Understanding the role of hxc-type II secretion system of *Pseudomonas aeruginosa* in pathogenesis

Submitted by

Philemon Gyasi-Antwi, MSc.

Thesis submitted to the University of Nottingham for the degree of

Doctor of Philosophy

December 2018

Declaration

I do hereby declare that the work presented is the outcome of my own research, and has not been submitted elsewhere for the award of a higher degree. Other people's work has been duly acknowledged.

……..

(14/12/2018)

Philemon Gyasi-Antwi, MSc

PhD Immunology Candidate

University of Nottingham

Abstract

Cross-kingdom interactions are ubiquitous in nature and play a key role in the survival of species in polymicrobial environments. The communication between the organisms in mixed infections are always complex with the antagonistic or synergistic nature of this interaction difficult to determine. *Pseudomonas aeruginosa* and *Candida albicans* are two opportunistic human pathogens commonly found together *in vivo.* These two organisms have been associated with host colonisation in several diseases including cystic fibrosis (CF), severe burn wounds and ventilator-associated pneumonia; where the nature of their interactions potentially influences virulence properties of individual populations and in turn the eventual outcome of the polymicrobial disease. Notwithstanding the frequent co-isolation of *P. aeruginosa* and *C. albicans* from mixed infections, the nature of their interactions and the molecules involved are still unclear. Towards this aim we developed a fungal culture system in the presence of supernatants from different strains of *P. aeruginosa* to identify potential changes in fungal behaviour by measuring optical density (OD) at 600 nanometres (nm).

Our preliminary data showed that wild type (WT) *P. aeruginosa* Lausanne strain (PAO1-L) induce filamentation of *C. albicans*, a key virulence attribute of this fungus. Using appropriate *P. aeruginosa* mutants, data showed that this observation was not due to the

production of rhamnolipids, exotoxin A, or dependent on the virulence regulator *ToxR*. Interestingly, promotion of filamentous growth was attenuated when using supernatants from a PAO1 strain, PAAMP1. PAAMP1 has a 58kb chromosomal deletion including genes encoding the Hxc-Type II secretion system (Hxc-T2SS), low molecular weight alkaline phosphatase (LapA), cyclic-di-GMP phosphodiesterase (BifA), and ToxR; indicating that the gene(s) responsible for promoting hyphal growth in *C. albicans* was encoded within this region.

Using biochemical approaches, the hyphal-inducing activity in WT PAO1-L supernatant was shown to be heat-labile (95°C, 10 min) and was retained in ultra-filtrates of relative molecular weights (rMW) above 10 kDa. Furthermore, protein gel analysis showed the presence of a band with rMW between 25-30 kDa specific for WT. Although the hyphal induction effect of PAO1-L secretions was lost in the course of the study and thus the observation inconclusive, the preliminary data put together suggest that WT POA1-L possibly secretes a protein capable of hyphal growth induction in *C. albicans in vitro*.

Alongside employing biochemical techniques to identify the molecular nature of the hyphal-inducing agent(s) in *P. aeruginosa* secretion, genetic approach was used to ascertain gene(s) involved in the

hyphal-inducing activity. Based on the preliminary data showing hyphal induction to be independent of *ToxR* gene regulation and the active agent having a rMW between 25–30kDa, it was hypothesised that the hyphal-inducing agent in the WT supernatant was Hxc-T2SS dependent LapA, which has a rMW between 38-to-40 kDa. Towards this aim, an in-frame deletion mutant lacking the hxc-operon (Δ*hxc*) was constructed in a PAO1-L background. Phenotypic characterisation showed that bacterial growth and virulence traits like biofilm formation and pyocyanin production were not detectably affected by the *hxc* deletion. Addition of exogenous phosphate to culture medium significantly increased bacterial growth as well as biofilm formation, but pyocyanin secretion was markedly reduced in both the mutant and the wild type PAO1-L.

Transcriptional analyses of the *hxc*-operon using a bioluminescent reporter mini-CTX-*lux* inserted into PAO1-L (this thesis) confirmed an earlier study by *Ball et al* (2002) that the operon is tightly regulated by two promoter regions, P_{hxccV} and P_{hxcT}. However, contrary to previous findings that P_{hxcV} transcriptional activity is five times less than P_{hxcT} under phosphate limiting conditions, this work demonstrated that P_{hxcV} is highly expressed, though earlier, and peaks at 5 hours of incubation while P_{hxcT} peaks at 10 hours. Further, the data indicated that expression of both promoters is sustained longer when bacteria is in contact with epithelial cells or in complete

media such as X-vivo[15] or DMEM-F12 compared to LB rich medium or phosphate-limiting proteose peptone medium. This supports earlier reports that the hxc operon is expressed during acute infection.

Using an *in vitro* infection model, it was demonstrated that PAO1-LΔ*hxc* detectably thrives better than the WT after contact with differentiated and undifferentiated human bronchial epithelial cells at a multiplicity of infection of 50. Assessment of bacterial-induced cytokine production showed that bronchial epithelial cells respond to *P. aeruginosa* infection through the production of cytokines and chemokines including TNF-α, GM-CSF, G-CSF, IL-6 IL-17C and IL-8. This pattern was the same for WT and Δ*hxc*.

Overall, the findings from this study show tight regulation and high dependency on culture conditions for the transcription of genes encoding the Hxc-T2SS, which implies that this system plays an important role on the adaptation of *P. aeruginosa* to its environment. Considering that the *P. aeruginosa* devotes Hxc-T2SS to a single substrate whose expression is not controlled by quorum sensing, this study provides a platform for further research into the hxc operon and how it may contribute to *P. aeruginosa* fitness under different conditions.

Acknowledgement

I am most grateful to God, for His sustenance and grace in seeing me through to the end of this PhD. I sincerely thank my principal supervisor, Dr. Luisa Martínez-Pomares, for her guidance, kindness, patience and continuous support throughout this study. My appreciation also goes to my co-supervisor Professor Miguel Cámara, for being supportive and providing great insight in the Pseudomonas study. I thank my thesis committee members, Dr Karl Wooldridge and Dr Alan Huett for their thoughtful advice during the early days of this PhD and their continuous support. To my Postgraduate Student Adviser, Dr Martin Gering, I thank you for your kind advice and support.

My deepest appreciation also goes to the University of Nottingham for the generous award of the Vice-Chancellor's Scholarship for Research Excellence (International), without which I wouldn't have been able to pursue this PhD. I am grateful indeed.

I appreciate Dr James Lazenby and Dr Stefan Heeb, as well as Dr Matthew Fletcher and Dr Manuel Romero for training me on molecular cloning techniques. I am most grateful to Dr Simon Avery and his lab for providing the wild type Candida strain and allowing me to use their facility for the co-culture assays. And to Dr Rebecca Hall at Birmingham University, thank you for providing clinical isolates of Candida and giving me the chance to carry out some of the study in your lab.

To the past and present members of the Lectin Biology Group, Darryl, Sonali, Tamanna, Farah, Raed, Sirina, Mohammad, Diego, Oliver, Yasir, and

Nora, thank you making life in and outside the lab both enjoyable and academically stimulating.

I would like to dedicate this thesis to my mom, Elizabeth Nkrumah and my dad, Kwabena Oti-Antwi; I couldn't have asked for better parents. Thank you for believing in me; your daily prayers and unconditional love encourages me greatly. To my siblings, Owusu, Antwi and Birago; your encouragement carried me through to the end. I am grateful to my Nottingham family, Mr Baah Acheanfour, mom Ruby, Eddie, Barbara, Wendy, Mr & Mrs Ntiamoah, Auntie Dorcas, and the entire members of Nottingham Church of Christ; your love and support cannot be quantified. To my Ghanaian colleagues and friends, Dennis, Kwadwo, Afrakoma, Kumako, Dr Osman and Dr Okyere, thanks for being there. To the Nottingham University Ghanaian Students' Society, I am grateful for giving me the opportunity to serve as your President for 2 years!

Finally, to the love of my life and my constant inspiration, Sarah Ago Acheampong, your love brightened my darkest moments. It is your thoughtfulness and patience that have sustained me to accomplish this dream. Many thanks also to your parents, and siblings, Ansong, James and Joyce, for their unflinching support. You are my Frangipani! To my PhD princess, Phoebe Pokua Gyasi-Antwi, how sweet to hold you and feel all the sighs and joys you bring!

Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Abbreviations

3-oxo-C12	*N*-(3-oxo-dodecanoyl)-homoserine lactone
AHL	acyl-homoserine lactone
ANOVA	analysis of variance
AQs	alkyl-4-quinolones
ALI	air-liquid interface
bp	base pair
C4-HSL	butyl-homoeserine lactone
CA	*Candida albicans*
CCL	chemokine (C-C motif) ligand
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony forming unit
CMC	chronic mucocutaneous candidiasis
COPD	chronic obstructive pulmonary disease
DMEM-F12	Dulbecco's modified Eagle's medium /Nutrient Mixture F-12
ETA	exotoxin A
FCS	foetal calf serum
GM-CSF	granulocyte macrophage colony-stimulating factor
Hxc-T2SS	hxc -type II secretion system
Δhxc	*hxc* gene deletion mutant in PAO1 Lausanne subline
IL	interleukin
IM	Inner membrane
IFN	interferon
kDa	kilo Dalton
LapA	Low-molecular-weight alkaline phosphatase
LasB	elastase B
LB	Lysogeny Broth

LCC	liquid covered culture
LDH	lactate dehydrogenase
LPS	Lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
Mbp	million base pairs
MOI	multiplicity of infection
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OD	optical density at a wavelength of 600 nm
OM	Outer membrane
PA	*Pseudomonas aeruginosa*
PAO1-L	*Pseudomonas aeruginosa* PAO1 obtained from Lausanne
PAO1-N	*Pseudomonas aeruginosa* PAO1 obtained from Nottingham
PHA	phytohaemoagglutinin
PPM	proteose peptone medium
PRRs	pattern-recognition receptors
PQS	Pseudomonas quinolone signal
QS	quorum-sensing
rcf	Relative centrifugal force
RIG-1	Retinoic acid-inducible gene I
rMW	relative molecular weight
Rpm	revolutions per minute
SCFSM	synthetic cystic fibrosis sputum medium
SNP	single nucleotide polymorphism
TLR	toll-like receptor
TNF-α	tumour necrosis factor-alpha
WT	Wild type
Xcp-T22	xcp-type II secretion system
YEPD	Yeast extract peptone dextrose

Chapter 1: General Introduction

1.1 General Introduction

Organisms in their natural habitats engage in competitions and social interactions that determine their survival in complex communities [1]. Experimental research on host-pathogen interaction usually focuses on a particular organism in isolation, thus not taking into consideration the importance of cross-kingdom communication and its influence on microbial pathogenesis. In recent times, experimental mixed cultures such as multi-strain probiotics are being explored in the food industry, which is revealing how for instance synergistic interactions between *Bifidobacterium breve* and *Lactobacillus plantarum* allow the two bacteria to grow in extreme acidic medium [2].

The aim of this study was to deepen our understanding of the mechanisms involved in cross-kingdom communication between prokaryotes and eukaryotes by first focusing on *Pseudomonas aeruginosa* and *Candida albicans*, two organisms commonly found together in diverse milieus, particularly in the lungs of cystic fibrosis (CF) patients [3, 4]. In order to contextualise and justify the present study, this general introduction focuses on the biology of *P. aeruginosa;* with an emphasis on its virulence factors and secretion machineries, and how these contribute to acute and persistent infection in a living host.

1.2 Genus *Pseudomonas*

First described in 1894 by Migula, the genus *Pseudomonas* is one of the most complex and diverse bacterial genera described [5]. Genus *Pseudomonas* belongs to the Gammaproteobacteria class under the phylum Proteobacteria, a group of bacteria with an outer membrane mainly composed of lipopolysaccharides (Gram negative). The Gammaproteobacteria class includes several medically important families of bacteria such as Enterobacteriaceae (*Escherichia coli, Salmonella, Yersinia pestis* etc) and Vibrionaceae (*Vibrio cholerae*) [6].

Presently, the number of published species of genus *Pseudomonas* is 244, including 18 subspecies [7]. This makes the genus to have the highest number of species amongst Gram-negative bacteria [5, 8]. The metabolic diversity and broad adaptability to several environmental niches by members of the genus Pseudomonas increase their ability to associate not only with animals and humans, but also with plants and non-living substratum [9]. Among members of the genus, *P. aeruginosa* is the most virulent species in humans while the most phytopathogenic species is *P. syringae. P. fluorescens* is considered a classic plant commensal [9-11].

1.2.1 *Pseudomonas aeruginosa*

Amongst the genus Pseudomonas, only *P. aeruginosa* can cause serious infections in humans. *P. aeruginosa* can exist as a motile mono-flagellated bacterium or as a sessile organism within biofilms [12]. Having the ability to assimilate nutrients from diverse carbon sources, *P. aeruginosa* is able to thrive and colonise many ecological niches. In the healthcare setting, *P. aeruginosa* has been recovered from ventilators, sinks, hydrotherapy pools, and various other sources in Intensive Care Units; making the bacterium a common cause of nosocomial infections [13].

P. aeruginosa grows readily on almost all media under aerated conditions and can survive anaerobically when supplied with nitrate. The bacterium optimally grows at 37 °C with the ability to persist at temperatures between 4 and 42 °C [14].

The 25th bacterial genome to be completely sequenced, *P. aeruginosa* is one of the most complex bacteria in nature having a genome of approximately 6.3 million base pairs (Mbp) [15]. Genome sizes of *P. aeruginosa* strains range between 5.5 and 7 Mbp (Table 1.1) [16]. Comparatively, the genomic length of *P. aeruginosa* laboratory strain PAO1 (~6.3 Mbp) is larger than those of *Bacillus subtilis, Escherichia coli* and *Mycobacterium tuberculosis*, which possess genomes ranging between 4 and 5 Mbp [12].

Table 1.1| Features of some sequenced *P. aeruginosa* strains

Strain	Source	Genome size (Mbp)	Reference
PAO1	Clinical; non-respiratory	6.264	[15]
PA14	Clinical; burn wound	6.538	[17]
PA7	Clinical; non-respiratory	6.588	[18]
PA2192	Cystic Fibrosis isolate	6.905	[19]
LESB58	Cystic Fibrosis isolate	6.602	[20]

P. aeruginosa has about 5570 putative genes, the majority of which are regulatory in function, with a large number also associated with metabolic pathways, multidrug efflux pumps, and transport and motility systems [21, 22]. These features contribute to the bacterial adaptability, virulence, and generally high intrinsic resistance to antibiotics including most β-lactams such as penicillin [23].

1.2.2 Pathogenesis and virulence factors in *P. aeruginosa*

As an opportunistic pathogen, *P. aeruginosa* causes severe infections in persons with dampened immune defence. Hence, patients with severe immunosuppression as in the case of people with Human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS), cancer patients undergoing chemotherapy, individuals with grave neutropenia and those undergoing solid organ and bone marrow transplants generally suffer from *P. aeruginosa* as well as other bacterial and fungal mix-infections [24-26]. However, three main maladies specifically associated with *P. aeruginosa* include

persistent lung infections in cystic fibrosis (CF) patients, sepsis in severely burnt or trauma patients, and acute ulcerative eye infections in individuals who wear soft contact lenses extensively [27]. Additionally, *P. aeruginosa* is one of the commonest causes of nosocomial infections especially in patients with endotracheal ventilators, urinary tract catheters and those on ambulatory peritoneal dialysis [24]. About 11 to 13.8% of all nosocomial infections are attributed to this bacterium which causes 70% to 80% mortality rate particularly in paediatric patients suffering from *P. aeruginosa* pneumonia [24, 28, 29].

P. aeruginosa virulence is multifactorial, combining a variety of cell-associated structures and extracellular secretions [21]. Some of these virulence factors are discussed in the subsequent sections.

1.2.2.1 Lipopolysaccharide

Lipopolysaccharide (LPS), also referred to as endotoxin or lipo-oligosaccharide, is part of the outer membrane of Gram-negative bacteria [30]. Acting as one of the first barriers protecting the bacteria from host defence or deleterious agents such as antibiotics and cationic antimicrobial peptides, LPS is one of the most important virulence factors for *P. aeruginosa* [31, 32]. Variations in the composition and phenotype of *P. aeruginosa* LPS is believed to affect colonisation and subsequent chronic infection of the host.

LPS is made up of three components namely: lipid A, core oligosaccharide (core OS), and O antigen. Lipid A comprises a disaccharide backbone which anchors LPS into the outer membrane of the bacteria through several fatty acid chains. Covalently attached to Lipid A is an eight to ten-carbon oligosaccharide which forms the core of LPS. The third LPS component, O antigen, is the most variable domain of LPS, which consists of a chain of carbohydrate polymers attached to the core [30, 31]. Most *P. aeruginosa* strains have been shown to simultaneously produce two different forms of O antigen: common polysaccharide antigen (CPA) and O-specific antigen (OSA). CPA consists of a homopolymer of D-rhamnose while OSA is a heteropolymer with a repetitive three to five carbon chains [33]. Although OSA-containing LPS is reported to be more dominant amongst the two types of O-specific antigens, CPA is selectively expressed on the bacterial outer membrane during chronic lung infection in CF patients. This is as a result of selective pressure against the bacterial OSA due to its high immunogenicity in eliciting strong antibody response [33]. CPA is also thought to promote *P. aeruginosa* adherence to host tissues, as bacterial mutants deficient in D-rhamnose biosynthesis was shown to have a reduced capacity to adhere to cultured human bronchial epithelial cells [33, 34].

Additionally, *in vivo* studies have revealed that *P. aeruginosa* strains recovered from late stage of CF chronic infection are able to thrive

and cause persistent infection in the lungs of infected mice unlike the early strains. This is attributed to changes in the structure of LPS of the late strains, such as loss of O antigen chains and lipid A modifications, which enables *P. aeruginosa* to dampen leukocyte recruitment in the lung [35].

1.2.2.2 Flagella and pili

Flagella and pili are two major cell-associated factors that facilitate movement and primary attachment of the planktonic form of *Pseudomonas* to substrate [36]. *P. aeruginosa* possesses a single polar flagellum which is a filamentous appendage made up of protein subunits called flagellin. Through rotation, the flagellum generates propulsive force that facilitates swimming motility. In addition to flagellum, the bacterium possesses multiple pili and fimbriae which are smaller appendages on its surface. Whereas fimbriae ensures surface attachment of *P. aeruginosa*, pili allow twitching motility which enable the bacterium to spread along hydrated surfaces [37].

Through binding to the epithelial surface glycolipid, asialo-GM1, flagella and pili facilitate the initial tethering and irreversible attachment to host epithelium which is important to the eventual bacterial colonisation and biofilm formation in the lungs of CF patients [37]. These structures do not only facilitate nutrient acquisition but

as in the case of burn infections, pili and flagella enable *P. aeruginosa* to persist at the wound site and spread through the lesion [38].

Flagella also induce inflammatory responses through interaction with toll-like receptor 5 (TLR5) and 2 (TLR2)resulting in the release of interleukin-8 [39]. The immunogenic nature of flagella requires that after colonisation of host epithelium, *P. aeruginosa* rids this appendage in order to escape host immune response and maintain a persistent infection. As infection models in animals have shown that anti-flagella antibodies protect against *P. aeruginosa*, this feature is being explored in the development of *P. aeruginosa* flagella vaccine for CF patients [40].

1.2.2.3 Major secretory exoproteins

P. aeruginosa secrets extracellular factors such as elastase, exoenzymes, exotoxin A, haemolysins, iron-binding proteins, and alkaline proteases, which cause tissue destruction and facilitate blood sepsis and systemic dissemination [41]. *P. aeruginosa* mutants lacking exotoxin A, exoenzyme S, elastase, or alkaline protease production are less virulent *in vivo* [41]. After colonization, exoenzymes including Exo S, Exo T, Exo U and Exo Y through the action of a type III secretion system invade host cells. Within cells, these toxins interrupt intracellular signal transduction and actin cytoskeleton rearrangement [42-44]. Exoenzyme S, an ADP-ribosyl-

transferase, specifically adds ADP-ribose moieties to GTP-binding proteins which directly causes tissue destruction in lung infections [45, 46].

Exotoxin A, a secreted product expressed by most *P aeruginosa* strains blocks protein translation in host cells also by catalysing ADP-ribosylation. This leads to cell lysis, bacterial invasion and eventual tissue destruction [41, 47]. Purified exotoxin A has been shown to be highly toxic for mice [48].

Phospholipase C and rhamnolipid are two additional *P aeruginosa* extracellular virulence factors. They are haemolysins thought to work in synergy to break down phospholipids in lung surfactant, thereby predisposing the lungs to atelectasis linked with chronic and acute *P. aeruginosa* infection [41, 49]. Additionally, rhamnolipids impair function of the cilia linings of the human respiratory epithelium [41].

1.2.2.4 Quorum sensing molecules and biofilm formation

With the ability to produce diffusible quorum signalling molecules, *P. aeruginosa* do not only co-ordinate cell-to-cell activities but also cross-talk with other organisms. These auto-inducers regulate bacterial gene expression in response to cellular density and also modulate the behaviour of competing microbes; thereby enabling the bacteria to thrive within a given environment [3, 50].

Currently, there are four quorum sensing (QS) systems found in *P. aeruginosa:* two LuxR and LuxI-type systems called LasR and LasI; PqsR-controlled quinolone system RhlR and RhlI, and the IQS system that functions under phosphate-limiting conditions [51-53]. The las system is driven by the production of N-3-oxododecanoyl homoserine lactone (HSL) [51], while the central molecule in the rhl system is N-butyryl-L-homoserine lactone (C_4-HSL) [51]. Signalling circuits of *P. aeruginosa* QS system is illustrated in Figure 1.1.

Quorum sensing is initiated with the production of the diffusible signalling molecule at a minimum concentration consistent with low cell population. The concentration of the QS molecule increases with increasing bacterial population until it reaches a maximum threshold. At this level, the signalling molecule activates a signal transduction cascade by binding to its target protein, either LasR or RhlR, resulting in the activation or repression of target genes [50, 51].

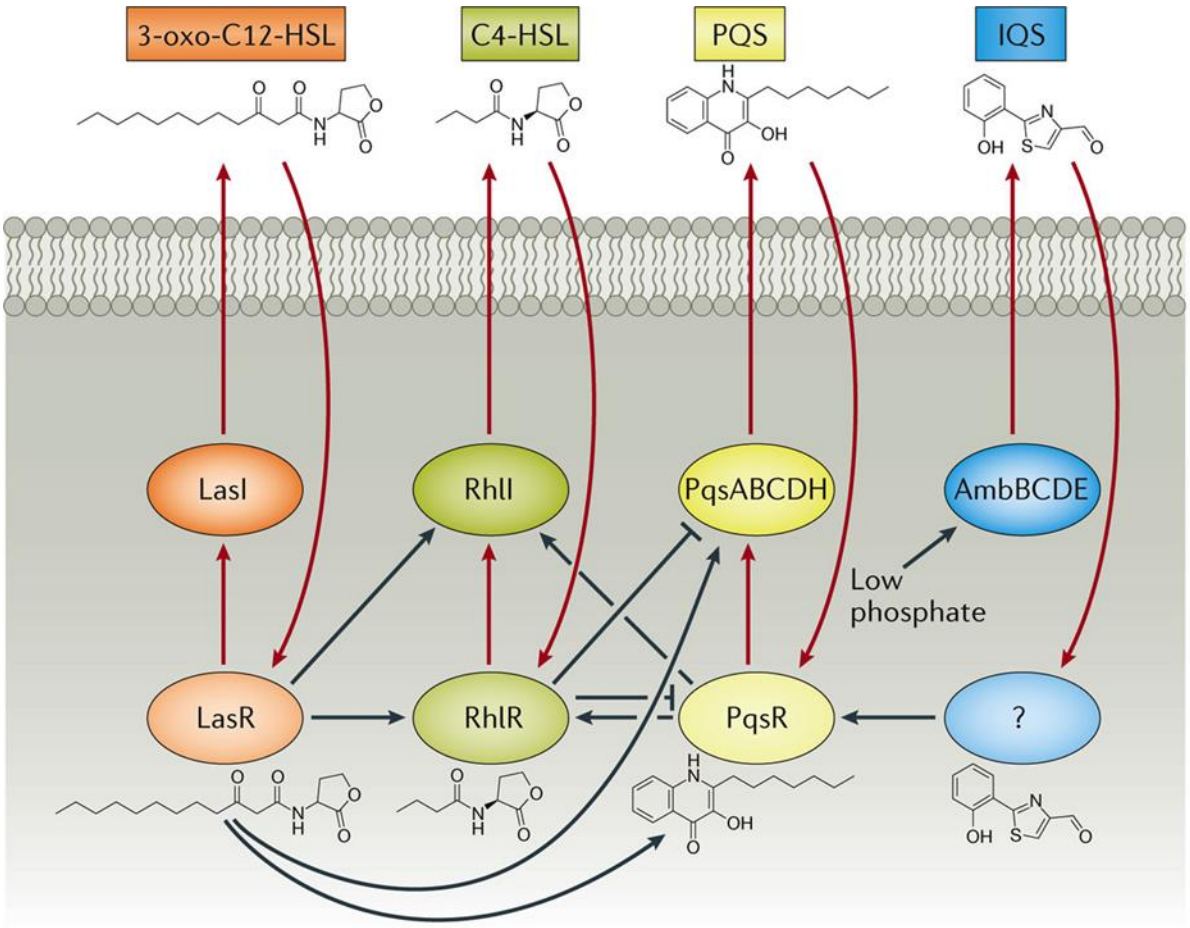


Figure 1.1| Quorum sensing signalling circuits in *P. aeruginosa*. The four autoinducer synthases, LasI, RhlI, PqsABCDH and AmbBCDE, produce the autoinducers, 3-oxo-C12-homoserine lactone (HSL), C4-HSL, 2-heptyl-3-hydroxy-4-quinolone (PQS) and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS), respectively. 3-oxo-C12-HSL, C4-HSL and PQS, are recognized by cytoplasmic transcription factors. The receptor for IQS is currently unknown. The production of the IQS signal is induced under phosphate starvation. The individual circuits are highly interconnected and involve autoinduction (red arrows). Figure taken from [52].

A *P. aeruginosa* las-deficient strain has been reported to lack the ability to disseminate from an infected wound, confirming the importance of QS in bacterial pathogenesis [27]. Table 1.2

summarises major virulence factors of *P. aeruginosa* controlled by QS.

Table 1.2 | Major virulence factors of *P. aeruginosa* under the regulation of quorum sensing [54].

QS regulated gene	Protein or virulence factor	Effects to host during infections	Benefits to *P. aeruginosa*	References
lasB	Elastase	Degradation of elastin, collagen, and other matrix proteins	Extracellular iron acquisition from host proteins	[55, 56]
lasA	Protease	Disruption of epithelial barrier	Staphylolytic activity, host immune evasion and enhanced colonization	[57]
toxA	Exotoxin A	Cell death	Establishment of infection; enhanced colonization	[58, 59]
aprA	Alkaline protease	Degradation of host complement system and cytokines	Immune evasion and persistent colonization	[60]
rhlAB	Rhamnosyl-transferases (rhamnolipids)	Necrosis of host macrophage and polymorphonuclear lymphocytes	Immune evasion; biofilm development	[61]
lecA	Lectin (galactophilic lectin)	Paralysis of airway cilia	Establishment of infection; enhanced colonization	[62]
hcnABC	Hydrogen cyanide	Cellular respiration arrest; Poorer lung function	Enhanced colonization	[63]
phzABCDEFG*, *phzM	Pyocyanin	Oxidative effects dampen host cellular respiration and causes oxidative stress; Paralysis of airway cilia; Delayed inflammatory response to *P. aeruginosa* infections through neutrophil damage	Establishment of infection; enhanced colonization; immune evasion	[64]

Studies have shown that *P. aeruginosa* QS coordinates biofilm formation in host tissues that facilitate resistance to antimicrobial therapy [65]. As compact aggregates of bacterial colonies embedded in an exopolysaccharide mesh, biofilms protect *P. aeruginosa* from

the host inflammatory response [66]; enabling the bacteria to persist in infected tissues and in-dwelling medical devices [65]. Numerous studies have focused on molecular mechanisms, maintenance, and antimicrobial resistance of *P. aeruginosa* biofilms, due to its association with the progression of diseases such as CF, endocarditis, and ventilator-associated pneumonia [67]. Understanding of these mechanisms will provide insight into effective treatment options of *P. aeruginosa* infections.

1.2.3 Chronic *P. aeruginosa* infections in cystic fibrosis patients

With a global prevalence of about 100,000 affected individuals, CF represents the commonest deadly disease among Caucasians associated with a genetic defect [68]. CF is an autosomal recessive disorder caused by a mutation in the CF-transmembrane conductance regulator (CFTR) gene, which encodes for a cyclic-AMP-dependent chloride channel. The abnormal chloride conductance caused by the defective CFTR promotes extensive absorption of Na^+ and water from the lumen of the respiratory, gastrointestinal, and reproductive epithelial tracts [69]. The decreased paraciliary fluid in the bronchial lining of the respiratory tract results in a rather unusual thick viscous mucosal layer, which impairs clearance of inhaled microbes and particles. Bacteria accumulate in the lower respiratory airways over time, with *P. aeruginosa* as the most virulent and persistent [70].

The prevalence of *P. aeruginosa* infection increases with age where positive cultures for the bacterium can be observed from approximately 80% of patients by their late twenties [71].

Several studies suggest that most CF patients acquire *P. aeruginosa* through casual contact with natural reservoirs such as lakes, streams, and on vegetables [71-73]. Some CF patients are also thought to acquire *P. aeruginosa* either directly or indirectly from other individuals with CF, which has raised some concerns from medical practitioners [71].

Defective CFTR channels have been shown to increase the expression of asialylated glycolipids on the surface of CF airway epithelium. These glycolipids are thought to serve as receptors for *P. aeruginosa* flagellum, which increases bacterial attachment and facilitates the early colonisation process of CF lung [74]. However, some studies have shown that CFTR mutant epithelial cells do not bind or internalise *P. aeruginosa* compared to WT epithelial cells [75, 76]. After colonisation, the bacterium switches its phenotype from a non-mucoid, antibiotic-susceptible to an alginate-producing mucoid form under anaerobic conditions [77]. Alginate is an exopolysaccharide that facilitates *P. aeruginosa* infection by increasing host cells' attachment and evasion of immune response through biofilm formation. Mutations in the *mucA* gene is believed to cause the

overproduction of alginate during chronic infection by enabling the bacterium to establish biofilms [78].

The characteristic feature of the disease is continuous lung deterioration and persistent *P. aeruginosa* infection, which drives exacerbated inflammatory episodes. It is believed that the overstimulated, yet ineffective, neutrophilic response leads to severe lung injury, thereby obstructing airflow and eventually leading to death [70].

1.2.4 Secretion systems in bacteria

Bacteria in a biotic or abiotic environment can engage in diverse associations, which can be a simple attachment to mutualistic, commensal or pathogenic associations. In order to survive in these environments, bacteria employ protein secretion systems [30]. The cell envelope in Gram negative bacteria is made up of two hydrophobic membranes spaced by the periplasm, thus secreted products from the cytoplasm which are usually hydrophilic need to traverse through these hydrophobic barriers [31]. The recent advancement of bacterial genome sequencing is revealing the complex mechanisms, which control bacterial protein secretions. At least there are now six known secretory mechanisms in Gram negative bacteria named Type I to Type VI (T1SS – T6SS); these systems mediate transportation of proteins across membranes of the

bacterium into the extracellular milieu and occasionally into the host cell [30]. Main features of the six secretory systems in Gram negative bacteria are summarised in Figure 1.2. Although the secretory machineries have different complexities, they are generally conserved across Gram negative bacteria, with some Gram positive bacteria also having similar secretory systems [30, 31].

Whereas some secreted proteins are transported at once across the inner and outer bacterial membranes as in the case of T1SS, T3SS, and T4SS; other exoproteins are secreted first into the periplasm via universal secretion (Sec) or twine arginine translocation (Tat) pathways before being translocated across the outer membrane via the T1SS, T2SS or T5SS [30, 31]. The bacterium produces a remarkable variety of toxins and enzymes which are released into the extracellular milieu through specific molecular secretory machines [79].

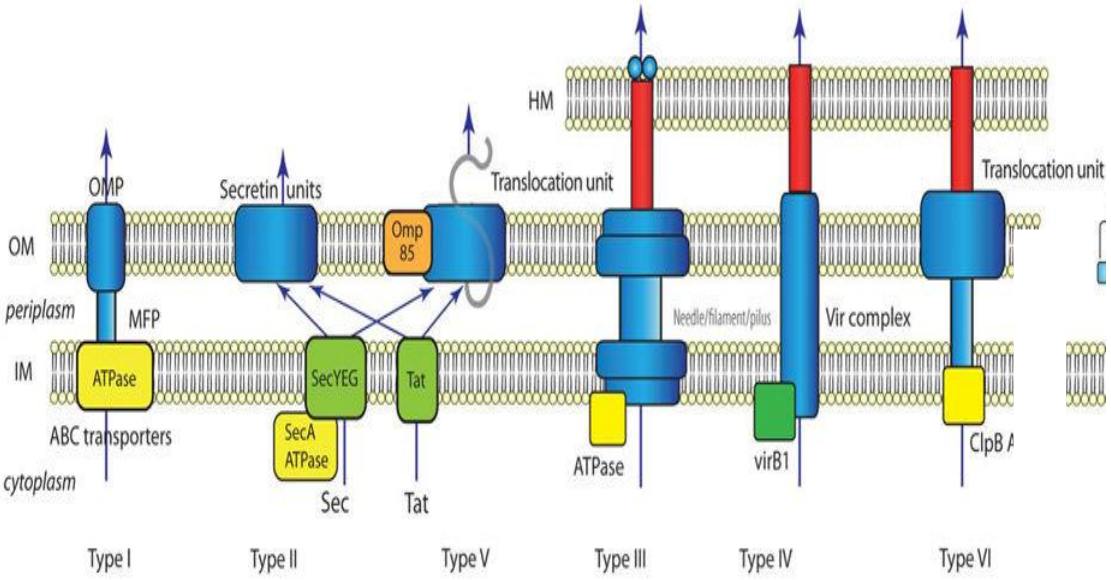


Figure 1.2| Schematic representation of known secretion systems in Gram negative bacteria. This diagram depicts each of the known secretion systems in their simplest forms. HM: Host membrane; OM: outer membrane; IM: inner membrane; MM: mycomembrane; OMP: outer membrane protein; MFP: membrane fusion protein. ATPases and chaperones are shown in yellow. Diagram adapted from [80].

Proteins secreted via the Sec pathway usually have hydrophobic N-terminal signal peptides which are recognised and cleaved by the SecA subunit of the translocase before being transported across the inner membrane in an ATP-dependent manner [30, 32]. Hence, proteins are translocated in an unfolded conformation via the Sec machinery using ATP hydrolysis and a proton gradient for energy [30]. Conversely, proteins that are destined for the Tat pathway are recognised by their N-terminal amino acid motif (S-R-R-x-F-L-K), and then get translocated in their folded form with a proton gradient as the main energy source [30].

Out of the six classes of secretion systems currently characterised, *P. aeruginosa* possesses, with the exception of T4SS, five of these secretion machineries, in some instances in multiple copies [81].

1.2.4.1 Type I secretory system (T1SS)

The least complex amongst the bacterial secretion systems is the T1SS, which consists of an Outer Membrane (OM) protein, an ATP-binding cassette (ABC) transporter which serves as a source of energy for the transportation process, and an adaptor protein which links the two components [37, 81]. The substrates of T1SS not only vary in sizes, ranging from 78- 8,682 amino residues, but are also functionally diverse [33].

There are two T1SS in *P. aeruginosa,* where the classical one is responsible for the secretion of alkaline proteases: AprA and AprX [79]. AprA is considered as a virulence factor in most *P. aeruginosa* infections where it is involved in the degradation of some components of the host immune system including complement C1q and C3, interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) [60]. AprA also inhibits TLR5 activation by cleaving bacterial monomeric flagellin but the function of AprX is currently unclear [72, 81, 82]. The other T1SS in *P. aeruginosa* is involved in the secretion of a protein that mediates iron assimilation through the binding of haem from haemoglobin, known as HasAp. The equivalent protein in

in *E. coli* is called α-haemolysin, and in *Neisseria meningitidis* iron-repressible repeat-in-toxin (RTX)[34, 37, 73]

1.2.4.2 Type 2 secretory system (T2SS)

Possessed by many pathogenic and non-pathogenic Gram negative bacteria, the T2SS is the most prolific secretion system; and has been extensively researched in human pathogens such as *P. aeruginosa*, *Klebsiella spp., E. coli, V. cholera, and Yersinia enterocolitica* [83]. *Shewanella oneidensis,* a metal reducing bacterium, is one of the non-pathogenic bacteria that possess a T2SS [83, 84].

Heat-labile enterotoxin from enterotoxigenic *E. coli* and cholera toxin from *V. cholera* are two highly homologous substrates of T2SS [85]. The products secreted by *P. aeruginosa* T2SS are predominantly associated with many respiratory diseases such as bronchiectasis, CF, and diffuse panbrochiolitis [69, 79, 81].

The conventional T2SS in *P. aeruginosa* is the extracellular protein (Xcp) system. It consists of 12 components encoded by a set of 11 genes found in two different operons, *xcpP*-to-*Q* and *xcpR*-to-*Z*, with the 12th gene, *xcpA*/*pilD*, positioned outside of the 2 *xcp* operons [81]. This machinery is used by the bacterium to secrete diverse exoproteins such as elastase, lipase, phospholipases or exotoxin A (Table 1.3) [79]. These exoproteins are produced with an N-terminal

signal peptide which undergoes cleavage during membrane translocation from the cytoplasm into the periplasm via either the Sec or Tat export pathways [54, 79]. From the periplasm, the matured proteins are then translocated across the outer membrane via the T2SS [79, 86].

Table 1.3 | Some T2SS-dependent secreted products of the *P. aeruginosa* PAO1 strain [81].

Secretion system	Secreted protein	Characteristics
T2SS (Xcp)	LasB (PA3 724)	Metalloproteinase (Elastase)
	Las A (PA 1871)	Staphylolytic and elastolytic
	PlcH (PA0844)	Hemolytic phospholipase C
	PlcN (PA3319)	Non hemolytic phospholipase C
	PlcB (PA0026)	Phospholipase C specific of phosphatidyl-ethanolamine
	CbpD (PA0852)	Chitin-binding protein
	ToxA (PA1948)	AB Toxin, ADP-ribosyl transferase
	PmpA (PA0572)	Putative metalloprotease
	PrpL (PA4175)	Lysine specific endopeptidase (Protease IV)
	LipA (PA2862)	Triacyl glycerol acyl hydrolase
	LipC (PA4813)	Lipase
	PhoA (PA3296)	Alkaline phosphatase
	PaAP (PA2939)	Aminopeptidase
T2SS (Hxc)	LapA (PA0688)	Alkaline phosphatase

Aside the originally characterised Xcp T2SS, *P. aeruginosa* has another T2SS known as Hxc (**H**omologous to **xc**p) system [79]. The Hxc-T2SS is described in detail in Chapter 3.

1.2.4.3 Type 3 secretory system (T3SS)

The T3SS is a highly conserved complex system in Gram-negative bacteria that functions as an injectisome to deliver effector proteins from the bacterial cytosol through the eukaryotic cellular membrane

[87, 88]. Thought to share a common ancestor with flagellum, the T3SS machinery is made up of a multi-ring basal body and a needle-like structure. The basal body permeates the inner and outer membranes through the peptidoglycan layer, and serves as the platform on which the needle-like filament is attached to the bacterial envelope [88].

As found in *Yersinia*, *Salmonella* and *Shigella spp*., the hollow needle-like barrel of *P. aeruginosa* T3SS delivers effector molecules through the translocon into the host cell. The translocon apparatus made up of hydrophobic proteins (PopB, PopD) and a hydrophilic protein (PcrV), forms the delivery complex to mediate the translocation of toxins directly into targeted eukaryotic cells [89]. PcrV is an essential component needed to assemble the T3SS PopB/D translocon complex [88, 90]. Ventilator-associated pneumonia patients have recently been reported to suffer worse clinical symptoms when infected with bacteria encoding T3SS [91] and application of monoclonal anti-PcrV antibodies have been shown to improve survival in mouse models [92].

There are at least four known effectors secreted via the T3SS pathway namely ExoU, ExoS, ExoT, and ExoY; which are involved in various disruptive activities in the host cell such as disruption of actin cytoskeleton, inhibition of phagocytosis and hijacking of cellular

metabolism [88]. Serum samples from CF patients have been shown to have antibodies raised against T3SS proteins, and *P. aeruginosa* isolates show less ability to secrete T3SS effectors. This suggests that though T3SS plays a critical role in host cell invasion during acute infection, it may be repressed during chronic infection [78, 93]. Also, studies have shown that environmental isolates of *P. aeruginosa* have T3SS which inject effectors into environmental amoebae to kill them during co-cultivation [78]. Indicating that the T3SS pathway might have evolved as a protective response against environmental predators [78].

1.2.4.4 Type 5 secretory system (T5SS)

The T5SS is believed to be the simplest known bacterial secretion system which was first described by Pohlner *et. al.,* in 1987 for the IgA protease in *Neisseria gonorrhoeae* [94]. Proteins destined for surface localisation via the T5SS pathway mostly consists of three parts: an N terminal signal peptide, the secreted passenger domain and a C-terminal β domain. The cleavable signal peptide facilitates the translocation process from the bacterial cytoplasm by targeting the protein via the Sec system into the periplasm. From the periplasm, the C-terminal domain forms a β-barrel structure through which the passenger domain exits into the outer environment [94, 95]. The secreted protein is often referred to as autotransporter since the mechanism of translocation was initially thought to be a self-

directed process, with the secreted protein itself containing the information needed for its translocation, but not dependent on energy coupling and accessory factors as in the case of T1-T6SS [95, 96]. However, recent studies have shown that there are other accessory proteins particularly the β-barrel assembly machinery (Bam) complex consisting of a large integral outer membrane (OM) protein BamA and four lipoproteins: BamB, BamC, BamD and BamE, which are required for the organisation and insertion of β-barrel proteins into the OM and the eventual secretion of the passenger domains [96].

Translocated passenger domains have been associated with bacterial virulence where it has been shown that *P. aeruginosa* esterase EstA is essential for rhamnolipid production, cell motility, and biofilm formation [97, 98].

1.2.4.5 Type 6 secretory system (T6SS)

The T6SS was recently identified in *V. cholerae* [99] and *P. aeruginosa* [100]. The T6SS machinery is thought to be involved in the transport of effector proteins into prokaryotic and eukaryotic cells thereby contributing to the bacterial competitiveness and pathogenesis [96, 101, 102]. The T6SS is made up of 13 conserved proteins that form the core machinery and several other accessory proteins [103]. The machinery can be broadly divided into two main components: an inner complex made up of lipoproteins homologous

to components of the T4SS and a tail complex that consists of components showing evolutional resemblance to bacteriophage (Figure 1.3) [96, 104].

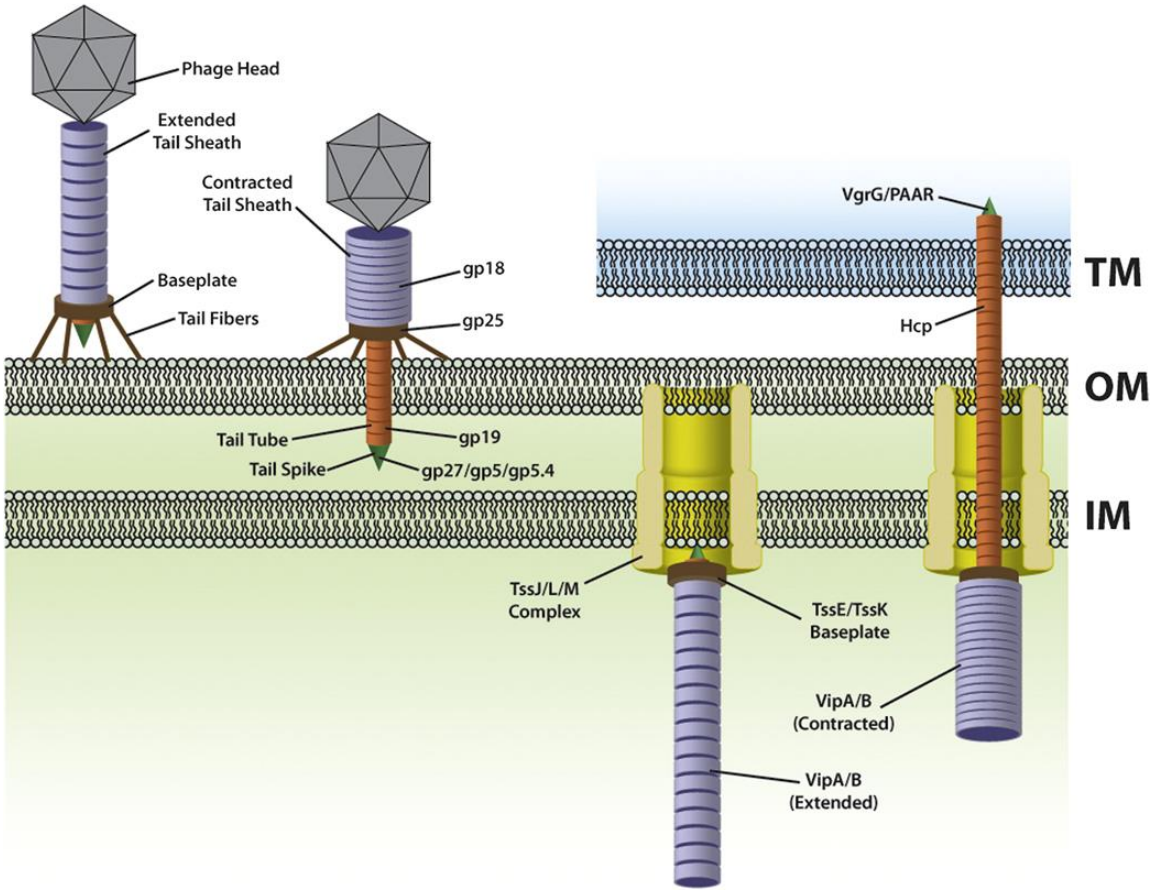


Figure 1.3| Structural and functional comparison of contractile bacteriophage tail and the proposed T6SS complex. T6SS and bacteriophages share common evolutionary structural components. Homologous components are represented in a same colour. In bacteriophage, tail fibres connected to the virion baseplate are used for the attachment to host outer membrane (OM) whereas in T6SS, the baseplate is attached to an inner membrane (IM) spanning the periplasm. Bacteriophage delivers the phage spike into target cell, while contraction of T6SS sheath forces the T6SS spike out of the cell and potentially across a target membrane (TM). Figure taken from Ho, B. *et al* [105].

