kDa [61], and some fungal trypsin-like proteases also have a similar molecular size [62, 63], the 30 kDa protein detected in the zymograms could belong to either of these two groups of proteases. However, as many elastases also fall within this size range [64], it is difficult to draw more detailed conclusions from the zymogram gels only. In the mucin-supplemented medium, the two proteolytic bands of about 70 kDa and 22 kDa were produced by the clinical isolate only (Fig 4 B). It could be speculated that these two proteolytic bands were produced by elastase-like, trypsin-like or aspartic proteases (Fig 3), secreted by the clinical isolate. Verification of these deliberations was attempted by MS/MS analysis (see below).

Identification of secreted proteases by mass spectrometry

After the broad profiling of protease activity, further studies focused on the secreted proteases produced by the clinical isolate WM 06.482 and the environmental isolate WM 10.136 in the mucin supplemented SCFM as more proteases were secreted by the clinical isolate in this medium and the composition of the medium mimics that of the lung fluid. Proteins of the day 4 culture supernatants were concentrated and separated on SDS-PAGE and each lane on the gel was cut horizontally into 10 pieces, as shown in Fig 5. The protein in each piece was digested by trypsin and GluC separately, in three biological triplicates. The extracted peptides were analyzed by LC-MS/MS and data were searched against a manually assembled database consisting of all available fungal protease sequences in NCBI, as only a non-annotated draft genome is available for *S. aurantiacum* at the moment [65, 66]. The search results from both trypsin and GluC digestion were combined together.

Identification of a protein was considered valid only when the protein was present in all three biological replicates and the total number of matched peptides was more than five. Six homologs of fungal proteases (Table 2) were identified from the clinical isolate and five from the environmental isolate. Three protease homologs were common for both isolates including a subtilisin protease S8, a putative leucine aminopeptidase and a PA-SaNapH-like protease. The NCBI data showed that these proteases were also present in *S. apiospermum*, which is placed in the same genus as *S. aurantiacum* [67].

The homolog of subtilisin protease identified by MS from the gel fractions numbered 8 and 18 (Fig 5), corresponds to the proteolytic band of around 30 kDa on the zymogram gel which showed up in all cultures (Fig 4 B). The fact that subtilisin-like protease activity was detected in all cultures with some difference between the clinical and environmental isolates (Fig 3) is consistent with the MS and zymogram analyses.

Homologs of putative leucine aminopeptidase and PA-SaNapH-like protease (Table 2) were identified by mass spectrometry from both strains. However, their corresponding proteolytic bands were not detected in the zymograms (Fig 4 B), indicating that these proteases were not capable of proteolysis of casein in the

zymogram gel or had lower specific activities compared to subtilisin since the proteolytic band of subtilisin was visible on the zymogram gels. These two protease homologs were identified from the sections of 5 and 6 (15 and 16 for environmental strain, Fig 5) respectively, and the ranges of their MW were similar to the MW of homologous proteins in *S. apiospermum* (Table 2).

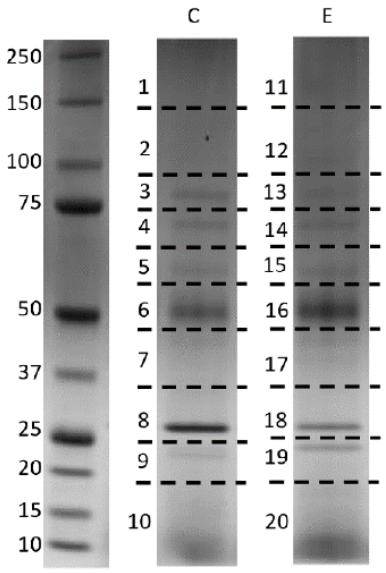

Fig 5. SDS-PAGE of proteins concentrated from the *S. aurantiacum* culture supernatants. 20 μg of protein was loaded in each well. The gel was cut horizontally into 10 pieces and the sections were numbered as shown. Note that some sections did not contain visible protein bands. Peptides from each single section were analysed by MS. Numbering of the bands corresponds to the numbers in Table 2. C: clinical strain, E: environmental strain.

A homolog of trypsin-like serine protease was identified from the cultures of the clinical isolate WM 06.482 (Fig 5, section 9), matching the 22 kDa proteolytic band on the zymogram gel (Fig 4 B), displayed only in the clinical cultures. Enzyme activity assays also demonstrated that the clinical isolate secreted higher amounts of trypsin-like activity than the environmental isolate (Fig 3); correspondingly, the proteolytic bands of around 22 kDa present only in the zymogram of the clinical s could be assumed to belong to potentially represent a trypsin-like protease (Fig 4 B) despite the fact that the day 2 culture supernatant that showed higher trypsin-like protease activity than the day 4-6 samples (Fig 3), produced only a faint proteolytic band (Fig 4 B).

In addition to homologs of a fungal subtilisin peptidase, putative leucine aminopeptidase, PA-SaNapH-like protease and a trypsin protease, the clinical isolate was found to secrete a peptidase C48 homolog (gi114192178), and a pepsin-like aspartate protease homolog (gi799243341). These proteases did not produce distinct bands on the zymogram gel (Fig 4), indicating that they were not able to degrade casein in the zymogram or their specific activity were quite low. The peptidase C48 was identified from section 10 with a MW < 20 kDa which was quite different from MW of the homologous *A. terreus* protein (100.3 kDa, Table 2). It may be that peptidase C48 has been degraded by other coexisting proteases in the culture supernatant, or because the peptidase C48 homolog identified from *S. aurantiacum* was only matching to one of the domains of the reference protein.

Table 2. Proteases identified by LC-MS/MS from day-4 culture supernatants of *S. aurantiacum* WM 06.482 (clinical isolate) and WM 10.136 (environmental isolate) grown in mucin supplemented SCFM. Full lists of identified proteins are given in supporting information S1 and S2 Tables.

Identified proteins	NCBI accession No.	Theoretical pI	Theoretical MW (kDa)	Score /log (e)	Homologous to	Protein matches	
						Section No.	No. of peptides found
Clinical Strain							
Peptidases S8, subtilisin family	gi|666867549|	8.2	41.2	-43.6	*Scedosporium apiospermum*	8	226
Putative leucine aminopeptidase, M28 Zn-peptidase	gi|666869274|	5.22	53.7	-28.9	*Scedosporium apiospermum*	5	15
PA SaNapH like protease	gi|666869012|	4.84	52.7	-10.1	*Scedosporium apiospermum*	6	14
Peptidase C48	gi|114192178|	10.46	100.3	-2.3	*Aspergillus terreus*	10	7
Pepsin-like aspartate, Asp	gi|79924334||	5.66	51.8	-2.2	*Hirsutella minnesotensis*	3	6
Trypsin-like serine protease	gi|573992599|	4.46	32.7	-2.3	*Cordyceps militaris* CM01	9	7
Environmental strain							
Peptidases S8, subtilisin family	gi|666867549|	8.2	41.2	-51.9	*Scedosporium apiospermum*	18	94
Putative leucine aminopeptidase, M28 Zn-peptidase	gi|666869274|	5.22	53.7	-15.1	*Scedosporium apiospermum*	15	15
PA SaNapH like protease	gi|666869012|	4.84	52.7	-11.3	*Scedosporium apiospermum*	16	17
Pepsin-like aspartic protease	gi|639569211|	5.75	103.4	-2.9	*Tilletiaria anomala*	14	7
KLLA0E06711p	gi|49643385|	6.94	83.1	-3.8	*Kluyveromyces lactis*	14	19

The number of peptides found represents the sum of peptides found in the three biological replicates. Log (e) value was calculated as the base-10 log of the expectation that any particular protein assignment was made at random (mean of three biological replicates). The SDS-PAGE gel with the numbered cut-out sections is shown in Figure 5.

Discussion

We have studied a clinical isolate *S. aurantiacum* WM 06.428 and an environmental isolate WM 10.136 with a view of establishing the profile of proteases secreted by these strains and detecting potential differences in protease production related to their lifestyle.

Similarly to the studies carried out with the opportunistic fungal pathogen *Aspergillus* [68, 69], the amount and type of proteases secreted by *S. aurantiacum* varied according to the medium composition (Fig 1 E, F, and Fig 3). Among all cultures, the highest overall protease activity was detected in the culture supernatant of the clinical isolate grown in mucin-supplemented SFCM (SCFM+M). Differently to the clinical isolate, the environmental strain produced the highest general protease activity in the casein-supplemented medium (SCFM+C). Elastase activity was primarily found in the culture supernatants of the clinical isolate whereas KLLA0E06711p, a protease homologous to a *Kluyveromyces lactis* protease showing metalloendopeptidase activity (http://www.uniprot.org/uniprot/Q6CP89), was only identified in the culture supernatant of the environmental strain.

High mucin concentration is one of features of CF, which makes the mucous lining of some organs thick and sticky favoring growth of microorganisms [70]. Accordingly, the clinical *S. aurantiacum* isolate produced over 2 fold higher total proteolytic activity in responding to the excessive mucin when compared to the environmental isolate (Fig 1 E, F). This ability also explained why the growth rate of the clinical strain was found to be the greatest on the mucin-supplemented SCFM medium where the growth also peaked earlier than in other cultures (Fig 1 A, B). It has been suggested that serine proteases, elastase-like in particular, are responsible for mucin digestion in the cystic fibrosis lungs [71]. In our study, elastase-like activity was mainly detected in the culture supernatant of the clinical isolate (Fig 3), which may also contribute to mucin degradation [71]. Indeed, serine proteases were the dominant protease class secreted by the clinical strain (Fig 2). Our study further demonstrated that the elastase-like and trypsin-like serine proteases as well as aspartic proteases displayed 2.5-75 fold higher activities in the clinical isolate compared to the environmental isolate (Fig 3).

Studies on elastase secreted by *A. fumigatus* showed that this enzyme could be inhibited by both PMSF (serine protease inhibitor, 100% inhibition) and EDTA (metalloprotease inhibitor 100% inhibition) [64]. These two compounds also showed the strongest inhibitory effect on the proteases produced by clinical isolate *S. aurantiacum* WM 06.482 (Fig 2). This points to the possibility that the *S. aurantiacum* protein exhibiting elastase activity may have similar properties to the *A. fumigatus* enzyme. Since some proteases, such as elastase, can be inhibited by multiple classes of inhibitors [64], the percentage values of inhibition cannot be simply added together (Fig 2).

The elastase-like activity produced by *S. aurantiacum* in the mucin medium, detected in class-specific substrate studies was not picked up by LC-MS/MS, although some peptides identified from section 7 of the protein gel (Fig 5) aligned with elastinolytic zinc metalloprotease (LasB) identified from various fungi. However, the identity score was very low and less than five peptides showed a match (S1 Table). On the other hand, the peptides displayed above aligned with the zinc metalloprotease (elastase) LasB (gi|398055407) of *Brevibacillus sp.* CF112 very well (S1 Table). This may indicate that either the amino acid sequence of *S. aurantiacum* elastase is different to other fungi but more similar to that from some bacteria or the elastase-like protease was of low abundance but had particularly high activity against the specific substrate N-Sue-Ala-Ala-Ala-MCA.

A trypsin-like serine protease homolog was also identified by mass spectrometry (Table 2) from the culture supernatant of the clinical strain grown on mucin-supplemented medium. This 22 kDa enzyme generated a clear proteolytic band on the zymogram gel (Fig 4 B). The finding is in accordance with the enzyme activity assays displayed in Fig 3. Fungal extracellular trypsin-like proteases may participate in the pathogenic process since a correlation between trypsin-like activity and fungal pathogenicity has been identified and almost all fungi containing genes encoding trypsin-like proteases are pathogens of plants, animals or other fungi [72-74]. Thereby the trypsin-like enzyme(s) produced by *S. aurantiacum* also deserve a further study.

Mass spectrometry revealed a number of proteases that were not detected in the current study by enzyme activity assays. This could be because of the range of selected substrates and focusing on more common fungal proteases. On the other hand, the trypsin like protease discussed above was only identified from the clinical strain by mass spectrometry even though trypsin-like activity was detected in both strains. This may be because of low abundance of the protein in the culture supernatant of the environmental strain. Availability of an annotated genome sequence of *S. aurantiacum* would help in resolving these questions in the future.

Conclusions

The clinical and environmental *S. aurantiacum* isolates differ in their capacity to produce secreted proteases. This was reflected both by the response of the two strains to the different culture media and the amount of a particular type of protease produced. The proteases were dominated by serine proteases, and activities of elastase-like and trypsin-like proteases produced by the clinical isolate on mucin-supplemented SFCM were up 2.5-75 fold compared to those produced by the environmental strain. There are indications that the elastase-like protease of *S. aurantiacum* might be different to other fungal elastases reported in the NCBI database or this protease was of low abundance but its activity was particularly high. To our knowledge, this is the first study on proteases secreted by *S. aurantiacum.*

Supporting Information

S1 Table. Identification data of the proteins from clinical isolate WM 06.482 listed in Table 2.

S2 Table. Identification data of the proteins from environmental strain WM 10.136 listed in Table 2.

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Author Contributions

Conceptualization: ZH LK HN.
Formal analysis: ZH HN.
Investigation: ZH.
Methodology: ZH LK HN.
Supervision: HN LK.
Visualization: ZH.

Writing ± original draft: ZH.
Writing ± review & editing: HN.

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Chapter 4

Growth and protease secretion of *Scedosporium aurantiacum* under hypoxia

4.1 Introduction

Hypoxia, deficiency of oxygen in the infected tissues, typically occurs in diseased organs such as lungs of cystic fibrosis patients [1-3]. This imposes a challenge for filamentous fungi such as *S. aurantiacum* that require oxygen for proliferation. Research has indicated that fungi adapt to hypoxia by using different energy metabolic pathways from those of normoxic growth, which results in changes in fungal biochemistry and physiology, including synthesis and transport of proteins [4-6]. To gain insights into protease production of *S. aurantiacum* under hypoxia, the clinical WM 06.482 and environmental WM 10.136 isolates were cultivated in an oxygen-controlled device under hypoxia, and growth and protease secretion were assessed. Along with slower growth, the total production of biomass was lower compared to normoxic cultivation. Proteases secreted by both strains were studied, including defining the protease class and activity; these proved to be different from those produced under normoxia.

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4.2 Contribution to manuscript 1

This manuscript was developed with supervision of my mentors at Macquarie University (Helena Nevalainen and Liisa Kautto). All experiments were performed by me and data were analysed with the assistance of Helena Nevalainen and Liisa Kautto. The manuscript was written by me and Helena Nevalainen and reviewed by Helena Nevalainen and Liisa Kautto.

3.1 Author contribution for manuscript 1.

	ZH	HN	LK
Experimental design	•	•	•
Data collection	•		
Data analysis	•	•	•
Manuscript	•	•	

* ZH = Zhiping Han, HN = Helena Nevalainen, LK = Liisa Kautto

4.3 Manuscript 1

Han, Z., L. Kautto, and H. Nevalainen, Growth and protease secretion of *Scedosporium aurantiacum* under hypoxia. Manuscript has been prepared for submission to Fungal Biology. Figures and figure captions are included as part of the text for easy reading.