Both T6SS identified in in *V. cholerae* and *P. aeruginosa* export Haemolysin-Coregulated Protein (Hcp) and a group of proteins named Valine-Glycine Repeats (Vgr) [103]. In *P. aeruginosa*, Hcp1 has been found in pulmonary secretions of CF patients with antibodies specific for Hcp1 detected in their sera; giving an indication that T6SS apparatus may be important to the pathogenesis of the bacterium in CF patients [100].

1.3 Aims and objectives

The possession of several secretion systems by *P. aeruginosa* and its high degree of adaptability to the environment provide the bacterium phenotypic plasticity to efficiently cause both acute and chronic infections in the human host. *P. aeruginosa* is considered the most predominant pulmonary pathogen in CF and *Candida albicans* being the commonest eukaryotic organism isolated from patients' sputum [106]. In this thesis and with an aim to increase our understanding of cross-kingdom communication between *P. aeruginosa* and *C. albicans*, chapter 2 explores the biology of *C. albicans*; molecules involved in the interactions between the two organisms, and morphological changes in *C. albicans* when in contact with supernatants from *P. aeruginosa*. The main aim of chapter 3 was to generate a *P. aeruginosa* mutant deficient in hxc-T2SS. This was to identify the impact of this secreton on cross-kingdom communication and investigate the transcriptional profile of the *hxc* operon under

different conditions. The aim of chapter 4 was to use an *in vitro* infection model to investigate the role of hxc-T2SS in bronchial epithelial infection, while chapter 5 summarises the work with general discussion and recommendations for future work.

Chapter 2: Identification of novel players in cross-kingdom communication between *Pseudomonas aeruginosa* and *Candida albicans*

2.1 Introduction

For the present chapter, the interaction between *P. aeruginosa* and *C. albicans*, two opportunistic human pathogens, was evaluated. In particular, the work focused on the effect of secretions of *P. aeruginosa* on the growth pattern of *C. albicans.* To achieve this, a fungal culture system in the presence of supernatants from different strains of *P. aeruginosa* was carried out to identify potential changes in fungal behaviour by measuring optical density (OD) at 600 nanometres (nm).

The following introduction sections will describe the nature of interaction between *C. albicans* and *P. aeruginosa* during mixed infection and explore the biology of *C. albicans* with emphasis on how its polymorphic nature plays a role in polymicrobial infections.

2.2. Evidence of *P. aeruginosa* and *C. albicans* co-infection in patients.

Polymicrobial communities of bacteria and fungi are commonly found in nature, where the interactions are thought to drive key biochemical pathways that contribute to soil nutrient cycling, bioremediation, food production and biocontrol; while also influencing health and diseases of plants and animals (Figure 2.1) [107]. Organisms within these polymicrobial environments develop strategies to safeguard their survival against harsh environmental or nutritional conditions as well

as against competing organisms. Hence, the behaviour of either the bacterium or fungus cannot be predicted easily based on the biology of the individual organism grown in single cultures [107].

Studies in recent years are exploring co-operative and social interactions which exist among interacting organisms, the molecular mechanisms involved in these interactions and their effect on human health [50, 108].

Figure 2.1| Types of applications and outcomes of bacterial-fungal interactions. Microbial communities are shaped by biotic and abiotic environmental conditions leading to selection for specific consortia of microorganisms. Bacteria and fungi frequently share habitats where their interactions either physical or molecular conversely affect the environment. Figure taken from [107].

P. aeruginosa and *C. albicans* have been associated with host colonisation in several cases where the nature of their interactions potentially influences their individual populations and virulence

properties as well the eventual outcome of the polymicrobial disease [109]. In ventilator-associated pneumonia (VAP), studies have shown that *C. albicans* tracheobronchial colonization increases *P. aeruginosa* associated infection in patients [110]. A follow-up study consequently showed that antifungal treatment reduces the risk of developing *P. aeruginosa*-related VAP [111].

P. aeruginosa is considered the most predominant pulmonary pathogen in CF with *C. albicans* being the commonest eukaryotic organism isolated from patients' sputum [106]. In CF patients, some of the predisposing factors to *C. albicans* infection include inhaled steroids, lifelong antibiotics and diabetes mellitus, which generally dampen the immune system of these patients [106]. As a strong indicator for lung transplantation, CF also presents an avenue for opportunistic infection of *P. aeruginosa* and *C. albicans* during the post-transplant period. Notwithstanding its frequent recovery from sputum, oral and vaginal swabs of CF patients, the contribution of *C. albicans* to the disease progression is not clear [106]. An epidemiological study examining the occurrence of bronchopulmonary fungal infection and the contribution of each fungus to disease progression in CF showed that, *C. albicans*, regardless of its frequent isolation was responsible for only one case of candidiasis [112]. Anti-fungal actions of bacteria co-colonising with

C. albicans in CF have been associated with this low prevalence of candidiasis [106, 112].

In the burn wound setting, *C. albicans* has become one of the key opportunistic pathogens whose colonisation has been linked with invasive infection of *P. aeruginosa* [38]. This observation has been demonstrated in murine studies, where mixed-challenge of sub-lethal *P. aeruginosa* and *C. albicans* in burned mice exhibited a high mortality rate compared with unburned mice similarly challenged, and burned mice challenged with only one test organism [113].

In vitro studies investigating pathogenic relationship between *P. aeruginosa* and *C. albicans* showed that *P. aeruginosa* attached to and formed dense biofilm on *C. albicans* hyphae which eventually killed the fungus [3]. Both fungal and bacterial QS signalling molecules are involved in the cross-kingdom communication (Figure 2.2)[108], whereas *P. aeruginosa* HSL represses hyphal formation of *C. albicans*, the fungal farnesol inhibits the release of *P. aeruginosa* pyocyanin and bacterial swarming motility [106].

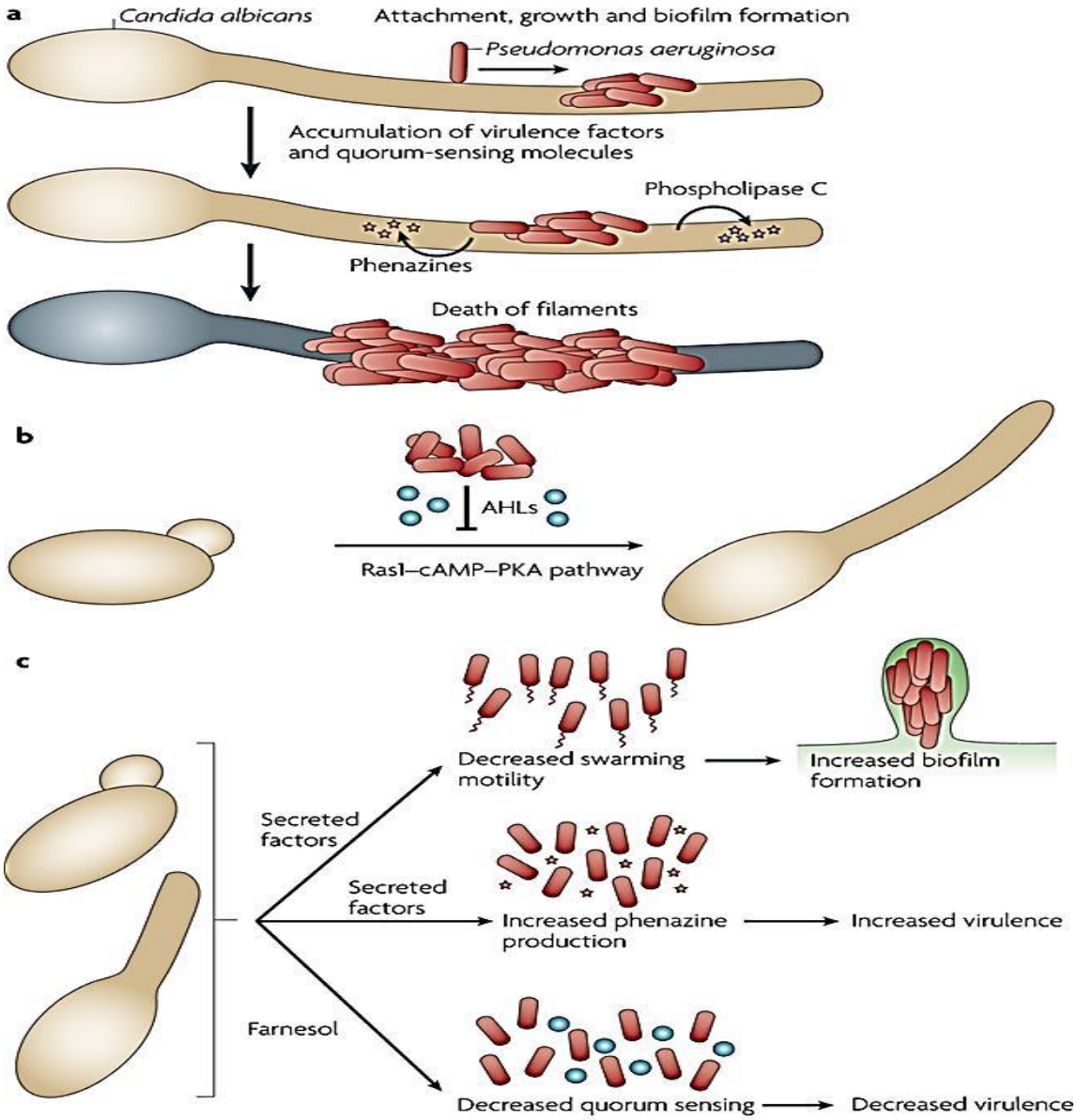


Figure 2.2| Molecular mechanisms involved in the interactions between *P. aeruginosa* and *C. albicans*. (a) *P. aeruginosa* selectively adheres to and forms biofilm on the surface of *C. albicans* hyphae, but not yeast cells. The bacterium secretes virulence factors such as phospholipase C and phenazines, which kill *C. albicans* filaments. (b) QS molecules released by both *P. aeruginosa* and *C. albicans* play key roles in autoregulation and interspecies interactions. HSL secreted by *P. aeruginosa* interferes with *C. albicans* filamentation by inhibiting the Ras1–cyclic AMP (cAMP)–protein kinase A (PKA) pathway (c) *C. albicans* produces QS molecule farnesol and other secreted factors that alter the behaviour of *P. aeruginosa*. Figure taken from [108].

Having discussed the biology of *P. aeruginosa* with an emphasis on its virulence factors in Chapter 1, the biology of *C. albicans* is discussed in the subsequent sections.

2.2.1 *Candida albicans*

The fungus *Candida albicans* was first identified as a human pathogen when detected as the causative organism of oral thrush. It was originally referred to as *Oidium albicans* by virtue of its ability to reproduce through budding [106]. Alongside other yeast species associated with human infections, the Genus *Candida* was adopted as the general taxon. Among the commonest species including *C. glabrata, C. krusei, C. parapsilosis* and *C. tropicalis*, *C. albicans* is the most frequently encountered fungal pathogen of humans [106, 114]. Phenotypically, *C. dubliniensis* is the most closely related yeast species to *C. albicans* where it shares many properties, including the ability to produce hyphae and chlamydospores [115] as well as the ability to form tight associations with some oral bacterial species [116]. However compared to *C. dubliniensis*, *C. albicans* has been shown to be more pathogenic, mainly due to its possession of many virulence-related factors such as cell-surface glycoprotein (ALS) and secreted aspartyl proteinase (SAP) [115]. *C. albicans* is among the first eukaryotic pathogens whose genomic library was sequenced; having a size of approximately 15.6 Mb [114, 117, 118].

C. albicans belongs to the commensal microbiota in healthy persons. The organism is mostly localised to human cutaneous or mucosal niches such as oropharyngeal airway, gastrointestinal tract and female genital tract, and can be detected in the oral cavity within 1 month from birth [119, 120]. As illustrated in Figure 2.3, the organism is a polymorphic fungus that can exist as yeast, filamentous or pseudohyphal forms. As an opportunistic organism, individuals undergoing immunosuppressive therapy, as well as those having HIV/AIDS, persons with indwelling devices and neonates are susceptible to *C. albicans* infection [119, 121].

2.2.1.1 Morphology and morphogenesis

Alternation between different morphological forms is one of the key features of *C. albicans*. Between unicellular budding yeast and polar filamentous forms, the fungus can grow as pseudohyphae and chlamydospore during its life cycle [122, 123]. Whereas true hyphae are formed from the continuous elongation of budding yeast followed by septation, pseudohyphal forms remain attached to the parent bud after septation; thus forming elliptical shapes with constrictions at their septa [122, 124].

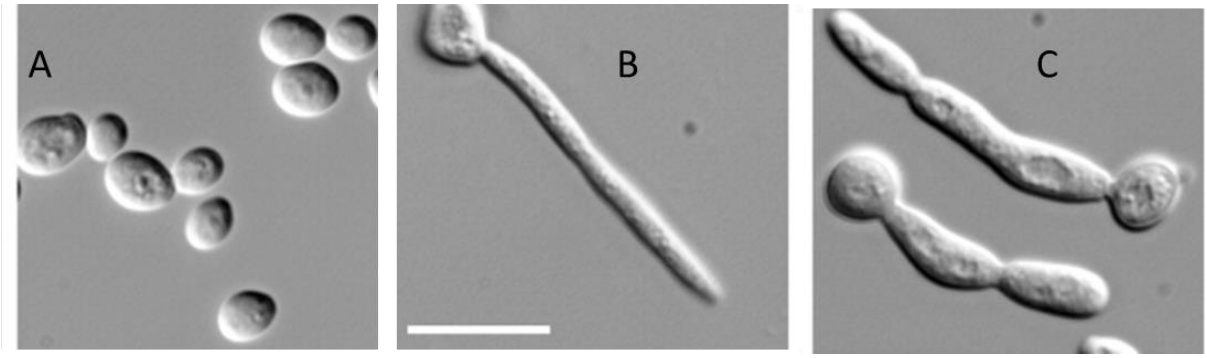


Figure 2.3| Different structural forms of *Candida albicans*. *C. albicans* is a polymorphic organism which can grow as a budding yeast (A), a parallel-walled hyphae (B) or as a pseudohyphae with constrictions (C). Scale bar in the B panel represent 10 μm. Figure adapted from [125].

These growth forms have been linked with *C. albicans* pathogenesis in immunocompromised individuals. The hyphae are thought to facilitate tissue infiltration and destruction [109].

In vitro studies involving reconstituted oral epithelial and endothelial cells show that *C. albicans* invades these tissues in its filamentous form [126]. Mucosal biopsies from individuals with mucosal candidiasis have been shown to harbour only the filamentous form of the fungus [127]. Moreover, yeast cells are thought to evade macrophage phagocytosis through morphological switch to hyphae [118, 128].

Although many studies have often considered a direct correlation between yeast-to-hyphal switch and virulence in *C. albicans* [124], some dimorphic human fungal pathogens such as *Blastomyces*

dermatitidis exist as budding yeast in infected tissues and exhibit filamentous growth forms in their natural environment [129].

Several environmental conditions trigger transition from yeast to filamentous growth in *C. albicans.* Most notable of these include a temperature of 37°C, presence of serum, neutral pH, and presence of *N*-acetylglucosamine (GlcNac) [130]. In medical microbiology, culture in the presence of serum at 37°C is used as a baseline diagnostic test for detecting *C. albicans* due to its potency in inducing hyphal growth [118].

C. albicans rapidly undergoes hyphal induction in the presence of serum notwithstanding the source [130]. *C. albicans* Ras-1 protein is reported to mediate signal transduction in response to serum during filament induction, and strains lacking *RAS1* fail to produce filaments after serum treatment [131]. Serum from albumin-deficient rat mutants efficiently promote filamentation, which indicate that hyphal induction in response to serum is not albumin-dependent [131]. Proline and GlcNac are two main hyphal inducers thought to be actively involved in the serum effect as they are by-products of glycoprotein degradation in serum [130].

DNA microarray has revealed about 61 putative genes significantly activated in *C. albicans* during yeast to hyphal switching in response

to serum and 37°C [124]. Genes encoding hyphal wall protein 1 (Hwp1), adhesin agglutinin-like protein 3 (Als3), secreted aspartyl protease 5 (Sap5) and hyphally regulated cell wall protein (HYR1), were the most highly expressed in response to the serum and temperature treatment [118, 124]. Almost half of the 61 hypha-specific genes were reported to be transcriptionally repressed in the yeast morphology by three main genes namely *Rfg1, Nrg1,* and *Tup1* [124]. Strains deficient in any of these regulatory genes constitutively grew as hyphae when exposed to non-filament-inducing conditions [132].

Several transcription factors such as Cph1, Cph2, Tec1, Rim101, Czf1, Flo8 and most notably Efg1, positively regulate hypha-specific gene expression in *C. albicans* [118]. Whereas Cph1 is required for hyphal induction only on solid media, Efg1 is essential for filament induction in liquid culture having serum, GlcNAc, CO_2, and neutral pH, as well as on solid media such as Spider medium, a nutrient-rich medium that strongly stimulates hyphal growth [118, 133, 134]. Efg1 is thus considered to be the key regulatory factor for hyphal growth in response to most conditions [118, 135].

C. albicans adenylyl cyclase (Cyr1) which is encoded by the *CYR1* gene plays a pivotal role during filamentation through the integration of many environmental cues of different sources. The enzyme forms

a functional three-dimensional apparatus with actin monomers, G-actin, and adenylyl cyclase-associated protein 1(Cap1) which initiates polarised cytoskeleton growth at the hyphal tips during filamentation [118, 136].

C. albicans encounters diverse microbial communities which impact on the morphological changes between yeast cells and filamentous forms. Through a quorum sensing (QS) mechanism, the fungus is able to sense its own cell density as well as surrounding bacterial cells, thereby changing its morphology [118]. Farnesol, an acyclic sesquiterpene alcohol, is one of the best characterised *C. albicans* secreted QS molecules known to inhibit hyphal growth in response to cell density and bacterial products [137]. Conversely, tyrosol another QS molecule of the fungus stimulates filamentation by shortening the lag phase of dormant yeast cells [118]. The various transcription factors and signalling pathways involved in hyphal induction or repression in response to diverse environmental cues are summarised in Figure 2.4.

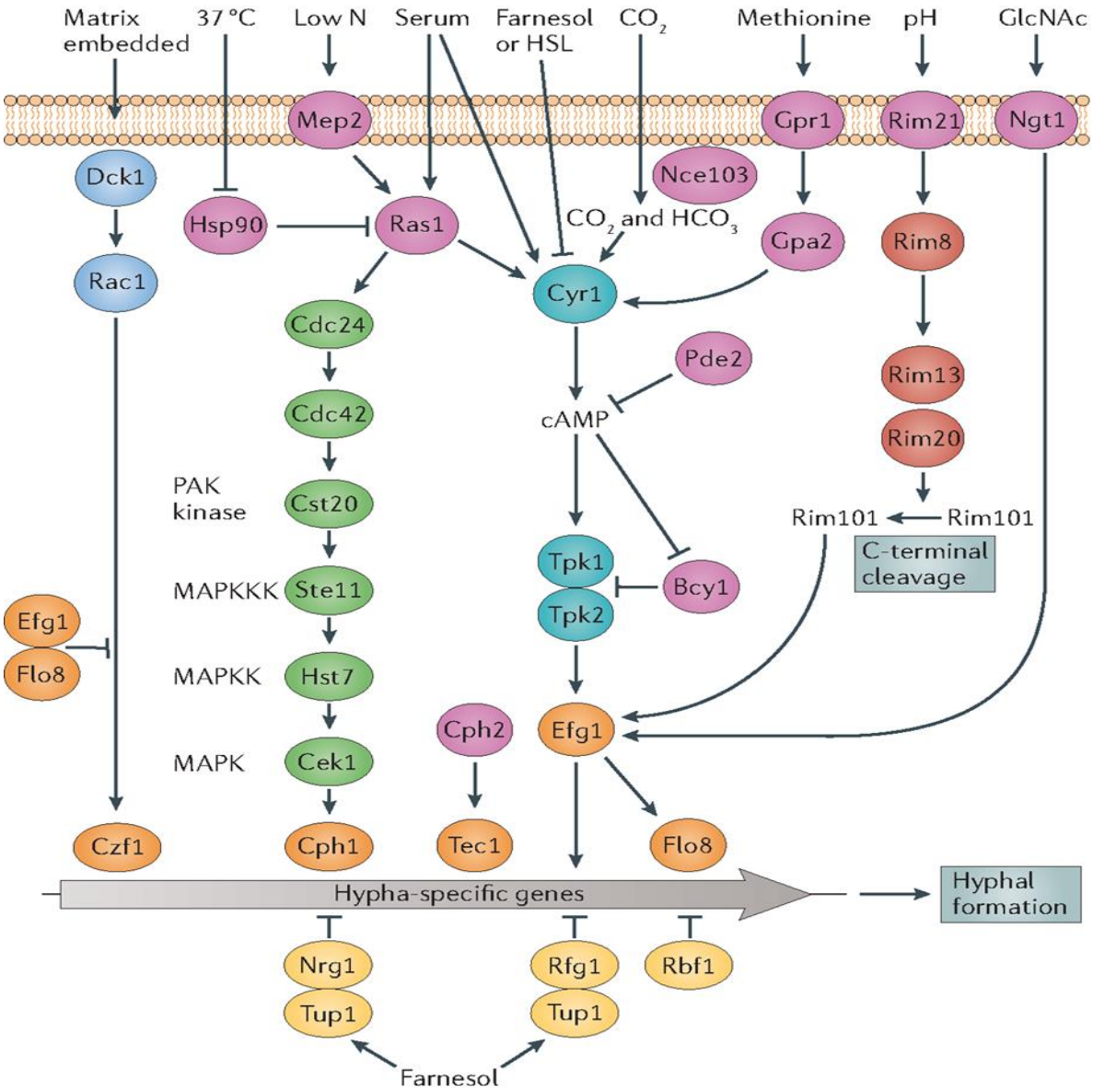


Figure 2.4| Signalling mechanisms controlling *C. albicans* hyphal induction or repression. A number of transcription factors are involved in the initiation of hyphal formation via multiple signalling pathways. Efg1 is one of the key transcription factors which meditate the response to several environmental cues through cyclic AMP-dependent pathway to induce filamentation. Hyphal repression induced by QS molecules such as farnesol or 3-oxo-C12 homoserine lactone (3OC12HSL) are mediated through negative regulatory factors Rfg1, Nrg1, and Tup1. Figure taken from [118].

2.2.1.2 *Candida albicans* infections

Although *C. albicans* belongs to the normal microflora, colonizing more than 50% of epithelial surfaces of healthy individuals [138, 139], it is one of the most commonly isolated human fungal pathogen capable of causing life-threatening infections when the host immune system is suppressed [139]. *C albicans* is also a major clinical problem in patients undergoing invasive clinical procedures as well as those on extended admissions in intensive care units. It is the fourth most common cause of nosocomial infections [119, 139].

One of the most frequently encountered invasive systemic infections linked with *C. albicans* involves the presence of prosthetic medical devices including totally implantable venous access devices (TIVADs), catheters, ocular lenses and other artificial implants [106, 140]. The fungus forms biofilm around these devices and releases hydrolytic enzymes including aspartyl-proteases and phospholipases resulting in systemic septicaemia [140-142]. Removing the device together with the administration of robust anti-fungal therapy leads to effective treatment [106].

In CF patients, some of the predisposing factors to *C. albicans* infection include inhaled steroids, lifelong antibiotics and diabetes mellitus, which generally dampen the immune system of these patients [106]. As a strong indicator for lung transplantation, CF also

presents an avenue for opportunistic infection of *C. albicans* during the post-transplant period. Notwithstanding its frequent recovery from sputum, oral and vaginal swabs of CF patients, the contribution of *C. albicans* to the disease progression is not clear [106]. An epidemiological study examining the occurrence of bronchopulmonary fungal infection and the contribution of each fungus to disease progression in CF showed that, *C. albicans*, regardless of its frequent isolation was responsible for only one case of candidiasis [112]. Anti-fungal actions of bacteria co-colonising with *C. albicans* in CF have been associated with this low prevalence of candidiasis [106, 112].

Aside being capable of causing severe invasive infections in immunocompromised patients, *C. albicans* causes other non-systemic infections such as genital candidiasis even in immunocompetent individuals with about 75% incidence rate [106, 141]. The fungus is also the causative organism of chronic mucocutaneous candidiasis (CMC), a persistent superficial infection involving mucous membranes, nails and skin epithelia [138, 143]. CMC has been associated with defects in cell-mediated immunity, specifically Th17, although the underlying mechanisms are yet to be fully explained [144]. This has been confirmed by recent data in patients with CMC where mutations in their STAT1, STAT3,

Interleukin (IL)-17 and IL-17RA correlated with reduced cytokine production by immune cells in response to *C. albicans* [138, 144].

Aside *P. aeruginosa*, there are other examples of cross-kingdom interactions between *C. albicans* and various bacterial pathogens. In mixed bacterial-fungal infections, the interaction can result in increased frequency or severity of disease; and in humans some of the diseases whose severity have been associated with polymicrobial infections include dental carries, keratitis, CF, burn wounds and urinary tract infections (Figure 2.5) [145, 146].

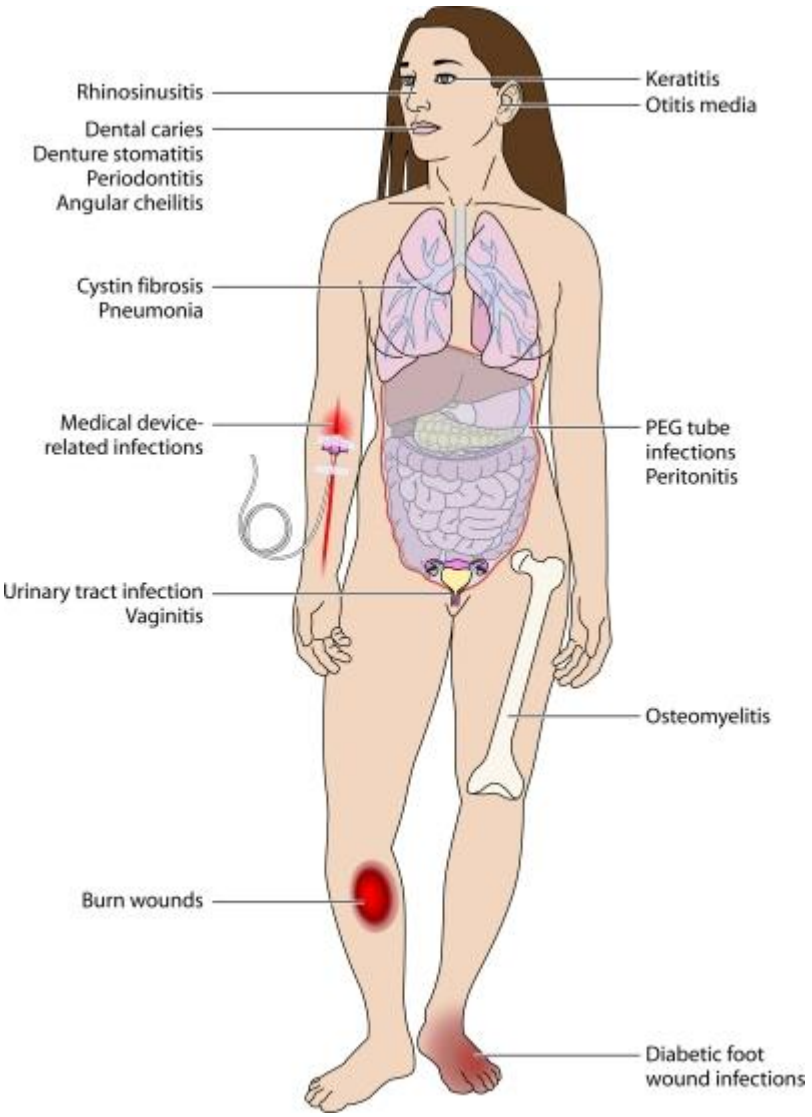


Figure 2.5| Representation of sites and types of diseases commonly associated with mixed fungal-bacterial infection in humans. Figure taken from [146].

Interaction between *C. albicans* and *Escherichia coli* has been shown to be cooperative, where prior urinary tract infection (UTI) with *E. coli* promotes the adhesion of *C. albicans* to bladder mucosa, thereby increasing the chances of fungal urinary tract infection [145, 147].

Likewise, inflammation of the oral mucosa in patients wearing dentures, referred to as denture stomatitis, is enhanced by the interaction between *Streptococcus gordonii* and *C. albicans* [145]. In this case, *S. gordonii*, a member of the microbiota in the oral cavity of the host, expresses cell surface polypeptides SspA and SspB (Antigen I/II family polypeptides) which anchors the bacterium to host cells [145, 148]. Subsequently, *C. albicans* binds to the surface-bound bacteria through protein-protein interactions, or through direct attachment to salivary proteins (basic proline-rich proteins, bPRPs) previously adsorbed by *S. gordonii* cells [145, 148-150].

As eukaryotes and prokaryotes employ variety of signalling mechanisms, the investigation of cross-kingdom interactions between *P. aeruginosa* and *C. albicans* is essential to understand the *in vivo* activities of the two organisms when in mixed infections.

2.3 Results leading to this project

The study of cross-kingdom communication in polymicrobial infections has gained attention due the influence of such interactions on microbial pathogenesis and ability to induce resistance to antimicrobial treatments. A number of reports have highlighted the co-existence of *P. aeruginosa* and *C. albicans* in opportunistic infections of humans, with much research still on going to fully

elucidate the molecular nature of the cross talk between these two organisms.

Purified exotoxin A has been shown to be highly toxic for mice [48], and a study conducted within our laboratory replicated this cytotoxic effect on human monocytes. Monocytes treated with supernatants from wild type (WT) *P. aeruginosa* strain (PAO1-L, Lausanne subline) displayed significant increased cytotoxicity compared to those treated with PAO1-LΔ*toxR* strain*,* which has a mutation in the regulatory gene that controls expression of both exotoxin A and rhamnolipid. PAO1-LΔ*toxA*, exotoxin A deficient strain, produced a comparable cytotoxic effect to that of PAO1-LΔ*toxR* and the negative control (Figure 2.6) which supports the role of exotoxin A as a major systemic virulence factor of *P. aeruginosa* [41].

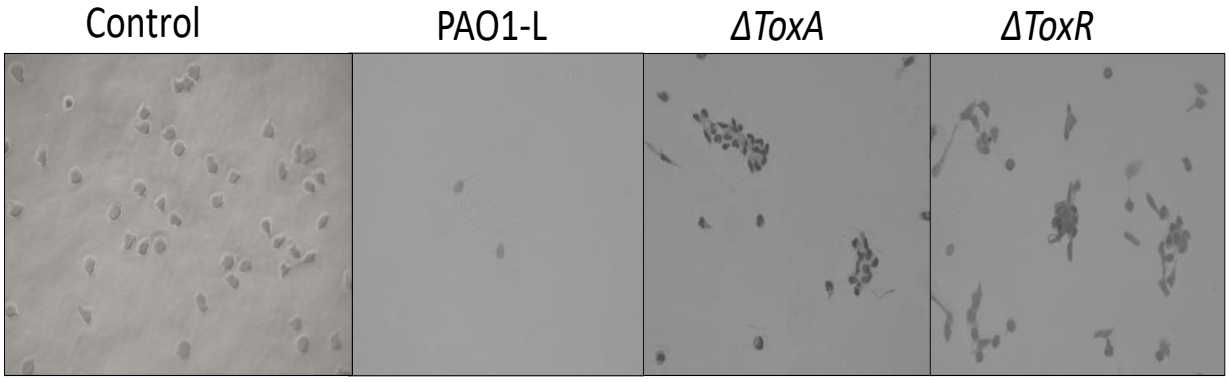


Figure 2.6| Exotoxin A dominates the cytotoxic effect of *P. aeruginosa* on human monocytes. Monocytes were seeded on glass coverslips and treated with supernatants (20% (v/v)) of PAO1-L, PAO1-LΔ*toxR* or PAO1-LΔ*toxA* for 16 hours. The samples were fixed with 4% Formalin and examined using Bright field microscopy. Data are representative from three independent experiments. Magnification=400x. (F. Hussain, PhD Thesis).

With the previous data from our group indicating that exotoxin A dominates the cytotoxic effect of *P. aeruginosa* on human monocytes, it was hypothesised that exotoxin A might confer an advantage to *P. aeruginosa* when in competition with *C. albicans*. This was based on the fact that expression of multiple virulence factors, including Exotoxin A, was upregulated in *P. aeruginosa-C. albicans* co-cultures [151]. These findings provided the preliminary data leading to the beginning of this work, and are reported in Farah Hussain's thesis.

2.3.1 Generation of PAAMP1 in PAO1 Lausanne background

A study in our laboratory which explored the role of RsmA (a post-transcriptional small RNA that regulates many virulence-related phenotypes), showed that PAO1- subline Nottingham (PAO1-N) behaved differently compared to PAO1-L (Mai-Prochnow, et al., manuscript in preparation). A *ΔrsmA* mutant in the PAO1-N background lost swarming motility, while swarming was maintained in a *ΔrsmA* mutant in PAO1-L background. Genomic comparison between the two strains revealed 58,569 base pair chromosomal deletion in PAO1-N, between bases 724103 – 780973, and that PAO1-L was closer to the PAO1 ancestor strain [152, 153]. Hxc-T2SS (PA0680 – PA0687), *toxR* (PA0707) and *bifA* are among the genes located in this region (Fig. 2.7).

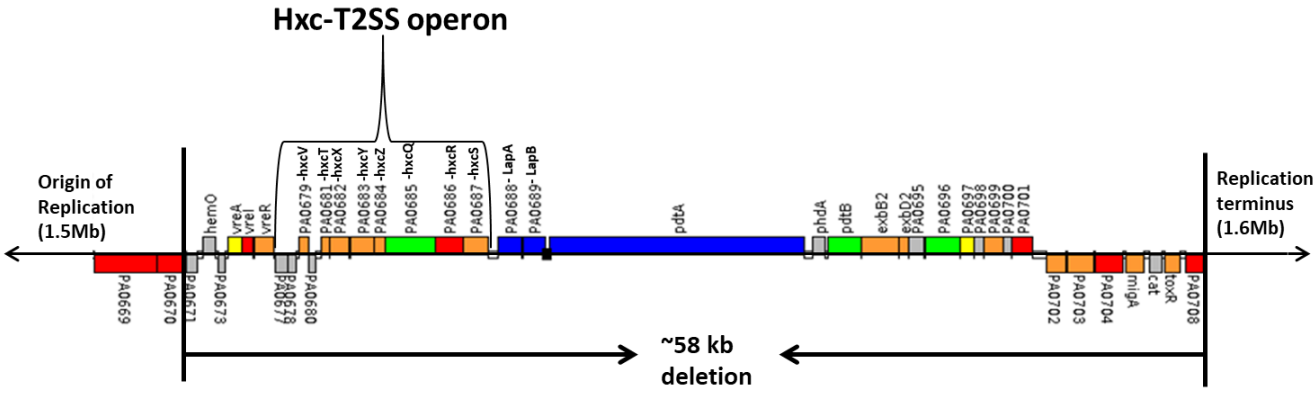


Figure 2.7| Genetic map of the 58 kb fragment deleted in PAO1-N strain. The diagram shows genes missing in the Nottingham strain of PAO1 in comparison to PAO1-L. Hxc-T2SS, *bifA*, *toxR* and genes encoding alkaline phosphatases *LapA* and *LapB* are among genes deleted in PAO1-N. Figure adapted from [154].

To understand how the absence of this genomic region affects bacterial virulence, a new in-frame deletion was constructed in the PAO1-L background and the new strain constructed was designated PAAMP1. The PAAMP1 strain was subsequently characterised, and its secretions tested on *C. albicans* by Farah Hussain (PhD thesis).

2.3.2 *P. aeruginosa* alters the growth of *C. albicans* under our experimental conditions

A fungal-bacterial supernatant culture system aimed at assessing the growth of *C. albicans* in the presence of *P. aeruginosa* secretions was carried out to identify potential changes by measuring OD_{600nm}. *P. aeruginosa* strains used in this study are described in Table 2.1 under Materials and Methods.

C. albicans cultures grown in the presence of WT PAO1-L supernatants reached lower OD_{600nm} compared to cultures treated with control medium. This observation was similar in *C. albicans* cultured in the presence of supernatants of *P. aeruginosa* strains deficient in the production of rhamnolipids, exotoxin A, pyocyanin, as well as the virulence regulator *toxR*. Interestingly, *C. albicans* cultures reached a higher OD_{600nm} comparable with that of the control when co-cultured with supernatants from PAAMP1 (Figure 2.8).

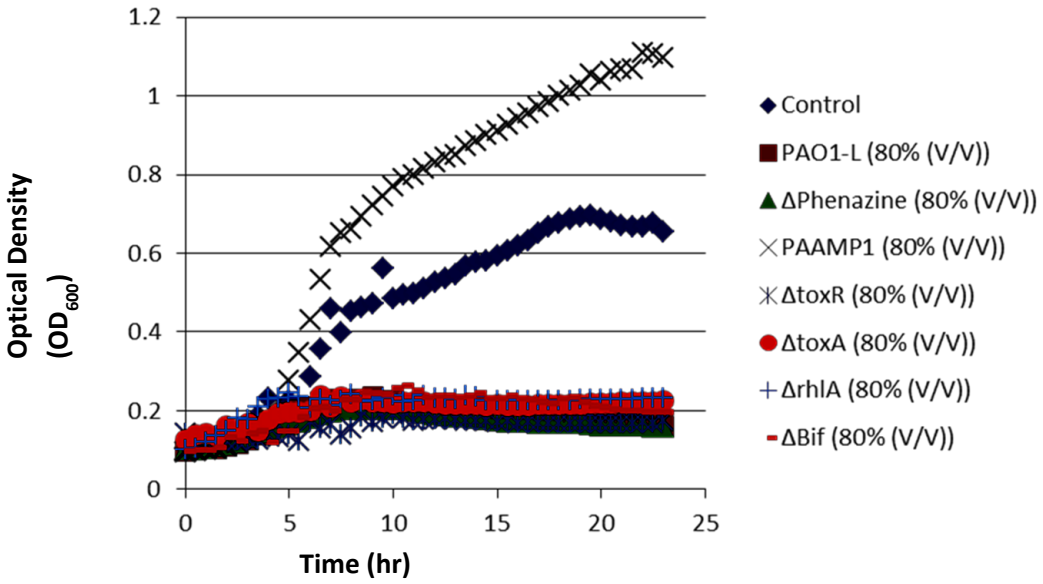


Figure 2.8| Growth response of C. albicans in the presence of supernatants from different *P. aeruginosa* strains. *C. albicans* cultures were incubated overnight at 37 °C in the presence or absence of 80% (V/V) bacterial supernatants prepared in X-vivo™ 15 or X-vivo™ 15 as control. Using a multimode microplate reader, the growth of *C. albicans* in each well was assessed by measuring OD_{600nm} (F. Hussain, PhD thesis).

2.3.3 Changes in *C. albicans* growth correlate with increased filamentation

To verify the morphology of *C. albicans* after the treatment with bacterial supernatant, cell suspensions were observed under light microscope. Microscopic examination of the cultures conditions revealed that *C. albicans* mainly grew as hyphae in the presence of PAO1-L supernatant and was not affected by secretions controlled by regulatory gene *toxR* (Arrows). Conversely, *C. albicans* treated with PAAMP1 supernatants typically grew as yeast similar to the control, with hyphae rarely observed in these cultures (Figure 2.9).

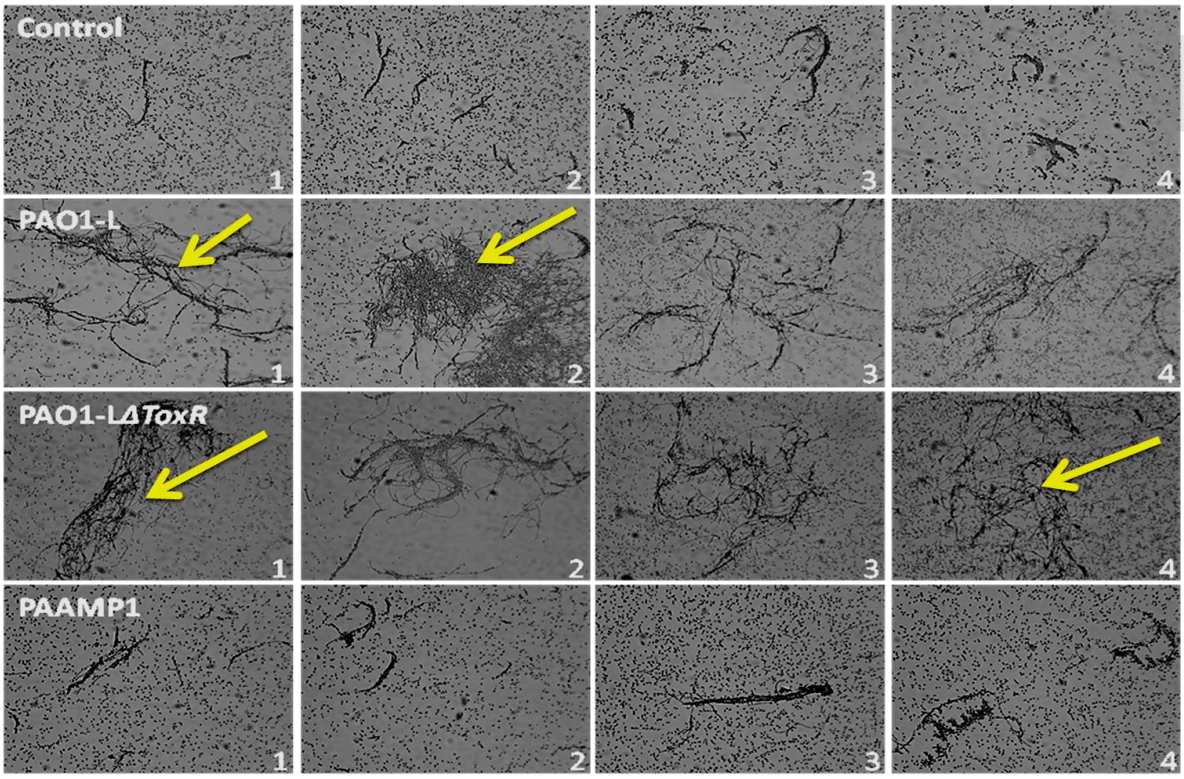


Figure 2.9| Changes in *C. albicans* growth correlate with increased filamentation. *C. albicans* was co-cultured with PAO1-L, PAO1-LΔ*ToxR*, or PAAMP1 supernatants in a rich YEPD medium at 37°C overnight. 10 µL of cell suspension was then observed using bright field microscope at ×400 magnification. (F. Hussain, PhD thesis)

To quantify the amount of hyphae produced by *C. albicans* after co-culturing with bacterial supernatants, 33 random microscopic images were taken at 20X magnification. Each image was divided into 99 squares and number of squares containing hyphae was quantified. Graphical representation of the hyphal quantification showed that *C. albicans* significantly grew as filament in the presence of supernatant from WT *P. aeruginosa*. On the contrary, PAAMP1 supernatant did not promote hyphal formation in *C. albicans* (Figure 2.10).

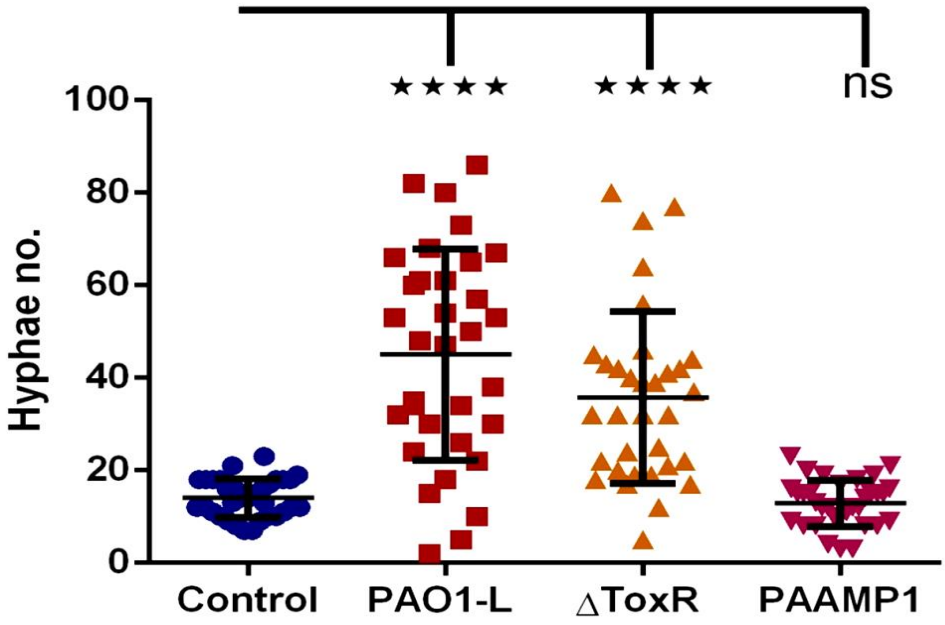


Figure 2.10| *P. aeruginosa* supernatant significantly induces filamentation in *C. albicans*. *C. albicans* was co-cultured with PAO1-L, PAO1-LΔ*ToxR*, or PAAMP1 supernatants in a rich YEPD medium at 37°C overnight. 10 µL of cell suspension was then observed under the light microscope with the ×20 objective lens. Results were obtained by counting number of squares containing hyphae out of 99 total squares in 33 random images per condition. One-way ANOVA and post-hoc multiple comparison test was used to calculate significance * * * * = P <0.0001. (F. Hussain, PhD thesis).

2.4 Hypothesis

Put together, the previous studies conducted by F. Hussain suggest that *P. aeruginosa* secretions induce filamentation of C. *albicans*, a key virulence determinant of this fungus [126]. Based on the absence of the hyphal inducing effect in PAAMP1 secretion, we hypothesised that the expression of the active hypha-inducing agent(s) depends on the presence of a 58 kb fragment within the *P. aeruginosa* genome.

2.5 Aim

To characterise and identify the active agent in *P. aeruginosa* secretions involved in inducing hyphal formation of *C. albicans*, biochemical separation approaches were used to determine its size and nature. This chapter also includes further optimisations of the fungal-bacterial supernatant co-culture system to address reproducibility challenges.

2.6 Materials and Methods

2.6.1 Preparation of *P. aeruginosa* supernatants

Table 2.1. |List of *P. aeruginosa* strains used in this study.

PAO1-L	Wild type, Lausanne subline	Dieter Haas' laboratory in Lausanne
PAO1-LΔ*phenazine*	Pyocyanin-deficient strain	Quorum Sensing Group
PAO1-LΔ*toxR*	PAO1-L mutated in regulatory gene that controls expression of both Exotoxin A and Rhamnolipid	Quorum Sensing Group
PAO1-LΔ*toxA*	*ToxA* chromosomal deletion mutant derived from PAO1-L	F. Hussain PhD thesis Lectin Biology Group
PAO1-LΔ*rhlA*	Rhamnolipid-deficient strain developed by *rhlA* in-frame deletion from PAO1-L	Quorum Sensing Group
PAO1-LΔ*Bif*	PAO1-L strain with a point mutation in the *Bif* gene	Quorum Sensing Group
PAAMP1	PAO1-L with a 58 kb chromosomal deletion between 724103 – 780973	Quorum Sensing Group
PAO1-LΔ*hxc*	*hxc* operon deficient strain in PAO1-L background	This study

Bacterial strains were streaked on Lysogeny Broth (LB) agar plate and incubated at 37 °C for 24 hours. A single colony was inoculated in 5 ml X-vivo[15] media (Lonza BE04-744Q) in a 50 ml falcon tube and incubated overnight at 37 °C and 200 rpm agitation. The overnight

cultures were diluted into 25 ml of fresh X-vivo™-15 media in 250 ml glass conical flask the next day, to optical density at 600nm (OD_{600nm}) adjusted to 0.01. The cultures were incubated at 37 °C, 200 rpm for 4 hours and subsequently centrifuged in a 50 ml falcon tube at 16,500×g (Beckman Coulter, Allegra X-30R) for 10 minutes at 4 °C. The supernatant was filtered with 0.22 µm filter to remove bacteria before use. Supernatants were routinely tested for the presence of live bacteria by plating 100 µl on LB agar.

2.6.2 Preparation of *C. albicans* culture

Wild type *C. albicans* strain SC5314 used in this study was kindly provided by Dr Simon Avery. *C. albicans* SC5314 is an invasive candidiasis isolate which has been studied worldwide as a reference strain of the fungus [155, 156].

The fungus was propagated on Yeast Extract Peptone Dextrose (YEPD) (Yeast extract OXOID, Bacteriological peptone OXOID, Agar SIGMA) agar plate and incubated at 37 °C overnight. A single colony was inoculated into 10 ml of YEPD broth in a 50 ml glass conical flask and incubated overnight at 37 °C, with agitation at 200 rpm. The next day, the overnight culture was adjusted to $OD_{600\ nm}$ = 0.5 in fresh 10 ml YEPD media to restart a new culture which was incubated at 37 °C, with agitation at 200 rpm for 4 hours. The 4 hour culture was treated with bacterial supernatants.

2.6.3 Co-culture of C. *albicans* in the presence of supernatants from *P. aeruginosa*

Cultures were set up in duplicates in a 48 well plate (Greiner Bio-One, Monroe, NC). The final culture volume per well was 300 µl with *C. albicans* growth adjusted to OD_{600nm} of 0.1. Plates were incubated at 37 °C with agitation at 200 rpm overnight using a Biotek PowerWave xs2 microplate reader (BioTek Instruments, Inc., UK) or Tecan Infinite® 200 PRO microplate reader (Tecan Group Ltd. Switzerland). *C. albicans* growth was measured at OD_{600nm}. The co-culture experiments were setup as summarised in Table 2.2.

Table 2.2. | Culture conditions of *C. albicans* treated with supernatants from *P. aeruginosa*

15 µl fungal growth (OD_{600} =2)+ 45 µl YEPD broth (2.33x) + 240 µl X-vivo™-15 (Control)
15 µl fungal growth (OD_{600} =2) + 45 µl YEPD broth (2.33x) + 240 µl supernatant (80% (V/V))
15 µl fungal growth (OD_{600} =2)+ 45 µl YEPD broth (2.33x) + 120 µl X-vivo™-15 + 120 µl supernatant (40% (V/V))
15 µl fungal growth (OD_{600} =2)+ 45 µl YEPD broth (2.33x) + 180 µl X-vivo™-15 + 60 µl supernatant (20% (V/V))

2.6.4 Fractionation of *P. aeruginosa* (PAO1-L, PAAMP1) supernatants

To assess the molecular size of the active agent(s) in supernatants from WT PAO1-L inducing *C. albicans* hyphal growth, supernatants were fractionated using Amicon® Ultra-15 centrifugal filters (Sigma-

Aldrich Co. LLC., UK) with 10 Kilo Dalton (kDa) pore size. The capped filter device was centrifuged in an angle fixed rotor at 3000×g (Beckman Coulter, Allegra X-30R) for 20min at room temperature. The concentrated supernatant remaining in the filter was recovered by inserting a pipette into the bottom of the filter device and withdrawing the sample using side-to-side sweeping motion to ensure total recovery. Both fractions (concentrate, >10 kDa, and flow through, <10 kDa) were stored at -80 °C until use.

2.6.5 Analysis of protein contents in supernatants by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

To analyse protein contents in supernatants from PAO1-L and PAAMP1 strains, 12 % SDS-PAGE was used. Below are compositions of the separating and running gels:

Table 2.3. |Separating Gel Solution (20 ml, 12% gel)

Materials	**Volume (Total= 20ml)**
H_2O	6.57 ml
1.5M Tris-HCl, pH 8.8	5 ml
Acrylamide/Bis-acrylamide (30:0.8)	8 ml
10% (w/v) SDS	200 µl
10% (w/v) Ammonium Persulfate (PSA)	200 µl
TEMED	30 ul

Table 2.4. |Stacking Gel Solution (Final volume: 10ml)

Materials	**Volume (Total volume= 10ml)**
H_2O	5.94 ml
0.5M Tris-HCl, pH 6.8	2.5 ml
10% SDS	100 µl
Acrylamide/Bis-acrylamide(30:0.8)	1.34 ml
10% (w/v) PSA	100 µl
TEMED	20 µl

45 µl of samples were mixed with 15 µl of 4× SDS-PAGE loading buffer (40% Glycerol, 240 mM Tris-HCl pH 6.8, 8% SDS, 0.04% bromophenol blue). As soon as the samples were added to the loading buffer they were boiled at 100 °C for 5 minutes in an Accublock digital dry bath (Labnet International, Inc., USA). The boiled samples were centrifuged for 20 seconds using an Eppendorf microcentrifuge (Fisher Scientific, UK). Using 6 µl of a 10-band multicolour recombinant ladder (Precision Plus Protein Kleidiscope, Bio-rad Lab. Inc., USA) as a molecular marker, 45 µl of sample were added onto the wells and the empty wells were filled with 1× SDS-PAGE loading buffer. Gels were run at 30 mA per gel at constant current using a Mini-PROTEAN Tetra vertical tank (Bio-rad, Life Sciences, UK). The gel was Coomassie stained (0.025% [w/v] Coomassie Brilliant Blue, 25% isopropanol, 10% acetic acid) and washed with several changes of de-staining solution (40% methanol, 10% acetic acid) until the background was transparent in order to analyse the resolved bands.

2.6.6 Heating inactivation of *P. aeruginosa* (PAO1-L, PAAMP1) supernatants

To determine if the active agent(s) in *P. aeruginosa* secretion inducing *C. albicans* filamentation was heat labile, supernatants prepared from PAO1-L or PAAMP1 strains were boiled in 1.5 ml Eppendorf tubes at 100 °C for 10min using an Accublock digital dry bath (Labnet International, Inc., USA). The supernatant was left to cool to room temperature before adding to *C. albicans* cultures as previously described. The heat-inactivated supernatants were tested for the presence of live bacteria by plating 100 µl on LB agar. No viable bacteria could be detected

2.7 Results

2.7.1 *P. aeruginosa* secretions affect the growth pattern of *C. albicans*

To determine if the hyphal inducing effect of *P. aeruginosa* secretion on *C. albicans* could be reproduced, a new co-culture experiment was independently setup using freshly prepared supernatants.

Propagation of *C. albicans* and preparation of *P. aeruginosa* supernatants as well as culture treatments were all done as described in materials and methods with the exception of the preparation of YEPD media which was done using HPLC-grade water instead of distilled water in the media prep room. This was to find out if the growth response of *C. albicans* was related to any background or signal interference with potential impurities in the water. A well-defined serum-free medium routinely used in our lab in infection assays of human macrophages with *P. aeruginosa,* X-vivo[15], was chosen to prepare the supernatants [107]. Using a multimode microplate reader, the growth of *C. albicans* in each well was assessed by measuring OD_{600nm} for 18 hours.

As shown in Figure 2.11, *C. albicans* cultures grown with supernatants from WT PAO1-L or PAAMP1 mutant reached OD_{600nm} values consistent with the preliminary data. This confirms the earlier

observation that *P. aeruginosa* secretions alter the growth pattern of *C. albicans*.

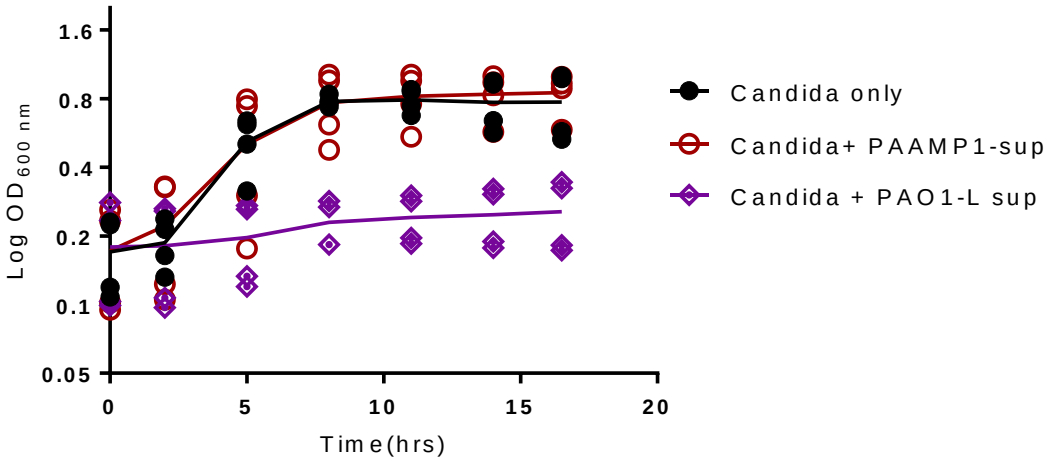


Figure 2.11 | *P. aeruginosa* secretions induce changes in growth in *C. albicans*. *C. albicans* was incubated overnight at 37 °C in a volume of 300 µl/well at an initial OD_{600nm} of 0.1 in the presence or absence of supernatants from WT PAO1-L, PAAMP1 strain or X-vivo™15 as a control. Bacterial supernatants were added at a concentration of 80% (V/V). Data represent individual replicates of two independent experiments with means connected.

Additionally, analysis of *C. albicans* growth after co-culturing with PAO1-L or PAAMP1 supernatants revealed that the active agent in PAO1-L secretions responsible for hyphal induction of *C. albicans* lost its effect at lower concentrations (Figure 2.12). This suggests that the hyphal inducing activity of PAO1-L secretions on *C. albicans* is concentration-dependent

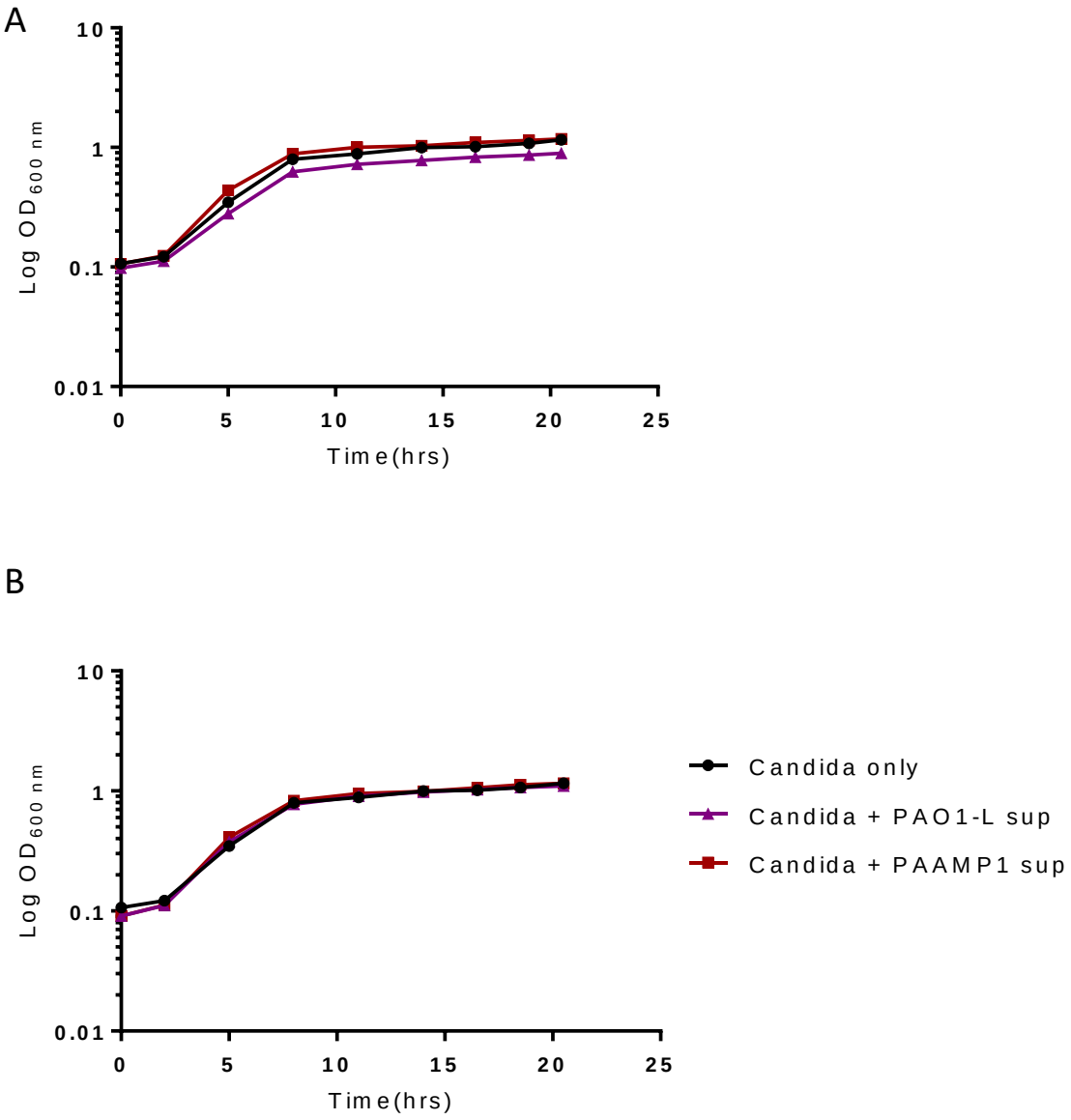


Figure 2.12 | Effect of *P. aeruginosa* secretions on *C. albicans* filamentous growth is concentration-dependent. *C. albicans* was incubated overnight at 37 ^{o}C in a volume of 300 µl/well at an initial OD_{600nm} of 0.1 in the presence or absence of supernatants from WT PAO1-L, PAAMP1 or X-vivo™ 15 as a control. Bacterial supernatants were added at a concentration of 40% (A) or 20% (B). n = 1

2.7.2 Identifying the active agent in *P. aeruginosa* supernatant responsible for stimulating hyphal growth in *C. albicans.*

With the above preliminary data suggesting that *P. aeruginosa* secretion alters *C. albicans* growth, and the observations showing that this effect was independent of the production of rhamnolipids, exotoxin A, or dependent on the virulence regulator *toxR*, a biochemical approach was utilised to determine the nature of the active component(s) in *P. aeruginosa* supernatant and their mechanism of action. Supernatants from PAAMP1 and the WT PAO1-L strain were fractionated using Amicon® Ultra-15 centrifugal filters having a cut-off pore size of 10 kDa. *C. albicans* was then co-cultured with different portions of the supernatant: above 10 kDa fraction diluted to 1X using X-vivo[15], below 10 kDa filtrate and non-fractionated supernatant.

Hyphal formation was induced after *C. albicans* was co-cultured with the above 10 kDa fraction of WT PAO1-L supernatant, which was comparable to that of the non-fractionated supernatant (Figure 2.13A). In contrast, the below 10 kDa fraction did not induce *C. albicans* hyphal growth (Figure 2.13B). Consistent with the previous observation, none of the fractions derived from PAAMP1 supernatant induced hyphal formation of *C. albicans.*

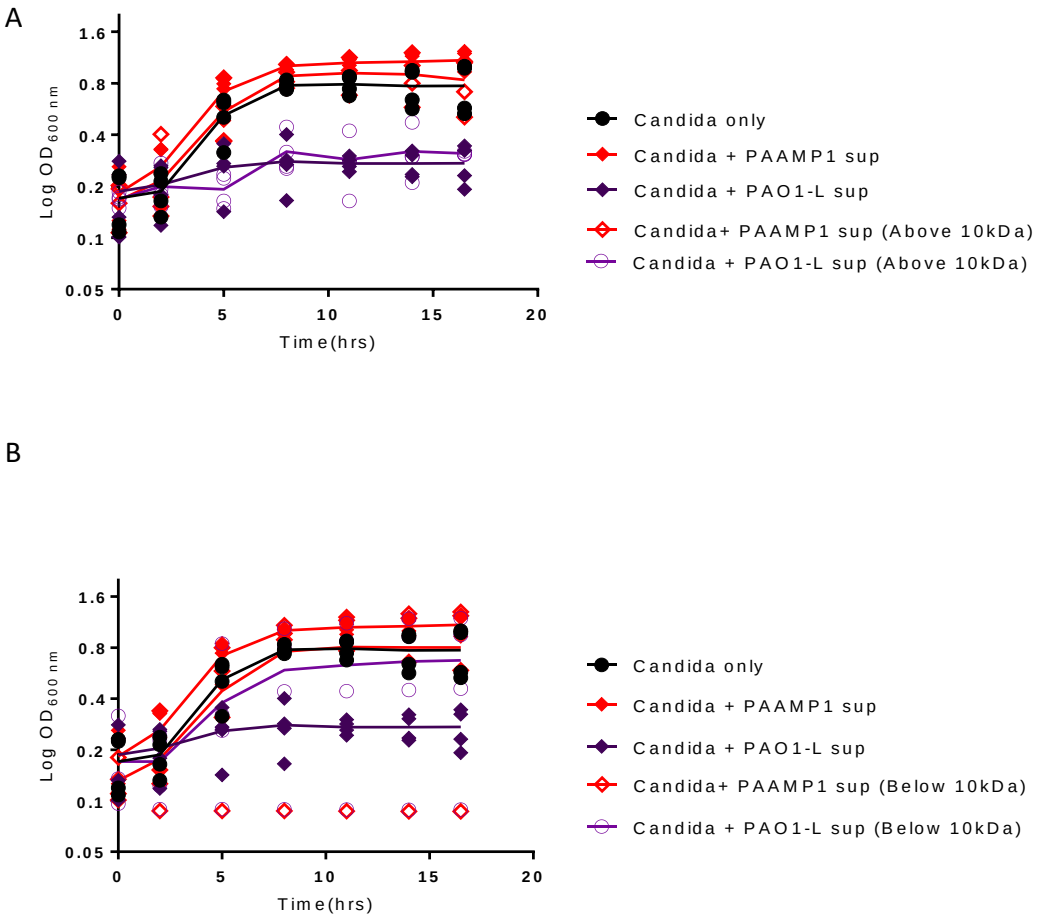


Figure 2.13 | The active agent in *P. aeruginosa* supernatant stimulating hyphal growth in *C. albicans* is above 10 kDa. Supernatants from WT PAO1-L or PAAMP1 strain were fractionated using Amicon® Ultra-15 centrifugal filters having a cut-off pore size of 10 kDa. *C. albicans* was then co-cultured for overnight at 37 °C in a volume of 300 µl/well and initial OD_{600nm} of 0.1 with different portions of the supernatants: (A) Above 10 kDa fraction or (B) Below 10 kDa fraction. Neat supernatants and X-vivo[15] as controls. Data represent individual replicates of two independent experiments with means connected.

2.7.3 The effect of WT supernatants on *C. albicans* filamentation is dominant.

In an attempt to determine the combined effect of supernatants from WT PAO1-L and the mutant PAAMP1 on *C. albicans*, the fungal cultures were incubated with different combinations of concentrated >10 kDa supernatant fractions (5x).

From Figure 2.14, consistent with previous observations, *C. albicans* cultured in the presence of concentrated WT PAO1-L supernatant (5×) reached a lower OD_{600nm} compared to the cultures treated with control medium or PAAMP1 supernatant (5×). *C. albicans* cultures grown in the mixture of supernatants from PAO1-L and PAAMP1 (5x final concentrations for both) had similar growth trend as those co-cultured only with the supernatants from the WT strain. The hyphal inducing agent in *P. aeruginosa* secretion is thus thought to have a dominant effect.

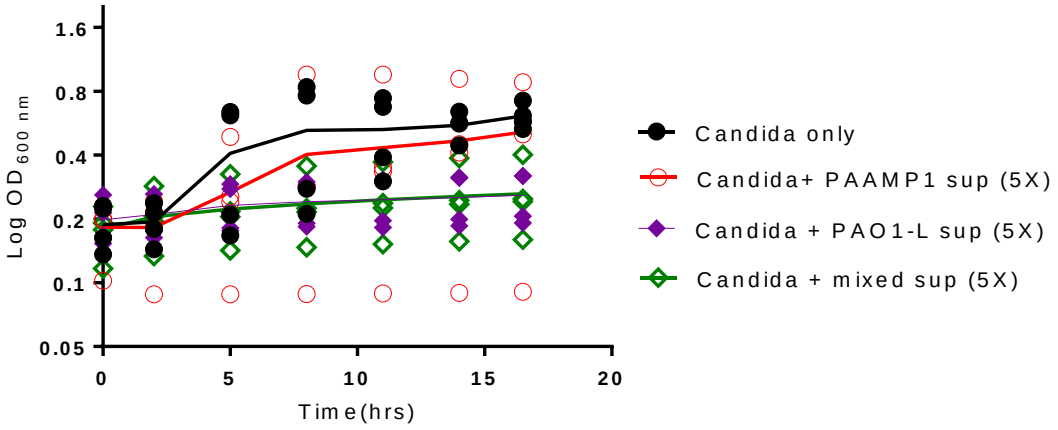


Figure 2.14| The effect of WT PAO1-L supernatants on *C. albicans* filamentation is dominant. *C. albicans* was incubated overnight at 37 ºC in a volume of 300 µl/well and initial OD_{600nm} of 0.1 in the presence or absence of 5X concentrated >10 kDa fractions of supernatants from WT PAO1-L and/or PAAMP1, or X-vivo[15] as a control. Data represent individual replicates of two independent experiments with means connected.

Further analysis of the fractionated supernatants showed that *C. albicans* cultures grown in the presence of higher concentrations of PAAMP1 supernatant (5×) resulted in lower OD_{600nm} of the fungal culture compared with *C. albicans* cultures treated with control media or intact supernatant from PAAMP1 (Figure 2.15).

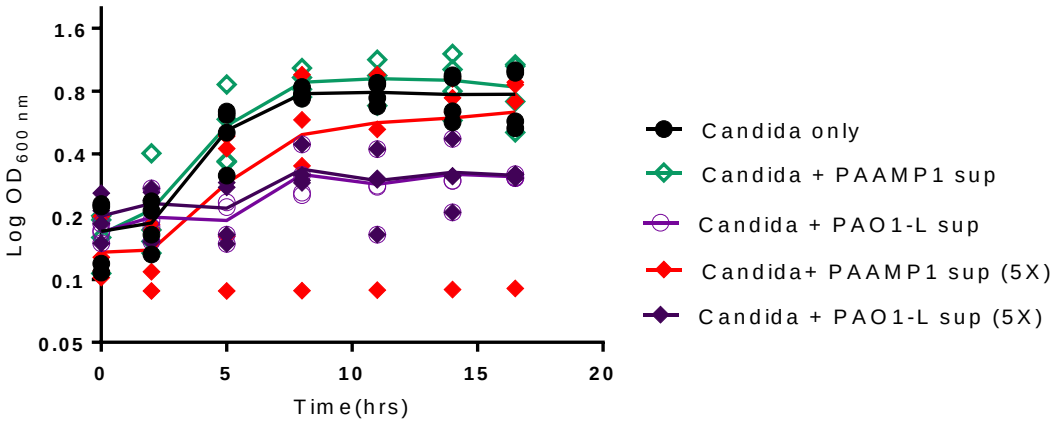


Figure 2.15| Concentrated supernatant from PAAMP1 strain alters *C. albicans* growth. *C. albicans* was incubated overnight at 37 °C in a volume of 300 µl/well and initial OD_{600nm} of 0.1 in the presence or absence of neat or 5x concentrated >10 kDa fractions of supernatants from WT PAO1-L, PAAMP1 or X-vivo[15] as a control. Data represent individual replicates of two independent experiments with means connected.

These results suggest that the active agent in *P. aeruginosa* secretions responsible for hyphal induction is possibly at a lower concentration in the PAAMP1 supernatant. Hence, once the supernatant from PAAMP1 strain is concentrated it reaches levels effective enough to promote hyphal growth of *C. albicans.*

2.7.4 Potential differences in protein content between WT and PAAMP1 supernatants

To identify proteins that are unique to the WT PAO1-L or the mutant PAAMP1 secretions, bacterial supernatants from the two strains were prepared and fractionated with a 10 kDa Amicon® Ultra-15 centrifugal filter as previously described. The above 10 kDa fraction of the supernatant was concentrated up to 10× the initial volume and

electrophoresed on a 12 % reducing gel at a 30 mA per gel and a constant current.

Analysis of bands resolved on the gel after Coomassie staining revealed a protein band with rMW between 25 – 30kDa specific for WT (Figure 2.16). Although the lanes were distorted, this preliminary result shows that there are potential protein candidates in the supernatants from PAO1-L which might be related to the induction of hyphal growth of *C. albicans*. The gel distortion (red arrow) is attributed to the presence of pharmaceutical-grade 66.5 kDa human serum albumin (HSA) in the X-vivo[15] medium used in supernatant preparation.

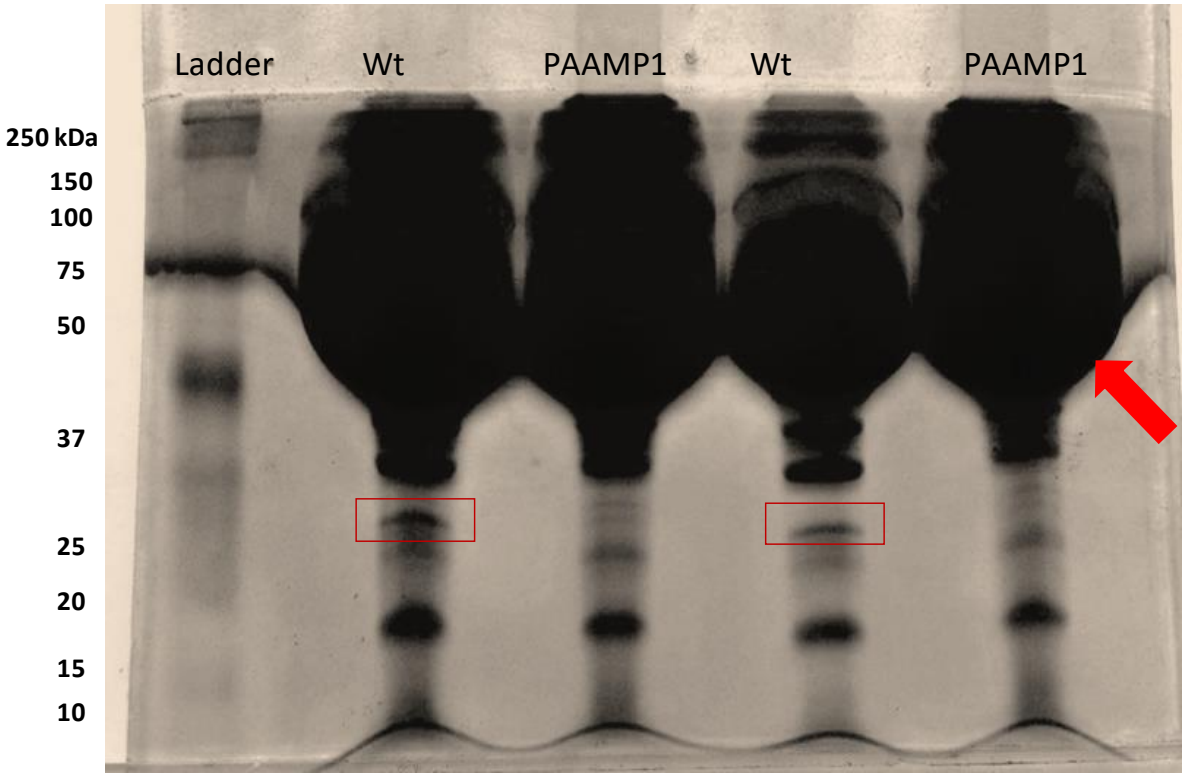


Figure 2.16 | Investigation of protein contents in supernatants from PAO1-L and PAAMP1. Supernatants from PAO1-L and PAAMP1 were prepared and fractionated as previously described and the above 10 kDa portions were examined for the presence of proteins by 12% SDS-PAGE. Bands were resolved on the gel after Coomassie staining. Red squares show bands unique to the WT PAO1-L supernatants. Gel also shows a disruption of the bands by human serum albumin (66.5 kDa, red arrow), a component of the culture medium X-vivo[15].

In an attempt to remove HSA from the bacterial supernatant to prevent it from disrupting the protein gel analysis, the supernatants were subjected to two-step filtration as illustrated in Figure 2.17. The supernatants were first filtered with Amicon ultra centrifugal filters of 50 kDa pore size to remove the 66.5 kDa HSA after which the flow-through believed to contain the putative hyphal inducing agent(s) was concentrated with 10 kDa filters.

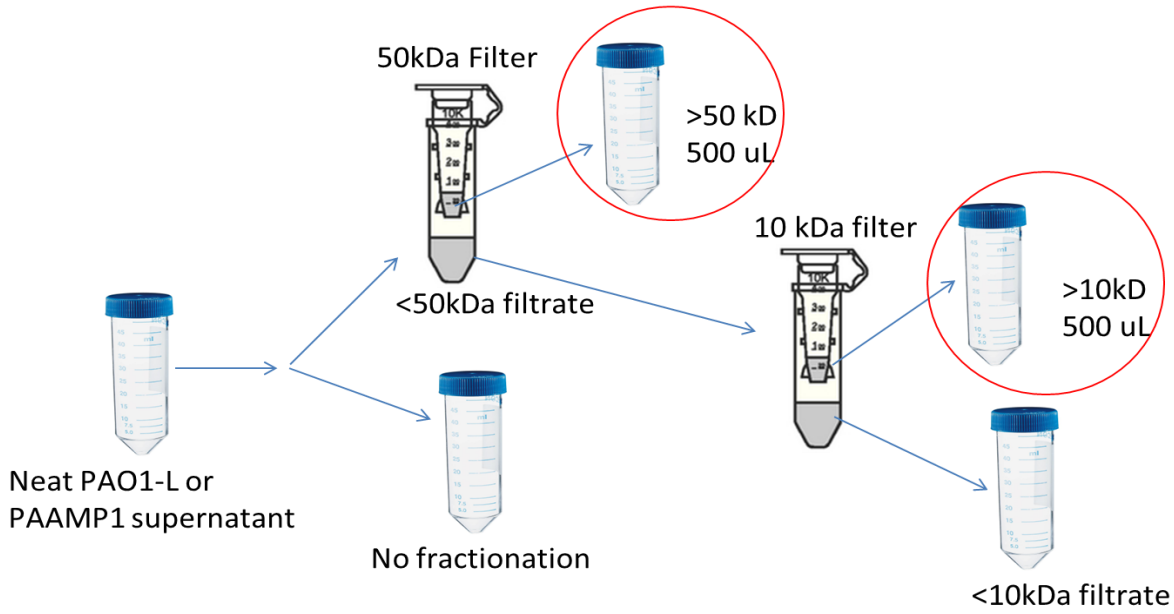


Figure 2.17| Illustration of two-step fractionation of *P. aeruginosa* supernatants. To prevent HSA protein from disrupting the SDS-PAGE gel analysis, supernatants from PAO-L or PAAMP1 strains were fractionated with a 50 kDa pore size Amicon filter before fractionating the filtrate with 10 kDa to concentrate the portion containing the proposed active agents. *C. albicans* were treated with portions highlighted with red circles.

When co-culture experiments were conducted with the fractions, hyphal inducing activity was retained in the 'above' 50 kDa concentrate (Figure not shown). It is thought that the active agent might be above 50 kDa, have aggregated to form a bigger molecule or the HSA might have blocked the filter thereby preventing the active agent to flow through.

2.7.5 **Heating *P. aeruginosa* supernatant inhibits hyphal induction activity**

To examine whether heat will have an impact on the active agent(s) in PAO1 WT supernatant, the supernatant was heated at 100 °C for 10 min and cooled, before adding to *C. albicans* cultures. The growth rate of *C. albicans* showed that hyphal inducing activity by PAO1-L supernatant was eliminated by heat as shown in Figure 2.18. As heat shock inactivates cellular proteins [152], this initial result confirms the possibility of the hyphal inducing agent being of protein nature.

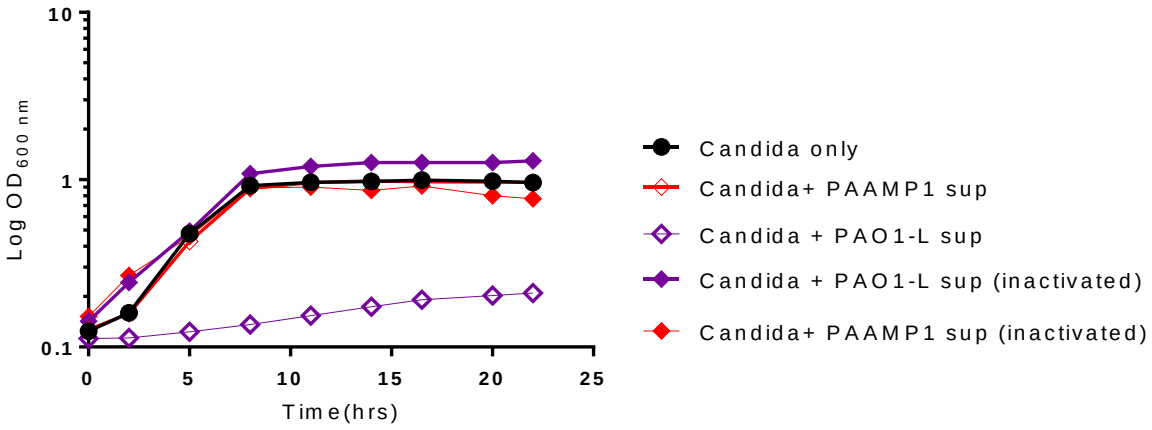


Figure 2.18| Active agent(s) in *P. aeruginosa* supernatant that alters *C. albicans* growth is heat labile. Supernatant from WT PAO1-L or PAAMP1 strain was heated at 100 °C for 10min. *C. albicans* was incubated overnight at 37 °C in a volume of 300 µl/well and initial OD_{600nm} of 0.1 in the presence or absence of heated or neat supernatants from WT PAO1-L, PAAMP1 mutant or X-vivo[15] as a control. n=1

Attempts to repeat this observation however did not yield the earlier result, as new preps of supernatant from PAO1-L comparable to

heated supernatant failed to induce *C. albicans* hyphal growth (Figure 2.19).

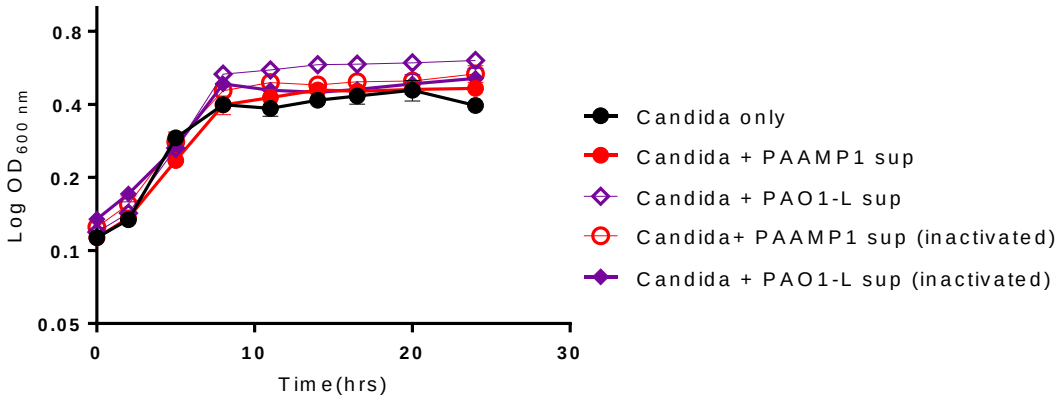


Figure 2.19| *P. aeruginosa* supernatant loses hyphal inducing activity Supernatant from WT PAO1-L or PAAMP1 strain was heated at 100 °C for 10min or left untreated. *C. albicans* was incubated overnight at 37 °C in a volume of 300 µl/well and initial OD_{600nm} of 0.1 in the presence or absence of neat heated or untreated supernatants from WT PAO1-L, PAAMP1 mutant or X-vivo[15] as a control. n=3

2.7.5 Loss of hyphal inducing activity in PAO1 supernatants

To reproduce the hyphal inducing activity of *P. aeruginosa* secretion on *C. albicans*, a number of steps as summarised in Figure 2.20 were taken including testing of different isolates of *C. albicans*.

Figure 2.20| Summary of steps taken to reproduce the hyphal induction of *C. albicans* by *P. aeruginosa* supernatant. To try to reproduce the preliminary observation of *C. albicans* filamentation in the presence of supernatants from WT PAO1-L, steps we took included among other things independent preparations of bacterial supernatants and fungal cultures, fractionation of supernatants, use of different isolates of *C. albicans* and use of different culture media.

Two people made supernatants of PAO1-L and PAAMP1 from new glycerol stocks while fungal inoculum from new *C. albicans* plate was kindly provided by Simon Avery's lab. The co-culture experiment was setup in a manner as previously described. As shown in Figure 2.21, the growth response of *C. albicans* to supernatants from PAO1-L and PAAMP1 strain were comparable.

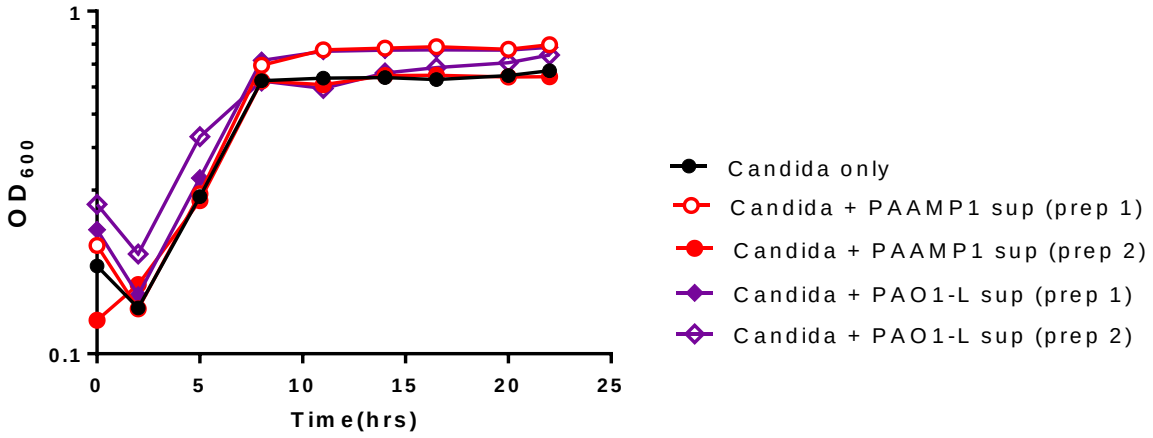


Figure 2.21| Loss of activity is not due to preparation of supernatants. Supernatants from WT PAO1-L or PAAMP1 strain were independently prepared and incubated with *C. albicans* overnight at 37 ºC in a volume of 300 µl/well and initial OD_{600nm} of 0.1 with X-vivo[15] as a control. n=1

Additionally, supernatants from the two strains were concentrated using Amicon® Ultra-15 centrifugal filters at a cut-off pore size of 10 kDa. *C. albicans* cultures were treated with the above 10 kDa fraction 5x concentrated. *C. albicans* showed no observable growth differences when co-cultured with supernatants from PAO1-L or PAAMP1 (Figure 2.22).

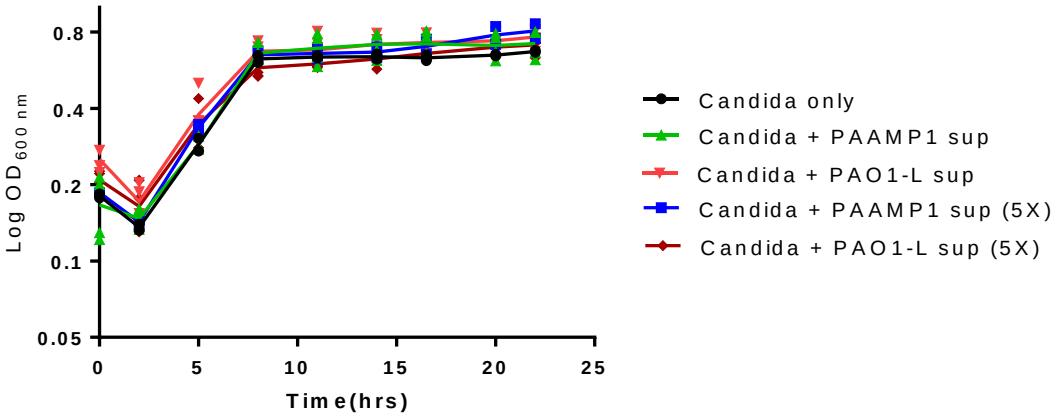


Figure 2.22| Loss of activity in *P. aeruginosa* supernatant cannot be compensated by concentration. Supernatants from WT PAO1-L or PAAMP1 were independently prepared and fractionated using using 10 kDa Amicon® Ultra-15 centrifugal filters. *C. albicans* was then co-cultured overnight at 37 ºC in a volume of 300 µl/well and initial OD_{600nm} of 0.1 with the above 10 kDa fractionate. Neat supernatants and X-vivo[15] were used as controls. Data represent individual replicates of two independent experiments with means connected.

To examine if culture conditions had an effect on the loss of activity, two different well-defined media, X-vivo[15] and DMEM-F12, were used to prepare the bacterial supernatants. The pH of the culture medium were measured to be at 7.2. *C. albicans* growth did not change in the presence of PAO1-L supernatant irrespective of the media used for the bacterial culture (Figure not shown).

Also, when two different plate readers, Biotek PowerWave xs2 microplate reader and Tecan Infinite® 200 PRO microplate reader, were used to measure the growth of *C. albicans* in an experiment independently setup by two people, no distinguishable growth differences were detected when the fungus was co-cultured with

supernatants from PAO1-L or PAAMP1 (Figure not shown). These results suggest that the loss of hyphal inducing activity by PAO1-L secretion was not due to sample preparation and handling, concentration of supernatant or growth conditions in the plate readers.

Furthermore, to determine if the hyphal induction of *C. albicans* is not strain specific, multiple strains of the fungus generously provided by Dr Rebecca Hall were tested at the University Birmingham. Bacterial supernatants from PAO1-L, PAAMP1 and PAO1-LΔ*hxc* (PAO1-L strain deficient of Hxc-T2SS, constructed as part of this study, Chapter 3) were prepared. *C. albicans* strains were cultured and setup in Birmingham by the collaborator using the established protocol in our lab. Growth patterns of all the *C. albicans* strains tested were similar showing no apparent differences when co-cultured with supernatant of PAO-L, PAAMP1 or PAO1-LΔ*hx* (Figure 2.23). This indicates that the loss of hyphal inducing activity by the PAO1-L strain was not only limited to the WT *C. albicans* strain originally used.