Growth and protease secretion of *Scedosporium aurantiacum* under hypoxia

Zhiping Han [1,2 *], Liisa Kautto[1,2] and Helena Nevalainen[1,2]

[1] Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, Australia

[2] Biomolecular Discovery and Design Research Centre, Macquarie University, Sydney, Australia

*Corresponding author

Email: zhiping.han@students.mq.edu.au (ZH)

Abstract:

One of the micro-environmental stresses fungal pathogens encounter *in vivo* is hypoxia, deficiency of oxygen in the infected tissues. *Scedosporium aurantiacum* is an opportunistic fungal pathogen colonizing human lungs and other organs. Yet, the impact of hypoxia on the growth and enzyme secretion of this fungal species is essentially unknown. In this work, we studied the implications of a hypoxic micro-environment (oxygen levels ≤1%) on the growth of a clinical *S. aurantiacum* isolate (WM 06.482; CBS 136046) and an environmental *S. aurantiacum* isolate (WM 10.136; CBS 136049) grown on a mucin-containing synthetic cystic fibrosis sputum medium, which mimics the cystic fibrosis lung sputum. Profiles of secreted proteases were compared between the two strains and protease activity was assessed using class-specific substrates and inhibitors. Overall, both strains grew slower and produced less biomass under hypoxia compared to normoxic conditions, and the microscopic features of fungal hyphae varied as well. The pH of the medium decreased to 4.0 over the culture time, indicating that *S. aurantiacum* released acid compounds into the medium. Accordingly, secreted proteases of the two strains were dominated by acidic proteases, including aspartic and cysteine proteases, with optimal protease activity at pH 4.0 and 6.0 respectively. The clinical strain produced higher aspartic and cysteine protease activities. Conversely, all serine proteases, including elastase-like, trypsin-like, chymotrypsin-like and subtilisin-like proteases, had higher activities in the environmental strain. Secreted proteases were identified by mass spectrometry by searching against other fungi in the NCBI database. Results from MS analysis were consistent with activity assays.

Key words: *Scedosporium aurantiacum*; hypoxia, proteases; class-specific substrates; mass spectrometry

1 Introduction

Fungi can grow on plants, insects and a wide range of other matters besides soil and mammals [1]. The conditions of these habitats are largely different, especially in terms of the availability of oxygen, which normally forms 20.9% of the gas in the environment (referred to as normoxia) [2]. Oxygen levels in mammalian tissues are found to be lower than the atmospheric levels [3, 4]. In unhealthy human tissues, oxygen levels may be further lowered due to inflammation, thrombosis or necrosis, leading to the occurrence of hypoxia (oxygen levels ≤1%) [5-7]. Hypoxic lesions have been well described in tumours, wounds and sites of necrotic tissue, and typically exist in lungs of cystic fibrosis patients [8, 9].

Fungal pathogens, therefore, must have developed metabolic pathways to generate energy under hypoxia. The ability of shifting from aerobic respiration to various forms of anaerobic respiration has been implicated as an important virulence attribute in fungal pathogens and has been studied with *Fusarium oxysporum*, *Aspergillus* spp and other fungi [10, 11]. Results show that fungi use multi-energy metabolic mechanisms under hypoxia to support growth. Lactic acid fermentation is performed in the presence of glucose, resulting in production of lactate. After glucose is utilized, ammonia fermentation is conducted, which reduces nitrate to ammonium; meanwhile, ethanol is converted to acetic acid to generate energy [12-14]. Fungal anaerobic respiration has been found to produce less energy than aerobic respiration. Accordingly, fungi decrease their growth rate and increase the cell wall width under hypoxia to save energy and defend against pH drops in the environment [15-18]. Other features involved in fungal growth also experience some changes. For example, glycolysis, tricarboxylic acid cycle and amino acid metabolism were increased in *A. fumigatus* as a response to hypoxia [18-20].

Expression of proteins is another feature that fungi may regulate in low oxygen conditions [21]. In a study of *A. fumigatus*, the abundance of 117 proteins was significantly changed under hypoxia in comparison to normoxia, for example, expression of many proteins involved in respiration increased to enhance the respiratory capacity [20]. In another study, in a hypoxic culture of *A. nidulans*, enzymes taking part in gluconeogenesis, tricarboxylic and glycosylate cycles, were downregulated while proteins involved in the pentose phosphate pathway, and metabolism of nucleotides and thiamine were upregulated [15].

Extracellular fungal proteases feature the families/subfamilies of elastase-, chymotrypsin-, subtilisin- and trypsin-like serine, aspartic, cysteine and metalloproteases [22-24]. These extracellular proteases have been found to contribute to fungal pathogenicity to humans in studies with pathogenic *Aspergillus* spp. [25, 26], *Candida* spp. [27, 28] and *Dermatophytes* [29]. One could think that secretion of proteases by fungi would be different under hypoxia in terms of the protease class and amount secreted; however, there are no published reports about this topic.

The opportunistic pathogenic fungus *Scedosporium aurantiacum* [30, 31] is the second most common fungus isolated from the sputum of the lungs of cystic fibrosis patients in Australia after *A. fumigatus* [32, 33]. *S. aurantiacum* was first identified in 2005 [34], but to date, little is known about its infection mechanism or virulence factors, which limits the development of treatment and preventative strategies for this specific fungal pathogen.

In our earlier study, we looked into the growth and protease secretion of *S. aurantiacum* grown in different media under normoxia. Results showed than *S. aurantiacum* grew well and produced proteases in mucin-containing synthetic CF sputum medium (SCFM). Furthermore, the activities of elastase-, trypsin-like and aspartic proteases secreted by the clinical *S.*

aurantiacum isolate were significantly higher than those of the environmental strain [35]. In this work, a clinical *S. aurantiacum* isolate and an environmental strain were cultivated under hypoxia and fungal biomass and protease secretion were assessed to gain an insight into fungal growth and enzyme production in low oxygen conditions.

2 Materials and methods

2.1 Fungal strains and liquid cultivation

Scedosporium aurantiacum strains selected for the study were WM 06.482 (CBS 136046; clinical isolate) isolated from broncho-alveolar lavage of a CF patient in Australia and WM 10.136 (INS1120; CBS 136049; environmental strain) originating from a valley near Innsbruck, Austria [36]. These strains were obtained from the culture collection of the Medical Mycology Laboratory, Centre for Infectious Diseases and Microbiology, Westmead Hospital, Sydney, Australia. Virulence of the strains used in the current study has been assessed using the invertebrate wax moth *Galleria mellonella* larval model, and results showed that the clinical isolate WM 06.482 was about three times more virulent than the environmental strain WM 10.136 [37]. Another study on the virulence status of WM 06.482 has been carried out using immunocompromised mice, where no significant virulence differences were found between this strain and environmental strains examined. However, the *S. aurantiacum* WM 10.136 was not amongst the strains tested in the mouse model [36]. To date, there are no published reports regarding comparison of *S. aurantiacum* WM 10.136 and WM 06.482 in a mammalian model to establish their relative virulence.

S. aurantiacum strains were grown in a mucin-containing synthetic CF sputum medium (SCFM+M) which was prepared as previously described [35, 38]. The medium composed of mineral salts, amino acids, 1% (w/v) glucose and 1% mucin (from porcine stomach, type III; Sigma-Aldrich, Australia) to mimic CF lung sputum. Mucin was dissolved in Milli-Q H_2O,

autoclaved separately and mixed with SCFM just before use. SCFM was sterilized by filtering through a 0.22 μm membrane (Millipore) and the final pH of medium was adjusted to 5.7 to support fungal growth [39].

2.2 Hypoxic culture of *S. aurantiacum*

Pre-cultures were prepared by incubating the fungus at 37 °C on malt extract agar plates for up to 10 days as described previously [35]. The spores were collected and inoculated into liquid medium to make a final concentration of 2×10^6 spores/ml. Hypoxic cultures were conducted in 250 ml flasks containing 50 ml liquid medium. The flasks were sealed with rubber stoppers and two sterilized hypodermic needles (DN 25 G × 1.0 LV, Livingstone, Australia) were inserted through each stopper, with one needle inserted deeper than the other. High purity Nitrogen gas (N_2), sterilized by filtering through a 0.22 μm filter (Millipore), was filled in the flasks through the deeper needle and the air inside the flasks flowed out through the other needle. The flow out pipes were connected with one-way valves to prevent air reflux. The oxygen levels in liquid cultures were maintained at 1% and monitored using a polarographic electrode coupled to an Activon® (Model 401) oxygen meter. All cultures were performed for up to 7 days at 37 °C on an orbital shaker at 200 rpm with three individual flasks dedicated for each time point. The hypoxia cultivation setting is shown in Supplementary Fig 1.

2.3 Growth and pH measurement

Measurements were conducted every 24 hours by taking out individual flasks. Contents were centrifuged at 4500 g for 30 min. The supernatant was collected and the pellet washed three times with 50 ml Milli-Q water by inverting the tubes. After washing, the tubes and their content were frozen at -30 °C for at least 4 h, and then freeze-dried. Biomass was calculated as the difference between the weight of the centrifuge tubes with and without dry mycelia. The day-0

biomass was recorded as the weight of the same amount of freeze-dried conidia inoculated in each flask.

Each supernatant was filtered through a 0.22 μm membrane (Millipore, Australia) at 4 °C and the pH of the cleared supernatant measured. The supernatants were then dialyzed overnight at 4 °C against sterilized Milli-Q H_2O using 10 kDa cut-off dialysis tubing (Amico Ultra-15, Millipore), aliquoted in 1.5 ml Eppendorf tubes and stored at -80 °C. Fungal protease inhibitor cocktail (0.05% v/v, Sigma-Aldrich, Australia) [40] was added in the culture supernatant samples used for proteomic analysis.

2.4 Light microscopy

Five μl of a shaken culture was placed onto a microscopy slide. Then, 5 μl of lactophenol cotton blue solution was pipetted onto the slide and mixed with the fungal sample for staining of the hyphae [41]. The slide was covered with a coverslip, and the edges of the coverslip were sealed with nail polish. The slides could be kept in a fridge for 2-3 days for further observation when required. Olympus BX 53 bright-field compound microscope with DP26 digital camera was used to observe the fungal culture samples. The width of fungal hyphae was measured using imaging software CellSen (V 1.14). Three to five slides were made of each fungal culture and over 10 images were taken of each slide.

2.5 Protease activity assays

General protease activity in thawed supernatants of 1-7 day cultures was assayed in both acidic and alkaline conditions following the methods of Rauscher [42] and Fedatto [43] with some adjustments. Haemoglobin (from bovine blood, acid denatured; Sigma-Aldrich, Australia) and azocasein (Sigma-Aldrich, Australia) were used respectively as substrates for the protease assay under acidic and alkaline conditions. Different buffers were used to achieve the assay pH: 0.05

$mol \cdot l^{-1}$ citrate-phosphate buffer pH 3.0-6.0, 0.05 $mol \cdot l^{-1}$ Tris-HCl buffer pH 7.0-9.0, and 0.05 $mol \cdot l^{-1}$ carbonate-bicarbonate pH 10.0-11.0. The reaction solution consisted of 75 µl of the supernatant and 50 µl substrate solution; the samples were incubated for 60 min at 37 °C in a 96-well plate. The reaction was terminated by adding 125 µl 5% (w/v) trichloroacetic acid (TCA). After centrifugation at 4000 g for 5 min, the supernatant was removed to a new plate. Absorbance was read at 280 nm for released tyrosine from hemoglobin or at 366 nm for released azo-dye from azocasein. Protease activity against hemoglobin (U_{hemo}) was calculated using a standard curve prepared with appropriate dilutions of tyrosine, and was given as µM of tyrosine released per min per mg of dry biomass. Protease activity against azocasein (U_{azoc}) was expressed as µg of azocasein digested per min, normalized against mg of dry fungal biomass. Protease-deactivated supernatants by heating were included in the assay for reaction blanks.

Protease inhibition study was performed at both pH 4.0 and pH 7.5. Inhibitors used included 1 mM PMSF (serine protease inhibitor; Sigma, Australia), 5 mM EDTA (metalloprotease inhibitor; Sigma, Australia), 10 µM E-64 (cysteine protease inhibitor, Sigma, Australia) and 100 µM pepstatin A (aspartic protease inhibitor; Merck, Nottingham, UK). The day-3 culture supernatants were pre-incubated with a protease inhibitor for 30 min at room temperature prior to performing the protease activity assay. Culture supernatants with no inhibitor added were also included in the assay for comparison.

To further characterize proteases in the day-3 culture supernatants, activities of serine proteases (including subfamilies of chymotrypsin-, subtilisin-, elastase- and trypsin-like proteases), collagenases, cysteine and aspartic proteases were tested using class-specific substrates. These substrates were synthetic peptides coupled with either 7-Amino-4-methylcoumarin (MCA) or ρ-nitroanilide (ρ-NA) (Peptide Institute Inc), dissolved in dimethyl sulfoxide (DMSO) to make a stock solution at 10 mM. They included N-Benzoyl-L-Tyrosine-ρ-NA for chymotrypsin-like

protease [44], Z-Ala-Ala-Leu- ρ-NA for subtilisin-like protease [45], N-Sue-Ala-Ala-Ala-MCA for elastase-like protease [46], Boc-Phe-Ser-Arg-MCA for trypsin-like proteases [47], Z-Arg-Arg-MCA for collagenase [48], Sue-Gly-Pro-Leu-Gly-Pro-MCA for cysteine protease [49] and Boc-Leu-Ser-Thr-Arg-MCA for aspartic protease [50]. All tests were performed at the optimal pH of each specific protease as described previously [35]. The reaction mixture contained 50 µl of culture supernatant and 0.5 mM substrate in 200 µl of appropriate buffer. After incubation at 37 °C for 4 min, the absorbance of released ρ-NA was read at 410 nm, or fluorescence of the released MCA was measured at λex =380 nm and λem = 460 nm at 37 °C. The amount of released ρ-NA or MCA was calculated using a standard curve prepared with appropriate dilutions of ρ-NA or MCA. Activities are given as nM of MCA or µM of ρ-NA released per minute per mg of dry biomass. All assays were performed in triplicate (technical replicates), using three biological replicates. Student's *t* test was conducted to investigate whether there was a significant difference in general protease activity between the clincal and environmental strains.

2.6 Sample preparation and identification of secreted proteases by MS

Proteins in the day-3 culture supernatants were precipitated by TCA, resulting pellets were washed twice using cold acetone (-30 °C) and resuspended in 50 µl Tris-buffer (50 mM, pH 8.0) [51]. Protein concentration was measured according to Bradford [52], and 20 µg of protein from each triplicate sample of the two strains were subjected to electrophoresis. Each lane on the protein gel was cut across horizontally into 10 slices. Proteins in each slice were digested by trypsin and GluC (Sequencing grade modified, Promega, Australia) separately and peptides were extracted following the method of Grinyer et al. [53]. After desalting and concentrating using C18 zip-tips (Millipore, Australia), peptides were subjected to reversed-phase nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC−MS/MS) using an LTQ-XL ion-trap mass spectrometer (Thermo, San Jose, CA). MS/MS data acquisition,

analysis, and representation were performed using Xcalibur software (version 2.06, Thermo, San Jose, CA) [54].