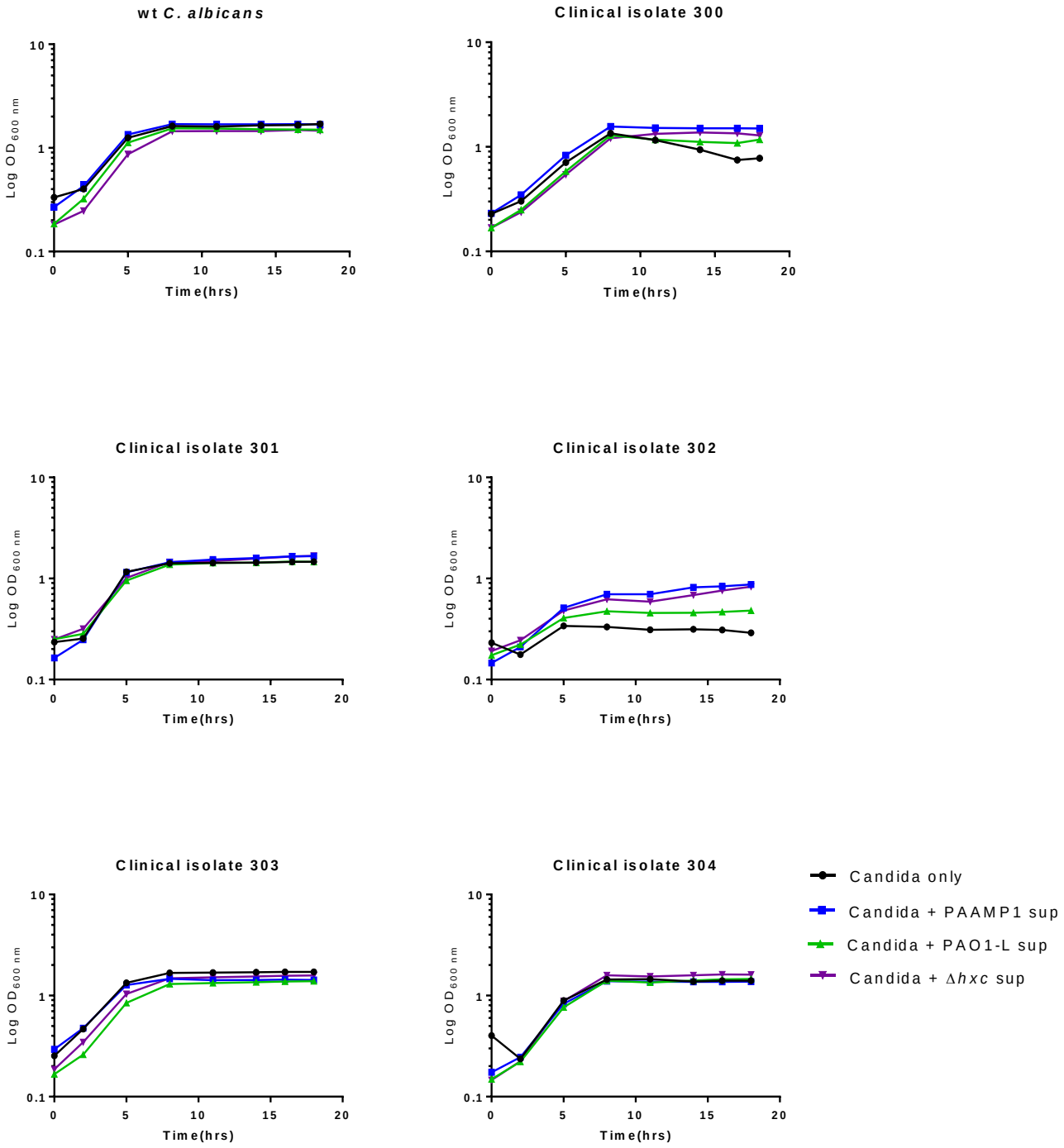


Figure 2.23 | Comparison of activity of *P. aeruginosa* supernatants on different *C. albicans* isolates. Supernatants from WT PAO1-L, PAAMP1 strain or PAO1-L*ΔHxc* mutant were independently prepared and incubated with different *C. albicans* strains overnight at 37 °C in a volume of 300 µl/well and initial OD_{600nm} of 0.1 with X-vivo[15] as a control. Clinical isolates 300 to 304 were kindly supplied by Dr Rebecca Hall.

2.8 Discussion

2.8.1 *P. aeruginosa* secretions affect the growth pattern of *C. albicans*

The characteristic feature of *P. aeruginosa* and *C. albicans* to co-localise and establish persistent infections in the same host niche highlights how these two organisms share many functional biological traits. Within the context of disease setting, both organisms cause opportunistic infections in immunocompromised patients, form biofilms on in-dwelling prosthetic devices, and evade host immune response through phenotype switching and secretion of QS molecules under stress conditions.

The relationship between these two taxonomically distinct organisms is very dynamic [108] and whether their interaction during infections is concerted or hostile to each other is generating interest, particularly with the emergence of drug resistant strains of these organisms during co-infection. It is therefore critical to understand the fundamental molecular mechanisms underpinning these cross-kingdom interactions and their significance to establishing persistent human infections [108]. History validates the significance of understanding such cross-kingdom interactions, notably the case of penicillin, which was discovered as a result of antibacterial action against fungal contaminants on culture plates [108, 157]

Based on the previous work in the lab by F. Hussain (PhD thesis), which showed that supernatants from *P. aeruginosa* WT PAO1-L induce filamentous growth of *C. albicans*, this study set out to validate the observation and also investigate the active agent(s) in the WT supernatants in comparison with supernatants from the PAAMP1 strain. It is worthy to note that this preliminary observation generated an interest as it was contrary to previous reports which had shown that yeast-hyphal transition of *C. albicans* is repressed when in mixed cultures with *P. aeruginosa* either by the bacterial QS signalling molecule HSL [158] or the fungal farnesol [119, 137].

C. albicans culture and *P. aeruginosa* supernatants were independently prepared, and using fungal-bacterial supernatant co-culture system, the growth of *C. albicans* was assessed by measuring OD_{600nm}. The initial results indicated that *C. albicans* cultured in the presence of supernatants from PAO1-L displayed lower OD_{600nm} compared with supernatants from PAAMP1, an observation consistent with the previous data. Microscopic examination of the co-cultures with supernatants from WT PAO1-L revealed a positive correlation between low OD_{600nm} with increased growth of filamentous forms of *C. albicans.* An *in vitro* study has demonstrated that *P. aeruginosa* selectively adheres and kills hyphae of *C. albicans*, but not the yeast cells [3]. Under our experimental conditions, the initial *C. albicans* hypha-inducing effect by PAO1-L secretions therefore could

presumably be a strategy by *P. aeruginosa* to out-compete the fungus by inducing filamentation and subsequently killing it. Also, since filamentation of *C. albicans* increases the invasiveness of host tissues by the fungus [122], it is suggested that *P. aeruginosa* stimulates hyphal growth of *C. albicans* to advantageously disseminate during polymicrobial infection by attaching to the hyphae.

The ability of *P. aeruginosa* to selectively kill hyphae has been reported to be dependent on both contact-mediated and secreted virulence factors such as type IV pili, haemolytic phospholipase C, and phenazine derivatives – pyocynanin and 1-hydroxyphenazine [3, 159, 160]. The preliminary work thus compared supernatants from different strains of *P. aeruginosa* lacking virulent factors such as rhamnolipids, exotoxin A, pyocyanin or the virulence regulator *toxR;* to study their effects on the fungal growth. *C. albicans* grew at lower OD_{600nm} values in response to each of the virulence-determinant deficient strain, which were comparable to the PAO1-L suggesting that these molecules are not responsible for this effect. Conversely, *C. albicans* cultured in the presence of supernatants from PAAMP1 strain remarkably grew at higher OD_{600nm} similar to the control medium. The 58 kb chromosomal deletion in PAAMP1 strain as mentioned earlier has the regulatory gene *toxR,* which controls cytotoxic molecules rhamnolipids and exotoxin A, amongst the deleted sequence. This is a further confirmation that extracellular

virulence factors regulated by *toxR* were not responsible for the hyphal induction of *C. albicans.* As *toxR* also regulates the Rhl QS pathway by controlling the expression of C4-HSL [161], this result raises the prospect that the active agent(s) in the WT secretion is possibly controlled by a *P. aeruginosa* locus independent of this QS system.

To identify the molecular nature of the hyphal inducing agent(s) in *P. aeruginosa* secretions, we fractionated supernatants from PAO1-L and PAAMP1 with a 10 kDa pore-sized filter and tested each fraction for hyphal inducing effect in a fungal-supernatant co-culture system. The hyphal inducing effect was retained in the above 10 kDa fraction of the WT supernatant. Interestingly, 5x concentrated above 10 kDa fraction of PAAMP1 supernatant induced *C. albicans* hyphal growth; indicating that the active agent(s) in PAAMP1 secretion might be produced by this strain but in lower amounts. As previously mentioned, *P. aeruginosa* has a relatively large genomic size, having multiple copies of secretion systems and overlapping secreted products [154]. It is thus suggested that other gene(s) outside the deleted domain may compensate the 58 kb deletion in PAAMP1.

P. aeruginosa is an active protein secretion bacterium with most of the products incidentally being released into the extracellular medium [94]. To examine whether *P. aeruginosa* secreted protein(s) were

involved in the hyphal induction of *C. albicans*, we brought supernatants of PAO1-L and PAAMP1 to 100 °C for 10 min before adding to the fungal cultures. Heat treatment removed the hyphal inducing activity from the supernatant compared to intact PAO1-L supernatant. Several studies have reported inactivation effect of heat stress on *P. aeruginosa* secretants. For instance, heating bacterial supernatant to 85 °C for 10 min was reported to eliminate its inhibitory effect on proliferation of mammalian dendritic cells and macrophages [162]. Also, degradation of inflammasome components such as caspase-1 by *P. aeruginosa* alkaline protease (AprA) was abolished when supernatant was heated at 98 °C for 30 min [163]. Put together, the above preliminary observations demonstrate that the ability of PAO1-L supernatants to induce hyphal growth may be due to a heat-labile protein(s).

2.8.2 Loss of hyphal inducing activity in PAO1 supernatants

Reproducibility of results is a fundamental basis in the conduct and validation of experimental science [153]. The ability to reproduce an observation is not only an attempt to show that an effect is not as result of chance or artefact, but also confirms the robustness of a particular experimental design. However, there are instances where one-time observations, though not reproducible, can point to an invaluable source of scientific information [153].

With the loss of hypha-inducing effect of PAO1-L supernatant on *C. albicans* during the course of this study, several attempts were made to understand the changes in the hyphal phenotype of *C. albicans* and to reproduce earlier observations. To eliminate the possibility of human error leading to the loss of effect, independent preparations of bacterial and candida cultures were carried out as well as the use of two different plate readers. Yet, *C. albicans* growth response to supernatants from PAO1-L and PAAMP1 was comparable to the media control. Fractionation of the supernatants to concentrate the hypha-inducing agent(s) did not compensate the loss of activity neither did the use of different media or different isolates of *C. albicans* produce the earlier observation.

Owing to its metabolic plasticity and ubiquity, *P. aeruginosa* PAO1 has been the most frequently used strain for research. Studies have reported genotypic diversity of PAO1 strains during its distribution worldwide with some sublines showing marked differences in fitness, antimicrobial susceptibility and virulence [164]. Comparison between reference sequence PAO1-UW (University of Washington) and sublines MPAO1 (widely used source of transposon library) and PAO1-DSM (German Collection of Microorganisms and Cell Cultures (DSMZ)) revealed major differences such as a 12 kb insertion in the 3’ end of a $tRNA^{Met}$ in PAO1-DSM and MPAO1, a 1 kb deletion about 12 kb downstream of the insertion and about 39 validated single-

nucleotide deviations (SNPs) [164]. Additionally, a study which compared 49 clinical isolates of *P. aeruginosa* recovered from various wound sites from individual inpatients and outpatients at the Queen's Medical Centre in Nottingham showed varied QS phenotypes. It was revealed that two of the isolates produced high levels of N-acylhomoserine lactone (AHL) QS signal molecules (QSSMs) and low levels of alkyl quinolone QS molecules, an observation previously not been found in clinical isolates of *P. aeruginosa* (Hardeep Naghra, PhD Thesis). As previously mentioned under Section, 2.3.1, PAO1-N was found to have lost 58 kb of genes compared to PAO1-L. Evidently, lab strains of PAO1 could be undergoing microevolution as a result of its intrinsic genomic features and laboratory procedures, which may trigger changes in phenotype [164]. However, in our case the findings point to a change in the culture conditions of *P. aeruginosa* or *C. albicans* that we cannot account for rather than genomic changes, since we could not reproduce the earlier observations even when original stocks of the organisms were tested. In summary, our observation of *C. albicans* hyphal induction by *P. aeruginosa* secretion will require further work to reach a conclusion.

Chapter 3: Generation and characterisation of a *Pseudomonas aeruginosa* mutant deficient of the hxc-Type 2 secretion system

3.1 Introduction

The ability of *P. aeruginosa* to inhabit many niches and cause infection has been associated with its metabolic versatility and the release of numerous toxins into the environment. To survive in the otherwise hostile environment of the host, *P. aeruginosa* relies on a number of proteins that are selectively produced by the bacterium which could remain attached to its cell surface, be released into the extracellular medium or be injected into the host cytosol [81]. Secretion of these proteins are facilitated by specific transport systems which *P. aeruginosa* possesses five of the six identified in Gram negative bacteria as previously mentioned.

Among the five secretion machineries, the type 2 secretion system, also referred to as secreton, is the most highly conserved in Gram negative bacteria; with prevalence not only in human pathogens but also in bacterial pathogens of plants such as *Pseudomonas fluorescens and Xanthomonas* species, and animal pathogens including *Aeromonas hydrophila* (Figure 3.1) [165]. The set of genes that encodes a single T2SS, designated as *gsp* (general secretion pathway), varies among species ranging from 12 in *P. aeruginosa* and *X. campestris* to as many as 15 in *Vibrio cholerae* and *Klebsiella oxytocae* [166, 167]. *Pseudomonas* and non-*Pseudomonas* T2SS genes have different nomenclature; for instance $gspE_R$ in *V. cholera, E. coli* ETEC or *A. hydrophilia* is denoted as $gspR_E$ in *P. aeruginosa or*

X. campestris [166]. Also, some bacteria have specific names for each T2SS gene cluster such as *xcp* or *hxc* for *P. aeruginosa* which could can be used to substitute *gsp*. In this thesis, *xcp* and *hxc* will be used when describing *P. aeruginosa* T2SS genes while *gsp* will be used when describing T2SS genes in Gram negative bacteria in general.

The T2SS genes are organised into operons made up of conserved 'core' genes denoted as $gspC_P$ to O_A and non-core extra genes such as *gspAB*, *gspN* or *gspS* found in some bacterial species (Fig. 1) [166]. Although rare exceptions exist, mutation in any of the *gsp* gene can inhibit the secretion of exoproteins thereby leading to their accumulation in the periplasm, [166]. For example in *P. aeruginosa*, a strain carrying a mutation in *xcpQ,* one of the core genes which encodes the secretin was still found to secrete T2SS-dependent exoproteins. This was attributed to the presence of another homologue, *xqhA* [168].

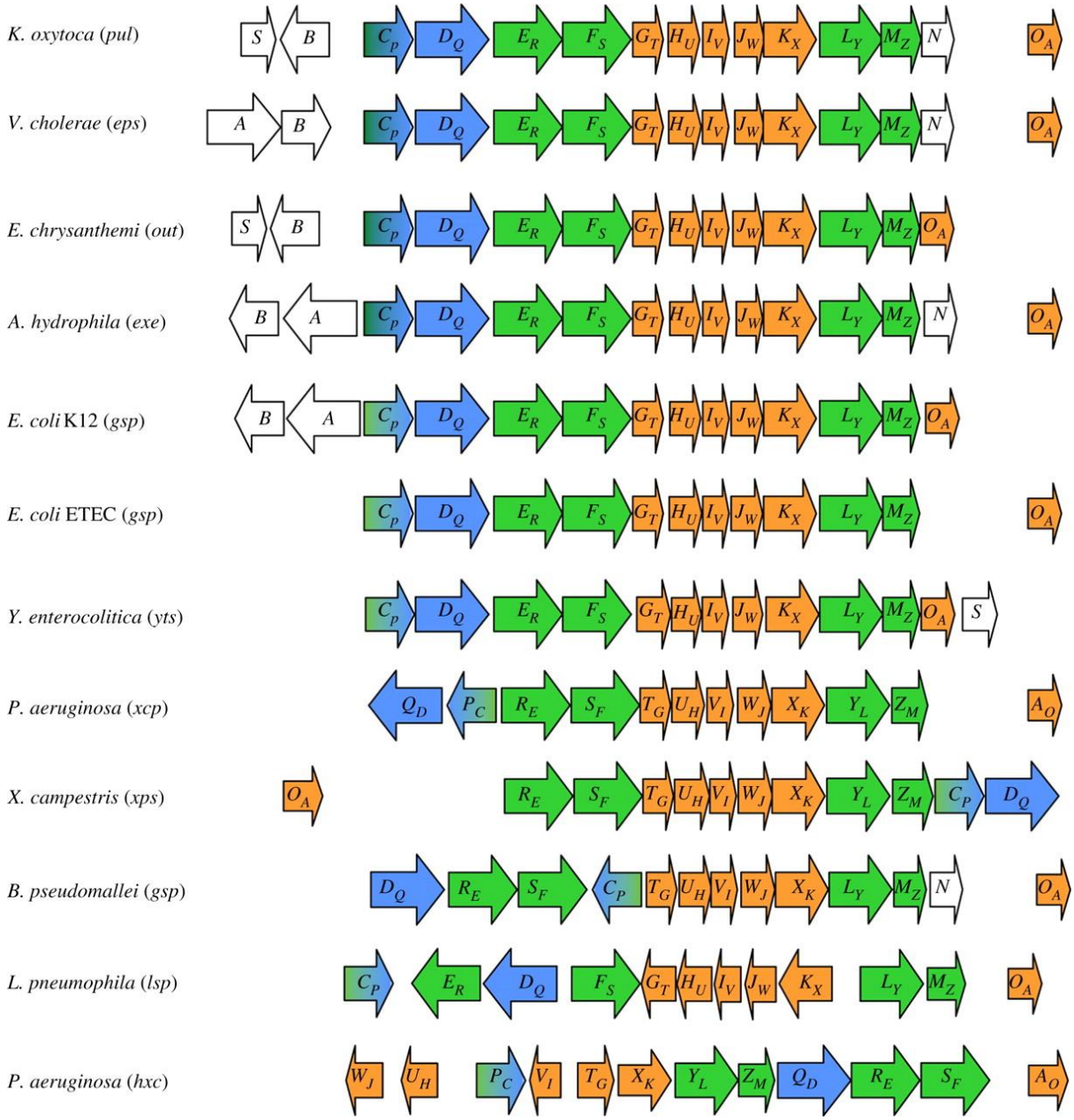


Figure 3.1 | Genetic organization of the T2SS clusters. The name of each T2SS gene cluster is shown in brackets beside the name of the bacterial species. Each gene is represented by an arrow and 'core' genes found in all T2SS clusters are coloured. The genes encoding components of the inner membrane platform are shown in green; the genes encoding pseudopilins and *gspO*$_A$ gene encoding the prepilin peptidase are shown in orange; the gene encoding the secretin is shown in blue; the gene encoding the trans-periplasmic protein is represented in shaded green and blue tones because GspC$_P$ is a component of the inner membrane surface which interacts with secretin in the outer membrane. The non-core genes: *gspA*, *B*, *N* and *S* are represented in white. Figure taken from [166].

3.1.1 The secretion pathway and structural organisation of the Type II secretion system

Encoded by 12 – 15 different genes, there are about 40 - 70 proteins that are involved in the assembly of the Type 2 nanomachine [83]. With such complexity, there are still many grey areas about the biogenesis and sequence of assembly of the components as well as substrate recognition by the secreton.

As illustrated in Figure 3.2a, proteins destined to be secreted into the extracellular environment via the T2SS undergo two-step processes, where they are first exported across the inner membrane (IM) into the periplasmic space before they are transported across the outer membrane (OM)[166]. Depending on its structural conformation, translocation of the cytoplasmic protein into the periplasm could occur either via Tat or Sec export system as previously described (Chapter 1, Section 1.2.4).

Once in the periplasm, the folded proteins are transported into the extracellular media through the T2SS which consists on a trans-membrane machinery made up of three sub-complexes, namely: inner membrane platform (IMP), the pseudopilus and the outer membrane secretin [166]. The structural arrangement of the secreton has been illustrated in Figure 3.2b. Briefly, the IMP consists of Gsp-C_P, -F_S, -L_Y and -M_Z proteins connected by a cytoplasmic traffic

ATPase $GspE_R$ at the base. Multi-domain proteins $GspM_Z$ and $GspC_P$ are believed to ensure the stability of the inner membrane complex while $GspE_R$ provides energy for the assembly of the machinery through its ATPase activity [166, 167].

The pseudopilus is the core of the T2SS which is a piston-like structure that pushes exoproteins through the OM channel. Six of the conserved *gsp* genes are involved in the formation of the pseudopilus where five of those genes, $gspG_T$-K_X, encode psuedopilins which assemble to form the pseudopilus, while $gspO_A$ codes for the prepilin peptidase required for the maturation of the psuedopilins [83, 166]. The proteins are named psuedopilins due to their amino-terminal sequence homology to type IV pilins and their dependence on prepilin peptidase for maturation [83]. They are initially produced as precursors having a short chain of 6-7 positively charged peptides which is then cleaved off by prepilin peptidase [166]. $GspH_U$, I_V, J_W and K_X are described as minor pseudopilins while $GspG_T$ is described as a major pseudopilin due to its abundance and ability to assemble into a long fibrillar structure called hyper pseudopilus (HPP) [166].

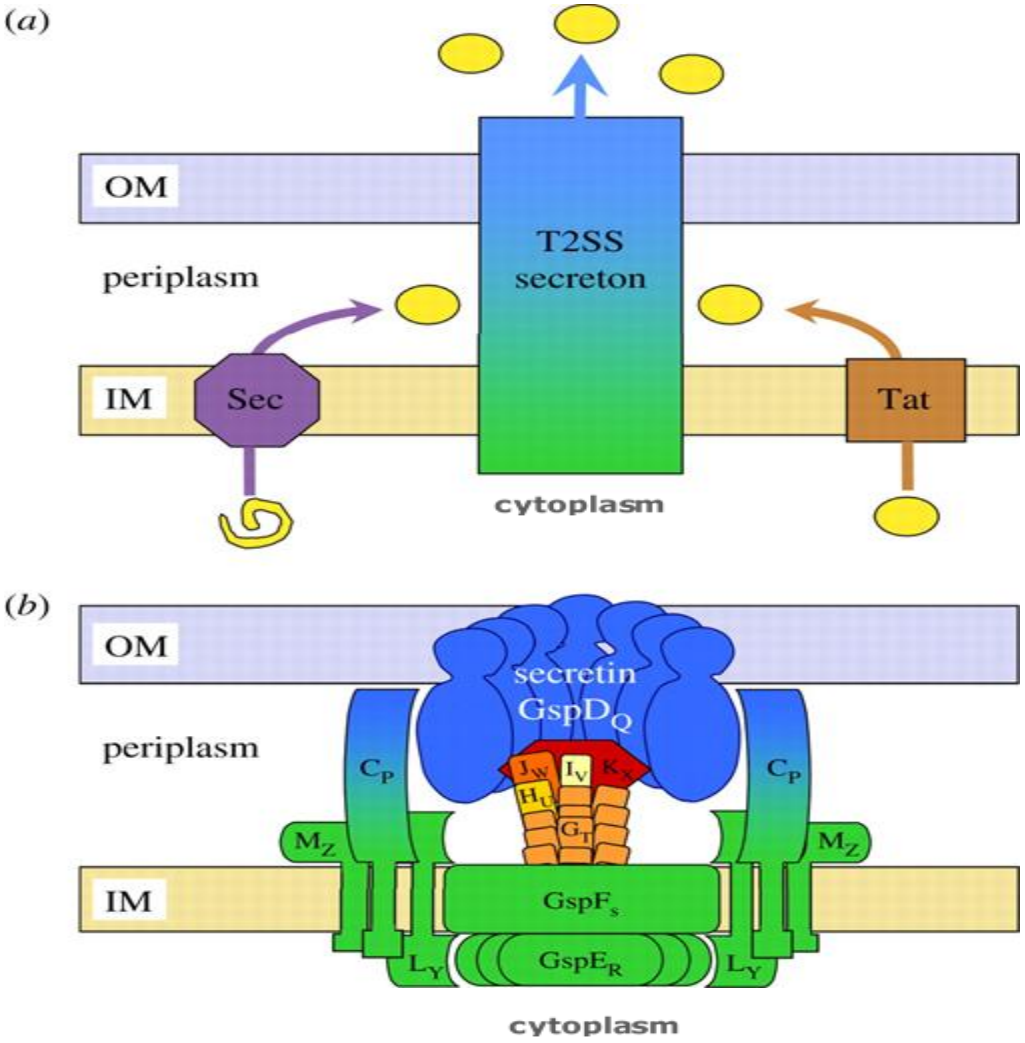


Figure 3.2 | Model of the secretion pathway and structural organisation of the Type II secretion system in Gram-negative bacteria. (*a*) The T2SS-dependent exoproteins (yellow circles) are first exported across the IM via the Sec (purple) or Tat (brown) machineries. The exoproteins are subsequently recognized and transported across the OM by the secreton (blue/green). (*b*) The secreton is divided into three sub-complexes: Secretin $GspD_Q$ (blue) forms a pore in the OM through its C-terminal domain whilst its N-terminal part protrudes in the periplasm. The IM platform and the traffic ATPase $GspE_R$ are coloured green. The transperiplasmic protein $GspC_P$ (blue/green) links the secretin to the IM platform. The pseudopilus, made up of major and minor psuedopilins is shown in orange and red. Figure adapted from [166].

Extending through the OM is the secretin which is made up of $GspD_Q$ protein homo-multimers which assemble as a ring-shaped structure to form a pore of 50–80Å in diameter [166]. $GspD_Q$ is not only involved in the assembly of T2SS, but also in T3SS, type IV pilus and the filamentous-phage assembly system [83]. Many bacterial OM proteins such as PilQ, a *Neisseria meningitidis* type IV pilus secretin, depend on β-barrel assembly machinery (BAM) complex for efficient insertion into the OM. In contrast, the insertion of T2SS secretin as in the case of *K. oxytoca* and *E. chrysanthemi* has been shown not to depend on the BAM complex but rather on small lipoproteins such as pilotin which is encoded by non-core gene *GspS* [83, 166]. Interestingly, species which do not possess the T2SS pilotin homologue are thought not to rely on any specific assistance by chaperones during their transport to the OM [166]. In this case, as observed in *P. aeruginosa*, insertion of the secretin HxcQ into the OM relies on its long N-terminal lipid anchor which targets the lipid group in the OM [169].

Based on studies on protein-protein interactions and information on structural arrangement of the secreton, a model has been proposed for its assembly and mode of action by Douzi *et. al,* (2012) [166]. They proposed that biogenesis of the T2SS begins with the insertion of secretin in the OM followed by the attachment of the trans-periplasmic protein GspCp, which allows docking of the IMP. The last

component suggested to join the machinery is the pseudopilus which emerges from the IMP into the secretin [166]. For substrate recognition by the secreton, they posit that GspCp protein first recognises the folded protein in the periplasm through a signalling mechanism which is still poorly understood. The substrate is then transferred to the secretin vestibule before being pushed and expelled into the extracellular environment by the pseudopilus [166]. Although this model provides insight into the assembly of the T2SS, there are still aspects of the biogenesis that remains unclear such as: the specificity of site of insertion of the secreton into the bacterial envelope, how the components of the secreton targets this site and specific signal needed for the recognition of the folded protein by the secreton [83, 166].

3.1.2 The *P. aeruginosa* hxc-Type 2 secretion system

Until recently, the Xcp-T2SS was the only well-studied T2SS of *P. aeruginosa,* and received much attention over the years due to its extensive use by the bacterium to secrete dozens of exoproteins. Following the completion of the *Pseudomonas* genome database, Ball *et al.,* (2001) first reported a cluster of *xcp*-like genes in *P. aeruginosa* PAO1, which was found to possess all known *xcp*-like genes and subsequently named as *hxc* (Table 3.1) [79]. Although the hxc-T2SS shows similarity to the Xcp system in terms of structural arrangement, its genetic organisation is completely

different [79, 81]. Unlike the *xcp* which is clustered into two operons, *xcpP*-to-*Q* and *xcpR*-to-*Z*, the *hxc* gene cluster is thought to have about four transcriptional units: *hxcU*-to-*W*, *hxcP*, *hxcV*, and *hxcQ*-to-Z (Fig. 1) [79]. The study also found that the two secretion systems were non-redundant. For example in the secretion of exoproteins, the HxcT which is 60% homologous to XcpT could not compensate for the loss of XcpT in *P. aeruginosa* [79]. Additionally, whereas XcpT is able to assemble and form HPP when overexpressed, HxcT pseudopilins does not; indicating key architectural differences between the two systems [165, 170].

Interestingly, studies have shown that the *hxc* genes are highly homologous to the *xcp*-like genes from *Burkholderia pseudomallei*, the two having almost an identical genetic organisation (Table 3.1) [79, 171]. This is supported by a related phylogenetic study which showed that in contrast to the xcp, the hxc system was acquired by *P. aeruginosa* PAO1 and other *Pseudomonas* species through horizontal gene transfer most likely from a Betaproteobacteria [165].

Table 3.1 | Characteristics of the *hxc* genes and gene products [79].

Gene name	PA number	Xcp/Gsp homologue	Protein size (amino acids)	Nearest homologue	Characteristics
hxcP	679	XcpP/GspC	143	ExeC/*A. hydrophila*	Bitopic IM protein
hxcQ	685	XcpQ/GspD	803	GspQ/*B. pseudomallei*	Secretin
hxcR	686	XcpR/GspE	469	GspE/*B. pseudomallei*	Traffic warden ATPase
hxcS	687	XcpS/GspF	404	GspF/*B. pseudomallei*	Polytopic IM protein
hxcT	681	XcpT/GspG	149	GspG/*B. pseudomallei*	Pseudopilin
hxcU	678	XcpU/GspH	149	GspH/*B. pseudomallei*	Pseudopilin
hxcV	680	XcpV/GspI	124	GspI/*B. pseudomallei*	Pseudopilin
hxcW	677	XcpW/GspJ	213	GspJ/*B. pseudomallei*	Pseudopilin
hxcX	682	XcpX/GspK	321	GspK/*B. pseudomallei*	Pseudopilin
hxcY	683	XcpY/GspL	399	GspL/*B. pseudomallei*	Bitopic IM protein
hxcZ	684	XcpZ/GspM	197	GspM/*B. pseudomallei*	Bitopic IM protein

Unlike the more general xcp-T2SS, the hxc-T2SS is involved in the extracellular secretion of a single substrate, the low-molecular-weight alkaline phosphatase (LapA) [79, 81]. The *hxc* gene cluster is expressed under phosphate limitation [79]. While expression of the *hxc* operon is thought to be independent of QS signalling, one study has reported that these genes could be regulated by a cell surface

signalling system designated as $PUMA_3$ [81, 172]. LapA is thought to be one of the virulence factors secreted by *P. aeruginosa* in outer membrane-derived vesicles (OMV) [173]. Through the binding of OMVs to lipid rafts in the host plasma membrane, these vesicles are able to release their contents directly into host cytosol, thus eliminating the need for the bacterium to directly interact with the host cell to cause cytotoxic effects [173]. It has been indicated that during infection, LapA localises to early endosomal compartments after entry into the airway epithelial cells [173]. Aside its potential direct cytotoxic effects, LapA is thought to promote *Pseudomonas* biofilm formation under phosphate-limiting conditions thereby facilitating colonisation of host tissues [5, 8, 174].

Environmental concentration of phosphate has been associated with changes in key virulent phenotypes in most bacteria. High phosphate conditions for example has been found to promote expression of *V. cholerae* genes encoding the colonisation factor toxin-co-regulated pilus (TCP) and the ADP ribosylating cholera toxin (CT) [175]. With *P. aeruginosa*, the bacterium has been found to secrete extracellular DNAses under phosphate-limiting conditions [176]. This not only enables the bacterium to use environmental DNA as a source of nutrient but also aid in host immune evasion through degradation of neutrophil extracellular traps [176, 177]. Pyocyanin and rhamnolipids are other virulence factors of *P. aeruginosa* whose secretion are

upregulated under phosphate-limiting conditions [178, 179]. As phosphate limitation is associated with expression of virulence factors, it is critical to understand the role of the *hxc* operon during *P. aeruginosa* pathogenesis. Also, understanding of phosphate acquisition and sensing by *P. aeruginosa* could reveal how the bacterium interacts *in vivo* with *C. albicans,* an organism that also depends on phosphate metabolism for survival in the host [180]. Although both organisms co-exist and interact in mixed infections as described under Section 2.2.4, the nature of this interaction is still under debate [106].

3.2 Summary of result leading to this work

For the present chapter, the contribution of hxc-T2SS and its substrate on *P. aeruginosa* pathogenesis was evaluated. To achieve this, a PAO1-L mutant deficient in hxc-T2SS was constructed. This was followed by an insertion of bioluminescent reporter mini-CTX-lux into PAO1-L to understand the genetic expression of the operon under different culture conditions. This work was a continuation to our earlier observation that supernatant from PAAMP1 strain was unable to induce hyphal growth response of *C. albicans* under our fungal-supernatant co-culture conditions. The PAAMP1 strain lacks, among other genes, the *hxc* operon, *toxR, and bifA,* a gene encoding cyclic-di-GMP phosphodiesterase, which is involved in swarming movement *of P. aeruginosa* PA14 [181]. Our preliminary observation had shown

that the lack of hyphal induction in PAAMP1 supernatant was not due to the absence of rhamnolipids, exotoxin A, pyocyanin, or the virulence regulator *toxR.* However, we observed that PAAMP1 supernatant lacked a unique protein band with rMW between 25 – 30kDa compared to the WT PAO1-L. In this regard, LapA was hypothesised to be a potential candidate for the hyphal inducing agent in the wild type supernatant. Thus the hxc-T2SS deficient strain of PAO1-L was generated to ascertain the role of this secreton during infection and interaction with *C. albicans*. It should be noted that, during the course of generating this mutant, we lost the hyphal induction effect in PAO1-L secretion (Chapter 2). Thus, what is reported subsequently is the characterisation of the *hxc* mutant and its regulation.

3.3. Materials and Methods

3.3.1 Bacteria strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 3.2. Both *E. coli* and *P. aeruginosa* strains were routinely grown in Lysogeny Broth (LB) at 37 °C, 200 rpm. Antibiotics were added to culture media when required: ampicillin (100 µg/ml) was added to maintain the cloning plasmid pBluescript KS II (+) in *E. coli* DH5*α*; tetracycline (25 µg/ml) was used to select for the suicide vector pME3087 in *E. coli* S17-1 λpir; tetracycline (150 µg/ml) was used for pME6032mCherry in *E. coli* DH5α; tetracycline (20 µg/ml) and carbenicillin (2,000 µg/ml) were used for the enrichment step during mutant generation to obtain tetracycline sensitive colonies. Unless otherwise stated, all general chemicals used in the cloning experiment were obtained from Sigma-Aldrich (UK). Primer pairs used in this study are described in Tables 3.3.

Table 3.2| List of bacterial strains and plasmids used in this study.

Strain	**Properties**	**Reference/Source**
PAO1-L	Wild type *P. aeruginosa* from Université de Lausanne, Switzerland	Dieter Haas' laboratory in Lausanne
PAAMP1	PAO1-L with a 58 kb chromosomal deletion	Quorum Sensing Group
PAO1-P	Wild type PAO1 laboratory strain from Parsek's laboratory. Normal cellular level of c-di GMP. Expresses Psl and Pel	[182]
PA176	PAO1-PΔ*wspF*. High cellular levels of c-di GMP. Expresses Psl and Pel	[183]
PAO1-LΔ*hxc*	*hxc* operon deficient strain in PAO1-L background	This work
PAO1-LmCTX::P_{hxcT}-*lux*	PAO1-L with *lux* a promoter fusion at the *hxcT* orientation	This work
PAO1-LmCTX::P_{hxcV}-*lux*	PAO1-L with *lux* a promoter fusion at the *hxcV* orientation	This work
E. coli DH5α	Cloning strain; F^- *endA1 hsdR17 supE44 thi-1 recA1 gurA96 relA1* Δ(*lacZYAargF*)U169 *deoR* λ(φ80*lac*ZΔM15)	[184]
E. coli S17-1λpir	TpR SmR recA thi pro hsdR17 (rK−mK+) RP4-2 [Tc::Mu Km::Tn7] _pir	[185]
pBluescript KH II+	Cloning vector; colicin E1 (ColE1) replicon; Ampicillin resistant	[186]
pME3087	Suicide vector, polylinker of pMMB67, ColE1-replicon; tetracycline resistance	[186]
pGEM-T Easy Vector	Multi-copy cloning vector linearised with EcoRV with a thymine overhang, within lacZ(Amp^R)	Promega, UK

Table 3.3| Oligonucleotide primers for generation of *hxc* deletion mutant

Primer	Sequence (5' to 3') with restriction site	Restriction Enzyme	Function
Hxc-UP-F (#1)	TATGAATTCGGTTGAGATCACCGGGAGCG	EcoRI,	To amplify upstream flanking region of the *Hxc-TXYZQRS* operon
Hxc-UP-R (#2)	TATAAGCTTGTCCTTTCGGGCTGGAGCTG	HindIII	
Hxc-DN-F (#3)	TATAAGCTTGGGGTGGTGCTGACCATCGT	HindIII	To amplify downstream flanking region of the *Hxc-TXYZQRS* operon
Hxc-DN-R (#4)	TATGGTACCGGTGACGGCCATGGCTTGGG	KpnI	

3.3.2 DNA manipulation

P. aeruginosa chromosomal DNA was extracted and purified using Wizard Genomic DNA purification kit (Promega, UK) using manufacturer's instructions. In brief, a single colony of *P. aeruginosa* on an LB plate was inoculated in 5 ml LB medium for overnight incubation at 37 °C with 200rpm shaking. Bacterial cells in 1 ml of the overnight culture were pelleted at 9504 rcf for 2 minutes, and genomic DNA precipitated through alkaline lysis and rehydration with nuclease-free water (Fisher, UK). Plasmid DNA isolation was routinely done using the Qiagen Miniprep kit (Qiagen, UK) or

GenElute plasmid miniprep kit (Sigma, UK) according to the manufacturer's protocol.

The size of DNA fragments and PCR products were regularly analysed using agarose gel electrophoresis. DNA samples were diluted 1:6 with 6x DNA loading buffer (Promega, UK) and loaded into 1% (w/v) agarose gel (Sigma-Aldrich, UK) in 1X TAE buffer (40 mM Tris, pH 8.0, 20 mM acetic acid, and 1 mM EDTA) supplemented with SYBR safe DNA gel stain (Fisher, UK) to a final concentration of 10 µg/ ml. The gels were run in 1X TAE buffer in a DNA electrophoresis tank (Bio-rad, UK) at 80 volts for 45 min and visualised using a GelDoc MP system (Bio-rad, UK). Standard molecular cloning techniques were used for restriction enzyme digestions and ligation reactions as described by Sambrook *et al* (1987) [187].

3.3.3 Generation of plasmid pBluescript KH II(+)Hxc (designated as pBSHxc)

The plasmid designated as pBSHxc was constructed by PCR amplification of a 671-bp fragment upstream (Hxc-Up) of the *hxc*-operon and a 675-bp downstream fragment (Hxc-Dn) using *P. aeruginosa* PAO1-L chromosomal DNA as template. The 671-bp Hxc-Up fragment was amplified using forward primer Hxc-UP-F carrying an EcoRI restriction site and the reverse primer, Hxc-UP-R; with a HindIII restriction site (Table 3.3). The forward primer Hxc-DN-F

containing HindIII restriction site and the reverse primer, which carries a KpnI restriction site (Table 3.3), amplified the 675-bp Hxc-Dn fragment.

The EcoRI and HindIII digested Hxc-Up PCR product was ligated into pBluescript KH II(+) digested with the same enzymes. The Hxc-Dn PCR product was then ligated into the pBShxcUp construct digested with HindIII and KpnI using T4 DNA Ligase (Promega). TOP10 *E. coli* strain was used to propagate the resulting recombinant plasmid on blue-white selection LB plates containing 40 µl of 0.1M IPTG, 40 µl of 10 mg/ml x-gal and 100 µg/ml ampicillin. Positive colonies carrying recombinant plasmids (pBSHxc) were sub-cultured and screened using GenElute Miniprep kit (Sigma-Aldrich). Recombinant plasmids were digested and run on agarose gel electrophoresis followed by nucleotide sequencing to determine if the concatamer was of correct construction.

3.3.4 Generation of plasmid pME3087Hxc

Recombinant plasmid pBShxc carrying the deletion concatamer of approximately 1300-bp was digested and gel extracted according to manufacturer's instructions (QIAquick Gel Extraction Kit - Qiagen). The resulting DNA fragment was cloned into pME3087 suicide plasmid at the EcoRI and KpnI restriction sites yielding pME3087Hxc; which was electroporated into *E. coli* S17-1λpir. Electroporation was

conducted in 2 mm electrode gap Gene Pulser cuvettes (BioRad, UK) using 2 µl of ligation reaction and 50 µl competent cells. A pulse of 2.5 kV was delivered for 4–6 millisecond using the BioRad Gene Pulsar connected to a BioRad pulse controller (BioRad, UK). Cells were resuspended in 950 µl of LB, transferred into 1.5 ml Eppendorf tubes and subsequently incubated for 2 hours at 37 °C, 200 rpm. The culture was then propagated on LB plates containing 25 µg/ml Tetracycline (Tc) at 37 °C for overnight. Colonies were screened for positive transformants using plasmid extraction, restriction digestion, agarose gel electrophoresis, and nucleotide sequencing as previously described. The resulting recombinant plasmid pME3087Hxc was used for mutant generation.

3.3.5 Mutation of the *P. aeruginosa* hxc Type II secretory system

The PAO1-L *hxc* in-frame deletion was constructed by allelic exchange using the suicide vector pME3087Hxc. The cloning strategy is illustrated in Figure 3.3. Briefly, the first homologous cross-over of *Δhxc* fragment with sequences on PAO1-L genome occurred through conjugation of PAO1-L as recipient and *E. coli* S17-1λpir:pME3087Hxc as the donor cells. The transconjugants were selected on LB plates supplemented with 150 µg/ml and 15 µg/ml Tc and nalidixic acid respectively. Next day, colonies on the selective plate were picked for purification on LB agar plate with the same antibiotic concentration,

cultured at 37 °C overnight. Successful chromosomal integration of the PME3087Hxc plasmid was confirmed through colony PCR using primer Hxc-UP-F1 (#1) and Hxc-DN-R (#4) with the desired PCR amplicon expected to be 1300-bp. To excise the suicide vector in order to delete the chromosomal hxc-T2SS, the transconjugants were selected for Tc sensitivity using a three-day continuous enrichment method. Purified colonies from the conjugation plate were grown overnight in 5 ml LB at 37 °C and 200 rpm without antibiotics. The cultures were diluted 1:100 in fresh LB the next day, and incubated at 37 °C with shaking at 200 rpm for 2 hours to a mid-log phase of OD_{600} between 0.05 and 0.1. Tetracycline was added to the culture to a final concentration of 25 µg/ml for 1hour incubation to arrest the growth of Tc-sensitive cells and also to make the cells resistant to metabolic poisoning. Carbenicllin was added to a final concentration of 2000 µg/ml and incubated at same conditions for 6 hours to kill actively dividing cells. Cells were tested for loss of Tc-resistance on LB plates containing 125 µg/ml of the antibiotic, and a mutant strain was confirmed to have the hxc-T2SS deletion by colony PCR analysis, gel electrophoresis and DNA sequencing; and was named PAO1-L*Δhxc*.

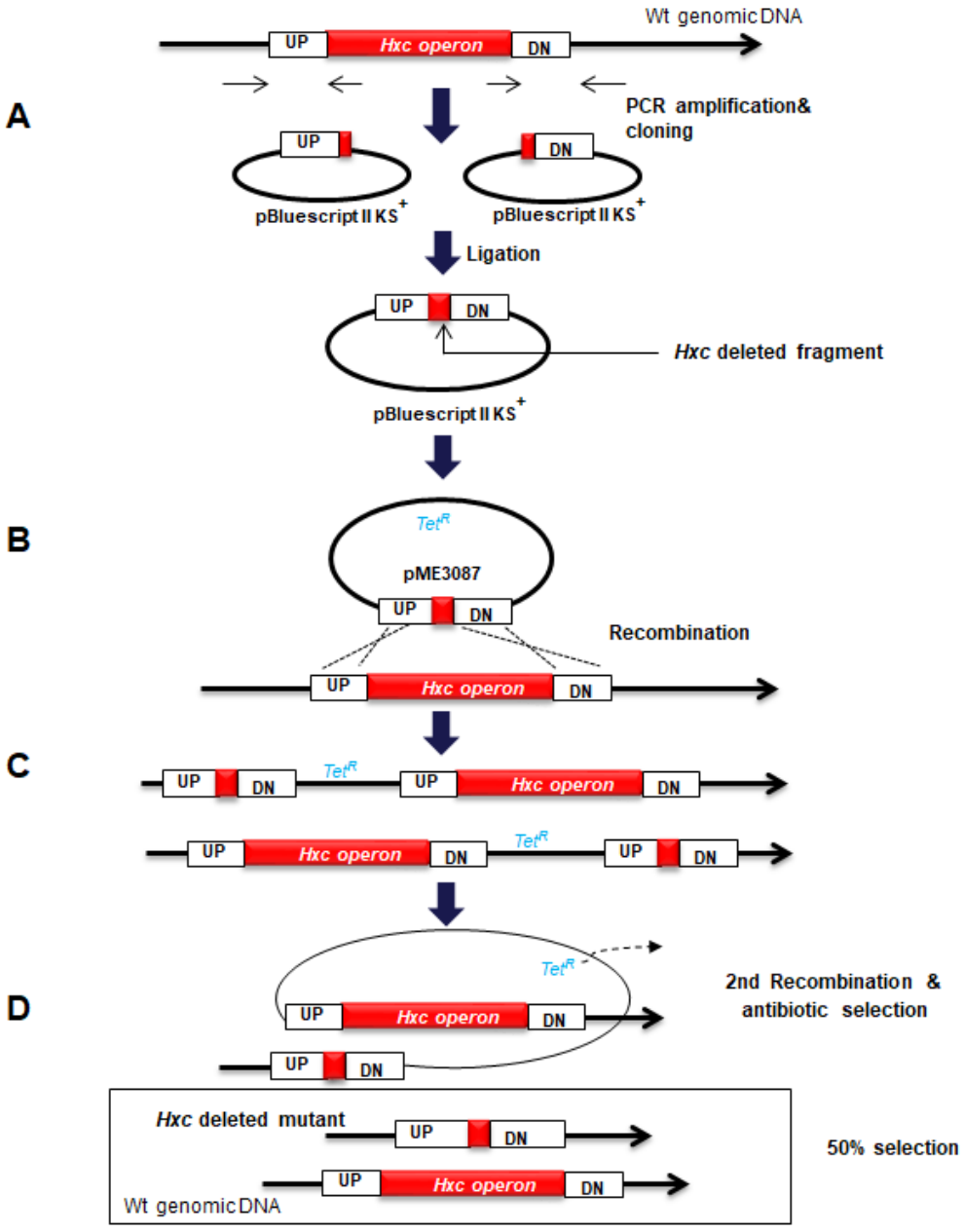


Figure 3.3| Schematic diagram showing strategy to construct PAO1-LΔhxc mutant. (A) The up and downstream fragments flanking the *Hxc-TXYZQRS* operon were amplified and cloned into pBluescript II KS+. (B) The resulting *Hxc* concatamer fragment was then cloned into suicide plasmid pME3087 and was subsequently conjugated to PAO1-L. (C) Homologous recombination occurred between the *Δhxc* fragment and PAO1-L chromosomal DNA through the insertion of pME3087Hxc. Successful conjugants were selected on tetracycline and nalidixic acid medium plates. (D) Conjugants were sub-cultured for a second homologous cross-over. Through antibiotic media enrichment, tetracycline resistant plasmids were removed from the *P. aeruginosa* genomic DNA resulting in two genotypes: WT PAO1-L (50 %) and *Δhxc* mutant (50 %).