The result raw files were converted to mzXML format and protein identification was carried out using the Global Proteome Machine software of the X!Tandem algorithm (GPM, http://www.thegpm.org). For each experiment, the 10 fractions were processed sequentially with output files for each individual fraction; also, a merged, non-redundant output file was generated for protein identifications with log (e) values less than -1. An in-house database was created from NCBI protein entries comprising 68,758 protease sequences of fungal species. The contaminants from human subjects like keratin and identifications with reversed protein sequences were excluded from the merged output file of each replicate. Reversed protein sequences were introduced to estimate false positive identification. Hence, matches showing reverse protein hits were omitted as false positives. Only those proteins that were common in the three biological replicates and had a total spectral count of at least 5 were considered as a valid protein identity [55]. Further searching for the sequences of identified peptides of a protein was performed using NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), and only the proteins which had been annotated as proteases in other fungi were included in the results.

3 Results and Discussion

3.1 Growth characterization

S. aurantiacum was capable of growing under hypoxia, like *Pseudallescheria boydii*, which is placed in the same genus as *S. aurantiacum* and reported to survive at very low partial pressure of oxygen [17]. As shown in Fig 1 A, the clinical and environmental *S. aurantiacum* strains showed a similar growth pattern under hypoxia. In the exponential phase of growth (0-3 days), production of biomass increased up to 3.5 $mg \cdot ml^{-1}$ while the medium pH decreased from 5.7 to

4.0. In the stationary phase, both the amount of biomass and medium pH remained unchanged. These results suggested a negative correlation between *S. aurantiacum* growth and pH of medium.

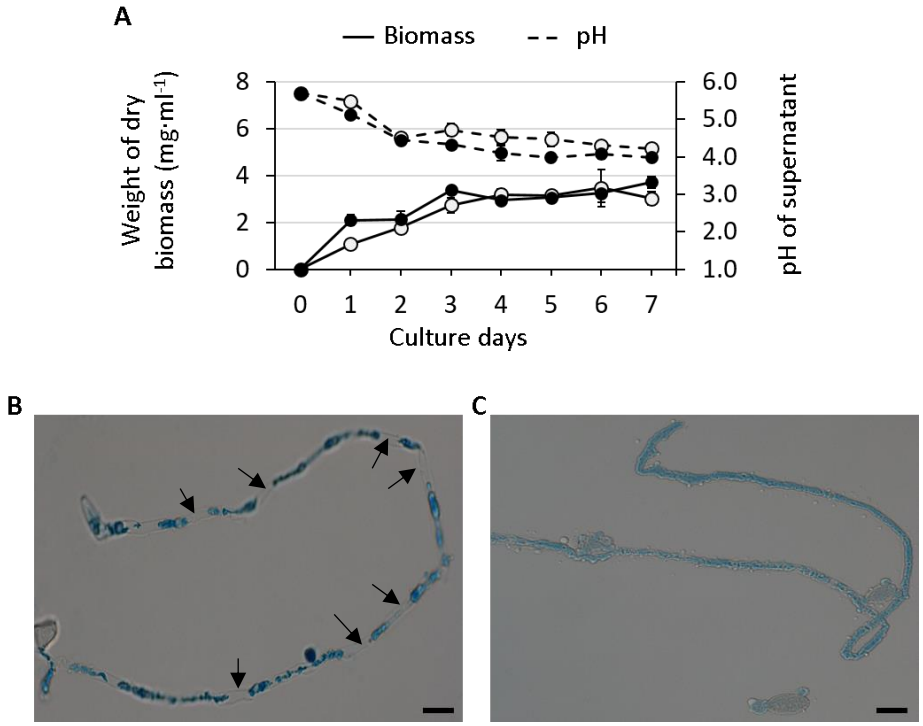

Fig 1. Growth of *S. aurantiacum* under hypoxia and pH changes in the medium. (A) Fungal biomass and pH of the culture supernatant over 7 days of cultivation. Data represent mean ± SD (n=3) of three biological replicates. ○: *S. aurantiacum* WM 06.482 (clinical isolate) ●: *S. aurantiacum* WM 10.136 (environmental strain). (B) and (C) Examples of hyphal morphology of *S. aurantiacum* under hypoxia at 72 h post inoculation. Arrows point to hollow spaces in the hyphae of *S. aurantiacum*. Bars = 10 μm. Magnification: 100 x.

S. aurantiacum strains grew slower under hypoxia compared to growth under normoxia, and production of biomass under hypoxia was 20-60% of that from normoxic cultures [35]. The same phenomenon has been observed in hypoxia-grown *A. nidulans* and *C. albicans*, which also experienced a considerable decrease in the production of biomass under hypoxia [15, 16, 56]. Takaya et al. used *F. oxysporum* as a model to study energy metabolism mechanisms of fungi growing in a low oxygen environment. They found that the fungus performed lactic acid

fermentation under hypoxia in the presence of glucose, and this pathway produced less energy [14]. As a result, fungal growth slowed down and energy-costly processes, such as protein synthesis, protein folding and transport, were downregulated [12, 14, 57]. In the current work, the culture period of *S. aurantiacum* was extended to 21 days, and results showed that the growth of both the clinical and environmental strains' abated after 12 days of growing under hypoxia (Supplementary Fig S2).

Both strains showed better growth rates when the medium pH was higher than 4. When the medium pH dropped to 4 on day 3, the amount of biomass did not improve but remained constant for the rest of the cultivation period (Fig 1 A). Studies on *A. niger* suggested that fungi could maintain a constant intracellular pH during growth by either pumping in or pumping out hydrogen ions when the extracellular pH deviated from their optimal level. However, if the extracellular pH deviates too much from the optimal range, it may become too difficult for the cell to maintain constant intracellular pH, and the enzymes may not function normally, leading to the failure of fungi to grow [58, 59].

A relationship between fungal pathogenicity and pH was first reported by Bateman and Beer in 1965. They found that the plant pathogen *Sclerotium rolfsii* caused acidification of the potato leaf tissue to facilitate enzymatic degradation of plant cell walls [60]. This mechanism has also been observed with other pathogenic fungi, such as *Penicillium* spp [61], *F. oxysporum* [62] and *A. niger* [63]. In our study, *S. aurantiacum* cultured under hypoxia caused a considerable drop in the pH of the medium, which may also function to acidify the compounds in the medium and further affect the production and activity of many hydrolytic enzymes.

Some *S. aurantiacum* hyphae contained large hollow spaces when growing under hypoxia (Fig 1B). Hollow spaces (vacuoles) in fungi play an important role in the regulation of cellular

homeostasis by transfer and storage of oxygen, ions and metabolites [64]. In this context, one could assume that the enlarged hollow spaces in the *S. aurantiacum* hyphae were to store oxygen for growth under hypoxia. Damage to hyphae was also observed in some samples (Fig 1C). A similar phenomenon was also seen with hypoxia-grown *A. fumigatus*; it is believed that this damage was attributed to changes in the composition of fungal cell walls under hypoxia [18]. In the current work, there was no apparent difference in the diameter of *Scedosporium* hyphae under hypoxia compared to normoxia (data not shown). This is different from the observation from *A. fumigatus* whose overall hyphal diameter decreased under hypoxia [18].

3.2 Protease activity assays

All protease activity assays were conducted under sub-saturating conditions. General protease activity of the *S. aurantiacum* culture supernatants was measured over seven days at pH 4.0 and 7.5 respectively (Fig 2A). pH 4.0 was selected because the pH of the fungal culture medium remained at approximately 4.0 from day 2 to the end of culture under hypoxia (Fig 1A). pH 7.5 was selected because it was deemed optimal for general protease activity in our earlier study of *S. aurantiacum* under normoxia [35]. As seen in Fig 2A, at pH 4.0, both strains produced the highest protease activity on day 3, and the protease activities from the clinical strain were significantly lower than those from the environmental strain on day 1, 2, and 5-7. At pH 7.5, the general protease activity from the environmental strain was significantly higher until day 6 with the highest peak on day 4 ($p<0.001$).

In the first three days post-inoculation, the general protease activity (per mg of dry biomass) kept increasing, accordingly to the increasing growth rates of the two strains. After day 3, the medium pH dropped down to almost 4.0 (Fig 1A), relating to a decrease in general protease activity, and cessation of fungal growth (Fig 1A and Fig 2A). These results suggested a connection between fungal growth, medium pH and secreted protease activity; speculatively,

fungal growth caused the medium pH to decrease, resulting in a decrease in the extracellular protease activity, which in turn limited fungal growth. Generally, low pH is optimal only for aspartyl proteases [22, 23], therefore the capacity for *S. aurantiacum* to digest nutrition decreased.

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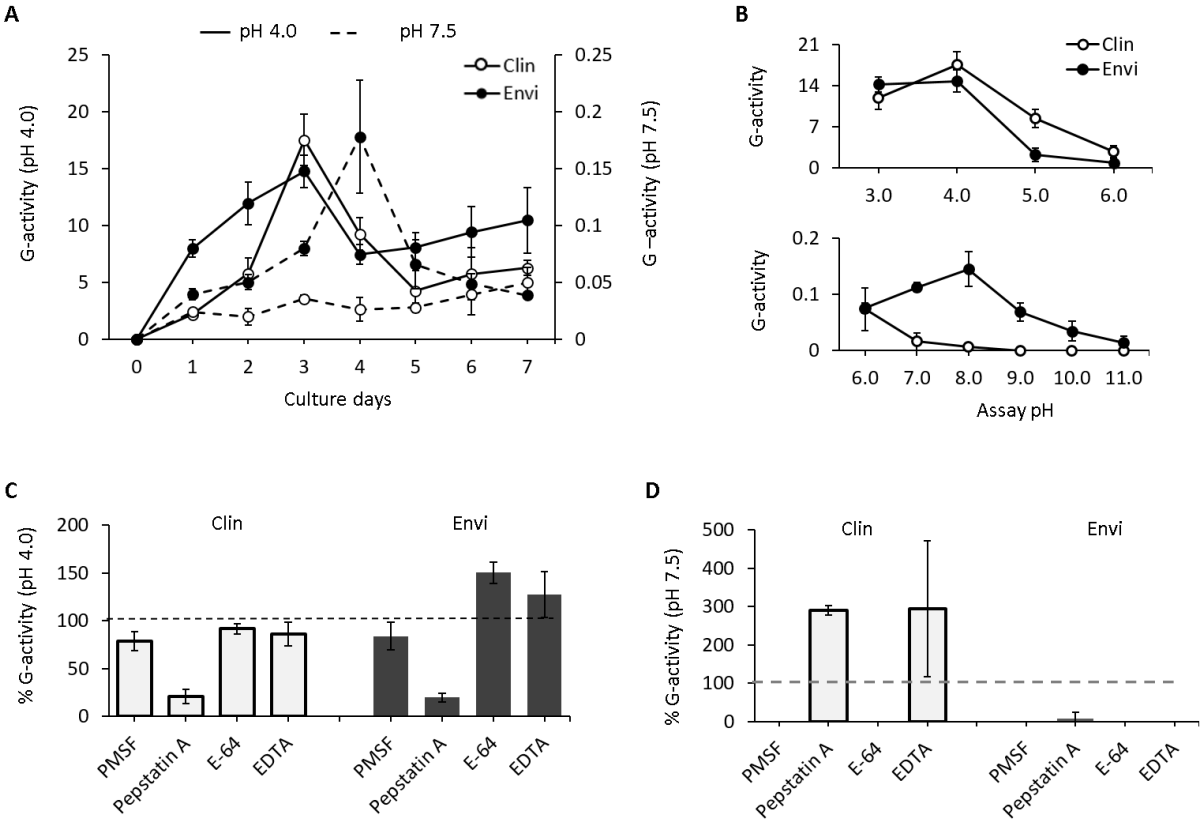

Fig 2. General protease activity assay of the culture supernatants of *S. aurantiacum* growing under hypoxia. (A) General protease activity (G-activity) at pH 4.0 and pH 7.5 over the culture time; (B) pH profile of general protease activity in day-3 culture supernatants; upper graph pH 3-6 and lower graph pH 6-11; (C) and (D) Protease inhibition assay at pH 4.0 and pH 7.5 in day-3 culture supernatants. Percentage changes in the protease activity in the supernatant with an inhibitor were relative to the non-inhibited control supernatant, set as 100% for each culture (dotted line). Data represent mean ± SD of three biological replicates. The activity was measured using two protease substrates, haemoglobin for pH 3.0-6.0 and azocasein for pH 6.0-11.0. Results were normalized against dry biomass.

In our previous study, the same *S. aurantiacum* strains were cultured under normoxia, where the pH of the medium increased to almost 9, and activities of secreted proteases were tested at pH 7.5 only. General protease activity from the samples of normoxic culture was at least 10 fold higher than that in samples from hypoxic cultures [35]. This suggested that the proteases produced under hypoxia were of low abundance, or some proteases might have become deactivated due to the acidified medium.

Additional protease activity measurements were thus performed at a pH range of 3.0-11.0 to further address the potential effect of medium pH on protease activity. As day-3 culture supernatants of both strains had the highest protease activities at pH 4.0 over the culture time, the pH profiles of day-3 supernatants were tested. Hemoglobin was used as a protease substrate in the acidic conditions since azocasein would precipitate in a low pH environment. As seen from Fig 2 B, when tested at acidic pH range, activity profiles at different pHs were similar for the clinical and environmental strains, with peaks at pH 4.0. In contrast, when tested at neutral and alkaline pH with azocasein as a substrate, protease activities produced by the two strains changed in a quite different way. Protease activities from the environmental strain were higher with a large peak at pH 8.0. On the contrary, protease activities from the clinical strain were only detected at pH 6.0, decreased greatly at pH 7.0 and could not be detected from pH 9.0 up. These results were different from those obtained under normoxic condition, where the clinical strain produced high protease activity in a pH range of 8.0-11.0 [35]. These results suggested that there was not much alkaline protease present in day-3 supernatants or they were deactivated.

Protease inhibitors were used to inhibit specific classes of proteases secreted by the two strains of *S. aurantiacum* in day-3 culture supernatants at pH 4.0 and 7.5 respectively in order to further explore the nature of proteases present in the supernatants. The inhibitor amount used was selected based on literature and previous testing in our laboratory (data not shown). As seen

from Fig 2C, when assayed at pH 4.0, the protease inhibition profiles of the two strains were quite similar. Both of them were inhibited predominately by pepstatin A (aspartic protease inhibitor), followed by PMSF (serine protease inhibitor). E-64 (cysteine protease inhibitor) and EDTA (metalloprotease inhibitor) may also have a slight inhibitory effect on proteases secreted by the clinical strain. These results indicated that the two strains secreted aspartic and serine proteases, whereas the clinical strain also secreted some cysteine and metalloproteases that were active at pH 4.0. These results were different from those obtained under normoxia, where *S. aurantiacum* mainly secretes serine proteases as well as a little aspartic protease in SCFM+M [35]. However, the inhibition study at pH 7.5 resulted in either 'zero protease activity' or 'over 100% protease activity' (Fig 2D). 'Zero protease activity' may reflect the fact that the overall protease activity was low to start with. The observation that in some cases, the addition of inhibitor in fact increased the protease activity indicates that while inhibiting one type of activity, the inhibitor may enhance activity of other proteases.