3.3.6 Phenotypic characterisation of PAO1-L*Δhxc*

3.3.6.1 Determination of bacterial growth

Bacterial growth was measured under both aerated and static conditions. For aerated conditions, WT PAO1-L or the PAO1-L *Δhxc* mutant were grown in 25 ml of LB, X-vivo[15] (Lonza, UK) or proteose peptone medium (BD Biosciences) containing 0.4% glucose (PPM) with or without 10mM exogenous Phosphate (KH_2PO_4) in a 250 ml flask overnight at 200 rpm and 37 °C. OD_{600} of the culture was measured using a spectrophotometer (BioMate 3S, Thermo Scientific, UK), and adjusted to 0.01 in 10 ml of fresh media. The new cultures were incubated at 37 °C, 200 rpm and OD_{600} was measured every hour for 9 hours under the aerated condition. For static condition, cultures were incubated in a 96-well plate (Greiner Bio-One, UK,200 µl per well in triplicate) and OD_{600} was read every 30 min for 16 to 24 hours using a microplate reader (Infinite® M1000 PRO, TECAN, UK).

3.3.6.2 Pyocyanin production assay

The pyocyanin quantification is based on a colorimetric method by measuring the absorbance of pyocyanin in acidic solution developed by Essar *et al* (1990)[188]. Bacteria strains were streaked on *Pseudomonas* Isolation Agar (PIA) plates and cultured overnight at 37 °C. A single colony from the PIA plate was inoculated in 5 ml LB and cultured overnight at 37 °C, 200 rpm. OD_{600} of the overnight

culture was measured the next day and adjusted to a final OD_{600} of 0.01 in 25 ml fresh LB media. The culture was incubated overnight at 37 C, 200 rpm. 5 ml of overnight culture was centrifuged in a 15 ml falcon tube at 3578 rcf (Allegra X-64R with C0650 fixed angle rotor, Beckman Coulter, UK) for 10 min at room temperature. The supernatant was transferred into a fresh tube and mixed thoroughly with 3 ml of chloroform through vortexing. The mixture was centrifuged and the chloroform organic layer was transferred to a fresh tube and mixed with 1 ml 0.2 M HCl (Sigma-Aldrich, UK). After centrifugation at 3578 rcf (Allegra X-64R, Beckman Coulter, UK) at 4 °C for 5 min, the top layer was collected to measure its absorbance at 520 nm. Concentrations of pyocyanin, presented as µg/ml, was determined by multiplying the OD_{520} value by 17.072 [188].

3.3.6.3 Crystal violet biofilm formation assay

Bacterial biofilm formation was quantified using the microtiter crystal violet assay according to O'toole, G.A., (2011) [189]. Bacteria strains were streaked on LB plates and incubated at 37 °C overnight. A single colony was inoculated into 5 ml of pre-warmed LB, X-Vivo[15] or PPM with or without 10mM exogenous phosphate and incubated overnight at 37°C at 200 rpm. 1:100 dilution of an OD_{600nm} = 1.0 overnight culture was inoculated into 20 ml fresh media in a sterile 250 ml conical flask and incubated at 37 °C, 200 rpm for 3 hours to get mid-log phase culture. The mid-log phase culture was diluted in a fresh

medium to obtain OD_{600nm} = 0.04 and kept on ice until inoculation. The culture was thoroughly mixed by vortexing and 100 µl was added to polystyrene microtiter 96-well plate (Fisher, UK) in triplicates and incubated at 37 °C in presence of 5 % CO_2 without agitation for 24 hours. The culture was removed the next day; each well was washed 3 times using HPLC water (Sigma-Aldrich, UK) and stained with 125 µl of 1 % of crystal violet solution. The plate was incubated for 1 hour at room temperature after which the crystal violet was removed and the wells washed with HPLC water 3 times. 200 µl of 70% ethanol (Fisher, UK) was added to each well to solubilise the crystal violet from the bacteria biomass and absorbance was measured at 595 nm using BioTek PowerWave HT Microplate Spectrophotometer (BioTek, USA).

3.3.6.4 Test for biofilm formation in synthetic CF media

The Synthetic cystic fibrosis sputum medium (SCFM) was kindly prepared by Daniella Spencer (PhD student) and Nora Wolff (MSc student) according to the method described by Palmer *et al.,* (2007) [190] . Briefly, bacterial cultures of PAO1-L, PAO1-L Δ*hxc*, PAO1-P, and PA-P176 were grown overnight in 5ml of Tryptone Soy broth (TSB) with or without 10mM exogenous phosphate KH_2PO_4 (Sigma-Aldrich) at 37 °C, 200 rpm. The next day, OD_{600} of the culture was adjusted to 5% in 1 ml ASM in a sterile 24-well cell culture plate (Corning® Costar®, Sigma-Aldrich) and incubated for 16 or 20 hours

at 37 °C with gentle shaking at 55 rpm. The culture was then fixed with formaldehyde to a 4% final concentration and left at 4 °C for a minimum of 48 hours before imaging.

3.3.6.5 Analysis of bacterial supernatants

Supernatants were prepared for the protein precipitation using trichloroacetic acid (TCA) technique as described in Ball *et al*., (2002) [79]. Liquid cultures of PAO1-L, PAAMP1 and PAO1-LΔ*hxc* were grown at 37 °C, 200 rpm in PPM or LB supplemented with or without 10mM exogenous phosphate for the bacteria to reach OD600nm of 1.5 in PPM (5 hours) or 2.5 in LB (4 hours). Bacterial cells were separated from supernatant by centrifugation (10,400 rcf, 10 min, 4 °C). Protease inhibitor was added to the supernatant in a ratio of 1:100 v/v from a x100 stock solution (Halt™ Protease Inhibitor Cocktail, ThermoFisher). Proteins were precipitated from the supernatant by addition of TCA to a final concentration of 12% and incubated at 4 °C for 2 hours. The samples were centrifuged as above and the pellets were washed in acetone and resuspended in a SDS-PAGE sample buffer (0.063M tris-HCL pH6.8, 2% SDS, 10% glycerol, and bromophenol blue). The samples were heated at 95 °C for 7 min and run on a 10.5% or 5-20% gradient gel. Protein bands were analysed after Coomassie blue staining (0.015% Coomassie blue in 25% isopropanol and 10% acetic acid) and destaining (25% isopropanol, 10% acetic acid).

3.3.6.6 Alkaline phosphatase activity assay

A single colony of PAO1-L, PAO1-LΔ *hxc* and PAAMP1 was inoculated in 10 ml LB, X-Vivo^{15} or PPM. The cultures were grown at 37 °C, 200 rpm with aeration to reach $OD600_{nm}$ of 1.5 in in LB (4 hours), PPM (5 hours) and X-Vivo^{15} (7 hours). Cells and supernatants were separated by centrifugation as previously described. Supernatants were subsequently treated with a cocktail of protease inhibitors before being concentrated four times using a 10 kDa pore size filters. The concentrated supernatants were serially diluted in a sterile 96-well plate (ThermoFisher) to a volume of 200 µl in duplicates. Alkaline phosphatase activity was determined using colorimetric Alkaline Phosphatase Assay kit (Sigma). Briefly, 50 µl of freshly prepared 1 mg/ml p-nitrophenylphosphate (pNPP) solution was added to each well and then incubated at room temperature in the dark for 30 minutes. A standard curve was prepared with 10 µl of alkaline phosphatase solution serially diluted in 1 mM pNPP. Reaction was stopped by the addition of 50 µl of 3M NaOH and absorbance was measured at OD_{405nm}.

3.3.7 Introduction of promoter fusions into PAO1-L *hxc* operon.

The procedure used for the generation of the promoter fusions followed the general principle of allelic exchange as described in Section 3.3.5 of this chapter. In brief, 418 bp DNA fragment comprising the intergenic region between the *hxcT* and *hxcV* genes (Figure 3.4) was PCR amplified in both orientations using primers described in Table 3.4. The fragments were cloned into mini-ctx-*luxCDABE* vector and subsequently electroporated into *E. coli* S17-1λpir. The resulting constructs, pHxcT and pHxcV, carrying the transcriptional fusions with the *hxcT* and *hxcV* promoter regions and *luxCDABE* as reporter gene were introduced into PAO1-L. Promoter activities were measured as bioluminescence using Tecan luminescence plate reader (Tecan, infinite 200Pro, UK).

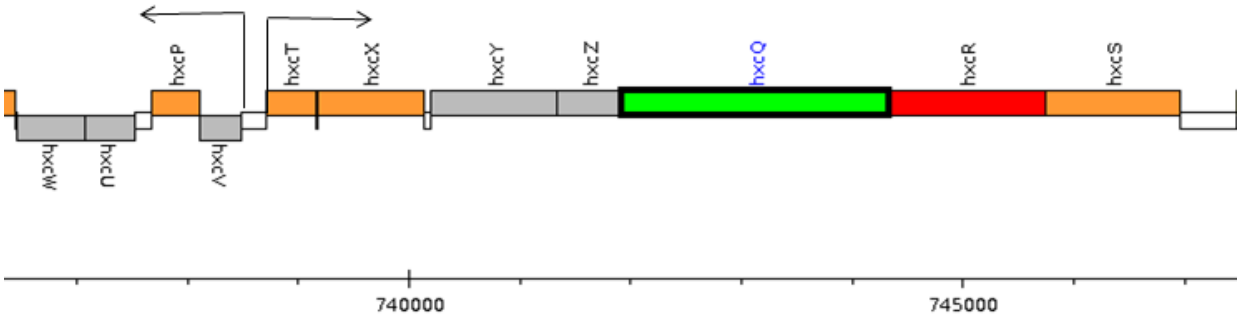


Figure 3.4| Genetic map position of the hxc operon. The intergenic region between *hxcT* and *hxcV* used for investigating promoter activity is bordered by two thin arrows. Figure adapted from [154].

Table 3.4 | Nucleotide sequence of the hxc promoter along with its forward and reverse primer sequences. Primers were designed to contain a restriction site for XhoI (Red) and EcoRI (purple) for PCR amplification of the 418 intergenic region (blue).

CTCGAGCGTTGCCGTCGGTCATCAGGCCCACCGCGCGGATCGCTGCG

GCCAGGGCGATGGCGACGATGGCCAGCGCGACCAGCACTTCGATCAG

GGTGAAACCGCGGGAAGACAGGCGCGTGGACATCGCGGAAAGGTCG

GCGAAAGGGGACTTTCAGGCTAGCCGGGAAATCTGACCGATCCCTGA

CAAAAGCTCGGGAGGGCGCCCCCGCGAGCCATCCCCGGCGCCTGCC

GGAAAGCCCGGCTCCAGGGCCTTGCGGGCGCCTCCGGAAGAGCGGC

GCACCCGAGGATTTTCAATATCGTTGACATATCTTCCTGAAACAATCCG

CGGCGAATCGAACCATGCCGAGGAGTGTCGAGATGGATGTCGTGCAG

TTCAGCTCCAGCCCGAAAGGACATCGAATTC

P_{hxcT} Primers

P_{hxcT}-FW: 5′ –TATCTCGAGCGTTGCCGTCGGTCATCAG – 3′

P_{hxcT}-RV: 5′ – TATGAATTCGATGTCCTTTCGGGCTGGAG – 3′

P_{hxcV} Primers

P_{hxcV}-FW: 5′ – TATGAATTCCGTTGCCGTCGGTCATCAG – 3′

P_{hxcV}-RV: 5′ – TAT CTCGAG GATGTCCTTTCGGGCTGGAG – 3′

3.4 Results

3.4.1 Generation of PAO1-L Δ*hxc* mutant

We had earlier observed that unlike the wild type PAO1-L, supernatants from PAAMP1 strain which lacked a 58 kb chromosomal region, failed to induce *C. albicans* filamentation. To identify the molecule (s) in PAO1-L secretions involved in the hyphae induction, a gene cloning approach was employed alongside biochemical tests described in Chapter 2. The choice of PAO1-L as the host to make a mutant deficient of the *hxc* gene cluster was based on the reasons specified in Section 3.2. The mutant was constructed using allelic gene exchange. Two primer pairs Hxc-UP-F and Hxc-UP-R, and Hxc-DN-F and Hxc-DN-R were designed to delete the target genes (Table 4). The upstream (Hxc-UP-F/Hxc-UP-R) and downstream (Hxc-DN-F/Hxc-DN-R) primer pairs were used to amplify PAO1-L genomic DNA as the template. This resulted in a 653-bp long amplicon containing EcoRI and HindIII at its 5' and 3' ends respectively for the upstream fragment and a 657-bp long amplicon containing HindIII and KpnI for the downstream fragment. The upstream and downstream amplicons were cloned individually into dephosphorylated pBlueScript KH II(+) plasmid digested with the relevant restriction enzymes, and propagated in TOP10 *E. coli*. To generate Hxc-Up and Hxc-Dn concatamer, the transformed pBShxcUp plasmid was digested with KpnI and HindIII creating a ligation site for the Hxc-Dn amplicon which contains the same restriction sites at the 3' and 5' ends

respectively. Five colonies were analysed and all showed the expected band of 1.3kb following restriction digestion of purified plasmid DNA with EcoRI and KpnI (Figure 3.5).

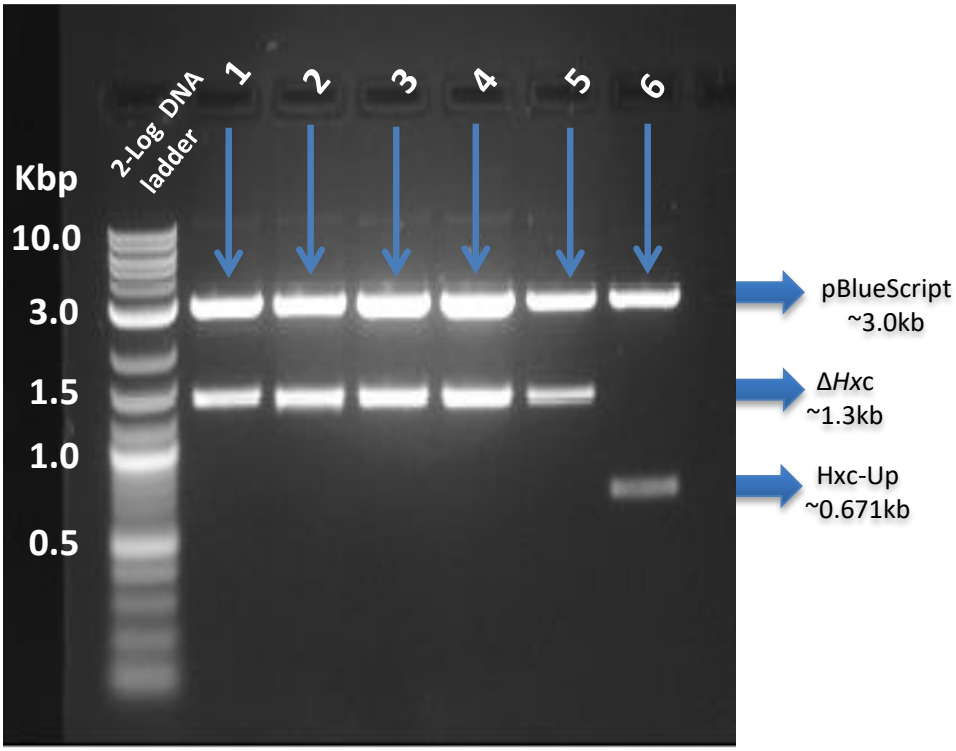


Figure 3.5| Cloning of 1.3kb Hxc deletion concatamer into pBlueScript KH II(+). Gel-extracted Hxc-Dn fragment was ligated into pBShxcUp plasmid digested with HindIII and KpnI. TOP10 *E. coli* was then transformed with the ligation mixture. Plasmids extracted from selected clones were digested with EcoRI and KpnI, and digested products were resolved on a 1% agarose gel to identify successful clones. Lanes 1-5 shows successful clones with 1.3kb Hxc deletion insert and lane 6 represent pBShxcUp (pBlueScript KH II(+) carrying only the Hxc-Up fragment) as a negative control.

The Hxc deletion fragment (Δ*hxc*) was then sub-cloned into the suicide vector pME3087 resulting in pME3087Hxc. *E. coli* s17-1 λ*pir* was used for the propagation of ligates and also facilitated homologous recombination between the pME3087Hxc and the PAO1-L genome through conjugation. Using a three-day continuous enrichment method, recombinant clones from the conjugation plates were purified and screened for tetracycline sensitive mutant candidates. Of nineteen colonies screened, thirteen showed the expected band of 1.3kb following colony PCR amplification (Figure 3.6). The Δ*hxc* mutant sequence was verified by nucleotide sequencing which showed that the last 20 nucleotides of the upstream fragment were successfully linked to the first 20 nucleotides of the downstream fragment with HindIII restriction site in between (Figure 3.7).

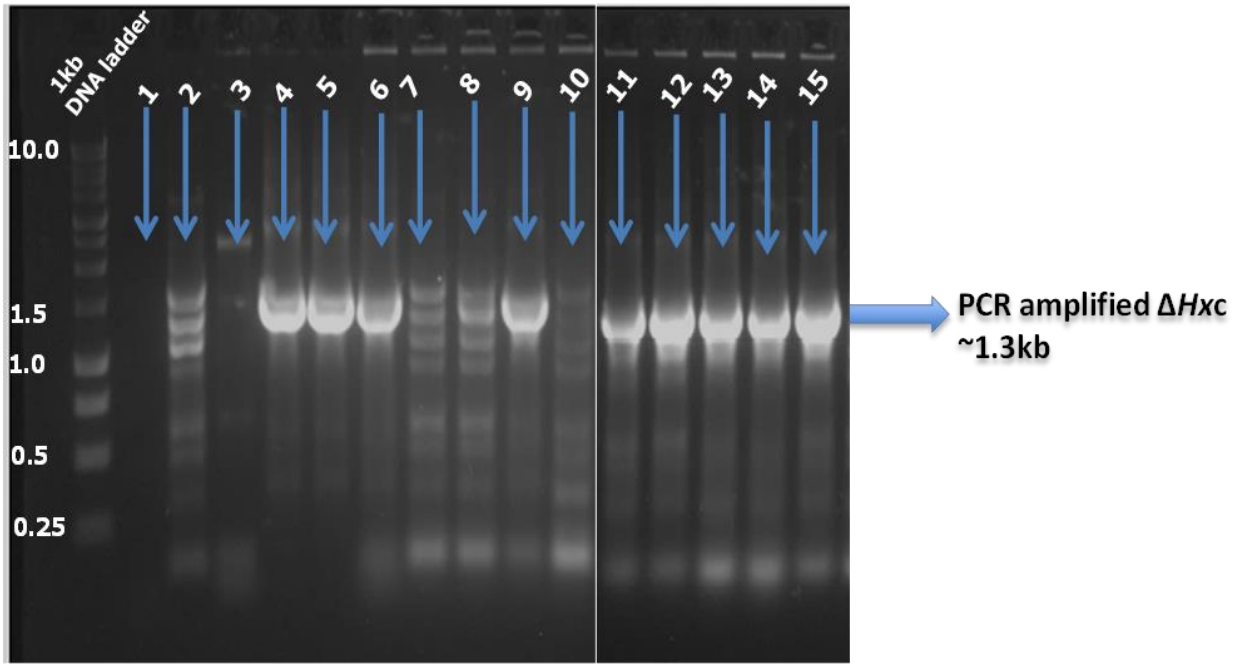


Figure 3.6| Colony PCR screening of the PAO1Δhxc deletion mutant. Several colonies from the conjugation plate were PCR amplified using primer pairs Hxc-UP-F (#1) Hxc-DN-R (#4), and the size of the amplicon was confirmed on 1% agarose gel. Lanes 1-3 represent negative controls: no template, PAO1-L genomic DNA and pBSHxc-Up respectively. Lanes 4-6 represent positive controls of pBSHxc (pBluescript carrying only the Hxc deletion fragment) and lanes 7, 8 and 10 represent negative clones. Lanes 9 and 11-15 show positive mutants with correct amplicon of 1.3kb.

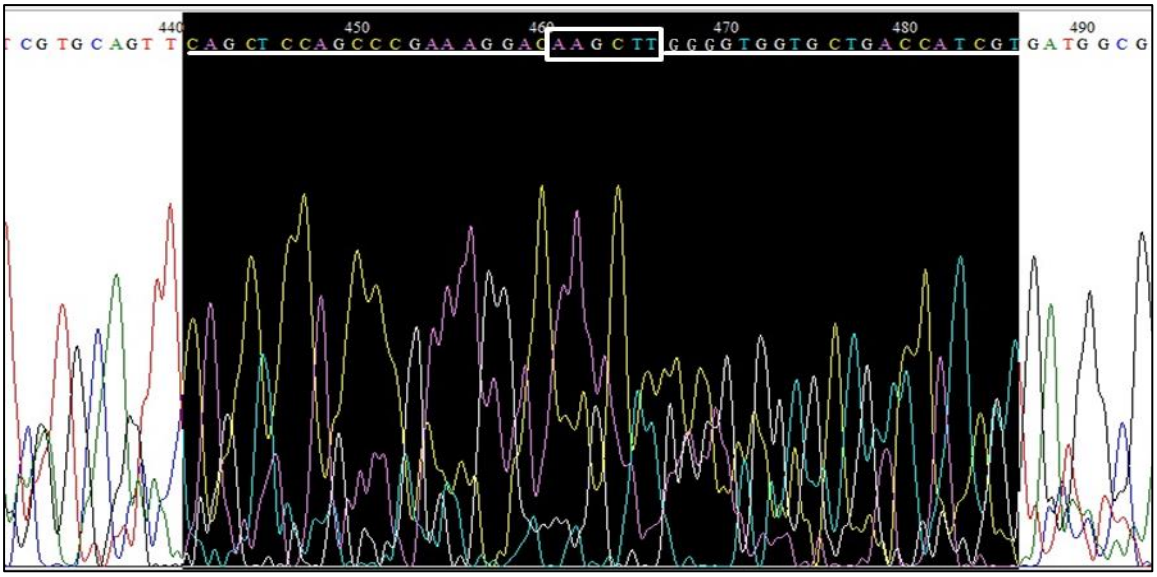


Figure 3.7| Nucleotide sequencing confirmed the deletion of the hxc gene cluster. DNA sequencing of the PAO1-LΔ*hxc* mutant was done using commercial M13 primer pairs. The first 20 nucleotides CAGCTCCAGCCCGAAAGGAC (underlined) of the downstream fragment and the last 20 nucleotides GGGGTGGTGCTGACCATCGT (underlined) of the upstream fragment were linked at the HindIII restriction site, making an *hxc* gene cluster deletion by knocking out 12100 nucleotides.

3.4.2 Phenotypic characterisation of *the* PAO1-LΔ *hxc* mutant

3.4.2.1 Bacterial growth

To examine whether the deletion of the *hxc* T2SS would affect fundamental metabolic processes of the bacterium, the growth of PAO1-L Δ*hxc* was compared to that of its isogenic WT strain PAO1-L. The two strains were cultured in LB medium, PPM or X-vivo[15] under static and aerated conditions where OD_{600} readings were taken every 30 minutes for 24 hours and every hour for 8 hours respectively. PPM is a phosphate-limiting medium and since the hxc-T2SS is thought to be expressed under low phosphate conditions [79], we decided to

examine the growth response of the mutant compared to the WT in such a medium. Also, with the aim of testing the PAO1-LΔ*hxc* strain on cultured human airway epithelial cells (Calu-3), X-vivo^{15} was also chosen as one of the appropriate media to test the bacterial growth. X-vivo^{15} is a well-defined serum-free medium routinely used in our lab in infection assays of human macrophages with *P. aeruginosa* [191].

Both the WT and *hxc* mutant showed comparable growth rates in all the three test media under static condition (Figure 3.8). This is consistent with previous studies which showed that the deletion of *hxcR* in the *P. aeruginosa hxc* operon did not affect growth and cell division in the bacterium [79].

Although addition of 10 mM exogenous phosphate significantly promoted bacterial growth under static condition in X-vivo^{15} ($p<0.001$), it was independent of the *hxc* operon (Figure 3.8A). No distinguishable growth differences were observed among these two strains when cultured under aerated conditions in all the test media with or without the addition of exogenous phosphate (Figure 3.9).

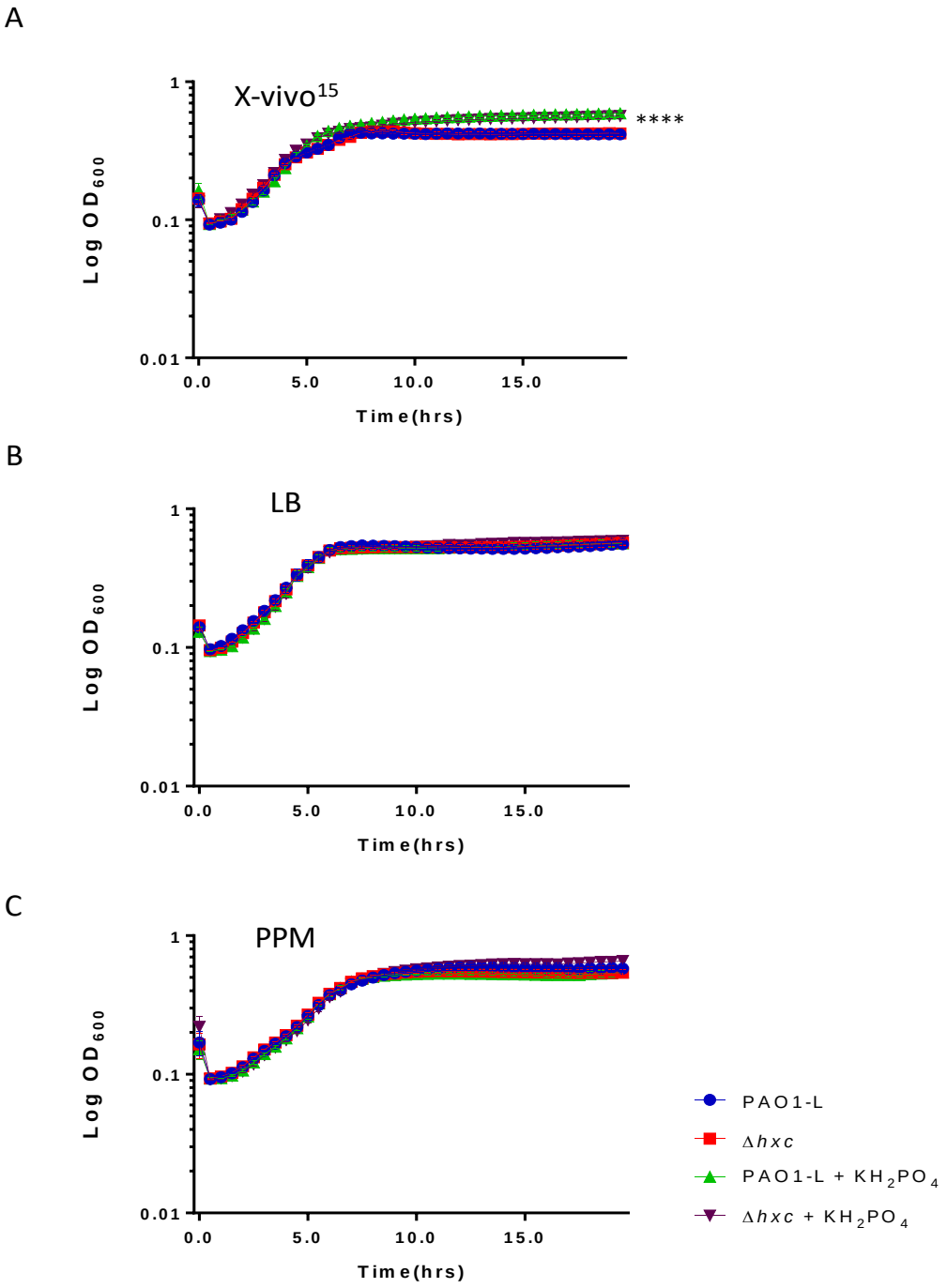


Figure 3.8| *P. aeruginosa* growth is not influenced by the hxc operon under static condition. Growth response of WT PAO1-L and the *hxc* mutant was tested in (A) X-Vivo[15], (B) LB or (C) PPM with or without 10mM exogenous phosphate under static conditions. 200 µl of cultures were incubated in a 96-well plate (in triplicates) and growth response was measured at OD_{600} for every 30 min for 24 hours using Infinite® M1000 PRO TECAN microplate reader (Tecan, UK). (*** $P < 0.001$, 95% CI of diff, Tukey's multiple comparisons test; Data points represent Mean ± SEM, n = 4)

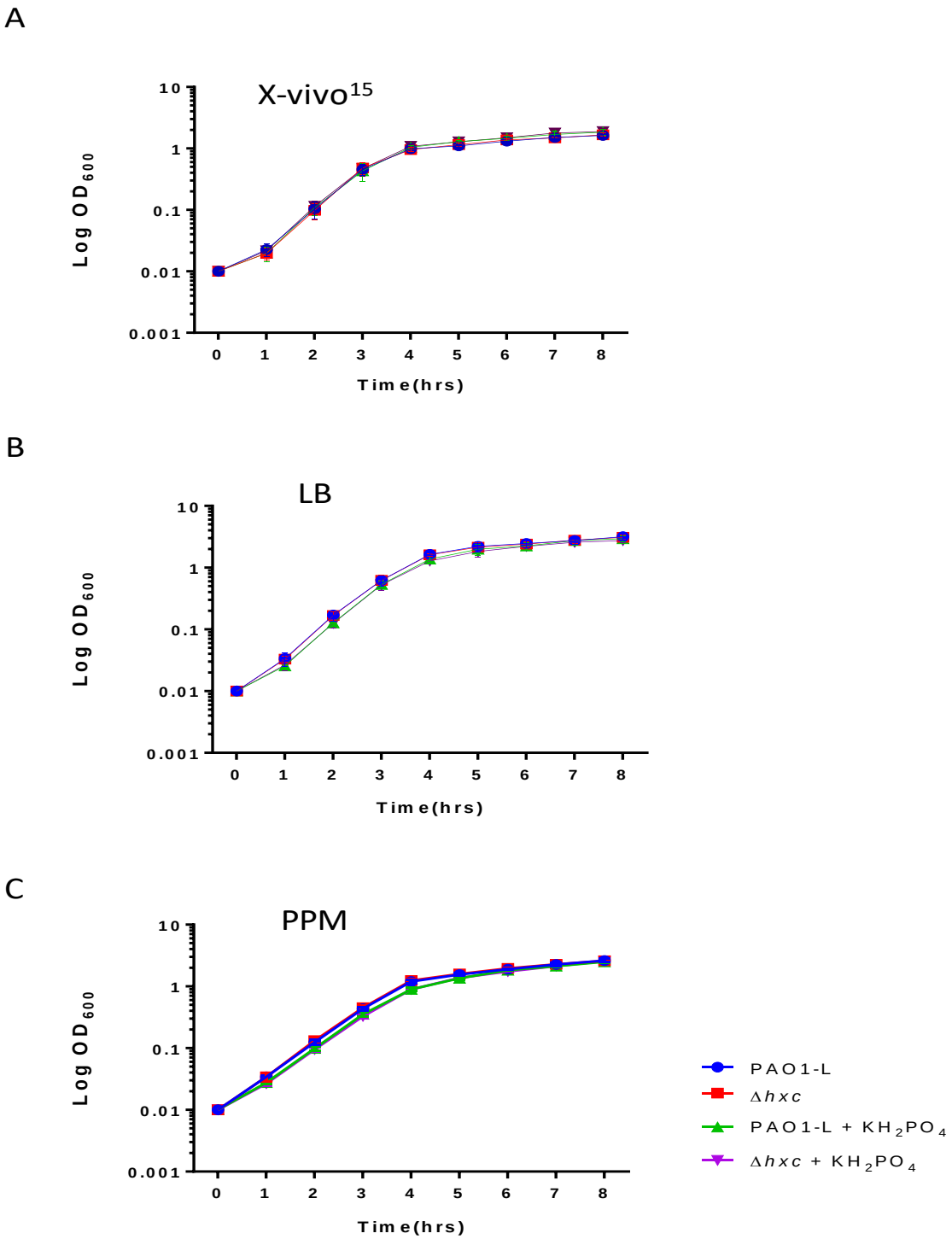


Figure 3.9| *P. aeruginosa* growth is not influenced by the hxc operon under aerated conditions. 10 ml 1% log-phase bacterial culture was set up either in (A) X-Vivo[15], (B) LB or (C) PPM with or without 10mM exogenous phosphate and incubated at 37 °C, at 200 rpm. Growth rate was measured at OD_{600} at every hour for 8 hours using spectrophotometer. Data points represent Mean ± SEM, n = 3

3.4.2.2 Biofilm formation

Biofilm formation is one of the key virulent features of *P. aeruginosa* which is coordinated by quorum sensing in order to persist and evade antimicrobial therapy [65]. Since the LapA alkaline phosphatase has been thought to promote biofilm formation [192], we tested whether this feature was affected by the deletion of *hxc* operon in PAO1-L using a 96-well Microtiter crystal violet biofilm assay [189]. Bacterial cultures were set in 1/10 strength LB (to prevent excessive biomass production [193]), X-vivo^{15} or PPM with or without 10mM exogenous phosphate. Both PAO1-L and the Δ*hxc* mutant produced very low biofilm with or without the addition of exogenous phosphate in both LB (Figure 3.10) and PPM (data not shown). They however produced a substantial amount of biofilm in X-vivo^{15} although there was no significant difference between the two strains. Addition of exogenous phosphate to X-vivo^{15} significantly promoted biofilm formation ($p<0.001$) which was comparable in both strains (Figure 3.10A). This observation is in agreement with the earlier assertion that exogenous phosphate is important in the bacterial cell division and that *P. aeruginosa* can assimilate phosphate during biofilm formation independent on the hxc T2SS.

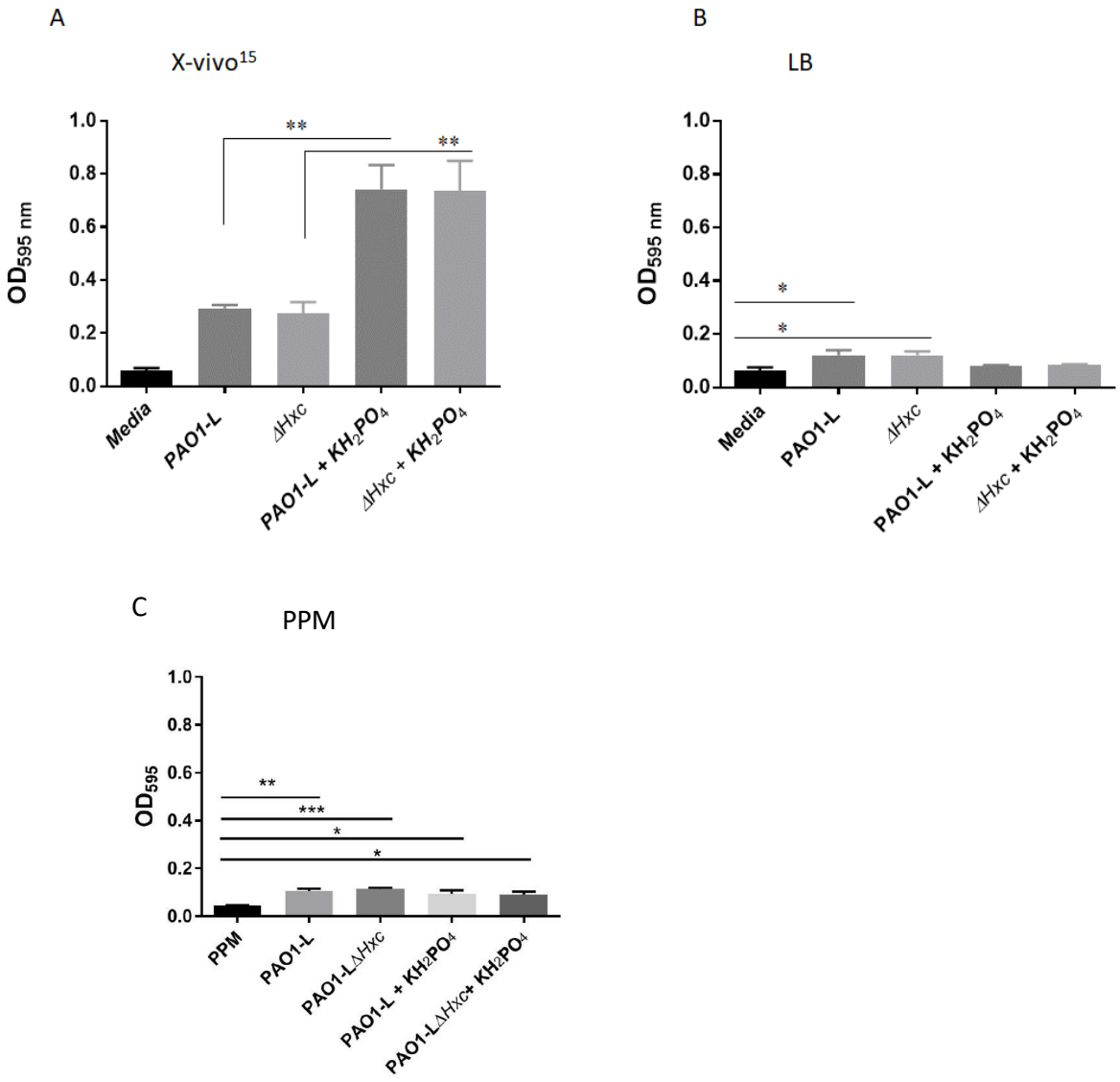


Figure 3.10| *P. aeruginosa* biofilm formation is promoted by exogenous phosphate and independent of the hxc operon. Bacterial suspensions were grown in X-Vivo[15], LB or PPM media with or without 10mM exogenous phosphate in a 96-well costar plate at 37 °C for 24hrs. Bacterial biomass was quantified by OD measurement at 595 nm after 1:2 dilutions in 70% ethanol. Data represent Mean ± SEM of 4 independent experiments performed in triplicates. (** $P < 0.001$, * p=0.024, 95% CI of diff, Tukey's multiple comparisons test)

3.4.2.3 Biofilm in synthetic cystic fibrosis sputum medium

Growth in CF sputum induces *P. aeruginosa* to display distinct phenotypes such as high-density growth, *Rhl*-dependent QS network, and repression of *fliC,* which encodes flagellin [190, 194]. To further investigate bacterial colony formation in a well-defined medium that nutritionally mimics CF sputum, the bacteria strains were grown in SCFM for 16 or 20 hours at 37 °C with minimum agitation. PA- Parsek (WT strain) and PA-P*ΔWspF* Parsek (overproduces Psl and Pel) were used as positive controls. All the test strains formed microcolonies, suspended dense aggregates, which increased with increasing incubation period (Figure 3.11). However, there were no clear distinguishable differences in microcolony formation between the PAO1-L Δ*hxc* mutant and the WT PAO1-L. As expected, the PA-PΔ*WspF* Parsek strain produced the highest amount of microcolonies. This strain overproduces Psl and Pel polysaccharides which are involved in surface attachment and maintenance of cell-cell interactions respectively during biofilm formation [195]. The addition of phosphate to the medium did not lead to detectable differences in microcolony formation by the bacterial strains. There was however, a clear difference in the colour of the media between the Lausanne and Parsek strains, in which the Lausanne strains were blue/green while the Parsek strains were not. This experiment was conducted only once by Nora Wolff, an MSc student, under the supervision of

Daniella Spencer, PhD student in Kim Hardie's lab (University of Nottingham).

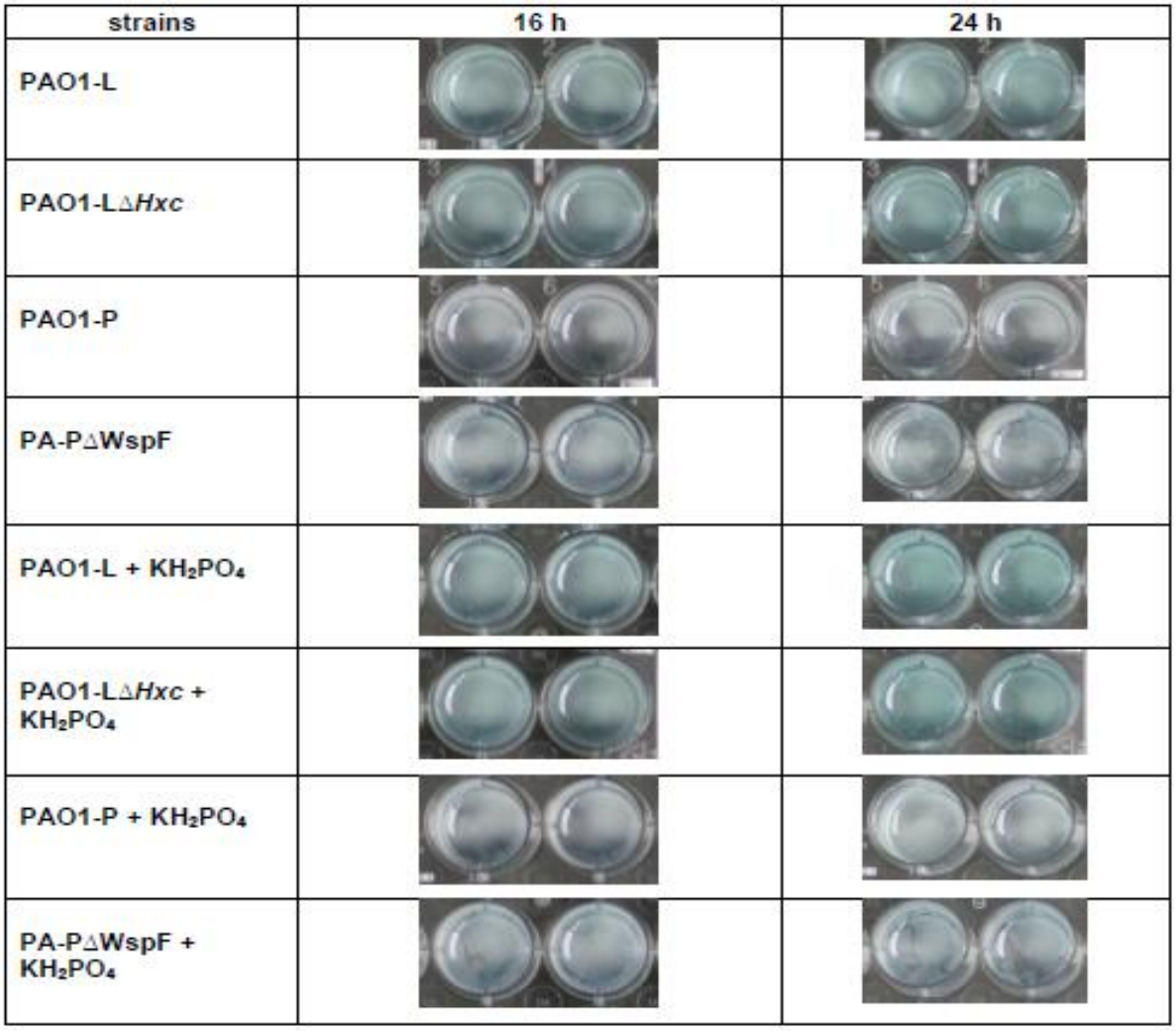


Figure 3.11| Investigation of biofilm formation by *P. aeruginosa* strains synthetic cystic fibrosis sputum medium. PAO1-L, PAO1-L Δ*hx*c, PAO1-P, and PA-PΔWspF were grown in quadruplicates for 16 h and 24 h in SCFM with and without additional phosphate at 37 °C. Following incubation, the microbial colonies were fixed using formalin and left at 4ºC for a minimum of 48 h before images were taken. Two samples are shown per each incubation time point. This data has been reported in Nora's MSc thesis.

3.4.2.4 Quantification of pyocyanin production

Pyocyanin is one of the exotoxin molecules secreted by *P. aeruginosa* under the control of QS. Considered as one of the characteristic

virulence factors, pyocyanin is essential for *P. aeruginosa* pathogenesis due to its ability to form reactive oxygen species such as O_2^- and H_2O_2, which can alter the electrochemical balance of host cells thereby resulting in tissue injury [196]. Further, pyocyanin has been shown to suppress growth of *C. albicans* isolated from CF sputum [159]. With our original objective to test the *hxc* mutant secretion on *C. albicans*, we assessed pyocyanin production by the mutant relative to WT.

Culture supernatant from the PAO1-L Δ*hxc* mutant was analysed for pyocyanin production in comparison to that of WT PAO1-L to determine whether its production was affected by the lack of *hxc* operon. As shown in Figure 3.12, there was no difference in the levels of pyocyanin produced among these two strains, suggesting that the hxc-T2SS does not detectably influence the secretion of this exotoxin. Interestingly, addition of exogenous phosphate to the culture medium significantly reduced pyocyanin production in both the mutant and the WT. This suggests that phosphate might have a partial inhibitory effect on the production of pyocyanin which is in agreement with previous reports that low phosphate culture media promote the highest levels of pyocyanin production by *P. aeruginosa* [197].