3.3 Classes and activities of specific proteases

Proteases present in day-3 culture supernatants were further studied using class-specific substrates. The clinical and environmental *S. aurantiacum* strains were found to secrete serine proteases (elastase-, trypsin-, chymotrypsin- and subtilisin-like), cysteine and aspartic proteases (Fig 3). Collagenase was not detected from either of the strains. Activity of each protease class varied between the two strains. The clinical strain produced higher cysteine and aspartic protease activities, whereas the environmental strain produced higher serine protease activity (including elastase-, trypsin-, chymotrypsin- and subtilisin-like activities) (Fig 3).

It has been shown that hypoxic growth promotes fungi to upregulate enzymes in the pentose phosphate pathway, and downregulate enzymes involved in the TCA cycle compared to normoxic conditions [57]. Our study demonstrated that protease secretion was yet another

factor that would change in hypoxic cultivation. In particular, activities of elastase- and trypsin-like proteases from both *S. aurantiacum* strains were significantly decreased during hypoxic growth [35], while subtilisin-like and aspartic activities were significantly increased compared to those obtained from normoxic culture [35]. Cysteine proteases were produced by *S. aurantiacum* only under hypoxia.

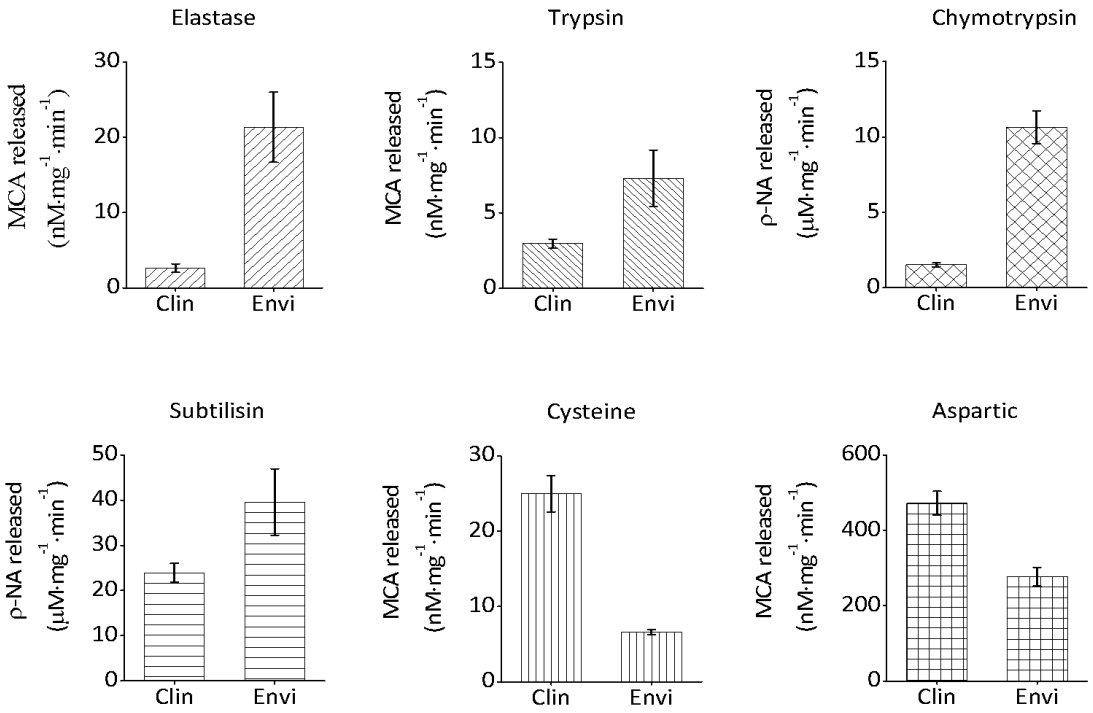

Fig 3. Classes and activities of specific proteases secreted by *S. aurantiacum* under hypoxia. Activities of specific proteases expressed as nM of MCA or μM of ρ-NA released per minute per dry biomass. Elastase: elastase-like protease, trypsin: trypsin-like protease, chymotrypsin: chymotrypsin-like protease, subtilisin: subtilisin-like protease. Collagenase was not detected (data was not shown). Data represent mean ± SD of three biological replicates.

Fungi typically produce neutral and alkaline proteases in a neutral environment, whereas acidic pH promotes production of acidic proteases [65, 66]. To find out the optimal pH of the proteases secreted by the clinical *S. aurantiacum* strain grown under hypoxia, pH profiles of each class of protease were assayed using class-specific substrates. As seen from Fig 4, all serine proteases (elastase-, trypsin-, chymotrypsin- and subtilisin-like) favoured neutral and alkaline

environment and their optimal pH was in a range of 7.0-9.0. Cysteine protease secreted by *S. aurantiacum* preferred weak acidic environment and had an optimal pH of 6.0. Aspartic protease secreted by *S. aurantiacum* was a strong acidic protease with optimal pH of 4.0. So it was expected that aspartic proteases were the predominant proteases secreted by *S. aurantiacum* under hypoxia since the medium pH remained at 4.0 after day 2 into culturing (Fig 1A and 2C).

Drawing a parallel between the optimal pH of each protease class and general protease activity assays, the clinical strain secreted less serine proteases (Fig 3), so its general extracellular protease activity in an alkaline environment was lower (Fig 2A, B). This strain produced higher cysteine and aspartic activities (Fig 3), corresponding to the higher general protease activity in an acidic environment (Fig 2A, B).

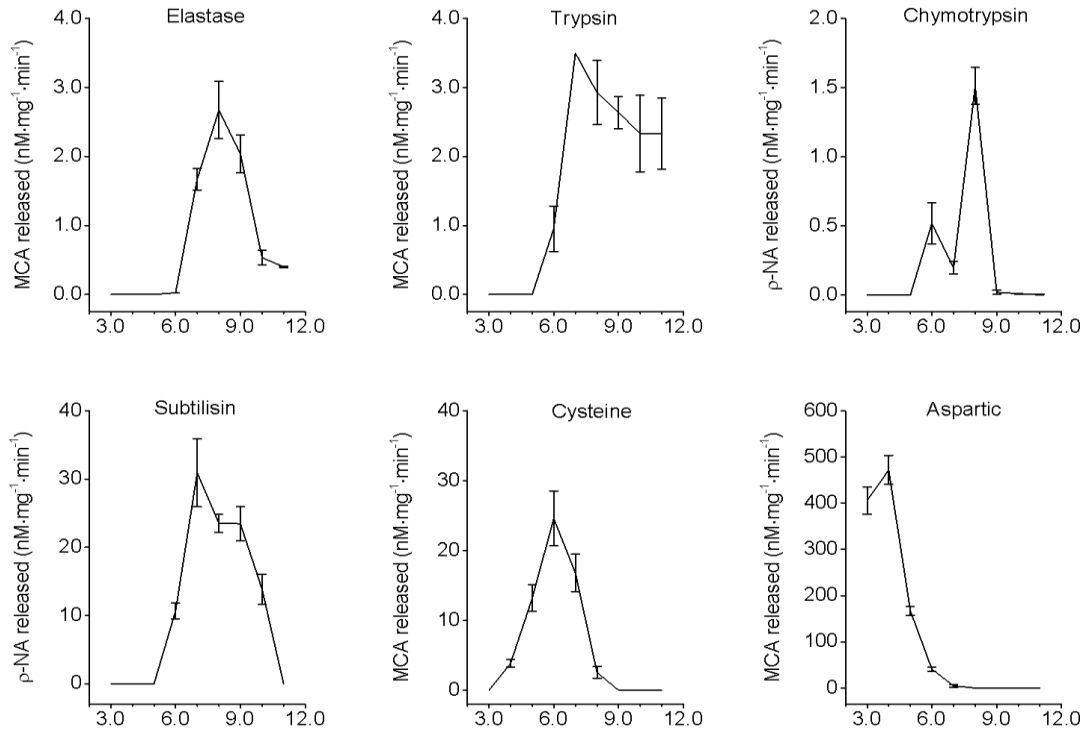

Fig 4. pH profiles of each protease class in the day-3 supernatant of the clinical *S. aurantiacum* isolate grown in hypoxia. Class-specific substrates were employed to test the activity of each protease class at different pH levels. Collagenase was not detected since it had no activity. Data represent mean ± SD (three biological replicates).

Low pH may be one of the reasons for the slow growth of *S. aurantiacum*. When pH values of the medium were higher than 4.5, the amount of biomass was increasing, however, when the medium pH decreased to 4.5 on day 3 post inoculation, the growth of *S. aurantiacum* paused (Fig 1A). Aspartic protease activity was high at pH 4.0 (Fig 4), suggesting that this protease was not a key for the growth under hypoxic conditions. Several other secreted proteases were active at this pH range, including trypsin-, chymotrypsin-, subtilisin-like and cysteine proteases (Fig 4). The secretion of chymotrypsin-like protease in the clinical strain was close to zero, and activity of trypsin-like protease largely decreased under hypoxia. In contrast, cysteine proteases were only secreted under hypoxia (Fig 3), and subtilisin-like and aspartic activity was higher under hypoxia when compared to findings in normoxia [35]. Thereby we could deduce that cysteine, aspartic and subtilisin-like proteases were the main proteases contributing to the growth of *S. aurantiacum* under hypoxia.

Biological roles of extracellular cysteine, aspartic and subtilisin-like proteases have been studied with other pathogenic fungi. Subtilisin-like proteases secreted by *Microsporum canis* were found to be involved in fungal adherence and invasion of keratinized tissues [67, 68]. Cysteine proteases secreted by *Aspergillus* species may cause damage to the attachment and viability of human cancer cell line A549 [69, 70]. Aspartic proteases have been found to facilitate hyphal penetration into the connective tissue layers of the host in aspergillosis, while aspartic proteases from *C. albicans* degrade complement components to escape host immune defence and also support hyphal attachment [27, 71]. In summary, all these proteases appeared to facilitate fungal attachment and penetration of host tissue. On this note, the function of cysteine, aspartic and subtilisin-like proteases secreted by *S. aurantiacum* deserve further study to understand their role as fungal virulence factors.

3.4 Identification of secreted proteases by MS

After profiling the protease activities, proteins in the day-3 culture supernatants from the clinical and environmental strains were concentrated and separated on SDS-PAGE. Each lane on the gel was cut horizontally into 10 pieces and proteins in each slice were digested by trypsin and GluC separately, in three biological replicates. The extracted peptides were analyzed by LC-MS/MS and data searched against a manually assembled database consisting of all available fungal protease sequences in NCBI, as only a non-annotated draft genome is available for *S. aurantiacum* at the moment [72, 73]. Further searching was performed using NCBI-BLAST and only the proteins that had been annotated as fungal proteases were considered as valid identification. The search results from both trypsin and GluC digestion were combined together.

As seen from Table 1, homologs of seven secreted proteases were common for both clinical and environmental *S. aurantiacum* strains. These included four fungal subtilase family peptidase S8 homologs and three 'pepsin-like aspartic proteases' with one of them being a pepsin retropepsin-like aspartyl protease.

Three proteases were unique in the clinical *S. aurantiacum* isolate, including a peptidase C48, a cysteine protease ATG4 and a protease-like homologue CYK3. In contrast, homologs of peptidase C19C and two ATP-dependent Lon proteases were unique in the environmental strain. Chymotrypsin- and elastase-like proteases were not identified by mass spectrometry, although their activities were detected in the culture supernatants (Fig 3). This is similar to observations concerning identification of proteases secreted by *S. aurantiacum* under normoxic condition, where the activities of chymotrypsin- and elastase-like proteases were high but neither of them was identified by MS [35]. This result may reflect either a low abundance of these proteases or limited protein identification due to non-availability of an annotated genome sequence or incomplete enzymatic cleavage into peptides using trypsin and GluC.

Parallels were drawn between protease activity assays using class-specific substrates (Fig 3) and protease identification via MS analysis (Table 1). Activities of subtilisin-like and aspartic proteases were detected in both strains and both were higher compared to those produced under normoxia [35], corresponding to the identification of these proteases by MS.

The MS analysis of the *S. auranticum* supernatant revealed peptides with high sequence similarlity to aspartic protease (gi|666867699) and subtilisin protease (gi|666867549|) from *S. apiospermum* (Table 1). This provides further support for identification of these proteases, as *S. apiospermum* is placed in the same genus as *S. aurantiacum* [74]. Identification of a cysteine protease from the clinical isolate only (Table 1) was in line with a higher enzyme activity detected in the culture supernatant of the clinical isolate (WM 06.482) compared to the environmental strain (WM 10.136).

Protein identification also demonstrated that the proteases secreted by *S. aurantiacum* under hypoxia were different from those under normoxia. M28 Zn-peptidase and PA SaNapH like protease homologs were only identified from the normoxic cultures [35], while homologs of cysteine protease, peptidase C19, peptidase C48 and ATP-dependent Lon protease were only identified from the hypoxic cultures. These results were consistent with the protease inhibition assay, which demonstrated that cysteine protease activity was detected only from hypoxic cultures.

Table 1. List of differentially secreted proteases from *S. aurantiacum* grown under hypoxia. The data include putative identifications based on homology to other fungal species. Full lists of all proteases identified in this study are included in the supporting data files (Table S1_H and S2_H). Clin: clinical isolate, Envi: environmental strain.

NCBI accession No.	Identified proteins	Homologous to	Log (e)	
			Clin	Envi
Common proteases				
gi|591477392|	Peptidase_S8, Subtilase familyProteases_S8_Kp43_protease	*Fusarium oxysporum*	-5.43	-5.5
gi|393247098|	Proteases_S8_5, Peptidase S8 family domain	*Auricularia subglabra*	-3.43	-5.93
gi|666867549|	Cuticle-degrading protease .Proteases_S8	*Scedosporium apiospermum*	-48.3	-53.8
gi|763730205|	Proteases_S8_5, Peptidase S8 family domain	*Hypholoma sublateritium*	-1.5	-7.33
gi|666867699|	Pepsin_retropepsin_like, Asp, Eukaryotic aspartyl protease	*Scedosporium apiospermum*	-9.13	-19.0
gi|648165636|	Pepsin-like aspartic proteases, Asp, Eukaryotic aspartyl protease	*Galerina marginata*	-4.03	-1.77
gi|658168160|	Pepsin-like aspartic proteases, Asp, Eukaryotic aspartyl protease	*Mixia osmundae*	-2.77	-4.5
Unique proteases in the clinical isolate (WM 06.482)				
gi|591496071|	Cysteine protease ATG4 .Peptidase_C54, Peptidase family C54	*Fusarium oxysporum*	-5.8	--
gi|160703044|	Peptidase_C48, Ulp1 protease family	*Parastagonospora nodorum*	-7.23	--
gi|768768045|	Protease-like homologuesCYK3, Cytokinesis protein 3	*Saccharomyces cerevisiae*	-1.53	--
Unique proteases in the environmental strain (WM 10.136)				
gi|465796362|	Peptidase_C19C, A subfamily of Peptidase C19	*Malassezia sympodialis*	--	-2.27
gi|575068326|	Serine protease, ATP-dependent protease La (LON) domainLON	*Heterobasidion irregulare*	--	-5.37
gi|628219077|	Subunit ChlI of Mg-chelataseLon, ATP-dependent Lon protease	*Capronia coronata*	--	-4.87

4 Conclusions

Both the clinical and environmental *S. aurantiacum* strains grew slowly under hypoxia, and the production of biomass appeared to stop increasing from day 3 after inoculation. Mainly acidic proteases were secreted, including aspartic and cysteine proteases, corresponding to the decrease in pH of *S. aurantiacum* growth medium. Cysteine proteases were secreted by *S. aurantiacum* only under hypoxia. These type of proteases play a role in destroying the attachment and viability of human cells which helps fungi to penetrate deeper into host tissues. Identification of secreted *S. aurantiacum* proteases demonstrated seven common proteases in the clinical and environmental strains and three unique proteases in the clinical strains; the results were consistent with protease assays using class-specifc substrates. Overall, *S. aurantiacum* was able to grow under hypoxia, and the secretion of proteases changed in response to the low oxygen concentration in the environment.

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Author Contributions

Conceived and designed the experiments: ZH, LK, HN. Performed the experiments: ZH. Analysed the data: ZH, LK, HN. Wrote the paper: ZH, HN.

Supporting data files

S1_H Table. Identification data of the proteins from the clinical *S. aurantiacum* isolate WM 06.482 grown under hypoxia listed in Table 1.

S2_H Table. Identification data of the proteins from the clinical *S. aurantiacum* isolate WM 06.482 grown under hypoxia listed in Table 1.

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Supplementary data

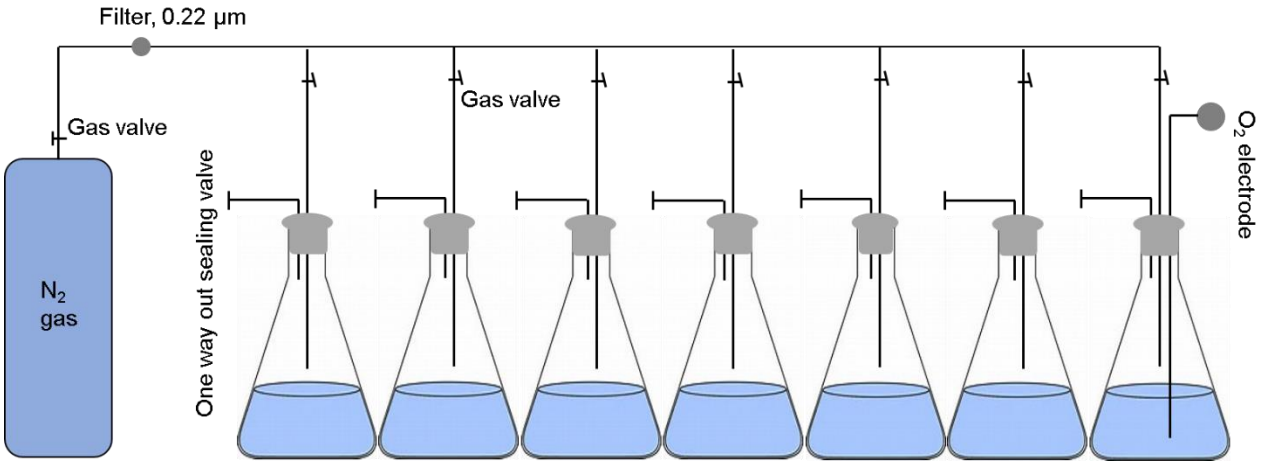

Fig S1. Design of hypoxia setting. A series of 250 ml Erlenmayer flasks containing 50 ml SCFM+M was set up. High purity nitrogen gas was used to replace air at the top of the medium in the culture flasks and was sterilized using a 0.22 µm filter. Nitrogen gas flowed into each flask through parallel pipes which had been disinfected by washing with 1% (w/v) virkon and 80% (v/v) ethanol, and by drying under ultraviolet (UV) light in a fume hood. Each nitrogen-inlet pipe was controlled separately with a gas valve, and each air-outlet pipe was connected with a one way out sealing valve to prevent air reflux. The oxygen concentration in the medium was monitored using a polarographic electrode coupled to an Activon® (Model 401) oxygen meter.

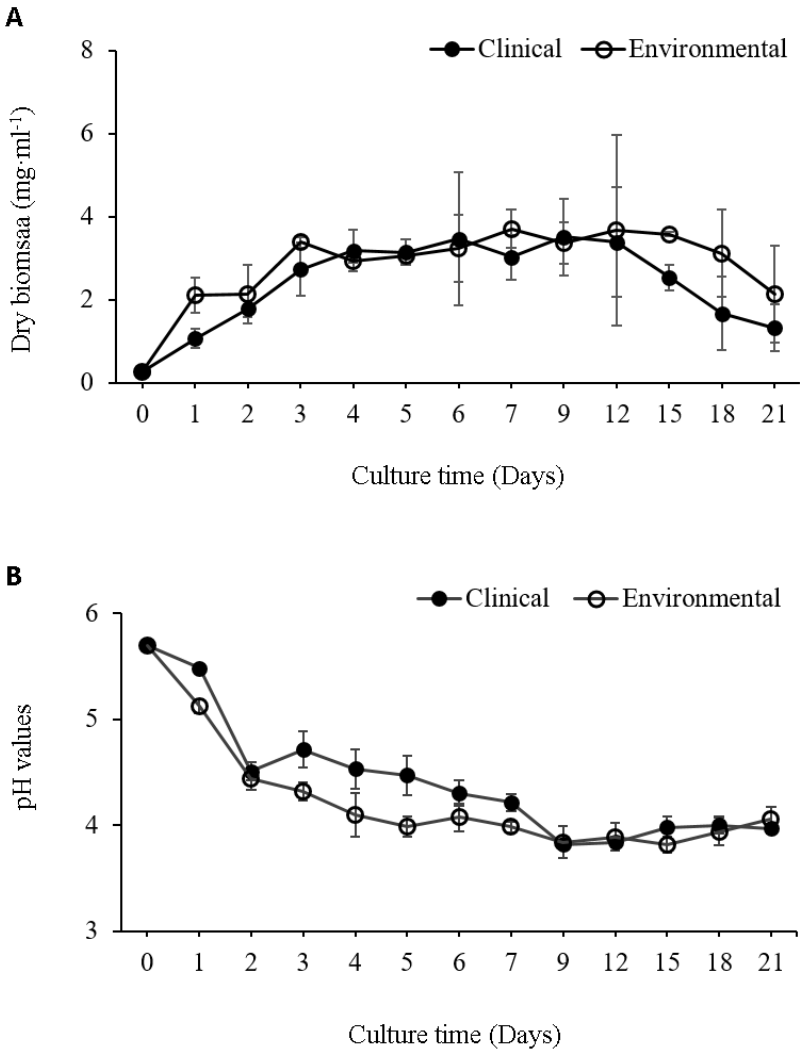

Fig S2. Growth of *S. aurantiacum* for 21 days in SCFM+M medium under hypoxia. A) Growth curve, and B) pH in the fungal culture medium. Data represent mean ± SD (three replicates).

Chapter 5

Effects of *Scedosporium aurantiacum* proteases on human lung epithelial cells

5.1 Introduction

In the two previous chapters, proteases secreted by *S. aurantiacum* were profiled and identified. The results showed that proteases were produced differentially in the clinical and environmental strains and varied between the normoxic and hypoxic conditions. In this chapter, the effect of these different proteases in *S. aurantiacum* pathogenicity was explored. Culture supernatant containing proteases secreted by the clinical *S. aurantiacum* isolate was freeze-dried, redissolved and fractionated using size exclusion chromatography (SEC). The classes of proteases present in each SEC fraction were determined using protease class-specific substrates. Six SEC fractions containing a varying amount of protease activity (10-50% v/v) were co-cultured with human lung epithelial cell line A549 and the effect of these SEC fractions on A549 cell attachment and viability was studied. Three of the fractions containing chymptrypsin-like, elastase-like and cysteine protease activity showed an effect on the attachment and viability of the human lung epithelial cells advancing the quest to learn more about the role of *S. aurantiacum* proteases in the infection and invasion of human cells.

5.2 Contribution to manuscript 2

This manuscript was developed with supervision of my mentors at Macquarie University (Helena Nevalainen and Liisa Kautto). All experiments were performed by me and data were analysed with the assistance of Helena Nevalainen and Liisa Kautto. The manuscript was written by me and Helena Nevalainen and reviewed by Helena Nevalainen and Liisa Kautto.

3.1 Author contribution for manuscript 2.

	ZH	HN	LK
Experimental design	•	•	•
Data collection	•		
Data analysis	•	•	•
Manuscript	•	•	

* ZH = Zhiping Han, HN = Helena Nevalainen, LK = Liisa Kautto

5.3 Manuscript 2

Han, Z., L. Kautto, and H. Nevalainen, Effects of *Scedosporium aurantiacum* proteases on human lung epithelial cells. Manuscript has been prepared for submission to Experimental Cell Research. Figures and figure captions are included as part of the text for easy reading.

Effects of *Scedosporium aurantiacum* proteases on human lung epithelial cells

Zhiping Han [1, 2*], Liisa Kautto[1, 2] and Helena Nevalainen[1, 2]

[1] Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, Australia

[2] Biomolecular Discovery and Design Research Centre, Macquarie University, Sydney, Australia

*Corresponding author

Email: zhiping.han@students.mq.edu.au (ZH)

Abstract

Scedosporium aurantiacum, an opportunistic fungal pathogen of clinical importance, is associated with chronic airway infections in patients with cystic fibrosis. At the moment, very little is known about the infection mechanism of this fungus. We have previously described the types of proteases secreted by *S. aurantiacum*, but the effects of these proteases on human lung cells are unknown. In this work, proteases secreted by *S. aurantiacum* under both normoxic and hypoxic conditions were separated using size exclusion chromatography, and protease activities present in each fraction were determined using class-specific substrates. Effects of those fractions demonstrating protease activity on the attachment and viability of A549 human lung epithelial cells were assessed *in vitro***.** Of the six major classes of proteases, the elastase-like protease from the *S. aurantiacum* supernatant was able to reduce the cell viability, chymotrypsin-like protease was associated with cell detachment, and cysteine proteases were able to destroy both cell attachment and viability. The effects increased with the increase of protease activity in the test fraction but could be specially reduced by addition of class-specific protease inhibitors. Cysteine and elastase-like proteases reduced cell viability in different ways. Cysteine proteases rounded cells up and destroyed the integrity of the cell membrane, leading to the release of the cell content into the environment. Our findings suggest that *S. aurantiacum* could breach the human alveolar epithelial cell barrier by secreting proteases that decrease cell viability and attachment of the cells to each other.

Key words: *Scedosporium aurantiacum*; proteases; human alveolar epithelial cells, cell attachment, cell viability

1 Introduction

Scedosporium aurantiacum is an opportunistic fungal pathogen especially threatening immunocompromised patients [1]. Symptoms caused by *S. aurantiacum* infection are similar to those caused by *Aspergillus fumigatus* in terms of variety and severity [2, 3]. *Scedosporium* infection is initiated by either inhalation of air-borne conidia or penetration of the host tissue at the site of injury [4]. Infection sites include the eye, ear, central nervous system, internal organs and more commonly the lungs, especially the lungs of cystic fibrosis patients [5]. Mortality from this infection is high in the mouse model (60-100%) [1]. In Australia, a population-based surveillance has revealed that *S. aurantiacum* is highly prevalent in human-impacted regions and accounts for at least 15.8% of all the fungal species isolated from 183 phenotypically-speciated isolates at clinic [1, 6]. At the moment, very little is known about the infection mechanism of *S. aurantiacum*.

Proteases secreted by fungi have been demonstrated to play a role in fungal pathogenicity by degrading and breaching the extracellular matrix barrier in the host cells [7]. For example, secreted serine and cysteine proteases from *A. fumigatus* were able to breach the alveolar epithelial cell barrier by disruption of the actin cytoskeleton and sites of focal attachment in human lung cancer cells *in vitro* [8]. Elastase secreted by *A. fumigatus* and *A. flavus* was involved in breaking down the major structure component (elastin) of host lungs to enable fungal germination and penetration into mice lungs [9]. Aspartic proteases secreted by *Candida albicans* were found to aid fungal attachment, colonization and penetration of host tissue by degradation of molecules that protect mucosal surfaces such as mucin and secretory immunoglobulin A (IgA) [10].

In previous work, we profiled the types of proteases secreted by *S. aurantiacum* grown under both normoxic and hypoxic conditions. It was found that serine (including chymotrypsin-,

subtilisin-, trypsin- and elastase-like) and aspartic proteases were secreted under both conditions; however, serine proteases were responsible for the majority of proteolytic activity under normoxia while aspartic proteases dominated under hypoxia. Cysteine proteases were secreted only under hypoxia [11]. The roles of these secreted proteases in the pathogenicity of *S. aurantiacum* are, so far, unclear.

Lungs connect directly to respiratory tract while the lung epithelium cells act as a barrier, which prevents inhaled pathogens gaining access and are the initial point to be affected by fungal growth [12, 13]. Cell attachment and viability were two important indexes in cell proliferation, which are involved in the process of assembly of individual cells into tissues and the the ability of a cell or a tissue to maintain its function respectively [14-16]. Cell attachment helps a damaged tissue to become repaired, and is mediated by interactions between molecules of the cell surface, including multiprotein complexes, such as integrin, immunoglobulin and proteoglycan [17]. Reduction of cell viability can result in necrosis or cell death caused by toxic effects of foreign compounds or a decrease in cellular proliferation [14].

The A549 cell line has a high degree of metabolic and morphological similarity to lung alveolar epithelial cells, and thus has been widely used as a model in *in vitro* studies. For instance, A549 cells were used to study the effects of *A. fumigatus* culture filtrate on human lung epithelium cells; the results showed that death of the cells was initiated by necrosis but factors responsible for the necrosis have not yet been identified [18, 19]. Thakur et al. used the A549 cell line to investigate the effects of *Paecilomyces hepialid* on human lung adenocarcinoma *in vitro*, and found that the extract from this fungus limited cell proliferation, induced apoptosis, and caused cell cycle arrest [20]. In the current work, effects of proteases secreted by *S. aurantiacum* on the viability and attachment of A549 cells were examined with a view of investigating a potential link between protease production and fungal pathogenicity.

2 Materials and Methods

2.1 A549 cell culture

The model human cell line used in this work was A549 (ATCC CCL 185, derived from a human lung carcinoma). RPMI 1640 medium (Life Technologies, Australia) supplemented with 10% v/v FBS (Fetal bovine serum, Life Technologies, Australia) and 1 mM glutamine (Life Technologies, Australia) was used for culture of A549 cells. All media for the cell culture were pre-warmed to 37°C. Brifely, cell stock was thawed and washed twice with supplemented RPMI 1640 medium, transferred into T-25 flasks (Thermofisher, Australia) containing 5 ml of the medium, and then cultured at 37 °C in a humidified 5% CO_2 atmosphere. When the cells were 70-90% confluent, the culture medium was discarded, cells washed twice with warm 1 x PBS, after which 1 ml of 1 x PBS solution containing 0.25% (w/v) trypsin and 0.53 mM EDTA was added to detach cells from the culture flask. Cell suspension was divided into new culture flasks. Detection of *Mycoplasma*, a common contaminant in cell cultures, was performed according to the protocol from the Garvan Institute of Medical Research (Sydney, Australia, https://www.garvan.org.au/) using a PCR-based method which analyses the 16S-23S rRNA.