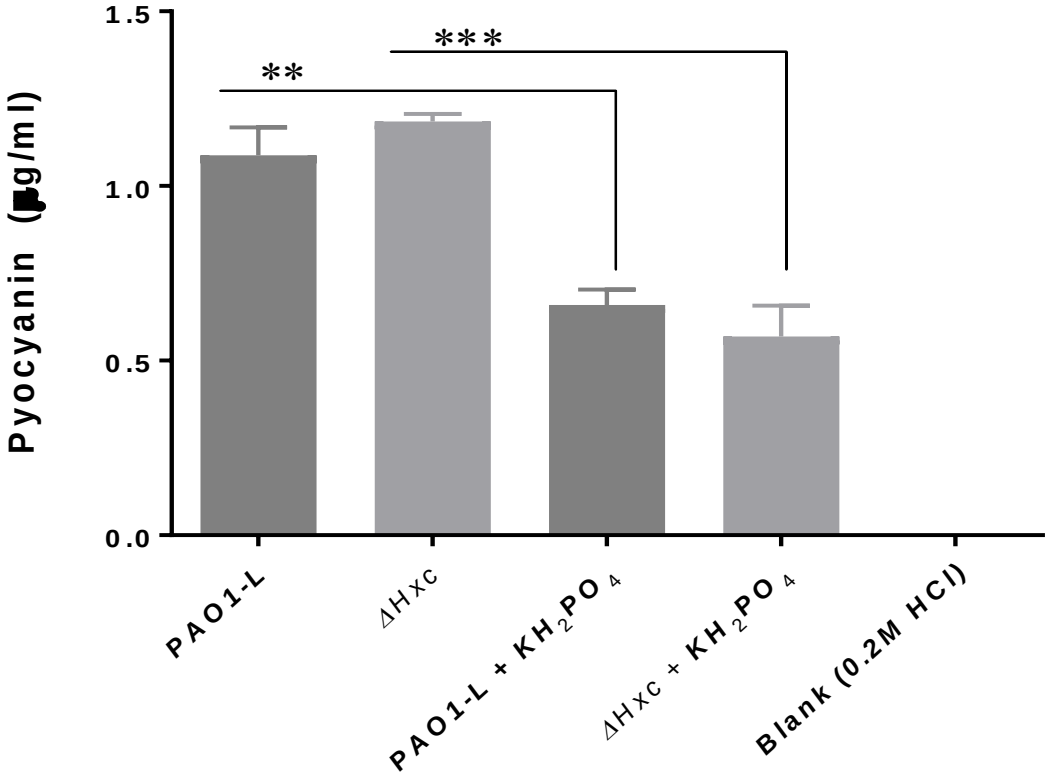


Figure 3.12| Production of pyocyanin in *P. aeruginosa* PAO1 strains. Pyocyanin in supernatants from overnight bacterial cultures in LB supplemented with or without 10mM exogenous phosphate was extracted with 4.5 ml chloroform. It was quantified at OD_{520} nm in 0.2 M HCl acidic medium. Data represent Mean ± SEM of 3 independent experiments (***P = 0.0003, ** *P* < 0.001, 95% CI of diff, Tukey's multiple comparisons test)

3.4.2.5 No major differences between proteins secreted by PAO1-L WT and PAO1-L Δ*hxc*

We further analysed the supernatants of *hxc* mutant strain in comparison to the WT and PAAMP1 strain for protein content. The bacterial strains were grown under phosphate limitation at 37 °C, 200 rpm to an OD_{600} 1.5. Phosphate starvation was chosen because it is a condition reported to induce *hxc* expression [79]. Protein contents

in the supernatants were pelleted by acid precipitation and resolved in a 5 - 20% gradient gel after Coomassie staining (Figure 3.13). Gel profile of the PAAMP1 strain showed a marked reduction in protein content, which could be attributed to the deletion of about 58 kb from its genome. There was no detectable difference in the protein contents between the WT and the *hxc* mutant.

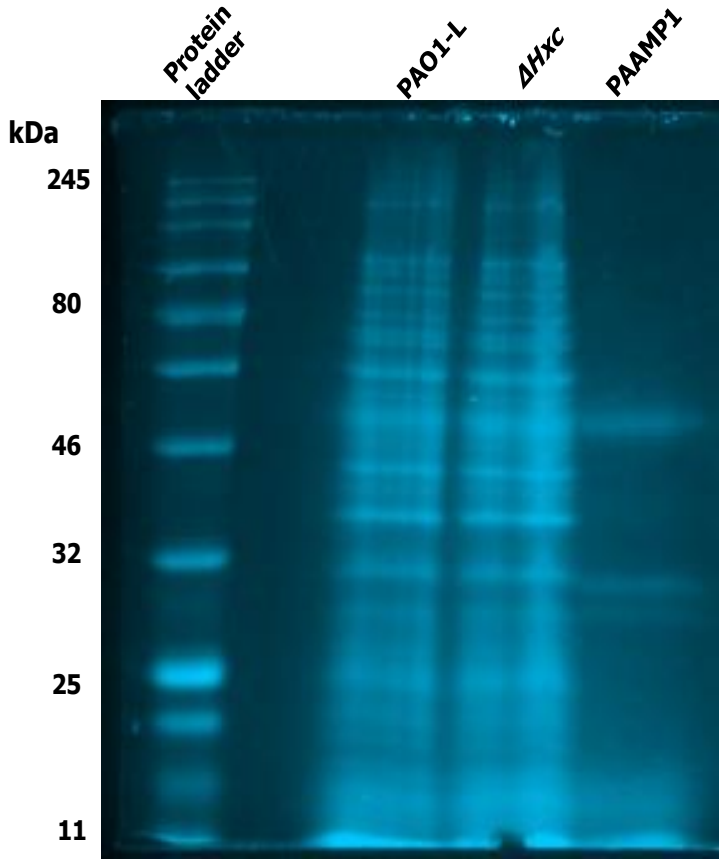


Figure 3.13| Protein gel analysis reveals no difference between wild type PAO1-L and the *hxc*-mutant. PAO1-L, the *hxc* mutant and PAAMP1 strains were grown under phosphate limitation in proteose peptone medium at 37 °C, 200 rpm to an OD_{600} 1.5. Protein contents in the supernatants were pelleted by acid precipitation and resolved in a 10.5% after Coomassie staining. While protein contents in the PAAMP1 strain having 58 kb deletion was markedly reduced, the WT and the *hxc* mutant did not show any difference in protein bands.

Protein analysis of the supernatants was repeated with PAO1-L and Δ*hxc* grown in PPM with or without 10mM exogenous phosphate to determine the effect of phosphate availability on protein secretion. Protein precipitation was also done at increasing concentrations of TCA (12%, 24%, and 48%). The results show that although increasing the percentage of TCA improved the concentration of proteins pelleted from supernatants, the addition of phosphate to the media however reduced protein secretion. The gel profiles did not show any visible differences between the WT and *hxc* mutant using this type of analysis and, due to time constraints, were not analysed further using more sensitive methods (Figure 3.14).

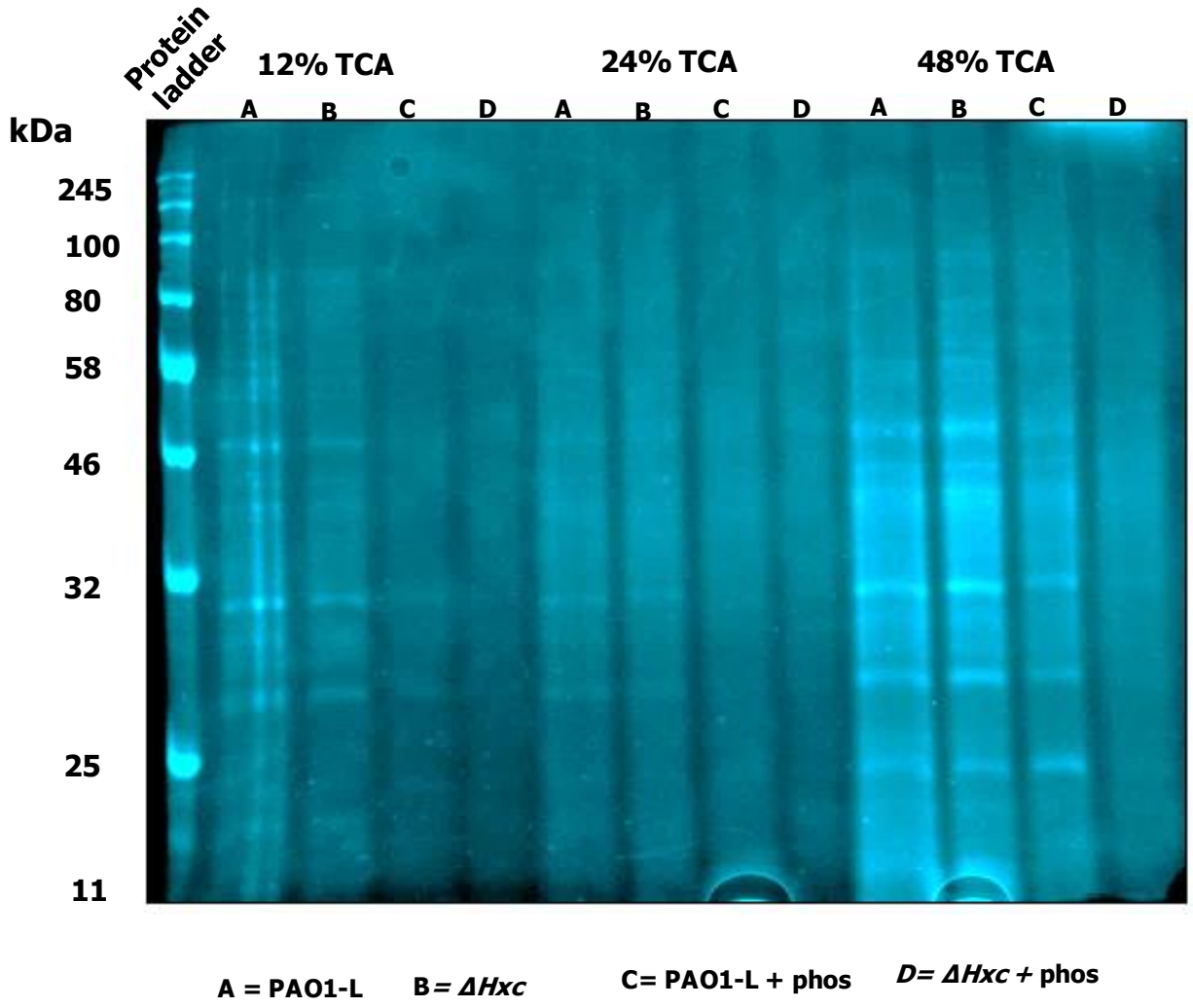


Figure 3.14| Protein gel analysis reveals no difference between wild type PAO1-L and the hxc mutant. Bacterial strains were grown in PPM with or without 10mM exogenous phosphate at 37 °C, 200 rpm to an OD_{600} 1.5. Protein contents in the supernatants were pelleted by acid precipitation and resolved in a 10.5% after Coomassie staining. There were no differences in the bands resolved by both the wild type and the *hxc* mutant with the addition of phosphate to the media reducing protein secretion.

3.4.2.6 Alkaline phosphatase activity in supernatants

P. aeruginosa secretes two alkaline phosphatases, *xcp*-dependent PhoA and *hxc*-dependent LapA. To identify and compare the activity of alkaline phosphatase in their supernatants, PAO1-L, *hxc* mutant and the PAAMP1 were grown in nutrient rich X-Vivo[15], LB or PPM. Cells and supernatant were separated through centrifugation, followed by concentration of the supernatants with 10 kDa ultrafilters. Alkaline phosphatase activity was detected by the addition of 1mg/ml pNPP and measured at OD_{405} nm. Alkaline phosphatase activity measured in the supernatants were standardised to 10 µl of alkaline phosphatase solution serially diluted in 1 mM pNPP (Figure 3.15A). Level of activity in X-Vivo[15] was 9% lower in the *hxc* mutant compared to WT PAO1-L, though not statistically significant (Figure 3.15B). The PAAMP1 strain produced no activity. Surprisingly, alkaline phosphatase activity was not detected in any of the bacterial strains when grown in PPM or LB. The addition of protease inhibitors before concentration of the supernatants did not change the observed trend.

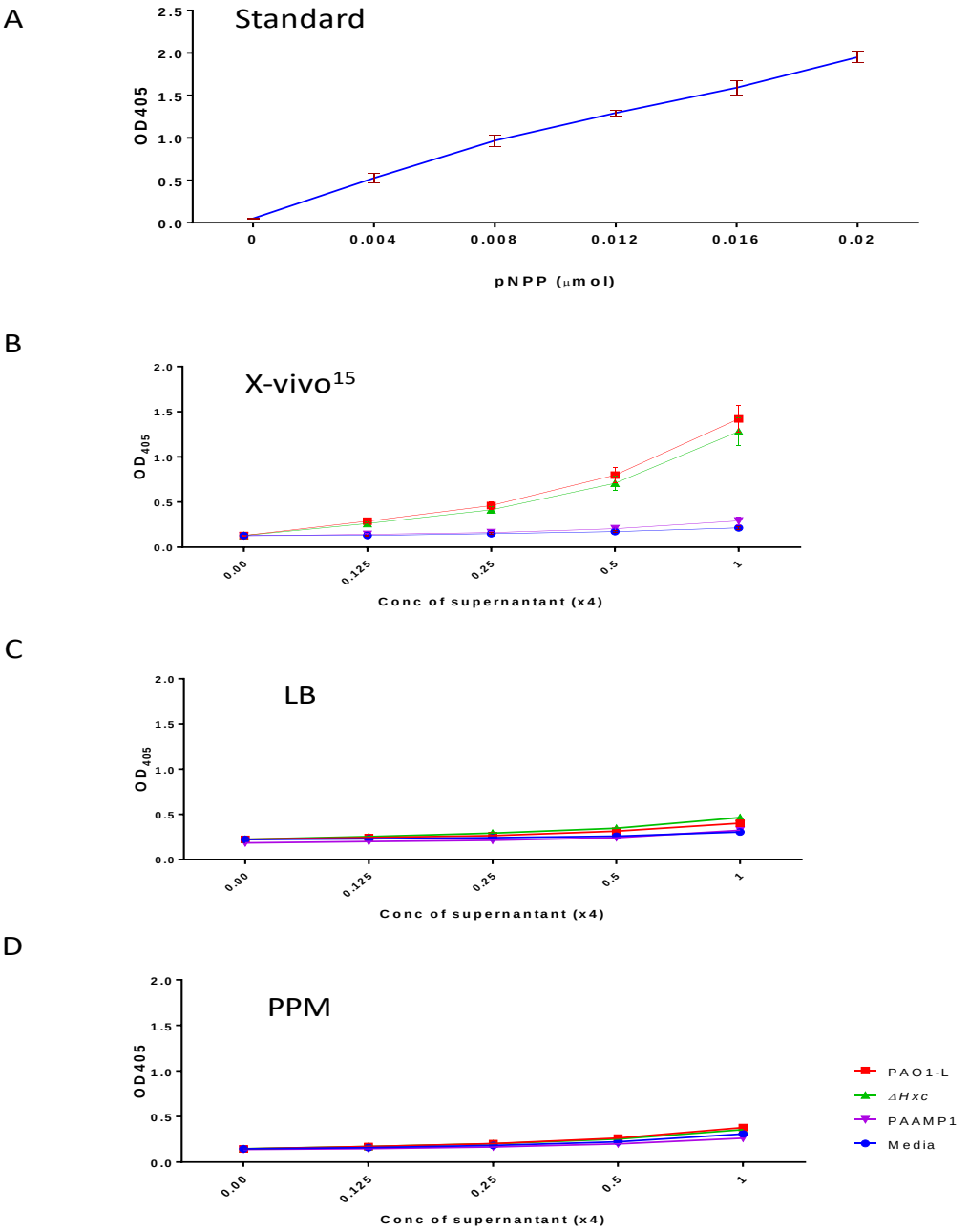


Figure 3.15| Alkaline phosphatase activities measured in the culture supernatant from various *P. aeruginosa* strains. Cells were grown in X-Vivo[15], LB or PPM. Supernatants from cultures of OD_{600} = 1.5 were concentrated and alkaline phosphatase activity was measured by adding 1mg/ml pNPP at OD_{405}. (A) Standard curve of enzymatic activity measured when 10 µl of alkaline phosphatase solution was serially diluted in 1 mM pNPP (B) In X-Vivo[15], level of alkaline phosphatase activity detected in both the *hxc* mutant and wild type were not significantly different. PAAMP1 strain produced no activity. No alkaline phosphatase activity was detected in any of the bacterial strains when grown in LB (C) or PPM (D). Data points represent Mean ± SEM, n = 3

3.4.3 Transcriptional analyses of the *hxc* operon with a bioluminescent reporter mini-CTX-*luxCDABE*

P. aeruginosa has two promoter regions controlling the *hxc* gene cluster located in the intergenic region between HxcV and HxcT. To investigate the expression of the *hxc* operon, two *lux*-based transcriptional fusions were constructed. The 418 bp DNA fragment corresponding to the above intergenic region was amplified and cloned in both orientations into mini-CTX-*lux* vector and subsequently introduced into PAO1-L, yielding PAO1-L mCTX::P_{hxcT}-*lux* and PAO1-L mCTX::P_{hxcV}-*lux.* Bioluminescent activities were measured in X-vivo[15], LB and PPM, with or without the addition of 10mM exogenous phosphate.

Bacterial growth of WT PAO1-L and PAO1-L mCTX::P_{hxcT}-*lux* were comparable in all the test media. As it has been observed earlier, growth rates were significantly higher when exogenous phosphate was added to X-vivo[15] but not in LB or PPM (Figure 13.6B, D, and F). Promoter activity measured from the PAO1-L mCTX::P_{hxcT}-*lux* was 2-fold in X-vivo[15] compared LB or PPM. Addition of exogenous phosphate however significantly inhibited P_{hxcT} promoter activity in X-vivo[15] ($p<0.0001$) and in LB ($p = 0.0427$) but not in PPM (Fig. 3.16A, C and E). This is in contrast to what was reported by Ball *et. al.,* (2002), where they showed that promoter activity was significantly abolished when inorganic phosphate was added in PPM [79].

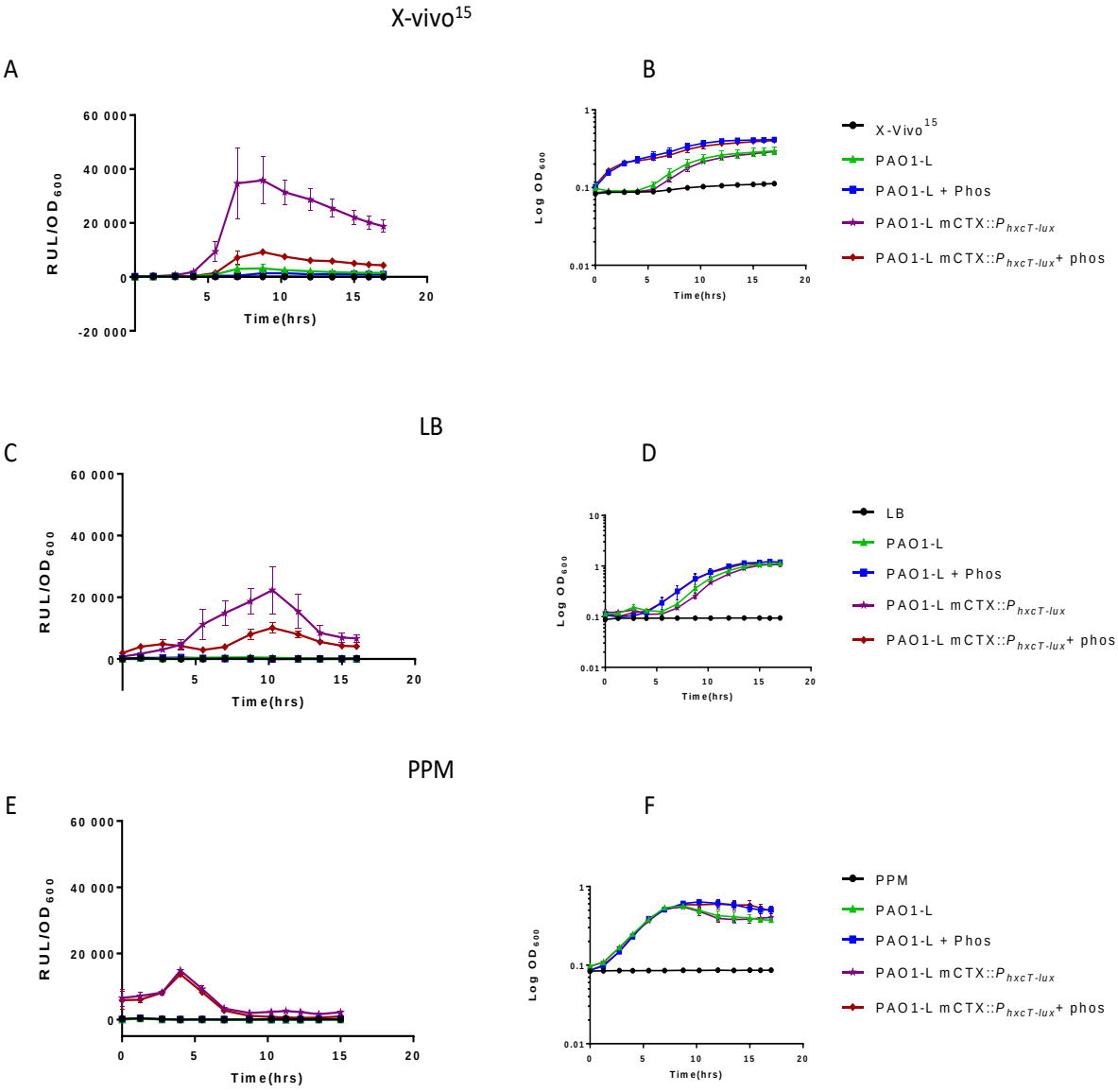


Figure 3.16| Transcriptional activity of the PhxcT promoter. The P_{HxcT} promoter transcriptional fusion to the *lux* genes was introduced into WT PAO1-L to obtain PAO1-L mCTX::P_{hxcT}-*lux*. The growth of WT PAO1-L and PAO1-L mCTX::P_{hxcT}-*lux* fusion was monitored in (B) X-vivo[15], (D) LB or (F) PPM with or without 10 mM exogenous phosphate at 37 °C. Promoter activities were measured in (A) X-Vivo[15], (C) LB or (E) PPM using Tecan, infinite 200Pro. (**** $P < 0.00001$, *p =0.0427, 95% CI of diff, Tukey's multiple comparisons test; Data points represent Mean ± SEM, n = 3)

In comparison with mCTX::P_{hxcT}-*lux,* promoter activity measured from mCTX::P_{hxcV}-*lux* was about 3 times more in X-vivo[15], and about 10 times more in LB and PPM (Figure 3.17A, C and E). This observation, again, is opposite to the findings of Ball *et. al.,* (2002), which reported that P_{hxcV} promoter activity is about five times less compared with the activity observed from P_{hxcT} promoter [79].

Our findings show that transcriptional activity from P_{hxcV} begins early and reaches peak at about 4 - 5 hours post-incubation while activity from P_{hxcT} peaks later at about 10 hours in X-vivo[15] or LB, and at about 6 hours in PPM. The results also show that promoter activity from PAO1-L mCTX::P_{hxcV}-*lux* similar to PAO1-L mCTX::P_{hxcT}-*lux* was sustained longer in X-vivo[15]. Moreover activity from PAO1-L mCTX::P_{hxcV}-*lux* was significantly reduced ($p=0.0006$) when exogenous phosphate was added to X-vivo[15] (Fig. 3.17A) unlike activity in LB or PPM, which remained unchanged.

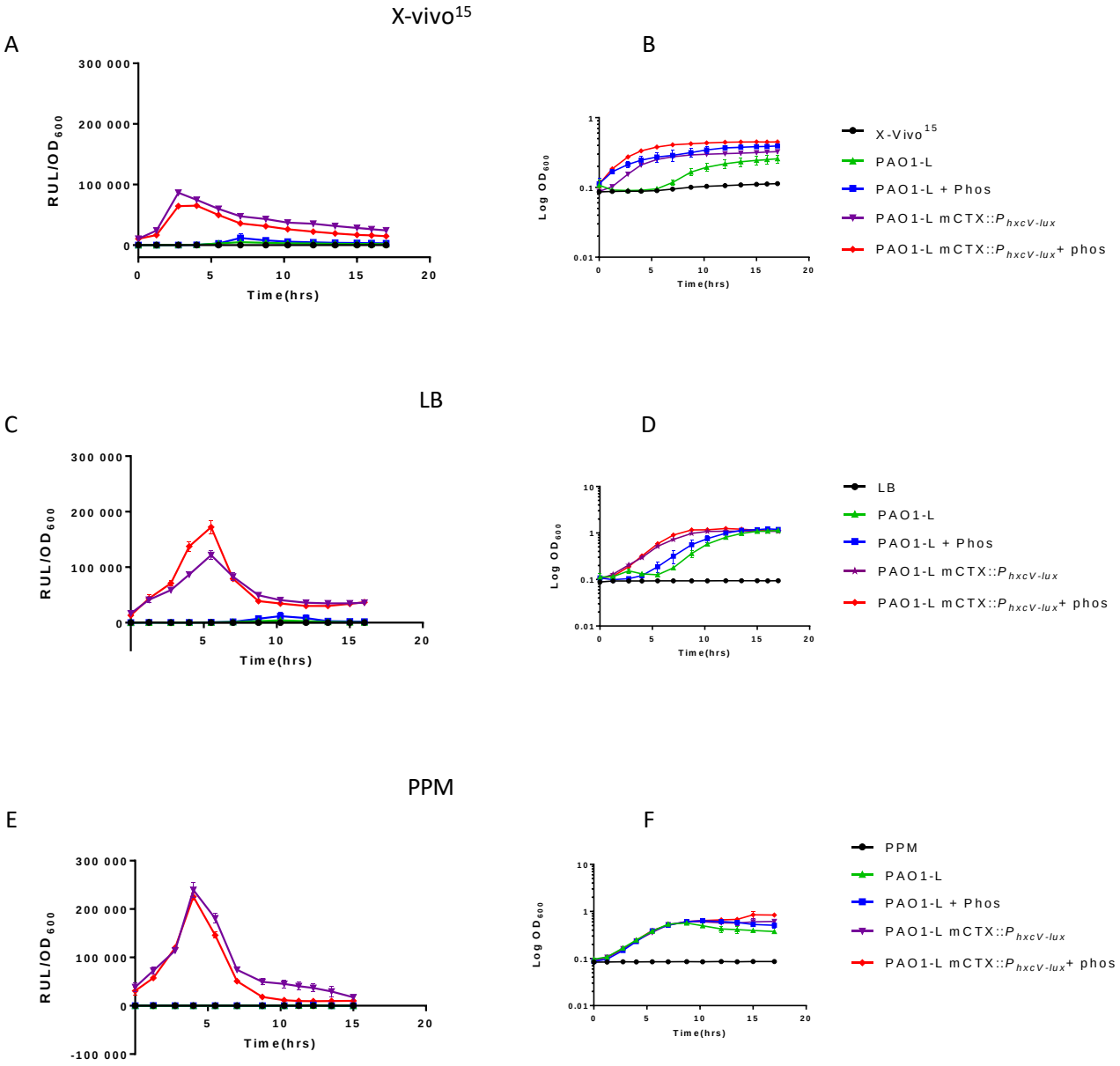


Figure 3.17| Transcriptional activity of the PhxcV promoter. The P*HxcV* promoter transcriptional fusion to the *lux* genes was introduced into WT PAO1-L to obtain PAO1-L mCTX::P*hxcV*-*lux*. The growth of WT PAO1-L and PAO1-L mCTX::P*hxcV*-*lux* fusion was tested in (B) X-Vivo[15], (D) LB or (F) PPM with or without 10 mM exogenous phosphate at 37 °C. Promoter activities were measured in (A) X-Vivo[15], (C) LB or (E) PPM using Tecan, infinite 200Pro. (*** *P*= 0.0006, 95% CI of diff, Tukey's multiple comparisons test; Data points represent Mean ± SEM, n = 3)

3.5 Discussion

P. aeruginosa employs diverse secretion systems which helps the organism to thrive in hostile environmental conditions by selectively releasing proteins to assimilate essential nutrients, to evade the host immune response and to eliminate other competing microbes [81]. Depending on the secretion machinery used, these proteins could remain attached to the bacterial cell surface, released into the extracellular milieu or be injected directly into the host cell [81].

P. aeruginosa possesses five secretion systems with the Xcp-T2SS being the most versatile machinery used by the bacteria and the well-studied [81, 94]. However, studies in the past decade have showed that the bacterium has a second T2SS gene cluster, the hxc-T2SS, which is involved in the secretion of LapA [79, 81, 94]. Although the Xcp machinery in *P. aeruginosa* has about fourteen characterised substrates including PhoA, LapA is the only substrate so far assigned to the hxc system (Table 1). The fact that *P. aeruginosa* has dedicated two independent systems for the secretion of two types of alkaline phosphatases is an indication of how important phosphate assimilation is to the bacterium. Yet, the full contribution of LapA to pathogenesis of *P. aeruginosa* or its involvement in interactions with *C. albicans* has not received much attention.

To understand the role of hxc-T2SS during *P. aeruginosa* and *C. albicans* co-infection, and to ascertain if the hyphal inducing agent in

PAO1-L supernatant is LapA, we constructed an in-frame deletion mutant lacking the *hxc*-operon (Δ*hxc*) in the PAO-L background using chromosomal gene exchange. Successful deletion of the *hxc* gene cluster in the PAO1-LΔ*hxc* mutant was verified by PCR and nucleotide sequencing.

In comparison to the wild type PAO1-L, the PAO1-LΔ*hxc* mutant was phenotypically assessed to further determine the function of the hxc-T2SS. Testing the impact of this system on bacterial growth, biofilm and microcolony formation, and pyocyanin production, we observed no changes in these phenotypes in the PAO1-LΔ*hxc* mutant. This observation is in agreement with the work of Ball et al (2002), which showed that deleting the *hxcR* in the *P. aeruginosa hxc* operon did not alter vital phenotypic expressions in the bacterium, such as twitching mobility, growth response or protein components of the bacterial outer membrane [79].

Remarkably, addition of exogenous phosphate to culture medium significantly increased bacterial growth as well as biofilm formation in X-vivo^{15} under static conditions, which was independent of the *hxc* gene cluster. Phosphate is an important component of many biomolecules such as lipids, nucleic acids, proteins and sugars, which plays crucial role in cellular activities [198]. The finding in this study is consistent with published work where addition of inorganic

phosphate was able to induce fragmentation and cell division in *P. aeruginosa* at 46 °C, a bacteriostatic condition [199]. The promotion of Δ*hxc* mutant growth with the addition of exogenous phosphate shows that *P. aeruginosa* is able to assimilate exogenous phosphate independent of the hxc T2SS. As the bacterium employs two independent T2SS, it is thought that the deletion of the *hxc* gene cluster might be compensated for by the Xcp-T2SS in terms of phosphatase secretion. It has been reported that only double deletion of *hxc/xcp* gene clusters completely eliminated extracellular alkaline phosphatase activity [79].

Although adding exogenous phosphate to our culture media significantly promoted bacterial growth and biofilm formation, results from our study indicate that pyocyanin secretion was markedly reduced in both the mutant and the WT PAO1-L under these conditions. Phosphate availability has been implicated in *P. aeruginosa* infection where some studies have reported that abundance of phosphate can reduce the bacterial pathogenesis [200]. Moreover, low phosphate culture medium has been shown promote the highest levels of pyocyanin production by *P. aeruginosa* [197].

Our investigation of culture supernatants from PAO1-L and the *hxc* mutant for changes in protein contents and alkaline phosphatase

activity did not reveal any noticeable difference when grown in phosphate limiting PPM, or nutrient-rich LB or X-Vivo[15]. From the work of Ball *et. al.,* (2002), a 40 kDa LapA protein was found in the WT PAO1 but not in the corresponding *hxcR* mutant when the bacteria were grown under phosphate limiting conditions, and that the *hxc* mutant retained only 20 % alkaline phosphatase activity compared to PAO1-L [79].

Having followed the procedure reported in Ball *et. al.,* (2002), without discounting the possibility of strain difference, we believe the lack of activity in our supernatants may be as a result of low level of alkaline phosphatase released into the media at the time point when supernatants were prepared. We harvested our supernatant from cultures of OD_{600} = 1.5 which corresponds to 4 hours in LB, 5 hours in PPM and 7 hours in X-Vivo[15] at 37 °C, 200 rpm. These time points are thought to be too early for the maturation and extracellular secretion of LapA. This suggestion is supported by our study of the transcriptional activities of the *hxc* operon which revealed that the expression of P_{hxcT} and P_{hxcV} promoters peaks at about 10 hours and 5 hours post-incubation respectively. It is thus not surprising that alkaline phosphatase activity was not detected in LB or PPM. It is also important to note that protein analysis were done using supernatants recovered from bacterial strains grown in PPM to the same OD_{600} =

1.5 and hence the lack of identification of any differences in protein profiles in the supernatants from WT and mutant strains.

With alkaline phosphatase activity detected when the bacterial strains were grown in X-Vivo[15], we posit that it was more of *xcp*-dependent PhoA and less LapA which was found in the supernatant. This probably explains why the *hxc* mutant retained about 90% of alkaline phosphatase activity compared to the WT.

Our investigation of the promoter activity confirms earlier studies that the *P. aeruginosa hxc* operon is controlled by two promoters in opposite orientation: P_{hxcV} and P_{hxcT}. We also show that transcriptional activities from the two promoters are sustained longer in X-vivo[15] compared to LB or PPM. X-vivo[15] is a well-defined serum-free medium routinely used to support proliferation of mammalian cell lines. Since transcription activity of the *hxc* operon seemed to be sustained in such eukaryotic cell media, we probed promoter activities of the mCTX::P_{hxcT}-*lux* and mCTX::P_{hxcV}-*lux* fusions when in contact with differentiated and undifferentiated human bronchial epithelial, which is fully discussed in Chapter 4 of this thesis.

The inhibition of transcriptional activity in X-vivo[15] by the addition exogenous phosphate confirms that the *hxc* operon is needed under phosphate limitation to improve secretion of alkaline phosphatase and inorganic phosphate acquisition as previously reported by Ball *et.*

al., (2002) [79]. It is not clear why addition of exogenous phosphate to LB or PPM did not reduce promoter activity, however we suggest our media may contain phosphate chelating agent(s) which possibly removed exogenous phosphate and thus prevented the bacterium from sensing it. An alternative possibility could be that the 10 mM exogenous phosphate added may have not been enough to repress transcriptional activity in LB and PPM. This reasoning might implicate that X-vivo[15] already contains phosphate, hence addition of 10 mM exogenous phosphate provided an optimal concentration to inhibit promoter activities. This cannot be confirmed as all components of X-vivo[15] are not publicly available.

Put together, we have successfully generated PAO1-LΔ*hxc* strain, and our phenotypic studies indicate that bacterial growth, biofilm formation and pyocyanin expression were not detectably different compared to WT PAO1-L. The hxc-and xcp-T2SS are believed to augment each other in terms of alkaline phosphatase secretion and inorganic phosphate acquisition. Therefore, the interplay between these two secretion machineries might be important in the release of substrates specific to a particular environmental cue [79]. Also, our work has confirmed that phosphate limitation or availability may have an effect on the expression of *P. aeruginosa* virulence factors such as biofilm formation or secretion of pyocyanin.

Phosphate depletion in humans has long been associated with infections of Gram negative bacteria [175, 176, 201]. In *P. aeruginosa*, virulence factors such as PA-I lectin, biofilm, pyocyanin and PstS, a phosphate scavenging protein, were shown to be highly expressed *in vivo* in surgically injured mice [202]. It was identified that local depletion of phosphate was a key environmental signal to which intestinal *P. aeruginosa* responded by increasing the expression of multiple virulence traits [176, 202].

Another study which examined multi-drug resistant (MDR) clinical isolates of *P. aeruginosa,* found the expression of appendage-like structures on the bacterial cell surfaces which contained PstS. Formation of PstS was found to be induced under phosphate limitation. Directly injecting MDR strains into intestinal tract of surgically injured mice was reported to cause 60%–100% mortality rates, while restoration of gut phosphate was found to prevent mortality [203]. Interestingly, construction of MPAO1 mutant deficient of *hxcR* significantly reduced PstS surface expression. PstS rich appendages were found to be involved in adherence to and destruction of epithelial cells by the bacterium. While the mechanism is not clear, this gives an indication that the hxc-T2SS might play a role in the conferment of virulence phenotype to *P. aeruginosa* MDR isolates [203].

With evidence pointing towards the correlation of phosphate depletion to infectious-related mortalities and the increasing prevalence of MDR strains of *P. aeruginosa* [176], it is important to understand the biology of the hxc-T2SS and explore it as a potential therapeutic target.

Chapter 4: Contribution of *hxc* to the interaction of PAO1-L with human bronchial epithelial cells.

4.1 Introduction

The epithelial lining of lung airways have cellular composition whose topography fulfils the functional needs of mucociliary clearance, hydration, host defence, and gas exchange [204]. Disturbance of epithelial linings because of damage by mechanical devices or, as in the case of CF, because of the presence of a thick mucus layer, can lead to severe infections by opportunistic pathogens such as *P. aeruginosa*. The colonisation of such niche often initiates a complex cascade of events that influence the immediate and long-term outcome of this interaction [204, 205]. With regards to CF, numerous patients suffer from chronic respiratory infections caused by *P. aeruginosa* which drastically increase mortality and morbidity [71].

In addition to providing an effective barrier against infections in the lung, bronchial epithelial cells produce broad spectrum antimicrobial agents such as β-defensins, lactoferrin, and hydrolytic lysozyme [206]. Moreover, recognition of pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs) expressed on bronchial epithelial cells can alert the immune system of the presence of pathogens through the production of cytokines and chemokines. *P. aeruginosa* PAMPs such as LPS, flagellin and peptidoglycan are recognised by PRRs expressed by epithelial cells including toll-like receptor (TLR)-4, TLR5/asialoGM1, and TLR2/nucleotide binding and

oligomerisation domain (NOD1/2) respectively [207, 208]. Ligand-receptor binding on epithelial cells leads to activation of intracellular signals (Figure 4.1) that results in the production of pro-inflammatory cytokines such as Tumour necrosis factor alpha (TNF α), Granulocyte-colony stimulating factor (G-CSF), Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interleukin (IL)-33, IL-27, IL-17C, IL-6 and chemokine IL-8 [206, 208].

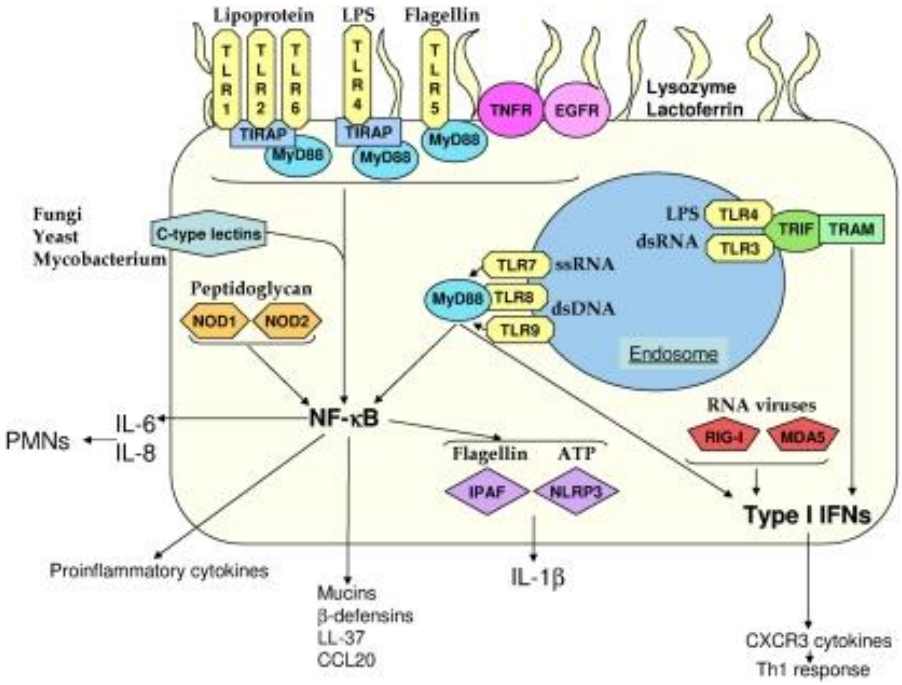


Figure 4.1 | Pathogen recognition and innate immunity by the respiratory epithelial cells. Airway epithelial cells express PRRs that can be divided into (1) transmembrane surface receptors including TLR1-6, (2) intracellular receptors such as endosomal receptors TLR3, 4, 7-9 and (3) cytosolic receptors such as retinoic acid inducible gene (RIG-I), melanoma differentiation–associated protein 5 (MDA5), NOD1,2, IL-1β-converting enzyme protease activating factor (IPAF) and NOD-like receptor pyrin domain (NLRP3). Ligand-receptor binding activates intracellular signals that leads to the production of inflammatory cytokines. Figure taken from [208].

There has been an increase in our understanding of the development and maintenance of the pulmonary epithelium thanks to advances in cell isolation, *in vitro* culture techniques and clinical microbiology [204], but there are still grey areas on the molecular mechanisms that underpin the colonisation by *P. aeruginosa* [205].

Work leading to this Chapter focused on the generation of a mutant in the Hxc-T2SS and analysis of factors that could influence its transcription. Data so far shows tight regulation and high dependency on culture conditions for the transcription of genes encoding this secretion apparatus which imply that this system plays an important role on the adaptation of *P. aeruginosa* to its environment. Previous data indicated that the *hxc* operon is expressed and sustained in eukaryotic cell media such as X-vivo[15]. Further, the *Hxc* operon has been shown to be upregulated during the interaction of *P. aeruginosa* with lung epithelial cells [209]. In light of these findings, we investigated the contribution of Hxc-T2SS to the interaction of *P. aeruginosa* with human bronchial epithelial in an acute infection setting.

The selection of Calu-3 cells as an infection model for this study was due to the capacity of this cell line to differentiate into a thick pseudostratified layer with tight junctions, microvilli and secretory granules; comparable to properties found in human airway epithelial

cells. This has been demonstrated in our lab where scanning electron microscopy analysis showed that differentiated Calu-3 cells produced cilia-like apical protrusions at day 21 in air-liquid interface (ALI) cultures (Figure 4.2) (Yi-Chia Lui, PhD Thesis, 2014). Calu-3 human bronchial epithelial cells were originally derived from bronchial adenocarcinoma in a 25-year-old male [210]

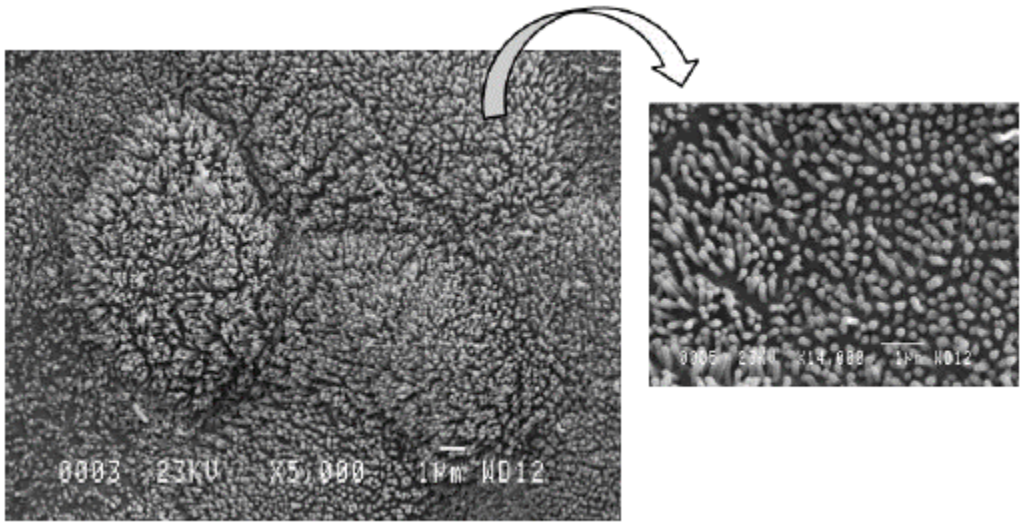


Figure 4.2| Calu-3 cells at ALI for 21 days exhibited microvilli at their apical surface. Calu-3 cells were seeded in an ALI at a density of 100,000 cells per well for 21 days at 37°C, 5% CO_2. Scanning electron microscopic images showed that Calu-3 cells displayed short and thick microvilli at their surface. Image shown on the left is at 5,000x magnifications and individual microvilli were clearly observed when image was zoomed to 14,000x magnifications (right). Figure taken from (Yi-Chia Lui, PhD Thesis, 2014).

4.2 Hypothesis and objectives

We hypothesise that the hxc operon could play an important role during the infection of bronchial epithelial cells by *P. aeruginosa*.

To test this hypothesis this chapter analyses the contribution of Hxc to the interaction of *P. aeruginosa* with Calu-3 cells. Towards this aim, Calu-3 cells were infected with PAO1-L and *Δhxc;* and bacterial growth, cytotoxicity and cytokine production were investigated. In addition, the reporter strains mCTX::P_{hxcT}-*lux* and miniCTX::P_{hxcV}-*lux* were exploited to investigate how Hxc transcription was regulated during infection under different conditions.

4.3 Materials and Methods

4.3.1 Preparation of *P. aeruginosa* inoculum for infection assay.

P. aeruginosa strains and promoter fusion constructs used in this study are listed in Table 4.1. Bacterial strains were routinely streaked on LB agar plates from -80 ^{o}C stocks and cultured at 37^{o}C overnight. A single colony was isolated the next day to prepare a 5 ml overnight culture in LB (37 ^{o}C, 220 rpm). Bacterial density was measured the following day at OD_{600nm} using a spectrophotometer and the inoculum was adjusted to 1% to inoculate a 25 ml culture in LB using a 250 ml conical flask. The new culture was incubated for 4 hours at 37 ^{o}C, 220 rpm, after which it was transferred to a sterile 50 ml centrifuge tube and centrifuged for 5 min at 3578 rcf (Allegra X-64R with C0650 fixed angle rotor, Beckman Coulter, UK) at 4 ^{o}C. Bacterial pellets were washed with cold Dulbecco's Phosphate-Buffered Saline (DPBS) with calcium and magnesium (Thermo Fisher Scientific) and then in Dulbecco's modified Eagle's medium (DMEM)/ Nutrient Mixture F-12 Hams (1:1) (DMEM-F12, Sigma-Aldrich, UK) under same centrifugation conditions. Bacterial pellets were resuspended in 5ml DMEM-F12, and cell density was measured at OD_{600nm} in an aliquot having 1:10 dilution in DMEM-F12. Bacteria suspension was diluted in a 10-fold fashion, 100 µl plated on LB agar and incubated at 37 ^{o}C for 18 hours. Bacterial inoculum was standardised by counting colony

forming units (CFU) from the dilution plate using the following formula:

Bacteria/ml = Total number of colonies counted x Dilution factor x Dilution point x 10

Table 4.1| List of *P. aeruginosa* strains used in this study

Strain	Properties	Reference/Source
PAO1-L	Wild-type *P. aeruginosa* from Université de Lausanne, Switzerland	Dieter Haas' laboratory in Lausanne
PAAMP1	PAO1-L with a 58 kb chromosomal deletion between 724103 – 780973	Quorum Sensing Group
PAO1-LΔ*hxc*	*hxc* chromosomal deletion mutant in PAO1-L background	This work
PAO1-L mCTX::P$_{hxcT}$-*lux*	PAO1-L with *lux* a promoter fusion towards *hxcT*	This work
PAO1-L mCTX::P$_{hxcV}$-*lux*	PAO1-L with *lux* a promoter fusion towards *hxcV*	This work

4.3.2 Calu-3 cell culture and differentiation

Calu-3 human bronchial epithelial cells (ATCC no. HTB-55) purchased from the American Type Culture Collection (Rockville, USA) were used between passage 19 and 35.