2.2 Fungal strain and growth medium

The *Scedosporium aurantiacum* WM 06.482 strain (CBS 136046; originally isolated from broncho-alveolar lavage of a cystic fibrosis patient in Australia), was obtained from the culture collection of the Medical Mycology Laboratory, Centre for Infectious Diseases and Microbiology, Westmead Hospital, Sydney, Australia.

Liquid cultures were performed in a synthetic cystic fibrosis sputum medium supplemented with mucin (SCFM+M) as previously described [11, 21]. The medium was composed of mineral salts, amino acids, 1% (w/v) glucose and 1% (w/v) mucin (from porcine stomach, type III; Sigma-Aldrich, Australia) to mimic CF lung sputum. Mucin was dissolved in Milli-Q H_2O,

autoclaved separately and mixed with SCFM just before use. SCFM was sterilized by filtering through a 0.22 μm membrane (Millipore) and the final pH of medium was adjusted to 5.7 to support fungal growth [22].

2.3 Normoxic and hypoxic culture of *S. aurantiacum*

Pre-cultures were prepared by incubating the fungus at 37 °C for up to 10 days on a malt extract agar plate, as described previously [11]. The spores were collected and inoculated into 50 ml of SCFM+M medium in 250 ml conical flasks to make a final concentration of 2×10^6 spores/ml. Incubation was carried out at 37 °C on an orbital shaker at 200 rpm for 4 days under normoxia and 3 days under hypoxia with three individual flasks dedicated for each condition. These culture times were selected based on time-dependent general protease activity measured from the culture supernatant in our earlier study [11]. At the end of culture, the supernatant was collected by centrifugation at 4500 g for 30 min and filtered through a 0.22 μm membrane (Millipore, Australia) at 4 °C. Cleared supernatants were then dialyzed against sterilized Milli-Q H_2O overnight at 4 °C using 10 kDa cut-off dialysis tubing (Amico Ultra-15, Millipore). Dialyzed supernatants were freeze-dried and stored at -80 °C until use.

Normoxic growth of *S. aurantiacum* was conducted at normal air atmosphere where oxygen content was approximately 21%. Hypoxic condition, with oxygen content ≤ 1% [23], was achieved by replacing air inside the flasks with high purity nitrogen gas. To set up the hypoxic culture, the flasks were sealed with rubber stoppers and two sterilized hypodermic needles (DN 25 G × 1.0 LV, Livingstone, Australia) were inserted through each stopper, with one needle inserted deeper than the other. High purity nitrogen gas, sterilized by filtering through a 0.22 μm filter (Millipore), was led in the flasks through the deeper-placed needle while the air inside the flasks flowed out through the other needle. The flow out pipes were connected with one-way valves to prevent air reflux. Oxygen levels in the hypoxic culture were maintained at 1%

and monitored using a polarographic electrode coupled to an Activon® (Model 401) oxygen meter.

2.4 Fractionation of fungal culture supernatant

Freeze-dried culture supernatants were resuspended in PBS buffer (pH 7.4) to achieve a final protein concentration of around 2 $mg \cdot ml^{-1}$ measured by the Bradford assay [24]. Aliquots of 0.5 ml (around 1 mg protein) were separated at 4 °C by size exclusion chromatography (SEC) using a 24 ml Superdex S-200 column (GE Healthcare Life Sciences, Australia) in filter sterilized PBS (pH 7.4) at 0.5 $ml \cdot min^{-1}$, and 1 ml fractions were collected and absorbance at 280 nm was measured using an AKTAPrime protein purification system (GE Healthcare Life Sciences, Australia). Fractionation was performed twice for two sets of freezed-dried fungal culture supernantants, and fractions with the same protease class were pooled together.

2.5 Classification of proteases

Types and activities of proteases present in each SEC fraction were tested using class-specific substrates. These substrates were synthetic peptides coupled with either 7-Amino-4-methylcoumarin (MCA) or ρ-nitroanilide (ρ-NA) (Peptide Institute Inc), including: N-Benzoyl-L-Tyrosine-ρ-NA for chymotrypsin-like protease [25], Z-Ala-Ala-Leu- ρ-NA for subtilisin-like protease [26], N-Sue-Ala-Ala-Ala-MCA for elastase-like protease [27], Boc-Phe-Ser-Arg-MCA for trypsin-like proteases [28], Z-Arg-Arg-MCA for collagenase [29], Sue-Gly-Pro-Leu-Gly-Pro-MCA for cysteine protease [30] and Boc-Leu-Ser-Thr-Arg-MCA for aspartic protease [31]. All tests were performed at the optimal pH of each type of protease as described previously [11]. The reaction mixture contained 50 µl of culture supernatant and 0.5 mM substrate in 200 µl of appropriate buffer. After incubation at 37 °C for 4 min, the absorbance of released ρ-NA was read at 410 nm, or fluorescence of the released MCA measured at λex =380 nm and λem = 460 nm at 37 °C. The amount of released ρ-NA or MCA was calculated using a standard curve

prepared with appropriate dilutions of ρ-NA or MCA. Activities are given as nM of MCA or μM of ρ-NA released per minute by per ml of sample. All assays were performed in triplicate, and statistical analysis was conducted using Excel or OriginPro 8.5 (www.originlab.com).

Protease inhibitors were added into each SEC fraction depending on the type of protease activity detected in each fraction. Concentration of protease inhibitors was determined according to the published literature and previous testing in our laboratory (Table 1). Each SEC fraction was pre-incubated with a selected protease inhibitor for 30 min at room temperature prior to performing a class-specific protease activity assay or adding to A549 cell cultures for cell viability and attachment assay. SEC fractions without inhibitors were also included in the assay for comparison.

Table 1. Protease inhibitors used in the study

Protease inhibitor and specificity	Final concentration	Suppliers
Boc-VF-NHO-Bz-Pcl for subtilisin protease	100 μM	Santa Cruz Biotechnology, USA
Elastatinal for elastase protease	50 μM	
Chymostatin for chymotrypsin protease	5 $mg \cdot ml^{-1}$	Sigma-Aldrich, Australia
Aprotinin for trypsin protease	100 nM	
E-64 for cysteine proteases	10 μM	
Pepstatin A for aspartic proteases	50 μM	
Galardin for metalloproteases	13 mM	
Protease inhibitor cocktail	0.05% v/v	
AEBSF [4(2-aminoethyl) benzenesulfonyl fluoride hydrochloride] for serine proteases	0.5 mM	

2.6 Cell attachment assay

A wash assay was performed to assess changes in cell attachment in a static culture, following the method of Kogan et al. [8]. Briefly, 1×10^5 A549 cells per well containing 3 ml of RPMI 1640 medium supplemented with 10% (v/v) fetal serum and 1mM glutamine were incubated in

a 6-well-culture plate (Corning Costar, Sigma-Aldrich, Australia) for 24 hours. After incubation, medium was discarded and cells were washed three times with 1 x PBS to remove detached cells and fresh medium consisting of 50% (v/v) supplemented RPMI 1640, and 10%, 20% or 50% (v/v) of a SEC fraction, made to 3 ml with 1 x PBS was added. After 4, 8, 12, 24, or 48 h incubation, the medium was discarded and cells were washed three times with 1 x PBS to remove the floating cells. The remaining cells were detached by trypsinization, stained with trypsin blue (Sigma-Aldrich, Australia) and counted using an automated cell counter (TC20, Bio-Rad, Australia). Cells grown in supplemented RPMI 1640 plus 1 x PBS (1:1) was included in the assay as a negative control for each time point independently. The assay with protease inhibitors was conducted in a similar fashion.

2.7 Cell viability assay

MTS assay is a colorimetric method for determining the number of viable cells. MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reduces to an orange formazan dye by dehydrogenase enzymes in metabolically active cells [32]. A sample of 5×10^3 A549 cells per well containing 100 µl of supplemented RPMI 1640 medium, prepared as described above for the attachment assay, were grown in a 96-well-culture plate (Corning Costar, Sigma-Aldrich, Australia) for 24 hours to allow cells to attach to the culture well. The liquid medium was discarded and cells were washed three times with 1 x PBS. After washing, fresh supplemented RPMI 1640 medium with 10%, 20% or 50% (v/v) of a SEC fraction made to up to 100 µl with 1 x PBS was added to each well. After 4, 8, 12, 24, or 48 h incubation, MTS Reagent (Promega, Australia) 20 µl/well was added to the cultures, followed by 2 h incubation at 37 °C in a humidified 5% CO_2 atmosphere. Absorbance of each well was read at 490 nm. The number of viable cells was calculated using a standard curve prepared by plotting the absorbance at 490 nm versus known concentration of viable cells. Cells grown in

RPMI 1640 plus PBS (1:1 volume) was included in the assay as a negative control for each time point independently. The assay with protease inhibitors was conducted in a similar fashion.

2.8 Light microscopy

Cell cultures in the multi-well plates were observed under optical Leica microscope (Leica DMIL, Germany). Images were acquired and processed with Leica Application Suite Version 4.3 software (Las v4.3).

3 Results

3.1 Separation and classification of proteases

In order to separate proteases secreted by the clinical *S. aurantiacum* isolate (WM 06.482; CBS 136046), proteins concentrated from the day-4 supernatant of the normoxic culture and from day-3 supernatant of the hypoxic culture were fractionated by size exclusion chromatography. These supernatants were selected because they had the highest general protease activity at the respective condition. Fractionation was performed at 4 °C to retain protease activity, and protein content in each fraction was monitored by reading the absorbance at 280 nm (Fig 1).

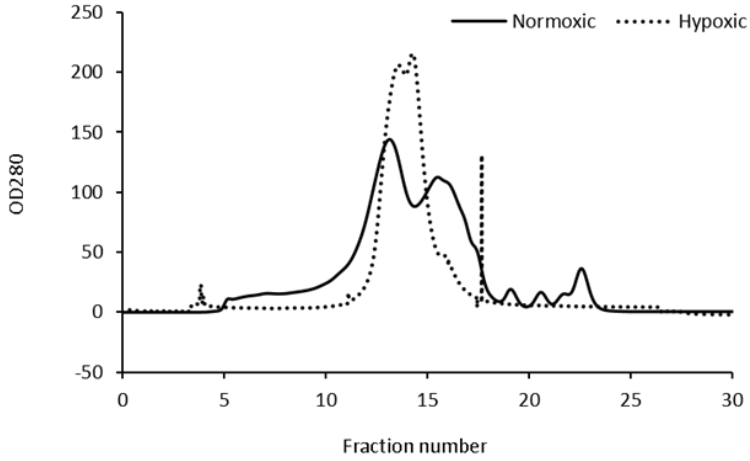

Fig 1. Fractionation of proteins secreted by *S. aurantiacum* under normoxia and hypoxia. One mg of protein was subjected to size exclusion chromatography and protein levels were measured continuously by UV absorbance at 280 m. One ml fractions were collected at 4 °C.

To determine the fractions containing protease activity, each fraction was assayed using the class-specific substrates listed in Materials and Methods. Subtilisin-, elastase- and trypsin-like protease activities were detected in fractions from normoxic cultures, while chymotrypsin-like, cysteine and aspartic protease activities were detected in fractions from hypoxic cultures (Table 1). Subtilisin-, elastase- and trypsin-like proteases secreted by *S. aurantiacum* were not separated well and were present across fractions. This may be due to their molecular size being fairly similar, in the range of 20-45 kDa, as shown in the studies of other fungal extracellular serine proteases [17, 33]. In this work, normoxic fractions 15, 17 and 19 showing elastase-, trypsin- and subtilisin-like protease activity, and hypoxic fractions 12, 16 and 20 representing aspartic, cysteine and chymotrypsin-like protease activity were selected for subsequent studies.

Table 1. Protease activity present in each fraction, tested using class-specific protease substrates

Protease	Culture	Fraction No.	Activity
Subtilisin	Normoxic	18,19	625 μM ρ-NA·ml^{-1}·min^{-1}
Elastase		15-18	32.4 μM MCA·ml^{-1}·min^{-1}
Trypsin		16-19	18.9 μM MCA·ml^{-1}·min^{-1}
Chymotrypsin	Hypoxic	19,20	566 μM ρ-NA·ml^{-1}·min^{-1}
Cysteine		15,16	6.1 μM MCA·ml^{-1}·min^{-1}
Aspartic		12	147.5 μM MCA·ml^{-1}·min^{-1}

3.2 Proteases involved in cell detachment

The six selected SEC fractions with protease activity were co-incubated with A549 cells, and detachment of cells from the culture wells was monitored (not shown). It turned out that only the chymotrypsin-like and cysteine proteases present in the hypoxic fractions were able to cause detachment of A549 cells by rounding the cells up (Fig 2A), and the rate of detachment increased with an increase of the volumetric concentration of the SEC fraction added into the cell culture. Addition of 10%, 20% and 50% (v/v) of the SEC fraction with chymotrypsin-like protease activity (hypoxic, fraction 20) into the cell culture medium caused detachment of

around 25%, 50% and 80% of cells respectively within 24 h. Addition of the above volumes of cysteine protease fraction (hypoxic, fraction 16) into the cell culture medium lead to approximately 10%, 20% and 50% detachment (Fig 2B). In addition, A549 cells began to detach from the surface of the culture wells within 30 minutes of addition of chymotrypsin-like protease fraction (data not shown). Approximately 60% of cells became detached after 4 h exposure to chymotrypsin-like protease, after which the rate of detachment slowed down (Fig 2C). The detachment of A549 cells caused by proteases could be decreased by class-specific protease inhibitors (chymostatin for chymotrypsin protease and E-64 for cysteine proteases), suggesting that the chymotrypsin-like and cysteine protease activity measured from the SEC fractions tested was the main factor for cell detachment. The relative abundance of individual proteases within the SEC fractions was not examined.