Newly thawed cell stocks were cultured initially in 75 cm^2 flasks (Corning Life Sciences, the Netherlands) in 15 ml medium. The medium consisted of DMEM-F12 containing 10% fetal calf serum (FCS) (Sigma-Aldrich, UK), and 1x non-essential amino acid solution (Sigma-Aldrich, UK) (thereafter designated 'complete media'). The cells were maintained at 5% CO_2, 37 °C and culture media was changed every 2 days until cells reached about 95% confluence. The cell cultures were regularly viewed under light microscopy at 200x Magnification. On the day of seeding, cells cultured in T-75 were washed with pre-warmed phosphate buffered saline (PBS) solution (Sigma-Aldrich, UK) and trypsinised with 1x trypsin solution (0.05% trypsin and 0.02% EDTA in PBS) at 37 °C for 15 min. Cells were detached from the flask and dispersed by pipetting up and down in 10ml DMEM-F12. The suspension was transferred into a 50 ml falcon tube and washed by centrifugation at 1000 rpm for 5 min. Supernatant was discarded and cells were resuspended in 10 ml complete media.

For undifferentiated Calu-3 culture system, cells were seeded at a density of 250,000 cells per well of a 24 well tissue culture plate

(Thermo Fisher Scientific) in 500 µl of complete media. The culture was maintained in 5% CO_2 at 37 ^oC until cells reach about 95% confluence for infection on day 8.

For differentiation, Calu-3 cells were seeded in 0.4 µM pore size membrane inserts (Corning Life Sciences, Netherlands) at a density of 100,000 cells per well in 500 µl of complete medium with 1 ml of medium in the basal chamber. After overnight culture at 37 ^oC, 5% CO_2, the medium in the insert was aspirated for the cells to differentiate under ALI conditions. The medium in the bottom chamber was changed every other day up to 21 days.

4.3.3 Infection of undifferentiated Calu-3 cells with *P. aeruginosa*

On the day of infection, culture medium was removed from the wells and the Calu-3 confluent monolayer was washed in complete media. The number of Calu-3 cells present in a well was counted after trypsinisation and estimated as $8.9x10^5$ cells per well. The cells were then infected with 250 µl of PAO1-L or PAO1-LΔ*hxc* at multiplicity of infection (MOI) of 50 in duplicate. Infected cultures were incubated at 37 ^oC, 5% CO_2 for 3 and 6 hours. Supernatant was collected after incubation, centrifuged at 225 rcf for 5 min and then 9504 rcf for 20 min in a refrigerated bench top centrifuge. The cell-free supernatants were stored at -80 ^oC for further analysis. To determine the number

of bacteria that associated with the epithelium, 250 µl of 0.1% triton X-100 in PBS (Sigma-Aldrich, UK) was added to lyse the Calu-3 cells. The triton/bacterial suspension from each well was serially diluted in complete media at 1:10 ratio before plating 100 µl on LB agar in duplicates. The plates were incubated overnight at 37 ^{o}C and colonies were counted the next day.

4.3.4 Infection of differentiated Calu-3 cells with *P. aeruginosa*

On day 21, differentiated Calu-3 cells present in the transwell were counted after trypsinisation and estimated as $9.2x10^{6}$ cells per well. Medium in the basal chamber was discarded and replaced with fresh complete media before addition of 100 µl of bacterial suspensions to the apical surface of the Calu-3 cells to achieve MOI 50 in duplicate. Schematic representation of this infection model is shown in Figure 4.3. Cultures were incubated at 37 ^{o}C, 5% CO_2 for 3 and 6 hours. Media at the basal chamber as well as the supernatant in the transwells were recovered post infection and centrifuged at 4^{o} C 225 rcf for 5 min and then 9504 rcf for 20 min in a benchtop centrifuge to remove cells and bacteria. Supernatants were stored at -80 ^{o}C for further analysis. Bacterial CFU in the transwells were quantified post infection after lysis of the Calu-3 cells as previously described.

Figure 4.3 | Diagram of the in vitro respiratory epithelium model for P. aeruginosa infection. Calu-3 cells were grown on 0.4 μM pore size membrane inserts at air-liquid interface at 37 °C, 5% CO_2 for 21 days. The cells in this condition differentiated into a pseudostratified mucociliary epithelium. On the day infection, *P. aeruginosa* was inoculated on the apical surface of the airway epithelium to mimic bacterial infection in the human bronchial epithelium. Figure adapted from Yi-Chia Liu (PhD thesis, 2014).

4.3.5 Detection of multiple biomarkers in supernatants from infection assay

Supernatants recovered from 6 hour infection of undifferentiated bronchoepithelium with PAO1-L and PAO1-LΔ*hxc* strains were assessed for the presence of cytokines including: TNF α, G-CSF, GM-CSF, Interferon beta (IFN β), IL-33, IL-27, IL-17C, IL-6, IL-8, IL-10, and IL-1β. Cytokine quantification was carried out using the Human Premixed Multi-Analyte Kit according to the manufacturer's instructions (R&D Systems, UK). Briefly, magnetic beads pre-coated with analyte-specific antibodies and fluorophores were reconstituted in assay buffer after which 50 μl was added to each well. After washing, 50 μl of diluted standards and sample supernatants were

pipetted into the wells in duplicate. The plate was incubated at 850 rpm for 30 min in a room temperature, washed three times in 100 µl wash buffer before the addition of 50 µl biotinylated detection antibody cocktail for 10 min under same conditions. Following a wash, 50 µl streptavidin-phycoerythrin conjugate (Streptavidin-PE) was added to each well and the plate was incubated in the dark for 10 min. After washing the plate three times, the beads were resuspended in 100 µl buffer and read using the Bio-Plex® 200 analyser (Bio-Rad, UK).

4.3.6 Quantification of cell cytotoxicity by measuring LDH release

Supernatants from 3 or 6 hour infection of undifferentiated Calu-3 cells with PAO1-L or PAO1-LΔ*hxc* were assessed for cellular toxicity using Lactate dehydrogenase (LDH) Cytotoxity Assay Kit according to the manufacturer's instructions (Thermo Scientific™, UK). Infection of undifferentiated Calu-3 cells was done as previously described, however FBS was omitted from the complete medium due to its high background effect (Yi-Chia Liu, PhD thesis, 2014). In brief, 50 µl of sample supernatants were mixed with 50 µl of reaction solution (diaphorase, NAD+ mixture, iodotetrazolium chloride, and sodium lactate) in a flat-bottom 96-well microtitre plate (Corning Life Sciences, Netherlands). Supernatant recovered from Calu-3 cells treated with 500 µl of 2% triton X-100 prepared in DPBS was used

as a positive control which represented 100% cell death. Absorbance was measured at OD_{490} nm and percentage cytotoxicity was calculated by comparison with the OD readings from the positive control.

4.3.7 Detection of alkaline phosphatase activity in supernatants from infection assay

Supernatants recovered post 6 hour infection of undifferentiated Calu-3 cells with PAO1-L or PAO1-LΔ*hxc* were assayed for alkaline phosphatase activity. Alkaline phosphatase activity was quantified using colorimetric Alkaline Phosphatase Assay kit (Sigma, UK) as previously described (see Section 3.3.6.6).

4.3.8 Transcriptional analyses of the *hxc* operon when in contact with undifferentiated bronchoepithelium

Calu-3 cells maintained under undifferentiated culture conditions were infected with PAO1-L constructs having promoter fusions. Calu-3 cells, bacterial culture conditions and infection were done as previously described (Section 4.3.3.). *Lux* activity was assessed using Hamamatsu light box (Hamamatsu photonics, Japan). In other tests, the promoter constructs were treated with supernatants recovered from infection assays. Under this condition, mid-log phase PAO1-L mCTX::P_{hxcV}-*lux* and PAO1-L mCTX::P_{hxcT}-*lux* were diluted to OD_{600} 0.01 in complete media or DMEM-F12. 175 µl of the bacterial

suspension was inoculated in a 96 flat bottom black Polystyrene microplates (Sigma, UK), after which 25 µl of supernatants assayed from 6 hour infection was added to each well (12.5% of total volume of 200 µl) in duplicate. Bioluminescence was measured at 37 °C for every 30min using Tecan luminescence plate reader.

4.4 Results

4.4.1 Optimisation of bacterial inoculum for the infection of bronchial epithelium

To ensure optimal viability of *P. aeruginosa* strains before infection, the bacteria were cultured in LB medium. The decision to use LB was due to its global use for the phenotypic characterisation of *P. aeruginosa* and also because growth of WT PAO1-L and PAO1-*LΔhxc* were comparable in LB. Since LB medium contains yeast extract and tryptone, which could potentially contain nucleic acids, endotoxins, PAMPs, and DAMPs capable of eliciting an immune response from the Calu-3 cells (Yi-Chia Lui, PhD thesis, 2014), bacterial cells pelleted from supernatants were thoroughly washed with DPBS containing $CaCl_2$ and $MgCl_2$ and complete media before finally being resuspended cells in complete media for infection.

To standardise the number of bacteria in the inoculum, the bacterial cells after washing were diluted to reach OD_{600nm} 1.21 which, according to previous work in the laboratory, corresponds to 4.135 x 10^8 bacterial cells per ml (Sirina Muntaka, PhD thesis, 2017). From Figure 4.4, our colony count showed that OD_{600nm} 1.21 corresponded to 7.15 x 10^9 cells/ml for PAO1-L while for PAO1-LΔ*hx*c corresponded to 8.35 x 10^9 cells/ml. Different handling of the bacterial culture was suggested as the reason why our PAO1-L numbers departed from the

reported figure. Thus the new estimates were used to calculate MOI values for the subsequent infection assays

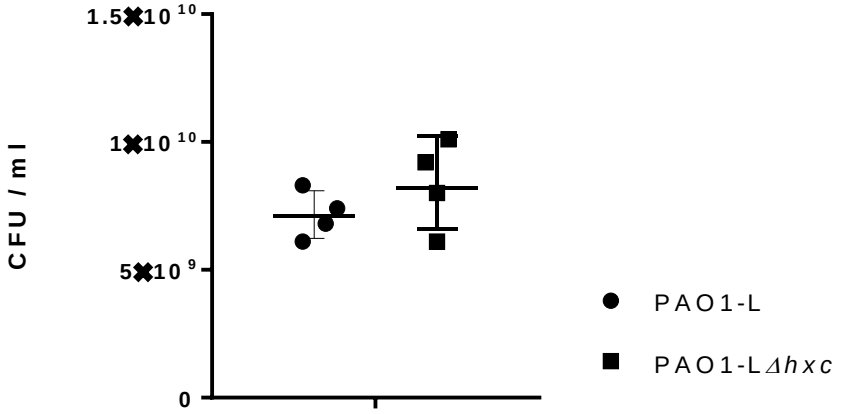


Figure 4.4| Optimisation of bacterial CFU in correlation to $OD_{1.21}$ nm.
Bacterial cells pelleted from 4 hour log-phase culture was standardised to OD_{600nm} 1.21. Serial dilutions were prepared and plated on LB agar to obtain CFU. Data represent individual replicates of two independent experiments with means connected.

4.4.2 Infection of undifferentiated bronchoepithelium with *P. aeruginosa* strains

Undifferentiated Calu-3 monolayers were first used to test the fitness level of the PAO1-LΔ*hxc* mutant compared with PAO1-L. The Calu-3 cells were grown in liquid-covered culture at 37 ^{o}C, 5% CO_2 and reached confluence in 8 says. Under these conditions the cells produced a dense monolayer with spindle-shaped striations (Figure 4.5).

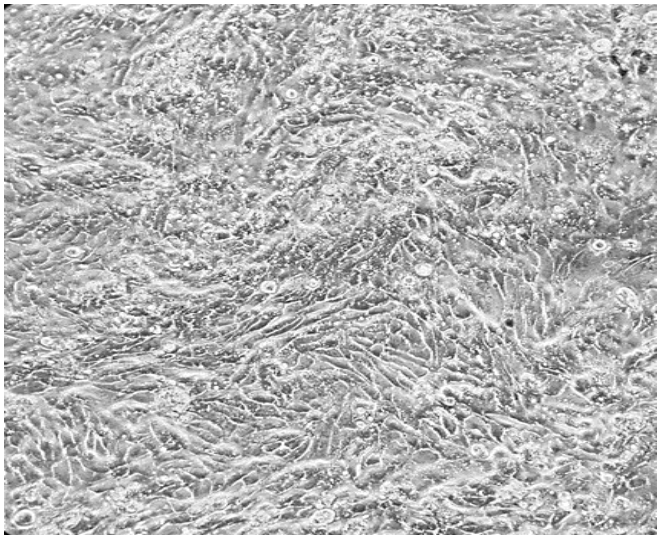


Figure 4.5| Morphology of undifferentiated Calu-3 cells monolayer. Calu-3 cells seeded at a density of 250,000 cells per well in a 24 well tissue culture plate were incubated at 37 ^{o}C, 5% CO_2. The cells reached confluence forming a spindle-shaped monolayer at day 8. Calu-3 cells were viewed under light microscopy at 200x Magnification.

On the day of infection, cells were infected at MOI 50 (1.87 x 10^8 CFU/ml, 250 µl per well, in complete media) in 5% CO_2, 37 ^{o}C incubator for 3 and 6 hours. First attempts of the infection assay although showed that PAO1-LΔ*hxc* mutant produced more colonies than PAO1-L, but there were difficulties in counting the CFU numbers due to poor spreading of the culture and subsequent poor resolution of colonies formed (data not shown). Consistent and reproducible counting was achieved in two independent experiments in duplicate which confirmed that PAO1-LΔ*hxc* mutant significantly produced more colonies after contact with Calu-3 cells than the PAO1-L ($p<0.0001$) at 3 hours but not at 6 hours ($p>0.05$) (Figure 4.6).

Compared with bacteria incubated under same conditions in complete media, both PAO1-L and Δ*hxc* produced more colonies after contact with Calu-3 cells than when cultured in complete media.

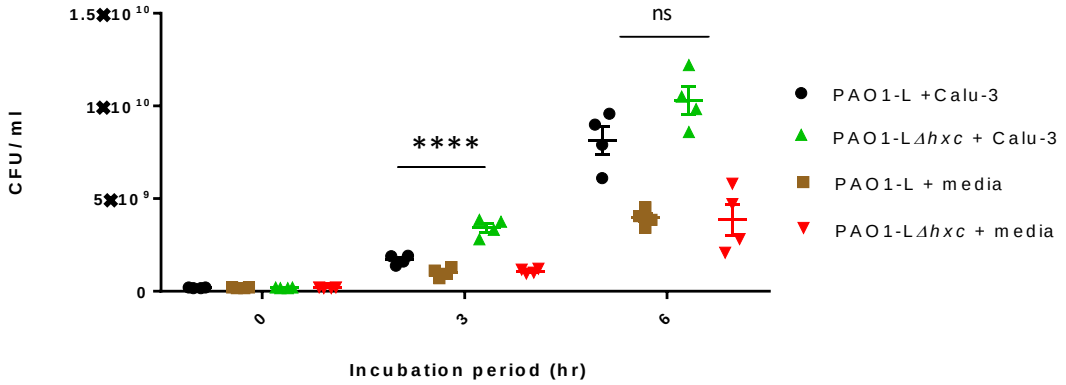


Figure 4.6| PAO1-LΔ*hxc* mutant grows better than the WT PAO1-L when in contact with undifferentiated epithelial cells. Calu-3 cells were grown in M24 tissue culture plate to form a monolayer. Infection was done with 250 µl bacterial suspension at an MOI of 50 for 3 or 6 hours incubation at 37°C, 5% CO_2. CFU were quantified after cell lysis to determine the number of viable bacteria after Calu-3 cell infection. Data represent individual replicates of two independent experiments with means connected.(**** $p<0.0001$, ns $p>0.5$, 95% CI of diff, Tukey's multiple comparisons test).

4.4.3 Infection of differentiated bronchoepithelium with *P. aeruginosa* PAO1-L and Δ*hxc*

After establishing that there was a significant growth difference between the two bacterial strains after contact with Calu-3 cells, the strains were tested on differentiated Calu-3 cells. Calu-3 cells were cultured and maintained in ALI for 21 days at 37°C, 5% CO_2. Under these conditions the cells were expected to form columnar epithelium

with a varied apical morphology, mimicking the physical properties of human bronchiolar epithelium [204].

On the day of infection, media in the basal chamber of the plate were replaced with fresh media before infecting the cells in the transwells at MOI 50 (4.6 x 10^9 CFU/ml, 100 µl per well) in 5% CO2, 37 ^oC incubator for 3 and 6 hours. From Figure 4.7, CFU performed post infection supported our earlier observation that Δ*hxc* grows better after contact with Calu-3 cells than PAO1-L but differences did not reach statistical significance (p>0.05).

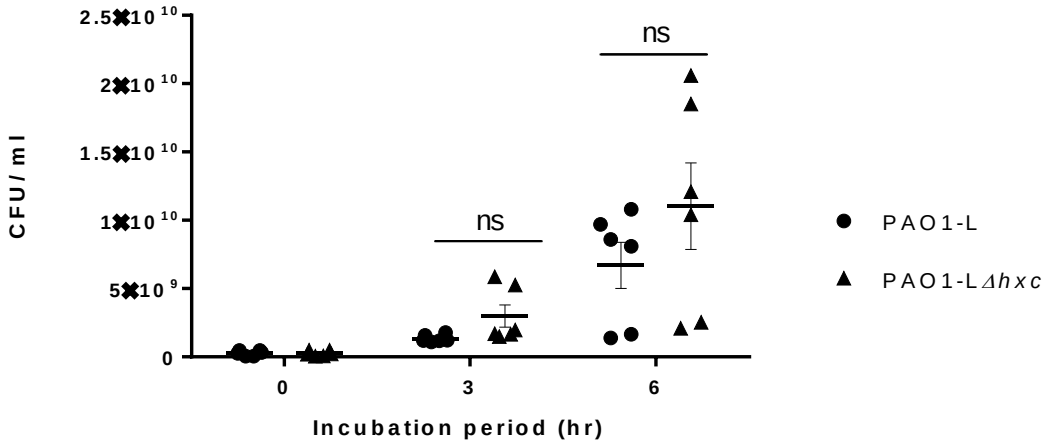


Figure 4.7| Δhxc mutant produces higher CFU than PAO1-L when in contact with differentiated epithelial cells. Calu-3 cells were seeded in an ALI at a density of 100,000 cells per well for 21 days at 37°C, 5% CO_2. Cells were infected at MOI of 50 for 3 or 6 hours at 37°C, 5% CO_2. CFU was performed after cell lysis to determine the number of viable bacteria after Calu-3 cell infection. Data represent individual replicates of 3 independent experiments with means connected. (ns p>0.5, 95% CI of diff, Tukey's multiple comparisons test)

4.4.4 Detection of multiple biomarkers in supernatants from infection assay

During infection, surface-associated or secreted bacterial products as well as the ability of *P. aeruginosa* to adhere to respiratory epithelial cells, result in production of inflammatory cytokines by the host [205]. To test for the presence of bacterial-induced cytokines during the infection of Calu-3 cells, supernatants recovered from the infection with PAO1-L or Δ*hxc* were analysed. Using Human Premixed Multi-Analyte Kit, 11 cytokines namely TNF α, G-CSF, GM-CSF, IFN β, IL-33, IL-27, IL-17C, IL-6, IL-8, IL-10, and IL-1β were quantified in four independent infection assays of undifferentiated Calu-3 cells. The result showed that the production of IL-1β, IL-27, IL-33, IFN-β and IL-10 by infected or uninfected Calu-3 cells were below the detection range of the assay therefore values shown (Fig. 4.8) are not accurate. In all instances, infection tended to increase detection of these cytokines (Figure 4.8).

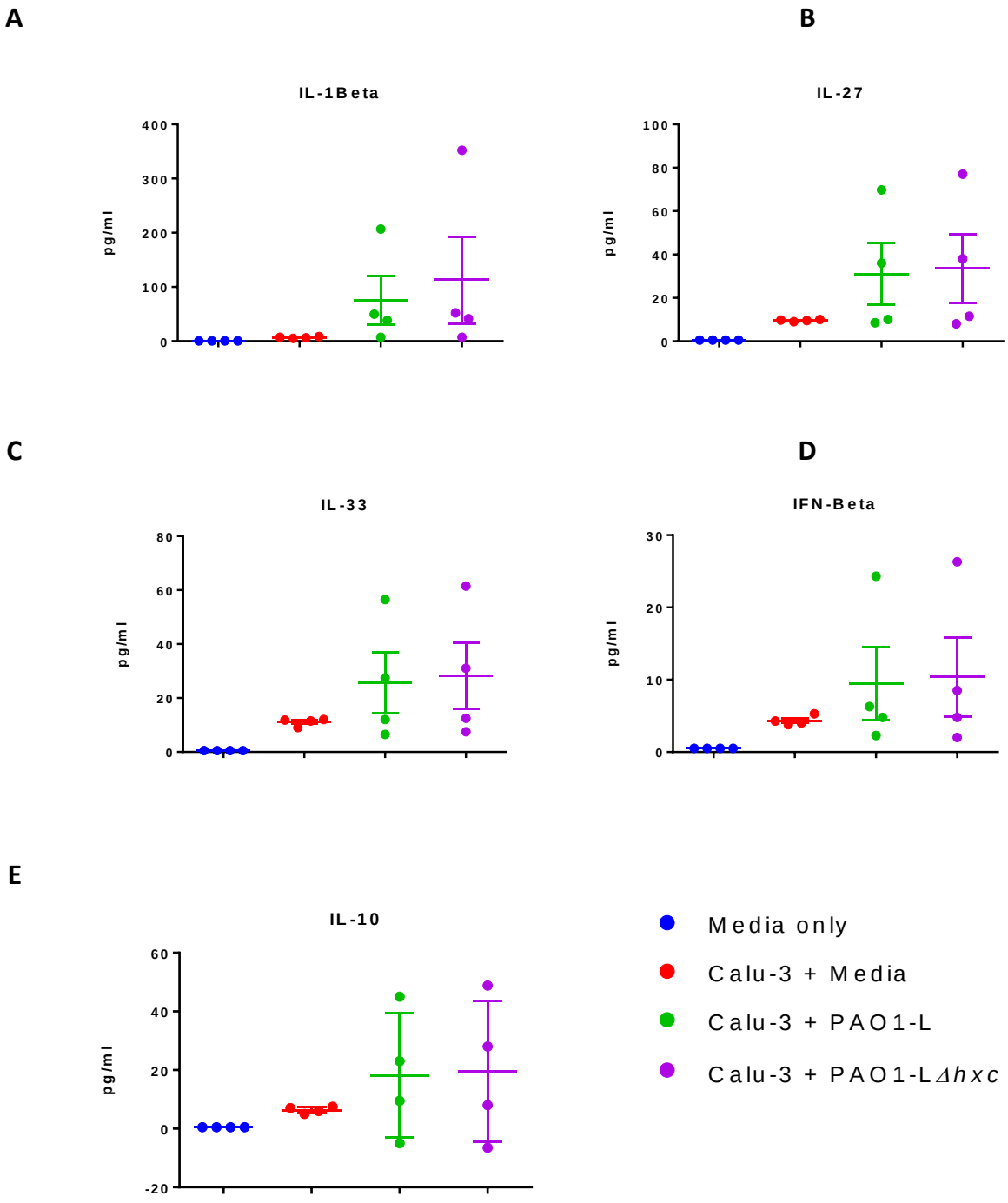


Figure 4.8| Quantification of cytokines released by Calu-3 cells in response to PAO1-L and Δ*hxc*. Supernatants from 6 hour infection of Calu-3 cells by PAO1-L and PAO1-LΔ*hxc* were analysed for cytokine responses. Compared to uninfected controls, the bacterial infection did not induce significant secretion of (A) IL-1β, (B) IL-27, (C) IL-33, (D) IFN-β or (E) IL-10. Each dot represents the mean from an independent experiment performed in duplicate.

In agreement with previous findings in the laboratory infected Calu-3 cells produced TNF-α, GM-CSF, G-CSF, IL-6 and IL-17C in response to PAO1-L infection but the levels were similar in cells infected with PAO1-L or Δ*hxc* (Figure 4.9) although high variability among assays makes comparison difficult. From all supernatants assayed, IL-8 was the only cytokine which was detected in uninfected cells. However, there was no difference in the production of this cytokine by infected or uninfected Calu-3 cells, suggesting that IL-8 may be constitutively released by these epithelial cells [211].

Even though there was no significant differences between cytokine response to PAO1-L and Δ*hxc*, the results taken together confirm that infection of respiratory epithelial cells by *P. aeruginosa* leads to inflammatory immune responses which facilitate recruitment of innate and adaptive immune cells to mediate clearance of microorganisms from the lung [191, 205, 212, 213].

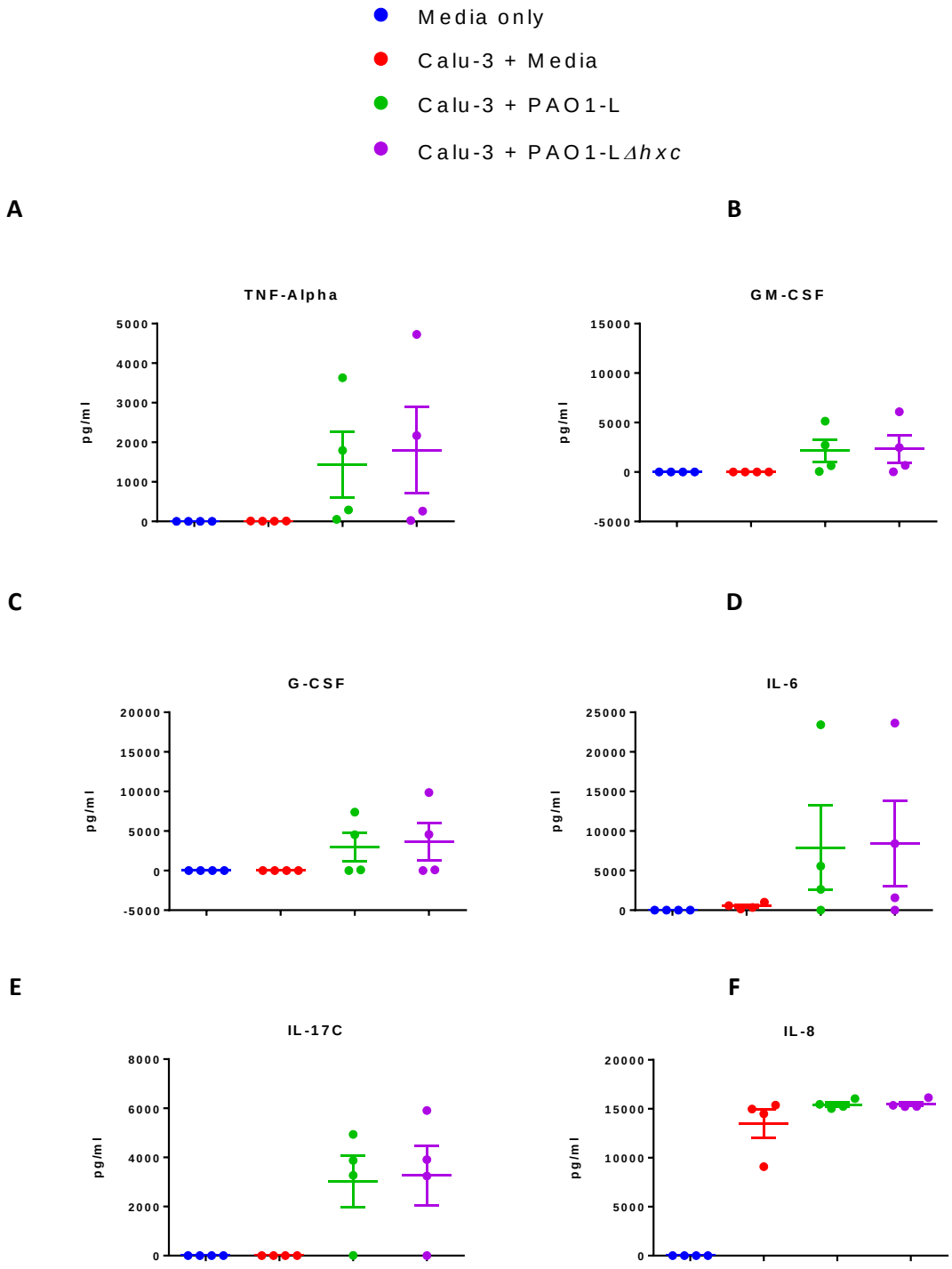


Figure 4.9| Quantification of cytokines released by Calu-3 cells in response to PAO1-L and Δ*hxc*. Supernatants assayed from 6 hour infection of Calu-3 cells by PAO1-L and PAO1-LΔ*hxc* were analysed for the presence of cytokines. Compared to uninfected controls, the bacterial infection induced the secretion of some pro-inflammatory cytokines although with high variation among experiments: (A) TNF-α, (B) GM-CSF, (C) G-CSF, (D) IL-6 and (E) IL-17C. IL-8 was constitutively released by the Calu-3 cells in all supernatants assayed (F). Each dot represents the result from an independent experiment performed in duplicate.

4.4.5 Quantification of cell cytotoxicity by measuring lactate dehydrogenase (LDH) release

Cytotoxicity in general encompasses cell death either by apoptosis or necrosis. As necrosis leads to permeabilisation of the plasma membrane, necrotic cell death can be quantified measuring the release of cytoplasmic LDH which is present in all cell types [214]. LDH firstly reduces nicotinamide adenine dinucleotide (NAD^+) to $NADH^+$ and H^+ during conversion of lactate to pyruvate. In the second step, the newly synthesised $NADH^+$ coverts a yellow tetrazolium salt to a red formazan product in the presence of a catalyst. The amount of formazan produced is then quantified through spectroscopy [214].

To determine the viability of Calu-3 cells after infection with PAO1-L or Δ*hxc* we assessed the release of the cytoplasmic enzyme LDH in our supernatants. LDH release from the infected cells was expressed as a percentage of LDH release by cells treated with 2% Triton X-100. We observed that Calu-3 cells underwent cell death, as measured by LDH release, when infected with PAO1-L or *Δhxc* with comparable kinetics (Figure 4.10). Level of cytotoxicity significantly increased with increasing duration of infection where PAO1-L caused about 19 and 29% cell death at 3 and 6 hours respectively ($p=0.0003$) while PAO1-LΔ*hxc* caused 20 and 31% respectively ($p <0.0001$). Although the level of cytotoxicity induced by PAO1-L or

LΔ*hxc* was not significantly different, there was an indication that the mutant caused slightly more cell death than PAO1-L (ns), corroborating our observation with the CFU analysis post infection.

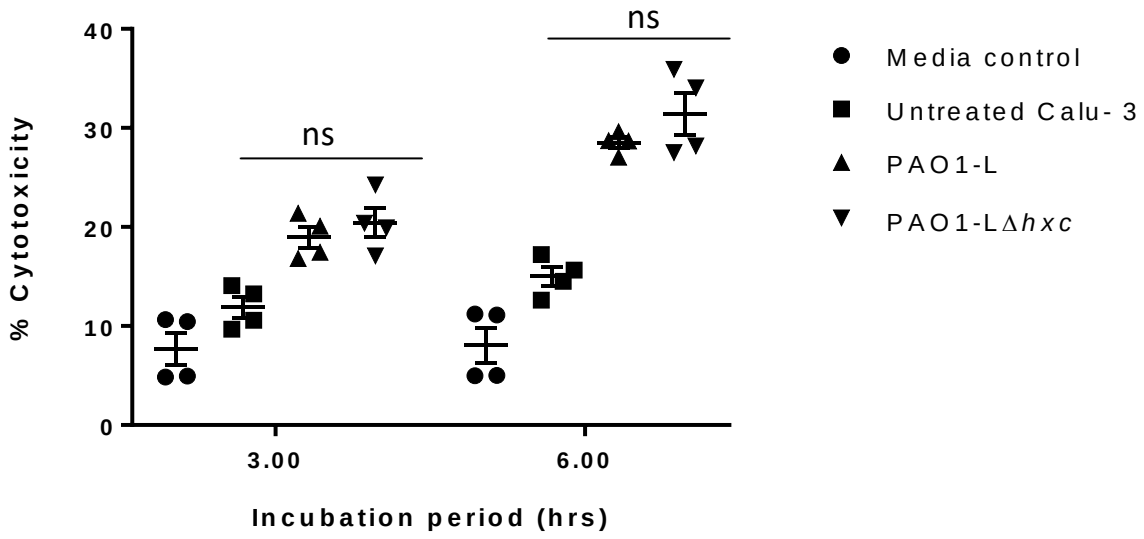


Figure 4.10| Calu-3 cell death during infection with PAO1-L and Δhxc. Cell death caused by PAO1-L and *Δhxc* infection increased from 18.94% and 20.41% at 3 hpi to 28.51% and 31.41% at 6 hpi, respectively. Cytotoxicity was assessed in comparison with LDH content released from 100% cell death prepared from cells lysed with 2% Triton X-100. Data represent Mean ± SEM of 2 independent experiments (ns p>0.5, 95% CI of diff, Tukey's multiple comparisons test)

4.4.6 Detection of alkaline phosphatase activity in supernatants from Calu-3 infection assays

Since the hxc operon is involved in the secretion of LapA, we investigated alkaline phosphatase activity in supernatants from the Calu-3 infection assays. Calu-3 cells were cultured for 8 days to form an undifferentiated monolayer before they were infected with PAO1-

L or Δ*hxc* under conditions previously described. As control, some Calu-3 cells were left untreated while 250 µl of bacterial suspension was put in empty wells (without Calu-3 cells, i.e. bacteria only cultures). The plate was incubated for 6 hours at 37°C, 5% CO_2, after which supernatants were assayed for alkaline phosphatase activity using a colorimetric Alkaline Phosphatase Assay. Alkaline phosphatase activity measured in the supernatants was generally low compared with the standard control (Figure 4.11). Analysis of the results showed that, Δ*hxc* supernatant from wells without Calu-3 cells had a small (6.5%) reduction of enzymatic activity compared with PAO1-L. This is in agreement with our earlier observation that alkaline phosphatase production is reduced in the *hxc* mutant (See Section 3.4.2.6). Activity measured in supernatants from infected Calu-3 cells was similar for both strains. No alkaline phosphatase activity was detected in supernatants from uninfected cells.

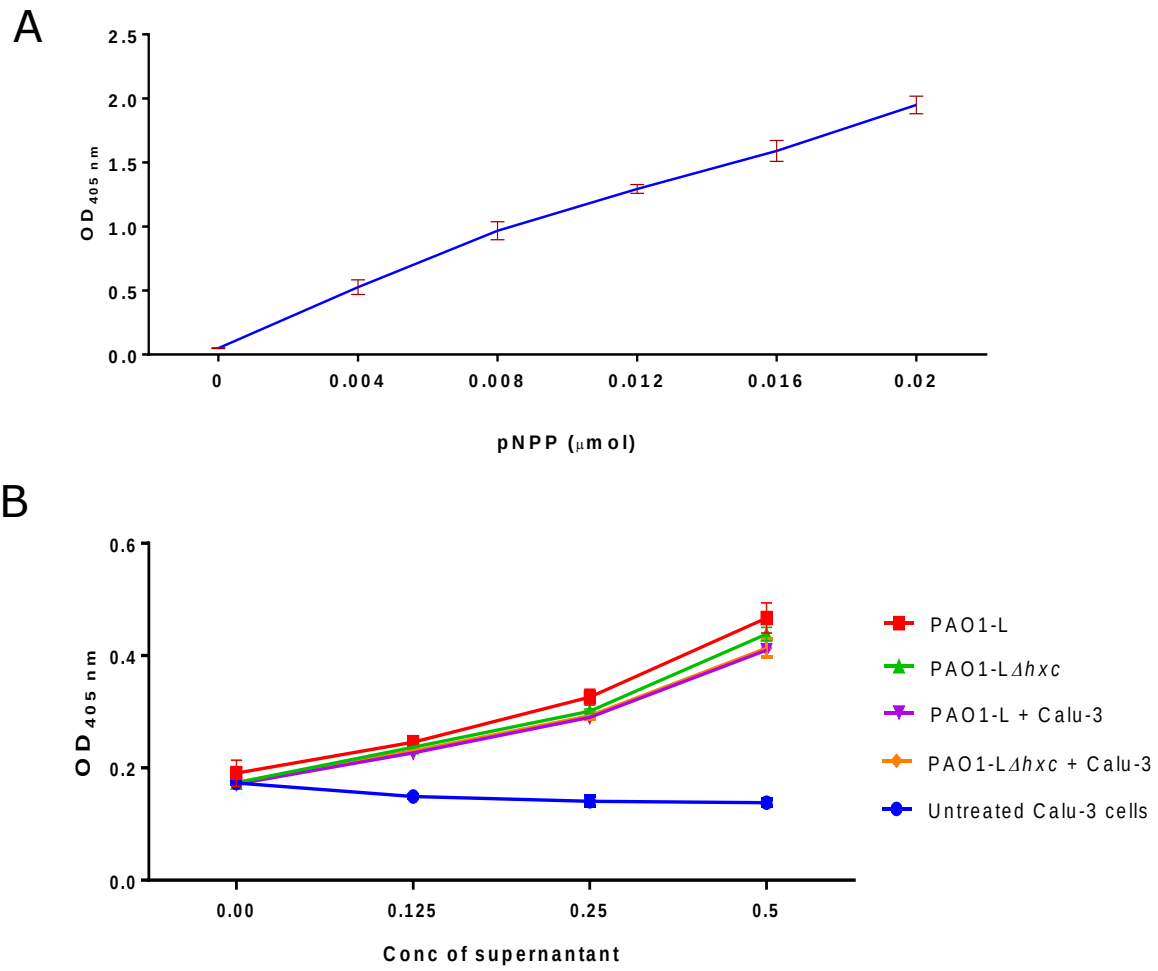


Figure 4.11| Alkaline phosphatase activity during the course of infection with WT PAO1-L and PAO1-LΔhxc. (A) Standard curve of enzymatic activity measured when 10 µl of alkaline phosphatase solution was serially diluted in 1 mM pNPP (B) Alkaline phosphatase activity quantified in supernatants recovered from infection of Calu-3 cells with PAO1-L and PAO1-LΔ*hxc*. Data represent Mean ± SEM from one experiment done in triplicate.

4.4.7 Transcriptional analyses of the *hxc* operon during infection of undifferentiated Calu-3 cells

Our earlier analysis of the hxc promoters P_{hxcV} and P_{hxcT} showed that the operon is expressed better and sustained longer in the eukaryotic cell medium, X-vivo[15]. With this background, we tested the

transcriptional activities of both promoters during infection of Calu-3 cells. Bacterial strains, PAO1-L and *Δhxc*, and promoter fusion constructs, PAO1-L mCTX::P_{hxcT}-*lux* and PAO1-L miniCTX::P_{hxcV}-*lux* were propagated on LB agar and in LB broth as routinely done. Bacteria pelleted from the log-phase culture was resuspended in X-vivo[15], LB, PPM, DMEM-F12 (without 10% FCS) and complete medium. This was followed by normalisation of the OD_{600nm} of the bacterial suspensions as previously described (Section 4.3.1). Undifferentiated Calu-3 monolayer was infected with the bacterial constructs at MOI 50 followed by incubation at 37°C, 5% CO_2. Bioluminescence was detected using a Hamamatsu light box every 30 minutes for 6 hours and after overnight incubation.

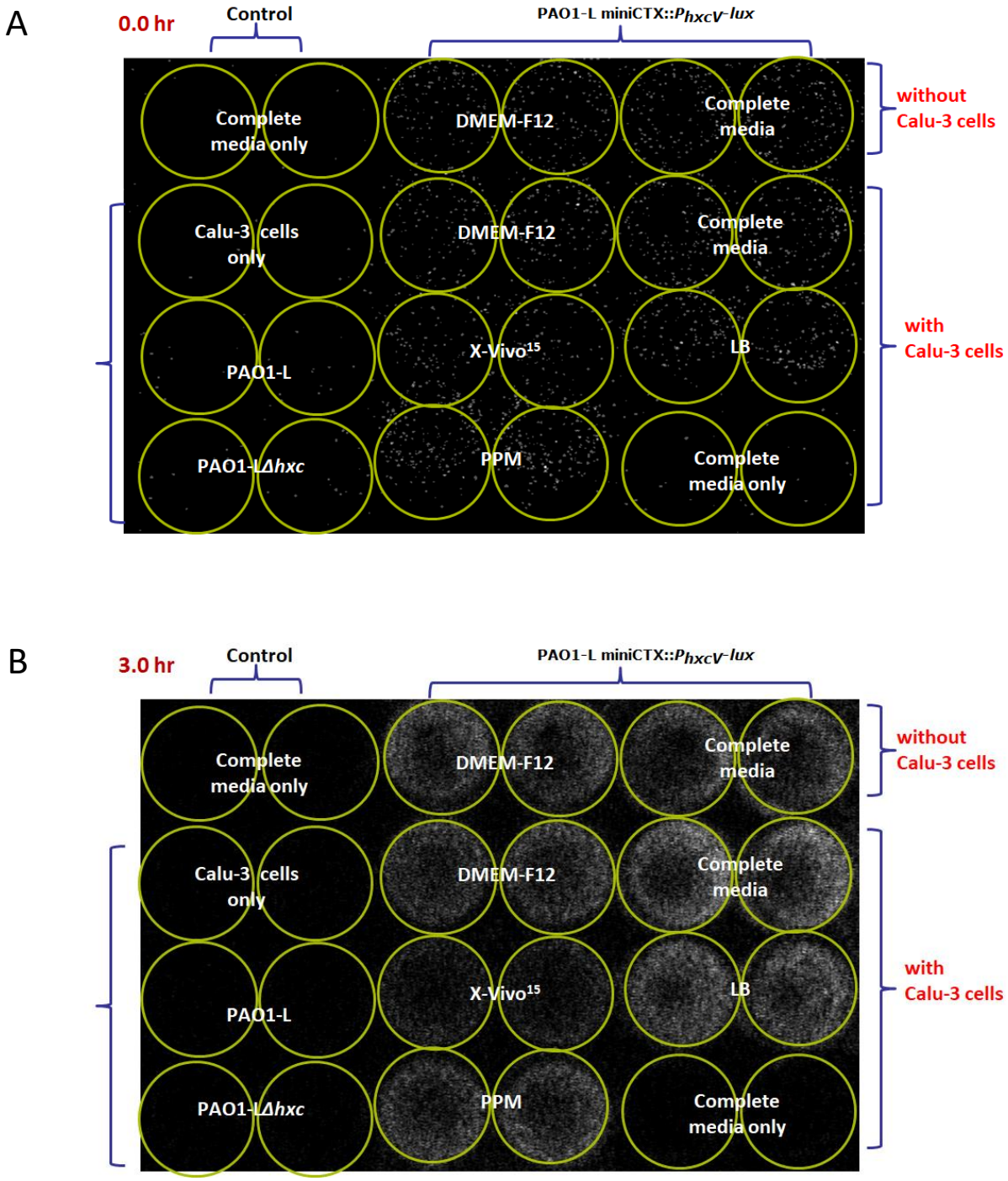
We observed that promoter activity of P_{hxcV} was rapid, producing substantial luminescence at 3 hours post infection (Figure 4.12A). Promoter activities from miniCTX::P_{hxcV}-*lux* reporter have been summarised in Table 4.2. Compared to all the other media treatments, P_{hxcV} expression at 6 hours was observed to be lowest in X-Vivo[15], while highest expressions were detected in LB and complete media with Calu-3 cells (Figure 4.12C). High promoter activity in LB and PPM was not surprising as we had previously shown that P_{hxcV} expression is twice in these media compared to X-vivo[15] (see Section 3.4.3).

Table 4.2| Transcriptional activity of the miniCTX::*P*$_{hxcV}$-*lux* reporter under different conditions.

miniCTX::*P*$_{hxcV}$-*lux* culture condition	Promoter activity quantified at incubation time points (hrs)		
	3	6	18
DMEM-F12 only	+	++	-
Complete media only	+	+	+
DMEM-F12 + Calu-3 cells	+	++	-
Complete media + Calu-3 cells	++	+++	+++
X-vivo15 + Calu-3 cells	-	+	+++
LB + Calu-3 cells	++	+++	++
PPM + Calu-3 cells	++	++	-

P$_{hxcV}$ expression was markedly reduced at 18 hours post infection in PPM, and DMEM-F12 with or without Calu-3 cells. Depletion of nutrient to support bacterial and Calu-3 cell growth was thought to be the reason for this observation. On the contrary, P$_{hxcV}$ remained highly expressed in X-vivo[15] and complete media. This supports our earlier observation that eukaryotic cell medium sustains the expression of P$_{hxcV}$; possibly due to the availability of nutrients to support bacterial viability. As one of the optimal growth medium routinely used to propagate *P. aeruginosa* globally, we posit that the seeming sustenance of P$_{hxcV}$ expression in LB was due to the easy assimilation of nutrients in this medium by the bacterium as well as

the presence of nutrients such as DNA and phosphates from dying Calu-3 cells.