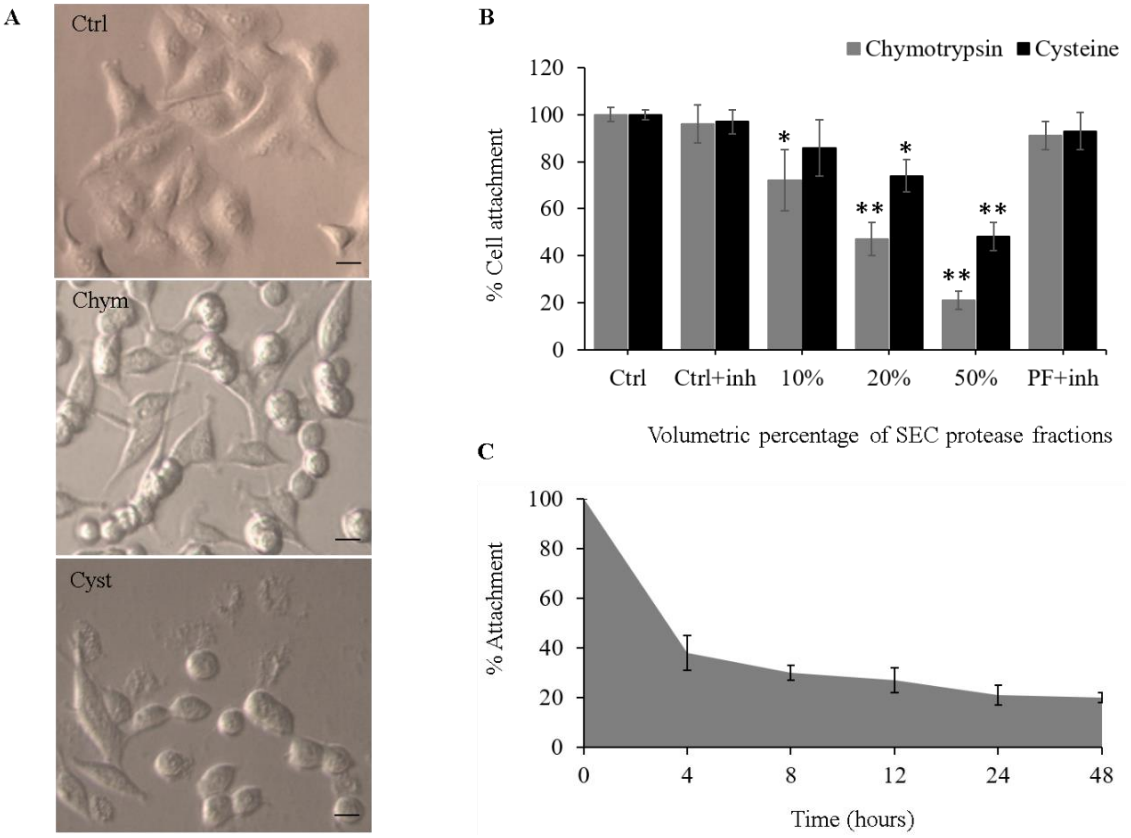

Fig 2. Proteases involved in cell detachment. (A) Microscopic images of detached A549 cells after 24-hour exposure to 50% (v/v) chymotrypsin-like and cysteine protease fractions. (B)

Effect of chymotrypsin-like and cysteine protease fractions (% v/v) on A549 cell detachment after 24 h exposure. (C) Time-dependent effect of 50% (v/v) chymotrypsin-like protease fraction on A549 cell attachment. Ctrl: cells growing in RPMI plus PBS (1:1 v/v) at each indicated time point; PF+inh: SEC protease fractions with addition of class-specific protease inhibitor; Ctrl+inh: control with addition of class-specific protease inhibitor; 10%, 20%, 50% v/v of a SEC fraction in the cell culture medium. Chym: hypoxic SEC fraction 20 with chymotrypsin-like protease activity, Cyst: hypoxic SEC fraction 16 with cysteine protease activity. Bars = 20 μm. Data represent a mean ± SD (three replicates). * means significant difference between the samples and Ctrl ($p < 0.05$), and ** means highly significant difference between the samples and Ctrl ($p < 0.01$). P-values were calculated using Student's *t* test.

3.3 Proteases associated with a decrease in cell viability

The effect of proteases on cell viability was assayed across the selected six SEC fractions showing protease activity (not shown). Results revealed that only cysteine (hypoxic, fraction 16) and elastase-like proteases (normoxic, fraction 15) could cause a decrease in A549 cell viability (Fig 3A), and rate of the reduction increased with an increase in the volumetric percentage amount of the protease fraction added into the cultures. Addition of 10%, 20% and 50% (v/v) of the normoxic SEC fraction 15 with elastase-like protease activity resulted in the loss of viability of around 20%, 35% and 80% cells. The same volumetric additions of the SEC fraction with cysteine protease activity (hypoxic, fraction 16) led to approximately 2%, 11% and 20% decrease in cell viability (Fig 3B). The A549 cells exposed to elastase-like protease primarily began to lose their viability after 4 h, after which the number of viable cells decreased gradually; about 12 h later, 50% of cells had lost their viability (Fig 3C). Cell viability assay was also conducted in the presence of trypsin-like protease inhibitor, because both trypsin- and elastase-like proteases were detected in the same SEC fraction. Since cell viability could not be reduced by addition of a trypsin-specific inhibitor, trypsin-like proteases were not associated with cell death.

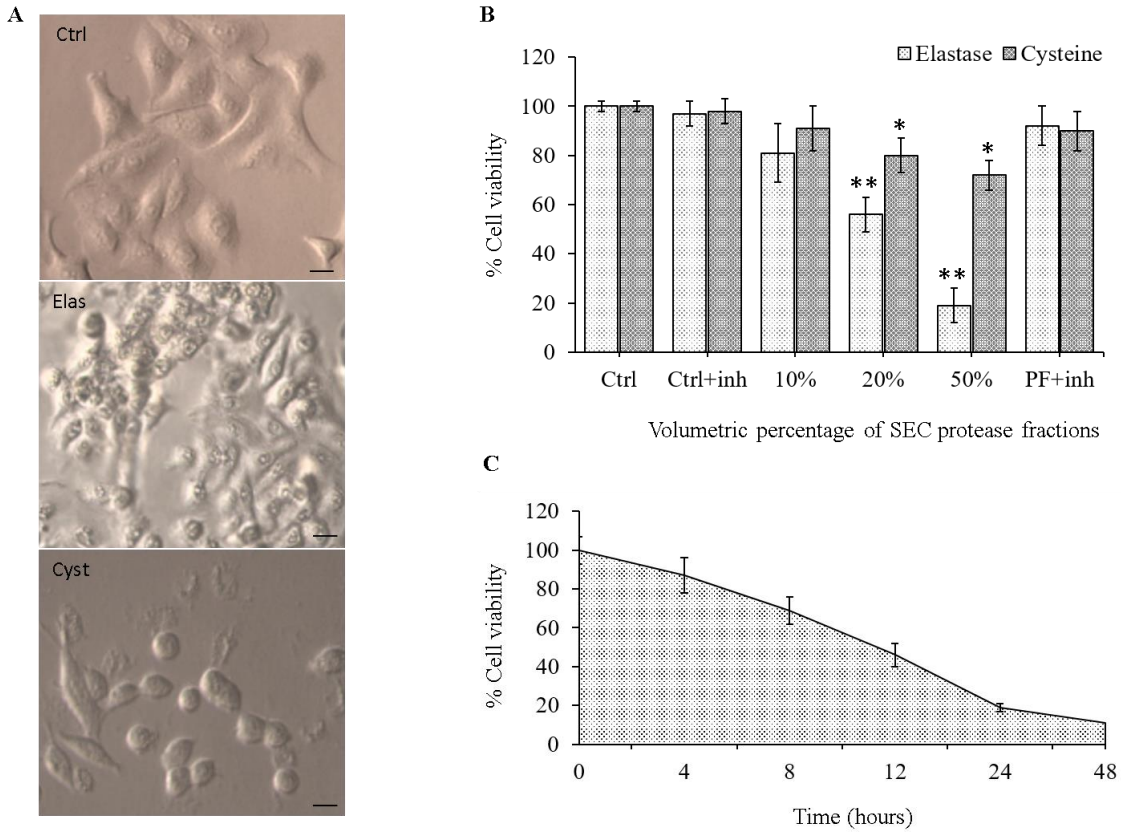

Fig 3. Proteases associated with loss of cell viability. (A) Microscopic images of A549 cells with decreased viability after 24-hour exposure to 50% (v/v) elastase-like and cysteine protease fractions. (B) Effects of elastase-like and cysteine protease fractions (% v/v) on A549 cell viability after 24 h exposure. (C) Time-dependent effect of 50% (v/v) elastase-like protease fraction on A549 cell viability. Ctrl: Ctrl: cells in RPMI plus PBS (1:1 v/v) at each indicated time point; PF+inh: SEC protease fractions with addition of class-specific protease inhibitor; Ctrl+inh: control with addition of class-specific protease inhibitor; 10%, 20%, 50%: volumetric percentage of a SEC fraction in cell culture medium (v/v). Elas: normoxic SEC fraction 15 with elastase-like protease activity, Cyst: hypoxic SEC fraction 16 with cysteine protease activity. Bars = 20 μm. Trypsin-like protease had no effect on cell viability (data not shown). Data represent a mean ± SD (three replicates). * means significant difference between the samples and Ctrl ($p < 0.05$), and ** means highly significant difference between the samples and Ctrl ($p < 0.01$). P-values were calculated using Student's *t* test.

Microscopic features of the cells, which had lost their viability were different between the cells exposed to elastase-like protease (normoxic, fraction 15) and cysteine proteases (hypoxic, fraction 16). A549 cells exposed to elastase-like protease were still attached to the surface of

the culture well and looked different to untreated cells. Cysteine protease rounded up A549 cells and cell content seemed to become released into environment (Fig 3A). This suggested that the mechanism by which the elastase-like protease and cysteine proteases affected cell viability was different.

4 Discussion

Studies on the effects of secreted fungal proteases on lung epithelium cells have primarily focused on major fungal pathogens, such as *A. fumigatus* and the yeast *Candida* spp. [8, 18, 34]. The aim of this study was to add to the existing knowledge by exploring the effects of proteases secreted by an emerging pathogen *S. aurantiacum* on the attachment and viability of human lung epithelium cells.

S. aurantiacum has been shown to invade human alveolar epithelial cells (A549 cell line) by initial attachment to the pneumocytes and then penetration of the cells [35], similarly to that of other fungal pathogens, such as *A. fumigatus* [36]. Our study demonstrated, for the first time, that secreted *S. aurantiacum* proteases could destroy the attachment and viability of A549 cells, and that the impact of chymotrypsin-, elastase-like and cysteine proteases was different.

Cysteine proteases secreted by *S. aurantiacum* under hypoxia caused cell detachment by rounding them up (Fig 2A); also, viability of a portion of the floating cells was reduced seemingly by release of cell content into environment (Fig 3A). A cysteine protease produced by *A. fumigatus* has been reported to have a similar effect on A549 cells [18]. Release of the cell contents may result in further damage to surrounding cells and an inflammatory response as indicated in the study of *A. fumigatus* using a mouse model [37]. In our study, secreted *S. aurantiacum* chymotrypsin-like protease also rounded up the A549 cells, leading to detachment of cells (Fig 2A), however, these floating cells were still viable (Fig 3B).

Disruption of cell attachment may result in deficiency in the normal cell function, such as failure in provision of a physical barrier and less effective cell-to-cell connection and communication. In turn, a fungal pathogen may benefit from cell detachment enabling the fungus to invade deeper into the tissue [8, 38]. Exact comparison of the effect of chymotrypsin-like and cysteine proteases on cell detachment would require purification of the enzyme proteins.

After 4 h exposure to secreted *S. aurantiacum* chymotrypsin-like protease, 60% of A549 cells detached from the bottom of the culture well (Fig 2C). However, protein concentration of the chymotrypsin-like protease fraction (hypoxic, fraction 20) was low. This indicated that the chymotrypsin-like protease secreted by *S. aurantiacum* was a potent enzyme. The observation by Kaur et al. that *S. aurantiacum* (WM 06.482) needed at least 4 h for the majority of conidia to attach to the A549 cells [35], support our findings that proteases secreted by *S. aurantiacum* destroy the physical barrier and cell-to-cell connection of A549 cells within 4 h to facilitate attachment of conidia to the cells to start the invasion.

Secreted elastase-like protease has been considered a virulence factor in *A. fumigatus*. The enzyme has been linked to germination and penetration of this fungus into murine lungs [9], deterioration of respiratory function [39], and lung injury [40]. Elastase protease secreted by the bacterium *Pseudomonas aeruginosa* caused death of A549 cells [41]. In the current study, we also demonstrated that *S. aurantiacum* elastase-like protease could decrease viability of A549 cells (Fig 3B), although these effects need to be further studied on animal models.

Addition of 50% (v/v) fraction of elastase-like protease activity could cause around 80% of the cells to lose their variability within 24 h (Fig 3B). This is similar to the findings of Kaur et al. who also observed that infection of A549 cells by *S. aurantiacum* (WM 06.482) would cause

cell death after 24 h incubation [35]. Together, these studies suggest that elastase-like protease secreted by the fungus was the main reason of cell death.

There was a visible difference in the morphology of A549 cells exposed to elastase-like proteases and cysteine proteases. Elastase-like protease failed to round cells up, yet some morphological changes were observed (Fig 3A). Cells exposed to cysteine protease were rounded up, lost their membrane integrity and released inner contents to the environment (Fig 2A). This is consistent to the study of Kaur et al., who found that the A549 cell line infected by the clinical *S. aurantiacum* isolate (WM 06.482) lost the membrane integrity (indicated by membrane blebbing and cell rounding) [35].

In summary, our study is the first exploring the effect of proteolytic enzymes secreted by the clinical *S.aurantiacum* isolate (WM 06.482) on cell attachment and viability *in vitro* using human alveolar epithelial cells (A549 cell line). The chymotrypsin-like protease caused detachment of cells and elastase-like protease was associated with loss of cell viability. A similar effect caused by elastase has been previously described for other fungi. Cysteine proteases were involved in destroying both the cell attachment and viability; however, the mechanism seemed different to that of elastase-like proteases. This study contributes to the understanding of the role of proteases in invasion of lung cells by *S. aurantiacum* paving the way for future studies aimed at assessing the effects of these proteases *in vivo*.

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Author Contributions

Conceived and designed the experiments: ZH, LK, HN. Performed the experiments: ZH. Analysed the data: ZH, LK, HN. Wrote the paper: ZH, HN.

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Chapter 6

Conclusive summary and future prospects

6.1 Summary of the work

S. aurantiacum is a fungal pathogen prevalent in cystic fibrosis (CF) lungs. The work described here is the first attempt to profile the proteases secreted by *S. aurantiacum* as well as study the *in vitro* effects of these proteases on human lung cell cultures. This is a step forward as most of the previous studies have focussed on the clinical, epidemiological and biological aspects of *S. aurantiacum*.

Chapter 1 reviews current literature in the field of research conducted in this thesis, with particular emphasis on *S. aurantiacum* and proteases secreted by fungi. Aspects discussed range from the big picture including geographic distribution of *Scedosporium* spp. to molecular and biological perspectives concerning this relatively recent emerging pathogen. Proteases secreted by fungi are introduced including approaches to their classification, analysis assessing their effect on human lung epithelial cells to provide background for the current research while addressing some deficiencies in the current knowledge on *S. aurantiacum* in particular.

Chapter 2 provides a guide to Materials and Methods used in the current work with a reference to the chapters in the Thesis.

Chapter 3 gives an overview of the growth of *S. aurantiacum* under normoxic conditions and profiling of extracellular proteases produced by two different *S. aurantiacum* strains: (WM 06.482; CBS 136046) isolated in the clinic and (WM 10.136; CBS 136049) isolated from the environment.

The two strains were grown in a medium mimicking the contents of sputum of CF patients (SCFM) with addition of casein (SCFM+C) and mucin (SCFM+M) to boost growth and induce protease production. Comparison between the two strains across the three media was conducted

in terms of measuring growth and changes in the pH of the medium, and profiling the extracellular proteases produced. The SCFM+M medium supported growth of *S. aurantiacum* the best; the clinical *S. aurantiacum* strain grew faster and attained the growth peak on day 2 with a biomass yield of 9.94 mg/ml. The extent of pH change was also larger in the SCFM+M medium compared to the other two media: the clinical and environmental strains showed similar changes in pH profiles in SCFM+M with a decrease on day 1 followed by an increase afterwards. This suggested that *S. aurantiaucm* conducted lactic acid and ammonia fermentation, as shown with other fungi, during the culture period in SCFM+M thus producing more lactic acid and alkaline ammonia than in the other two media tested [1, 2]. Efficient fermentation implies that more energy was produced, which in turn support the results that *S. aurantiacum* grew better in this medium.

The highest extracellular protease activity was also detected in SCFM+M, produced by the clinical *S. aurantiacum* strain, which was around two fold higher than in the environmental strain, with an amount of 4.73 μg azocasein digested per minute per mg of dry biomass. These results showed that the clinical *S. aurantiacum* strain was thriving in the presence of mucin, which is abundant in the CF lungs by growing well and producing proteases [3].

Protease inhibition assays demonstrated that serine proteases were the predominate proteases secreted by *S. aurantiacum* in SCFM+M, where the activities of the elastase- and trypsin-like serine and aspartic proteases were, overall, 2.5-75 fold higher in the clinical strain compared to the environmental strain. A homolog of trypsin and aspartic protease was identified by mass spectrometry (MS), however, even though enzyme activity was found, elastase protease was not identified by MS searching against other fungi in the NCBI database. This may indicate that the elastase-like protease of *S. aurantiacum* was of low abundance but had a particularly high activity against the class-specific substrate.

Chapter 4 describes the growth and protease secretion of the *S. aurantiacum* cultivated under hypoxia. Hypoxia is one of the characteristics of diseased human organs and has been found to impact the growth and enzyme secretion in studies with *Aspergillus fumigatus* and other fungi [4, 5].

The clinical (WM 06.482; CBS 136046) and environmental (WM 10.136; CBS 136049) *S. aurantiacum* strains were grown in SCFM+M under hypoxia (oxygen level ≤1%). The two strains exhibited great similarity in terms of growth patterns and pH changes in the medium under this condition. Both the biomass yield and medium pH were unchanged from day 3 post inoculation, with values of about 3.8 $mg \cdot ml^{-1}$ dry biomass and pH 4.0 respectively. The decrease in medium pH was probably due to lactic acid fermentation conducted by fungi under hypoxia in the presence of glucose; this energy pathway produces less energy than those used in normoxic growth [6]. This is consistent with the low growth rate of *S. aurantiacum* under hypoxia as growth is an energy-costly process. Other energy-costly processes, such as protein synthesis, protein folding and transport, have also been found to be downregulated in the studies of other fungi like *Fusarium oxysporum* and *Aspergillus nidulans* [6-8]. Acidification of the environment associated with fungal growth has been found to contribute to fungal virulence in the studies of other fungi, such as *Penicillium* spp, *F. oxysporum* and *Sclerotinia sclerotiorum* [9-11]. This may also be the case with *S. aurantiacum* although this hypothesis needs to be studied further.

The activity of proteases secreted by both strains was highest at pH 4.0 and consisted mainly from aspartic proteases. The clinical strain produced higher aspartic and cysteine protease activities all of which are acidic proteases. Conversely, all serine proteases, including elastase-, trypsin-, chymotrypsin-and subtilisin-like proteases, had higher activities in the environmental strain. Protein identification and quantification was performed using MS and a mass spectral

counting approach; results from these analyses were consistent with the activity assay. These results indicated that the clinical and environmental *S. aurantiacum* isolates did differ in protease secretion as a response to hypoxia, which may be linked to their different virulence level established in *Galleria mellonella* model [12]. The difference between production of specific proteases implies that there may also be differences in the genotype of the clinical and environmental isolates. In fact, when conducting the ITS typing of a series of *Scedosporoium* strains, Harun et al. [13] found differences in the genomes between the clinical and environmental strains used in this study. Whether these differences can be experimentally linked to virulence remains to be studied.

Compared to growth under normoxia, *S.aurantiacum* grew slower and produced 80-40% less less biomass over the 7 day culture period. The medium supporting fungal growth became acidic under hypoxia, opposite to normoxia where the medium became alkaline. Predominating protease activity also changed between the two conditions: serine proteases were abundant under normoxia and aspartic protease under hypoxia. Of all class-specific proteases, subtilisin-like and aspartic proteases showed higher activities under hypoxia, and cysteine proteases were only detected from hypoxic culture. These extracellular proteases have been found to demonstrate different biological roles in virulence studies with other fungi, such as *Microsporum canis, C. albicans* and *A. fumigatus*. Subtilisin-like proteases contribute to fungal adherence and invasion of keratinized tissues [14, 15], aspartic proteases digest cells and molecules of the host immune system [16, 17], and cysteine proteases may cause damage to adhesion and viability of the human lung epithelial cell line A549 [18, 19]. The function of proteases secreted by *S. aurantiacum* deserve further study to better understand their role as potential virulence factors.

Chapter 5 studies the effects of proteases secreted by *S. aurantiacum* under both normoxic and hypoxic condition on human lung epithelial cells. The A549 cell line was used as an *in vitro* cell culture model because it has a high degree of metabolic and morphological similarity with lung epithelial cells, and has been widely used as a model in previous *in vitro* studies [20, 21].

Proteins in *S. aurantiacum* culture supernatants with the highest general protease activity were concentrated and fractionated by size exclusion chromatography (SEC). Proteases present in each SEC fraction were profiled using class-specific protease substrates. Six types of proteases were detected, including chymotrypsin-, subtilisin-, trypsin- and elastase-like, aspartic and cysteine proteases. Studies into their effect on cell attachment and viability showed, for the first time, that the secreted *S. aurantiacum* chymotrypsin-, elastase- and cysteine proteases could destroy the attachment and viability of the A549 cells. This may contribute to the initiation of *S. aurantiacum* invasion, as previous studies have shown that *S. aurantiacum* invades human alveolar epithelial cells (A549 cell line) by initial attachment to the pneumocytes and then penetration of the cells [22], similarly to other fungal pathogens, such as *A. fumigatus* [23].

The ways each specific protease impacted on A549 cells were different. Chymotrypsin-like and cysteine proteases were involved in cell detachment by rounding the cells up. The rate of detachment increased with an increase of volumetric concentration of proteases to which A549 cells were exposed, and the detachment could be reduced by chymotrypsin-specific protease inhibitors. This is similar to the study of secreted *A. fumigatus* cysteine protease, which caused detachment of about 50% of A549 cells from culture wells after 24 h of incubation by disrupting cell focal adhesions [18]. The process of detachment may destroy the physical barrier and cell-to-cell connection of A549 cells and help *S. aurantiacum* conidia to attach to the A549 cells.

Exposure to elastase-like and cysteine proteases resulted in a decrease in cell viability. The decrease in cell viability was relative to the concentration of proteases present in the cell culture medium. After exposure to elastase-like protease for 12 h, cell viability decreased to 50%. The way elastase-like and cysteine proteases impacted on A549 cell line was different. Elastase-like protease did not destroy cell attachment. Studies of the secreted elastase-like protease produced by *A. fumigatus* have shown that this protease targeted the growing point of host (mouse) lung cells, and was associated with high mortality of the host [24]. Secreted *A. fumigatus* cysteine proteases were found to disorganize the actin cytoskeleton, leading to A549 cell death. In the current work, cysteine proteases also caused A549 cell death and cell contents seemed to become released to the outside environment.

In summary, in this work, a potential link between pathogenicity of *S. aurantiacum* and one of the putative virulence factors of pathogenic fungi - secreted proteases - was investigated aiming at increasing our understanding about the mechanisms underlying cell attachment and invasion by *S. aurantiacum.* Comparison was conducted between clinical and environmental *S. aurantiacum* strains grown under normoxic and hypoxic conditions. It turned out that protease secretion differed with respect to types and activity between the clinical and environmental strains, and also varied between normoxic and hypoxic conditions. In total, seven types of protease activities were detected, including chymotrypsin-, subtilisin-, trypsin- and elastase-like serine proteases, aspartic, cysteine and metalloproteases. Homologs of subtilisin-, trypsin-, aspartic and cysteine proteases were identified by mass spectrometry. Amongst these proteases, cysteine protease activity was only found from hypoxic cultures, and metalloproteases were only detected from SCFM+C medium, while serine and aspartic proteases were common in the clinical and environmental strains grown under either normoxia or hypoxia.

These differentially produced proteases may play various roles in facilitating *S. aurantiacum* to establish an infection and maintain it successfully afterwards. Secreted chymotrypsin-like and cysteine proteases destroyed the attachment of A549 cells thereby potentially destroying the physical barrier and cell-to-cell connection in lung cells, which may facilitate the *S. aurantiacum* hyphae to penetrate into the host tissue. Elastase-like and cysteine proteases secreted by *S. aurantiacum* under hypoxia led to a decrease in cell viability, which also may help the pathogenic fungus to invade the host tissue. The research presented in the thesis provides an insight into proteases secreted by *S. aurantiacum* that play a role in the invasion of a human host, and may assist in designing a strategy to prevent the establishment of *S. aurantiacum* infection.

6.2 Future directions

To our knowledge, this is the first study on proteases secreted by *S. aurantiacum*, and thus represents a start for more research to fully understand the roles of these proteases in *S. aurantiacum* pathogenicity.

Purification of the protease enzymes is an essential first step in understanding their function. From purified proteases, their amino acid order can be worked out, protein structure mapped, biochemical properties investigated, and the role of proteases in fungal pathogenisis assessed [25]. For example, Markaryan et al. purified a protease from *A. fumigatus* culture supernatants by affinity chromatography and gel filtration. The molecular and catalytic properties of this protease, such as molecular weight, substrate and inhibitor specificity, temperature and pH profiles of enzyme activity, and N-terminal amino acid sequence were assessed. On the basis of this characterization, the homology relationship of this protease with proteins from other organisms was investigated, and it turned out that the protease was an elastinolytic metalloprotease. Secretion of this protease in *A. fumigatus* infected murine lungs, tracked using

an immunogold labeling method, and it was found that the elastinolytic metalloprotease contributed to invading lungs of mice by degrading elastin of the lung tissues [26].

In the current work, the types of secreted *S. aurantiacum* proteases have been profiled by class-specific substrate assays, and homologs of some proteases were identified by MS analysis. Properties of each type of protease, such as molecular weight, substrate and inhibitor specificity, activity profiles under different temperature and pH conditions, and specific amino acid sequence, need yet to be investigated. Therefore, the way forward will be purification of the chymotrpsin-, elastase-like and cysteine proteases starting with large scale culture, protein concentration, and then purification of individual proteases by various chromatographic methods. Starting from pure proteins, one can determine the amino acid sequence, and investigate biochemical properties for a more detailed assessment of the role of particular proteases in fungal pathogenesis.

Given the proteases secreted by the clinical and environmental *S. aurantiacum* strains were different in terms of types and activities assessed *in vitro*, it will be important to determine their effects, and contribution to virulence *in vivo*. This question in mind, animal models have been used widely in the studies of extracellular proteases produced by *A. fumigatus in vivo*. In these studies, the proteases were usually first purified and characterized, and an antibody against each protein was prepared using rabbits. Finally, immunolabelling of the protease under investigation was conducted using the antibody to detect and localize the proteases in lungs of sacrificed mice under an electron microscope [24, 26].

Studies of the virulence status of *S. aurantiacum* have been conducted in a murine model and a *Galleria mellonella* larval model [12, 13]. However, research into virulence factors of *S. aurantiacum* is still largely missing, making it is impossible to understand the molecular

mechanisms underlying the established difference in virulence levels among *S. aurantiacum* strains. Assuming that proteases are virulence factors also in *S. aurantiacum*, the future could see a series of protease-knock out strains tested in a mouse model. In the future, prevention and control of fungal infections might be achieved by drugs specifically designed to inhibit proteases that have been shown to be important for *S. aurantiacum* virulence.

Apart from proteases, fungal pathogens also secrete other compounds into the outside environment, such as other enzymes, enzyme inhibitors and organic acids. These compounds facilitate fungal pathogenesis in many ways as shown with *Aspergillus* spp. and *Candida* spp., in helping the fungi escape from the host immune cells, facilitating fungal attachment to host lung epithelial cells and dissolving insoluble matters for hyphal absorption [27-30]. Research into these compounds secreted by *S. aurantiacum* can be carried out to achieve more comprehesive understanding of the nutritional requirements and pathogenicity of this fungus.

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Supporting Information

Table 1 Supporting data files included in the attached CD.

File name	File type	Description	Source
S1 Table.	XLSX	Identification data of the proteins from the clinical *S. aurantiacum* isolateWM06.482 grown under normoxia listed in Table 2	Chapter 3
S2 Table	XLSX	Identification data of the proteins from the environmental *S. aurantiacum* strainWM10.136 grown under normoxia listed in Table 2	Chapter 3
S1_H Table	XLSX	Identification data of the proteins from the clinical *S. aurantiacum* isolate WM 06.482 grown under hypoxia listed in Table 1	Chapter 4
S2_H Table	XLSX	Identification data of the proteins from the environmental *S. aurantiacum* strain WM10.136 grown under hypoxia listed in Table 1	Chapter 4

Appendix: Biosafety Approval

Macquarie University

Biosafety Committee

Notification of a 'Notifiable Low Risk Dealing' (NLRD)

Dear Prof Nevalainen,

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

NOTIFICATION OF A NOTIFIABLE LOW RISK DEALING (NLRD)

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.

Work with the GMOs described in your Notification form may continue provided that the following conditions for NLRDs are met:

- The NLRDs must be conducted in a facility certified by the Regulator to at least PC2 and of appropriate design for containing the particular type of GMO
- The dealings must be properly supervised and a record of the details of the dealings retained
- Any transport of the GMO must be conducted in accordance with the Regulator's *Guidelines for the Transport of GMOs* available at: http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/transport-guide-1
- If the dealing involves organisms that may produce disease in humans, the NLRDs must be conducted in accordance with the vaccination requirements set out in the Australian Standard AS/NZS 2243.3:2003 (Safety in laboratories: Part 3: microbiological aspects and containment facilities).

- The Chief Investigator must inform the Biosafety Committee if the work with GMOs is completed or abandoned.

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

Please note the following standard requirements of approval:

1. 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 study 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.

4. 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.

If you need to provide a hard copy letter of Final Approval to an external 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

MACQUARIE University
SYDNEY·AUSTRALIA

Zhiping Han zhiping.han@students.mq.edu.au

Approval [5201200092] Inbox x

Bio Safety <biosafety@mq.edu.au>
to me

Dear Zhiping,

Please see below for the approval that you requested. I can confirm that you are authorised to conduct research on this project.

Kind Regards,
Liette

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
Zhiping Han - Student
--
Dr. Liette Vandine
BMedSc(Hons), PhD
Macquarie University Biosafety Advisor & IBC Secretariat

Research Office
Level 3, Research HUB, Building C5C
Macquarie University
NSW 2109

Ph: +61 2 9850 4063

Email: biosafety@mq.edu.au