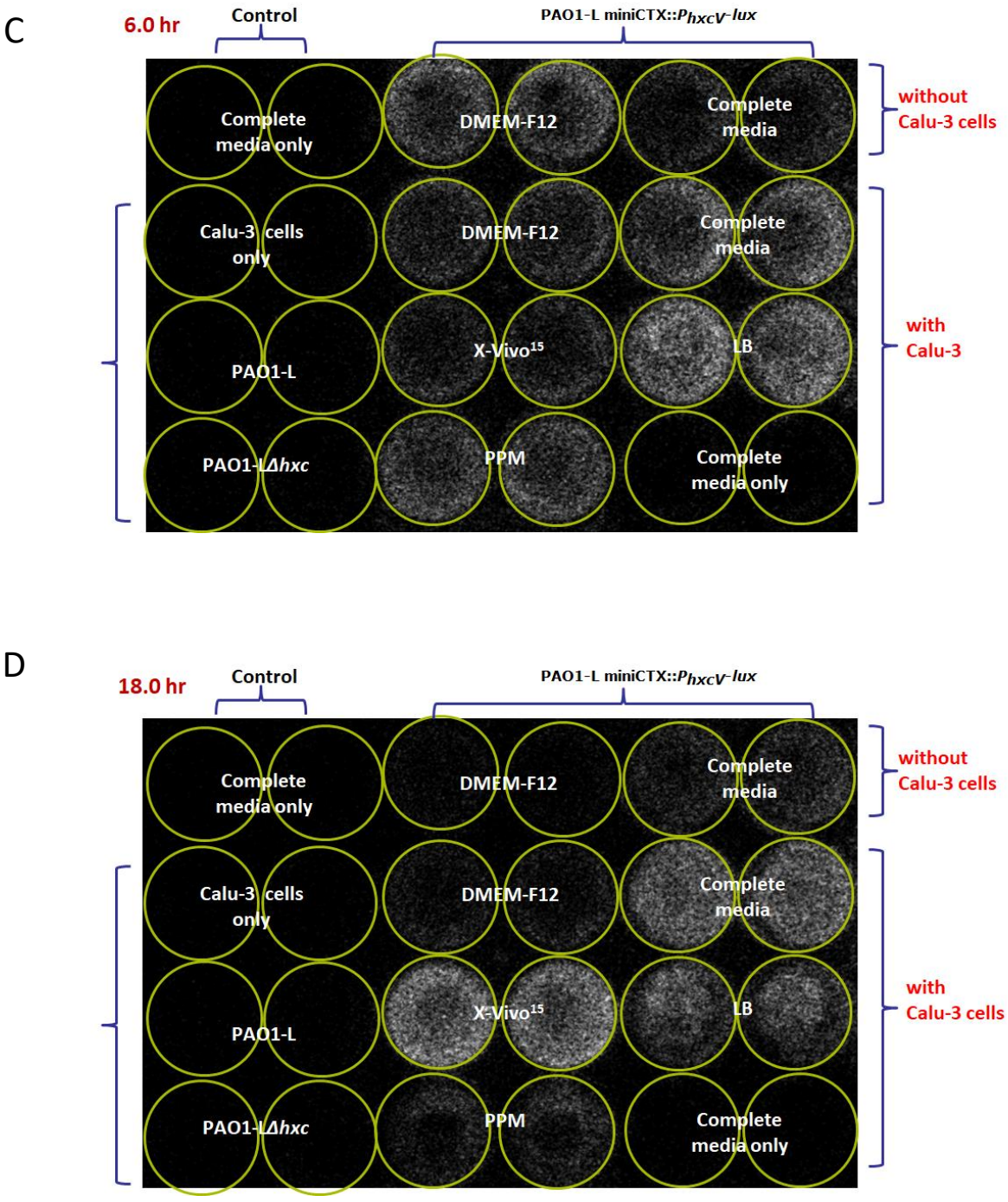
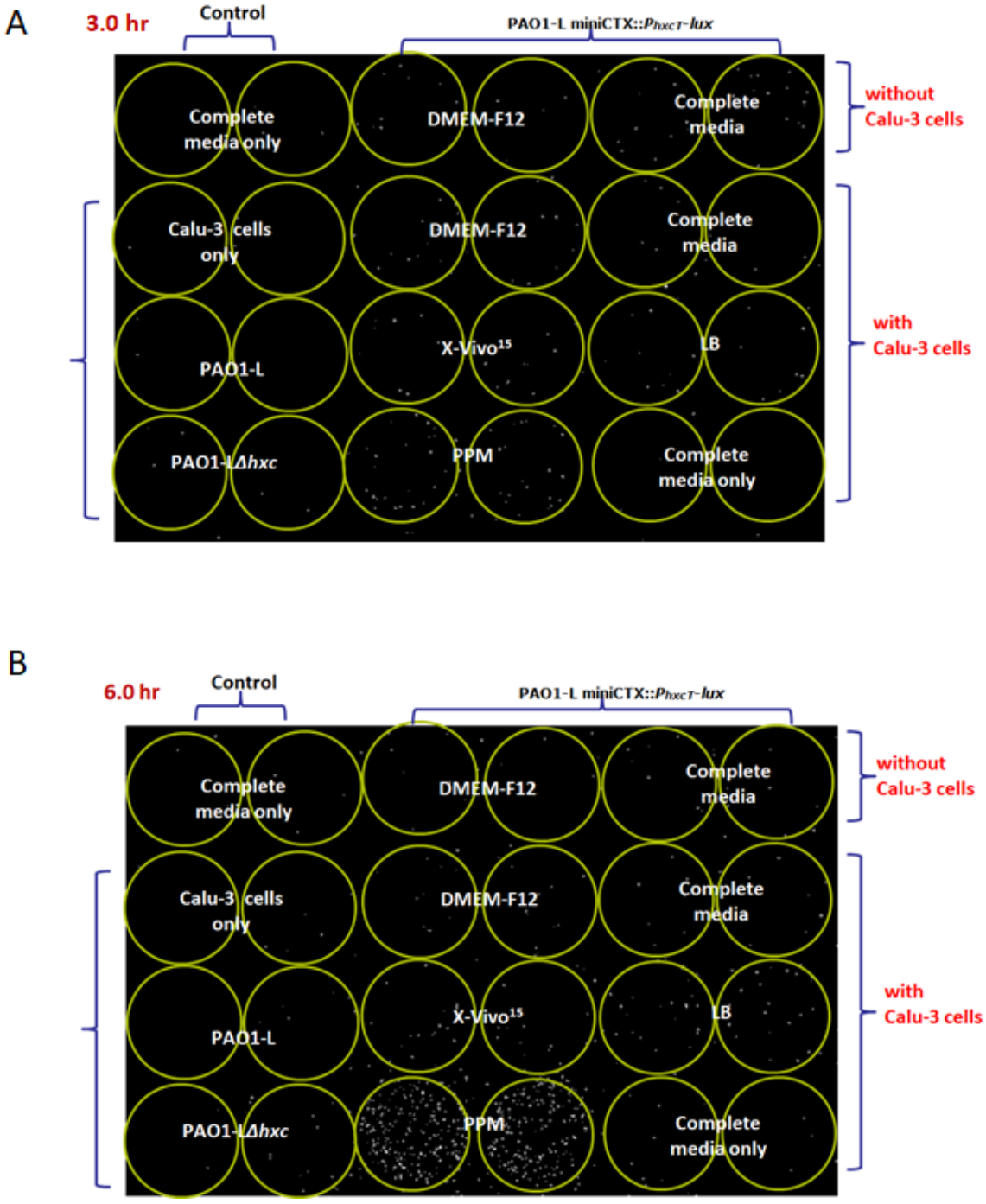


Figure 4.12| Transcriptional activity of the PhxcV promoter is sustained in eukaryotic media. The P_{hxcV} promoter transcriptional fusion to the *lux* genes was introduced into WT PAO1-L to obtain PAO1-L mCTX::P_{hxcV}-*lux*. Promoter activity of PAO1-L mCTX::P_{hxcV}-*lux* construct was tested in different media in the presence or absence of Calu-3 cells. Promoter activities were detected using Hamamatsu light box every 30 minutes for 6 hours and after overnight. Data is a representation of images taken at these time points. N=2.

In the case of PAO1-L mCTX::P_{hxcT}-*lux*, transcriptional activity in this orientation was very low and perhaps below the level of detection with this method (Figure 4.13). This observation confirms our earlier findings that the promoter activity from the P_{hxcV} orientation is about 10 times higher than for the P_{hxcT} orientation. Promoter activities is summarised in Table 4.3. Substantial luminescence was only detected at 6 hours in PPM supporting the earlier findings that P_{hxcT} promoter activity peaks earlier under phosphate limiting conditions. At 18 hours post infection, activity was detected in X-vivo[15] and complete media, a demonstration that expression of the *hxc* operon is sustained in eukaryotic environment.

Table 4.3| Transcriptional activity of the miniCTX::P_{hxcT}-*lux* reporter under different conditions.

miniCTX::P_{hxcT}-*lux* culture condition	Promoter activity quantified at different time points (hrs)		
	3	6	18
DMEM-F12 only	-	-	-
Complete media only	-	-	++
DMEM-F12 + Calu-3 cells	-	-	+
Complete media + Calu-3 cells	-	-	++
X-vivo[15] + Calu-3 cells	-	-	++
LB + Calu-3 cells	-	-	+
PPM + Calu-3 cells	-	+	-


A
3.0 hr
Control
PAO1-L miniCTX::Phxc-lux
Complete media only
DMEM-F12
Complete media
without Calu-3 cells
Calu-3 cells only
DMEM-F12
Complete media
PAO1-L
X-Vivo15
LB
PAO1-LΔhxc
PPM
Complete media only
with Calu-3 cells
B
6.0 hr
Control
PAO1-L miniCTX::Phxc-lux
Complete media only
DMEM-F12
Complete media
without Calu-3 cells
Calu-3 cells only
DMEM-F12
Complete media
PAO1-L
X-Vivo15
LB
PAO1-LΔhxc
PPM
Complete media only
with Calu-3 cells

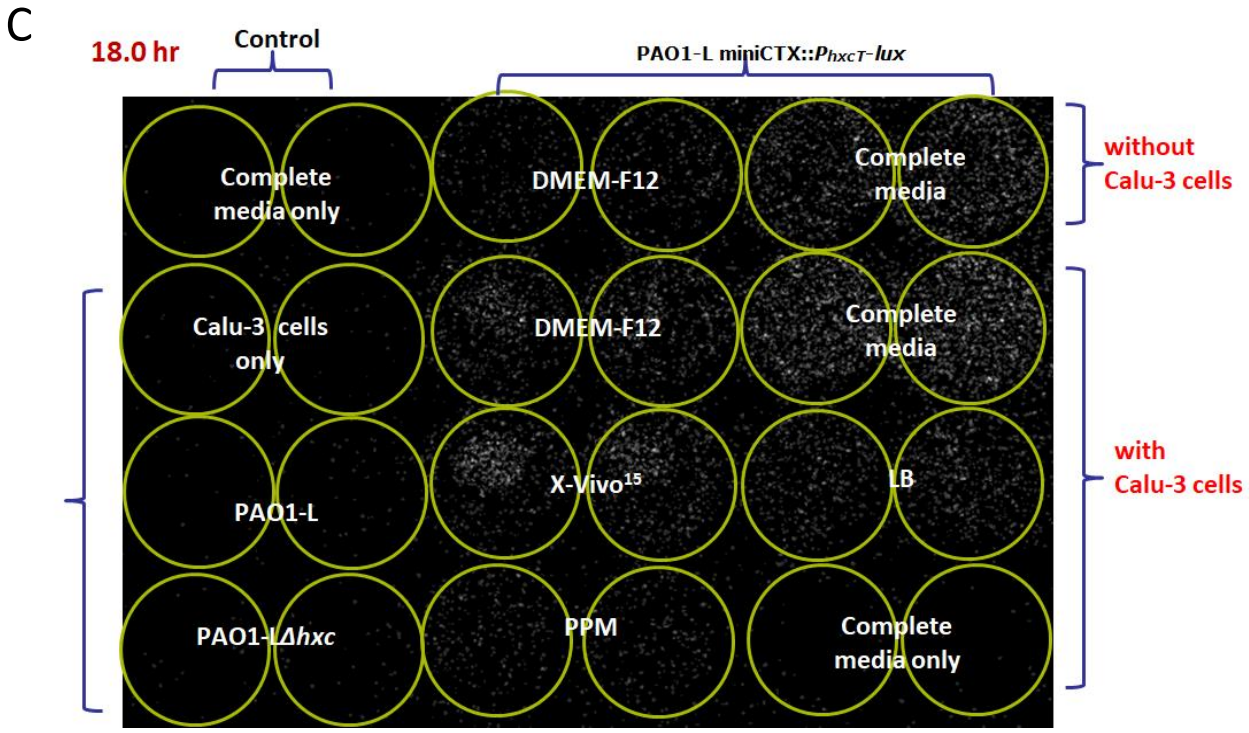


Figure 4.13| Transcriptional activity of the PhxcT promoter. The P_{hxcT} promoter transcriptional fusion to the *lux* genes was introduced into WT PAO1-L to obtain PAO1-L mCTX::P_{hxcT}-*lux*. Promoter activity of PAO1-L mCTX::P_{hxcT}-*lux* construct was tested in different media in the presence or absence of Calu-3 cells. Promoter activities were detected using Hamamatsu light box every 30 minutes for 6 hours and after overnight. Data is a representation of images taken at these time points. n=1.

In light of these findings, we further probed the promoter activities in the presence of supernatants recovered from infection of undifferentiated Calu-3 cells with PAO1-L as well as in the presence of different concentrations of serum. Bioluminescence from these treatments was measured using the Tecan luminescence plate reader. Control wells had bacterial suspensions treated with

supernatant from uninfected Calu-3 cells or neat supernatants from PAO1-L.

In complete media, promoter activity of untreated P_{hxcT} was found to be similar to that when treated with supernatant from uninfected Calu-3 cells (Figure 4.14). However, activity was found to be lower when treated with supernatants from Calu-3/PAO1-L infection (20% reduction) and from PAO1-L (39% reduction), giving an indication that P_{hxcT} expression was being modulated by PAO1-L secretion(s) (Fig. 4.14A). Promoter activity from the P_{hxcV} orientation on the contrary did not show any observable change under all the conditions tested (Fig. 4.14B).

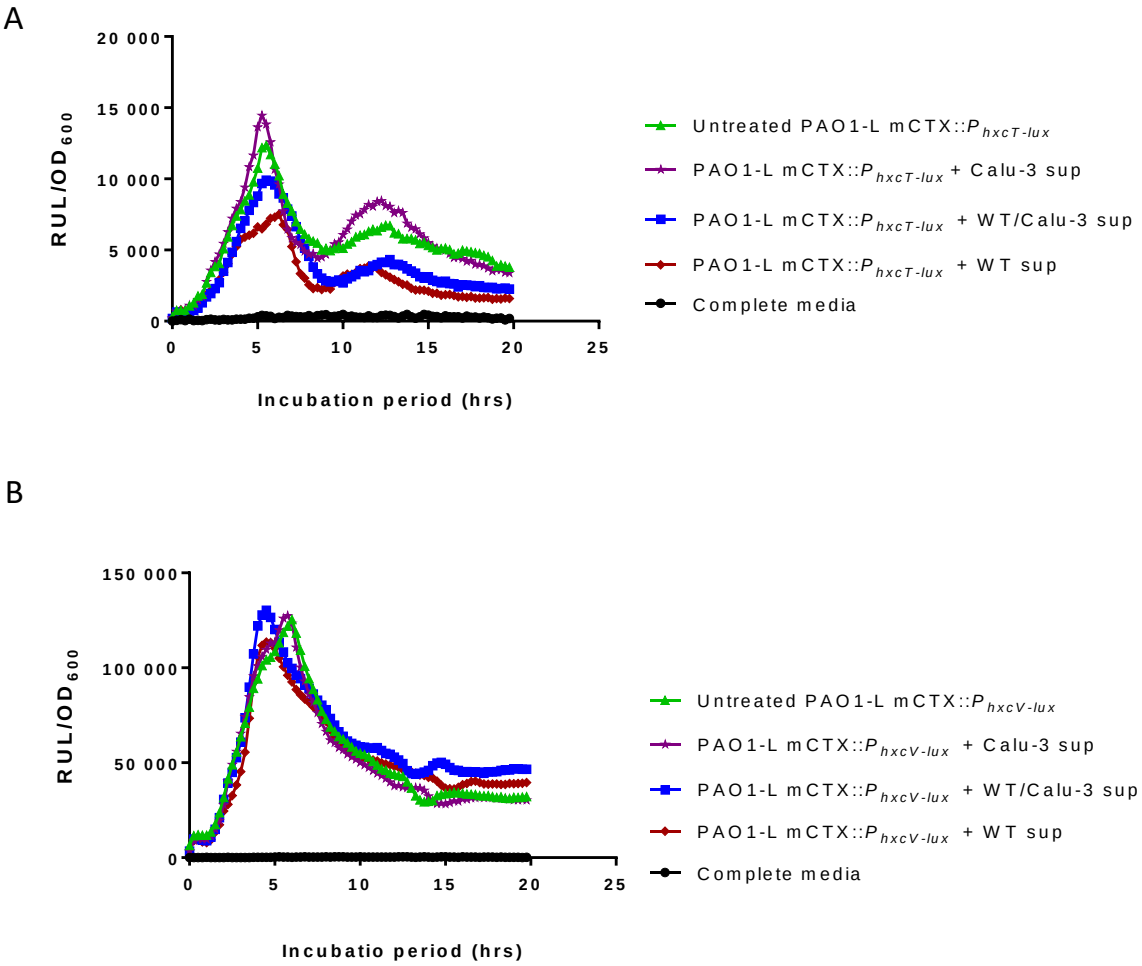


Figure 4.14| Transcriptional activity of the PhxcT and PhxcV promoter in complete media supplemented with different supernatants. The promoter fusion constructs were inoculated in microplates with or without addition of supernatants recovered from Calu-3 infection with WT PAO1-L. (A) P_{hxcT} promoter activity (B) P_{hxcV} promoter activity. Data represent Mean ± SEM of an independent experiment done in duplicate.

In DMEM-F12, P_{hxcT} activity in the presence of supernatants from Calu-3/PAO1-L infection or PAO1-L was reduced by 39% (Figure 4.15A) whereas P_{hxcV} activity although peaked earlier was reduced by 22% in the presence of supernatants from Calu-3/PAO1-L infection and 36% in the presence of supernatant from PAO1-L (Figure 4.15B).

This was in agreement with our earlier observation that *P. aeruginosa* may modulate the expression of its *hxc* operon during infection via a negative feedback mechanism.

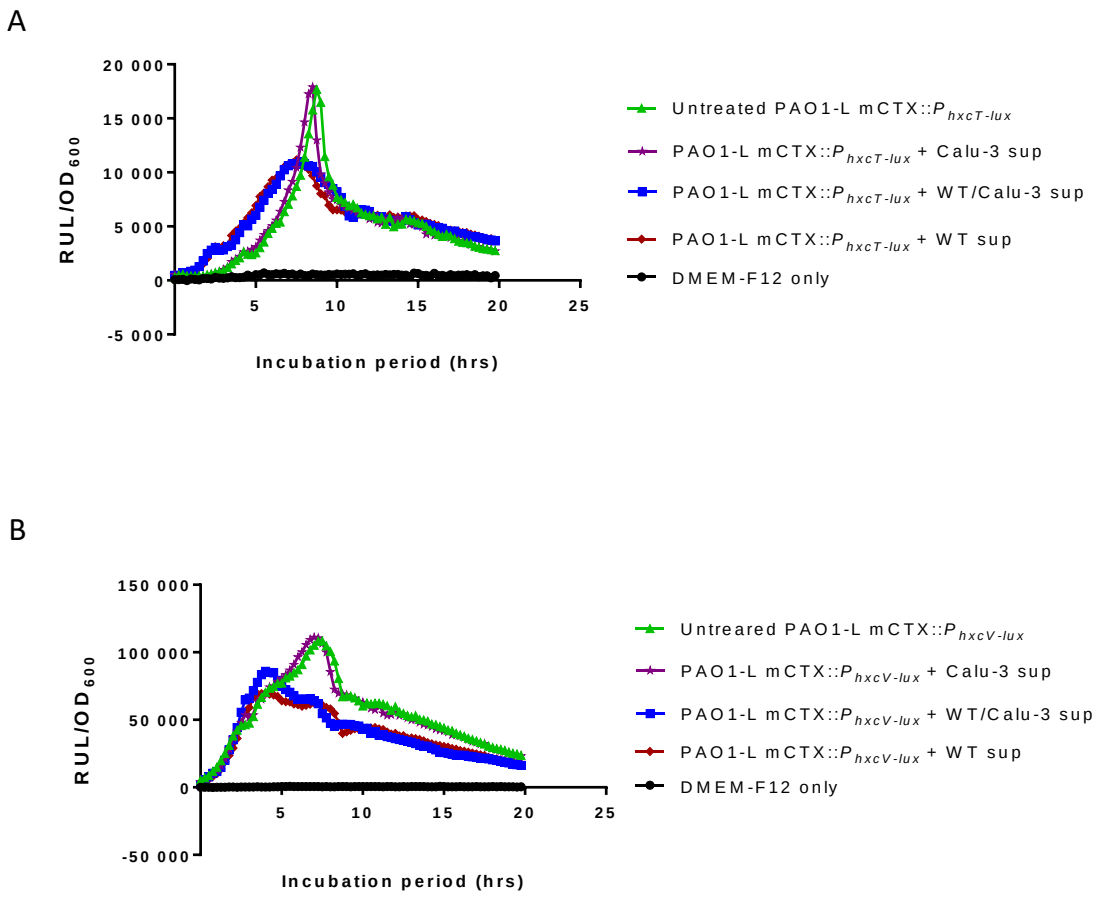


Figure 15| Transcriptional activity of the PhxcT and PhxcV promoter in DMEM-F12 supplemented with different supernatants. The promoter fusion constructs were inoculated in microplates with or without addition of supernatants recovered from Calu-3 infection with WT PAO1-L. (A) P_{hxcT} promoter activity (B) P_{hxcV} promoter activity. Data represent Mean ± SEM from one experiment done in duplicate

Although this experiment was conducted once, it gave an indication that the effect of the inhibitory agent secreted by *P. aeruginosa* may be neutralised in the presence of serum. Thus, we measured transcriptional activity of the two promoters in DMEM-F12

supplemented with different concentrations of FCS. The result did not show any clear pattern of the effect of serum, and was not pursued further (Fig. 4.16).

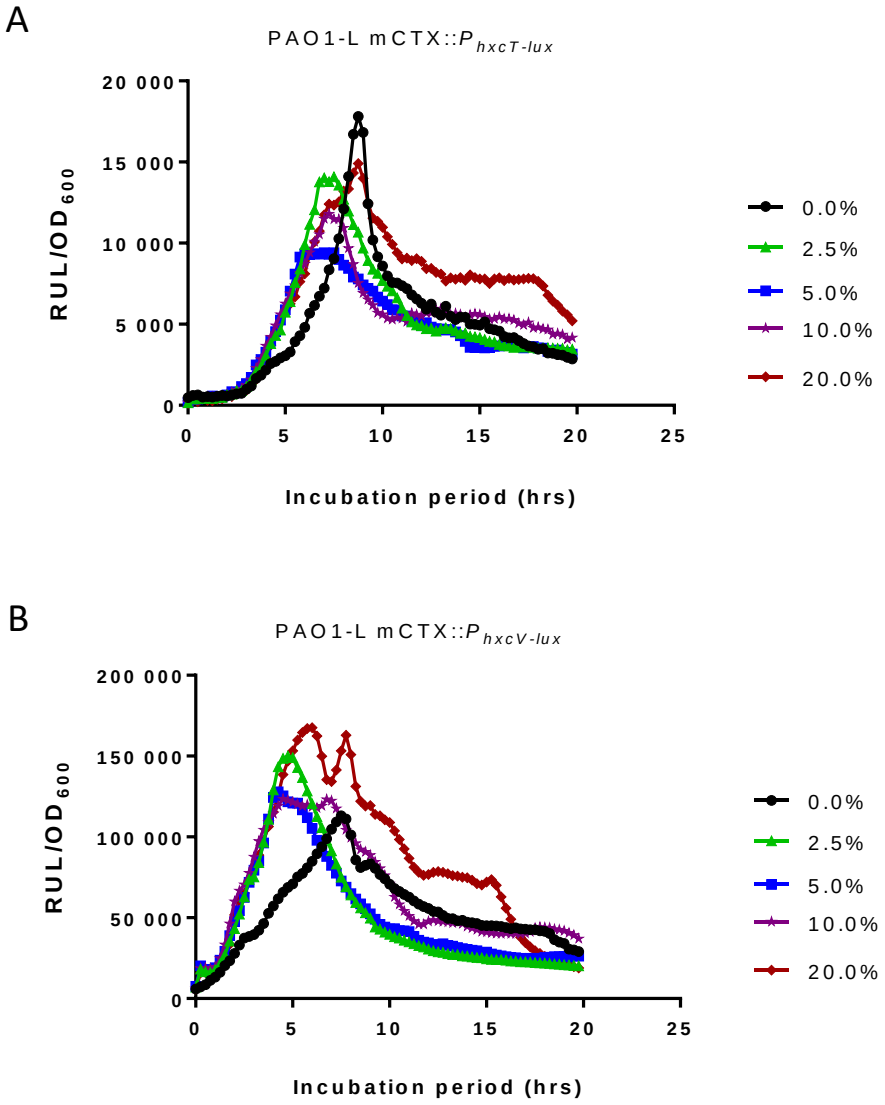


Figure 16| Transcriptional activity of the P_{hxcT} and P_{hxcV} promoter in DMEM-F12 supplemented with different concentrations of serum. The promoter fusion constructs suspended in DMEM-F12 were inoculated in microplates with different concentrations of serum (A) P_{hxcT} promoter activity (B) P_{hxcV} promoter activity. Data represent Mean ± SEM of an independent experiment done in duplicate.

4.5 Discussion

Several *P. aeruginosa* virulence factors have been shown to interfere with the integrity of epithelium. For instance, flagella mediate the transmigration of the bacterium across airway epithelium, while elastase B is thought to degrade tight junction proteins between alveolar epithelial cells [215, 216]. In this study, the study of how *P. aeruginosa* interacts with bronchial epithelial cells was further explored by examining the impact of Hxc-T2SS on this process. The Hxc operon is dedicated to the secretion of LapA, whose contribution to pathogenesis of *P. aeruginosa* or its involvement in interaction with human bronchial epithelial cells has not received much attention. We thus tested our *hxc* deletion mutant on human bronchial epithelial *cells in vitro* to assess the impact of this deletion on bacterial fitness during infection in comparison with its isogenic WT strain PAO1-L.

Similar to our phenotypic characterisation showing that the *hxc* mutant is comparable to PAO1-L in terms of growth, biofilm formation and secretion of virulent factor pyocyanin (Chapter 3), the two strains did not drastically differ in pathogenicity during infection of Calu-3 cells. Our CFU analysis post infection showed that both PAO1-L and Δ*hxc* seem to grow in the presence of undifferentiated Calu-3 cells, although *Δhxc* had higher colony counts than the PAO1-L. When the two strains were compared on differentiated Calu-3 cells, although there was a trend suggesting that the *hxc* mutant thrived better than

the PAO1-L, the differences in CFU was not statistically significant. The reason why the mutant produced higher CFU counts relative to the WT is not clear. We propose the Δ*hxc* strain may be overproducing virulence-associated exoproteins, including xcp-dependent phoA [79], to compensate the deficiency, hence the seeming fitness. On the other hand, the WT strain may be overburdened with protein secretion, as most genes associated with phosphate acquisition are expressed during acute infection [217], which could result in lower cell division rate.

Additionally, quantification of cellular toxicity as well as detection of bacterial-induced pro-inflammatory cytokines did not show significant differences between the PAO1-L and the *hxc* deficient strain. This demonstrates that the hxc operon might not influence *P. aeruginosa* infectivity or its role in the initial invasion of human bronchial epithelium by PAO1-L may be compensated by the overlapping xcp secreton. This is in agreement with a study conducted by Jeevan *et. al.,* (2011) which showed that it was the Xcp-T2SS which was involved in the secretion of factors responsible for death of infected mice but not HxcQ or the alternative secretin, XqhA [218].

The contribution of LapA to *P. aeruginosa* pathogenesis is still unclear especially as some isolates of the bacterium from blood (X13273) and

urinary tract infections (JJ692) have been shown to be deficient in *lapA* and *lapB,* which encodes a protein with high similarity to LapA [219-221]. Instead of LapA/LapB, these clinical isolates possess LapC which is secreted in an Hxc-dependent manner and has 56 and 53 per cent homology to LapA and LapB respectively. Whereas LapA and LapB show phosphatase activity and are secreted under phosphate limitation, LapC does not show phosphatase activity but rather bind and recapture phosphate from the extracellular medium. Its secretion is thought to be under the regulation of PhoB regulon [219]. While it is not clear why *P. aeruginosa* clinical strains replace LapA/LapB with LapC during infection, it is thought this change enhances the survival of the bacterium in a chronic setting.

There is increasing evidence suggesting that increased production of proinflammatory cytokines contribute to airway destruction and fibrosis of lungs in CF patients [222-224]. In addition serving as a protective physical barrier and producing broad-spectrum agents such as defensins and surfactant proteins against environmental challenges, airway epithelial cells are also a major source of chemokines and cytokines that lead to the recruitment of inflammatory cells to the site of infection [206].

Consistent with other studies, our results demonstrated that bronchial epithelial cells secrete proinflammatory cytokines TNF-α,

GM-CSF, G-CSF, IL-6 and IL-17C in response to *P. aeruginosa* (Yi-Chia Lui, PhD thesis, 2014)[225]. The cytokine TNF-α has been associated with many chronic inflammatory diseases including keratitis, Crohn's disease and pulmonary fibrosis [226-228]. The cytokine is considered as an obligate precursor for the production of other chemokines like IL-6, IL-8 and GM-CSF [226, 229]. TNF-α thus contributes to a critical step for the recruitment of neutrophils to the site of infection, and plays an important role in bacterial clearance or containment during pneumonia [228, 230]. Notwithstanding its beneficial role, TNF-α has been shown to alter intestinal tight junction integrity through the activation of myosin light chain kinase (MLCK) protein expression [227]. The cytokine has also been demonstrated to enhance the growth of *E. coli* in neutropenic mice and worsened the bacterial burden in the lungs [228].

GM-CSF plays an important role in proliferation, differentiation and mobilisation of myeloid cells. This growth factor is not only produced by bronchial epithelial cells but by other immune cells, such as macrophages, fibroblasts, and activated T cells [212]. GM-CSF contributes to the maintenance of surfactant homeostasis in the lungs, which in turns promote alveolar macrophages chemotaxis and secretion of proteolytic enzymes [231]. Studies have shown that in the presence of IFN-γ, GM-CSF promotes pro-inflammatory response of human macrophages to *P*. *aeruginosa* infection by increasing IL-6

and TNF-α production [191]. Also, GM-CSF deficient mice showed more susceptibility to lung infection with *P. aeruginosa* than WT mice due to impaired function of alveolar macrophages [232].

Although the recruitment of neutrophils to site of infection is important for bacterial clearance, their accumulation in CF lung is proven to be detrimental to the host due to extensive tissue damage caused by the exacerbated inflammation [222]. G-CSF is one of the key cytokines involved in the maturation and recruitment of neutrophils. Its expression is often upregulated during infection, and high concentrations of the cytokine have been recovered in inflammatory exudates and blood, indicating that G-CSF may be involved in both local and systemic neutrophil responses [233]. IL-6, similar to G-CSF, enhances the recruitment and efficiency of neutrophils during *P. aeruginosa* infection. Recognition of bacterial LPS and flagellin by TLR-4 and TLR-5, respectively, are known to upregulate the secretion of IL-6 in mouse alveolar macrophages and epithelial cells [234]. In IL-6 deficient mice, corneal infection by *P. aeruginosa* was shown to increase by 2-fold due to impaired neutrophil recruitment [235].

IL-17C is a member of the IL-17 family of cytokines that is selectively expressed in epithelial cells in response to bacterial challenge such as *P. aeruginosa* and *Haemophilus influenza* or inflammatory stimuli

like agonists for TLR-3 and TLR-5. Through an autocrine mechanism, IL-17C stimulates inflammatory responses from epithelial cells by binding to receptor complexes IL-17RA and IL-17RE which are expressed on epithelial cells [232]. IL-17C activated epithelial cells to secrete neutrophil chemoattractants including G-CSF, IL-8 and antimicrobial agents such as β-defensins. Other members of the IL-17 family include IL-17A to IL-17F. IL-17A is mainly produced by leukocytes such as T_H17 and is notable for maintaining homeostatic conditions in the gut mucosa. IL-17A induces the production of antimicrobial proteins such as Regenerating islet-derived protein 3γ (ReG3γ) by the gut epithelial cells which prevents dissemination of commensal bacteria from penetrating the epithelial barrier. Similar to IL-17C, uncontrolled expression of IL-17A can lead to human autoimmune diseases such as psoriasis, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease [236-238].

Additionally, the results showed that Calu-3 cells displayed a high level of constitutive IL-8 production that was not further increased by PAO1-L and *Δhxc* infection. IL-8 is a crucial neutrophil chemoattractant whose major source is bronchial epithelial cells [239]. Constitutive IL-8 production by bronchial epithelial cells have previously been reported in our lab (Yi-Chia Lui, PhD thesis, 2014) in agreement with other studies [211, 240]. Moreover, airway epithelial cells with dysfunctional CFTR have been reported to produce IL-8 *in*

vitro even in the absence of infection [241]. In cancer cell lines, constitutive production of IL-8 is associated with the mutation of p53 tumour suppressor gene in these cell lines [242]. This may explain the constitutive IL-8 production by Calu-3 cells since they are derived from a person with bronchial adenocarcinoma [210]. The results of the cytokine profiling demonstrated that *in vitro* human bronchial epithelial cells mount a robust proinflammatory response upon viable PAO1-L and *Δhxc* infection, demonstration that Calu-3 cell epithelia is a good infection model for respiratory diseases.

Although it is still not clear the initial determinant factors that drive a *P. aeruginosa*-epithelial cell interaction to either an acute or a chronic condition, it is proven that the bacterium expresses distinct sets of virulence factors in each condition [209]. Virulence factors such as type IV pili, flagella, and expression of T2 –and-T3SS drive acute infections while T6SS and exopolysaccharide (Pel and Psl) involved in biofilm formation contribute to chronic infections [243, 244]. Our findings from the reporter strains when in contact with Calu-3 cells showed that the *hxc* operon is expressed during acute infection; which is in agreement with a previous study [209]. It is thus not surprising that the deletion of *hxc* operon alone did not affect the infectivity of PAO1-L mutant since many virulent factors including phoA, which can compensate for LapA, are expressed during this acute interaction with bronchial epithelial cells.

Put together, we show that Calu-3 cells produce inflammatory respond to *P. aeruginosa* infection through the production of cytokines and chemokines capable of recruiting neutrophils and macrophages. The results also demonstrate that the hxc operon is expressed upon acute infection of bronchial epithelial cells, although its role during infection may not be detectably significant.

Chapter 5: General discussion and future perspectives

The first project

Polymicrobial diseases, caused by combinations of different viruses, bacteria, fungi and parasites are increasingly being detected and becoming a global concern [146, 245-247]. Van Leeuwenhoek demonstrated the idea of multiple organisms colonising human tissues early around 1680 from observation of his tartar with a primitive microscope [146, 248]. The microbes which van Leeuwenhoek described in his notebook including cocci, spirochetes, and fusiform bacteria are now considered as some of the most abundant bacteria in the mouth [248]. There are different categories of interactions that happen within human polymicrobial diseases. Firstly, there is microbial interference where the presence of one microorganism in a niche inhibits the colonisation of other microorganisms [246]. This is demonstrated in children with a history of recurrent acute otitis media, where nasopharyngeal carriage of *Streptococcus pneumonia* suppressed colonisation of *Staphylococcus aureus*. Vaccination of these children with pneumococcal-conjugate vaccine led to an increase in *S. aureus* infection [249, 250].

In synergistic polymicrobial interaction, the presence of one microorganism creates a favourable niche for the colonisation and infection of other microorganisms [246]. This interaction is mostly observed in situations where a viral infection leads to immunosuppression as in the case of human immunodeficiency virus

(HIV)/*Mycobacterium tuberculosis* co-infection, and Measles virus/*M. tuberculosis/S. aureus* co-infections [251-253]. This is also demonstrated in urinary tract infection, where prior infection of bladder mucosa by *E. coli* has been shown to increase *C. albicans* colonisation. Again, in CF and ventilator-associated pneumonia, *C. albicans* colonisation is considered to promote *P. aeruginosa* tissue invasion [3, 110, 111, 145]. In additive polymicrobial infections, colonisation of tissue by two or more often non-pathogenic microorganisms leads to infection. In burn wound for example, *P. aeruginosa* and *C. albicans* individually could not cause mortality in burned mice compared to mixed-challenge [113]. Development of polymicrobial biofilms in CF lungs is also considered to increase the pathogenicity of *C. albicans*, *S. aureus*, *and P. aeruginosa* [246, 254].

Notwithstanding the advancement in diagnostics and treatment of some of these polymicrobial infections, our understanding is still limited on how interactions of multispecies affect the scope, progression, and severity of diseases, as well as the mechanism involved in how the host immune system responds to these polymicrobial infections [146]. The purpose of this study was thus to investigate novel players involved in cross-talk during mixed infection of *P. aeruginosa* and *C. albicans*, two opportunistic organisms frequently found together in CF, burn wounds, and nosocomial infections. This was achieved by setting up fungal-bacterial

supernatant culture system from which we assessed the growth of *C. albicans* in the presence of *P. aeruginosa* secretions (Chapter 2). Data leading to this project had shown that supernatants from *P. aeruginosa* PAO1-L induced filamentation in *C. albicans*, which corresponded to lower OD_{600nm} compared to cultures treated with control medium. Further, it was shown that *C. albicans* filamentation was not due to the presence of rhamnolipids, exotoxin A, or pyocyanin in the supernatants, or the virulence regulator *toxR* gene. However, the hyphal induction effect on *C. albicans* was attenuated in supernatant from PAAMP1 strain, which had a 58kb chromosomal deletion including genes encoding the Hxc-T2SS, LapA, BifA, and ToxR. To the best of our knowledge, no previous work has reported the induction of hyphal growth of *C. albicans* by *P. aeruginosa* secretions independent of QS signalling. On the contrary, it had been reported that *P. aeruginosa* QS signalling molecule 3-oxo-C12 homoserine lactone rather inhibits yeast-hyphal transition of *C. albicans* in mixed cultures [158].

To ascertain the nature of the hyphal inducing agent(s) in *P. aeruginosa* secretion, separation analysis of PAO1-L and PAAMP1 supernatants were conducted using 10 kDa pore-sized filter. The results showed that the hyphal induction activity was retained in the above 10 kDa fractionate of PAO1-L supernatant. Concentration of fractionate from PAAMP1 (5x) produced hyphal induction of *C.*

albicans to the extent as WT strain. This observation suggests that the hyphal inducing agent(s) is above 10 kDa, and that in PAAMP1, the active agent(s) is secreted at a lower concentration. Since *P. aeruginosa* possesses multiple copies of secretion systems and overlapping secreted products [154], we speculate that the 58 kb deletion in PAAMP1 may be compensated by overlapping gene(s) independent of the deletion. In an effort to confirm that, the hyphal induction is due to a protein in the supernatant, we denatured the supernatant proteins at 100 °C for 10min; after which these inactivated supernatants we tested on *C. albicans*. It was found that subjecting the supernatants to heat stress abolished hyphal induction activity. Further, comparison of protein content in supernatants revealed a protein band with rMW between 25 – 30kDa specific for WT. The results put together pointed to the possibility that the active agent(s) is a heat-labile protein [162, 163].

In the course of this study, we lost the *C. albicans* hyphal induction effect in PAO1-L secretion. This prompted an evaluation of the fungal-bacterial supernatant culture assay, in which several steps were taking to reproduce our earlier observations. The PhD student who generated the preliminary data leading to this work (Farah Hussain, PhD thesis, 2016) did preparations of bacterial and candida cultures from the original glycerol stocks. The results did not show any growth differences when *C. albicans* was treated with supernatants from

PAO1-L, PAAMP1 or media control. To determine if the loss of effect was due to wrong OD measurements in the plate readers, we examined *C. albicans* growth using two different plate readers. We also concentrated the bacterial supernatants (5x) using 10 kDa pore sized filters, and treated *C. albicans* with the above 10 kDa fractionate as well as the filtrate. To determine if PAO1-L secretion was being affected by the growth medium, X-vivo[15], we cultured and prepared bacterial supernatants in another well-defined medium, DMEM-F12. Growth response of *C. albicans* in the presence of PAO1-L or PAAMP1 supernatants under all these test conditions did not show any difference compared to control media. We finally tested our supernatants on five clinical strains of *C. albicans* in comparison with the WT. Dr Rebecca Hall (University of Birmingham) kindly provided the *C. albicans* strains and helped in setting up the co-culture assay in her lab. The results did not show any difference in *C. albicans* growth in response to supernatants from WT, PAAMP1 and PAO1-LΔ*hxc*. Put together, our attempt to reproduce our earlier observation of *C. albicans* hyphal induction by *P. aeruginosa* secretion was not successful, and we did not proceed further.

While it is not clear why we lost the effect, we speculate there may be alterations in our culture conditions, media preparations or the co-culture that we cannot account for. We propose that in addition to measuring absorbance, *C. albicans* germ tubes can be quantified in

the co-culture plates as it might give a better indication of phenotypic changes of the fungus in response to *P. aeruginosa* secretions. There is a possibility that our lab strain PAO1-L like PAO1-N could be undergoing microevolution [Mai-Prochnow, *et al*., manuscript in preparation][164] that could account for changes in the bacterial phenotype; however high-throughput genome resequencing would be required to confirm this.

In summary, we demonstrated that *P. aeruginosa* secretion might induce filamentation in *C. albicans in vitro*; however, this is inconclusive and will require further work. The lack of reproducibility of the hyphal induction results demonstrate how difficult it may be to understand and replicate polymicrobial infection *in vivo*.

The second project

In our previous study, we focused on the growth response of *C. albicans* to *P. aeruginosa* secretion. Preliminary data had shown that supernatant from WT unlike PAAMP1 strain could induce hyphal growth response of *C. albicans* under our fungal-supernatant co-culture conditions. Data had shown that the lack of hyphal induction in PAAMP1 supernatant was not due to the absence of rhamnolipids, exotoxin A, pyocyanin, or the virulence regulator *toxR.* Further, we observed that heating PAO1-L supernatant abolished hyphal induction effect. Analysis of supernatant proteins showed a unique

protein band with rMW between 25 – 30kDa in PAO1-L. We hypothesised that the hyphal inducing agent in the wild type supernatant was Hxc-T2SS dependent LapA. LapA has a rMW between 38-to-40 kDa [79], and its gene is among the deletion in PAAMP1, including the *hxc* operon.

Most studies on *P. aeruginosa* type II secretory systems have focused on the Xcp-T2SS over the years. This is most likely due to the involvement of xcp-dependent exoproteins in many respiratory diseases such as exotoxin A (regarded as the most toxic virulent factor of *P. aeruginosa*), which is involved in host cell lysis by disrupting protein synthesis [47] and haemolytic phospholipase C, which causes vascular permeability, impairs lung surfactant and inhibits neutrophil respiratory burst activity [255]. To our knowledge, aside the work by Ball *et. al.* 2002 which first reported and described the Hxc-T2SS [79, 169], the functional role of this secreton in *P. aeruginosa* pathogenesis is poorly understood. Thus, the purpose of the current study was to conduct a comprehensive investigation of the involvement of *P. aeruginosa* Hxc-T2SS in: 1) expression of bacterial virulence factors *in vitro*, 2) cross-Kingdom communication with *C. albicans* during co-infection, and 3) how this secreton is regulated. This was achieved by constructing an in-frame deletion mutant lacking the *hxc*-operon (Δ*hxc*) in the PAO-L background (Chapter 3). The phenotype of Δ*hxc* was characterised, and showed

bacterial growth, biofilm and microcolony formation, and pyocyanin production, comparable to its isogenic WT strain. This was in agreement with past studies, suggesting that the *hxc* operon does not detectably affect vital virulence factors of *P. aeruginosa* [79]. Co-culture of *C. albicans* with supernatant from Δ*hxc* did not produce any different observation compared to WT or media.

The seminal work on the Hxc-T2SS had reported that the secreton is expressed under phosphate limitation [79]. To determine the influence of exogenous phosphate on Δ*hxc* phenotype, we supplemented our media with 10 mM exogenous phosphate. We observed that addition of exogenous phosphate to our culture media, independent of *hxc*, significantly promoted bacterial growth and biofilm formation, but not pyocyanin secretion, which was reduced in both the mutant and the WT PAO1-L. The importance of phosphate in promoting *P. aeruginosa* growth have been reported [199, 256]. The deletion of polyphosphate kinase (*ppk)* gene, which encodes PPK responsible for the synthesis of inorganic polyphosphate from ATP, has been shown to inhibit P. aeruginosa formation biofilm and colonisation of mouse tissues [257]. The ability of Δ*hxc* to respond to endogenous phosphate is thought to be due to the presence of a second alkaline phosphatase, PhoB, an Xcp-dependent secretant [79, 256]. Although phosphate assimilation by *P. aeruginosa* has been positively correlated with growth and expression of virulence factors,

other studies have reported the contrary [202]. Our observation of low pyocyanin production by *P. aeruginosa* in response to high exogenous phosphate is in agreement with a study which showed that pyocyanin as well as catalase and superoxide dismutase activities are enhanced under phosphate limitation but not in high-phosphate succinate medium [197]. We speculate that under stress conditions, the bacterium deploys exoproteins including pyocyanin into the extracellular environment which increases the chance of the bacterium to outcompete other microorganism for limited nutrients. Investigation of Δ*hxc* supernatant protein content and alkaline phosphatase activity showed no difference when grown in phosphate-limiting PPM or nutrient-rich LB when compared to PAO1-L. Lack of alkaline phosphatase activity in LB was consistent with the findings of Ball *et. al.* 2002, while our observation in PPM was contrary as they reported that a 40 kDa LapA protein was found in the WT but not in the corresponding *hxcR* mutant when the bacteria were grown in PPM. They also reported that *hxc* mutant retained only 20 % alkaline phosphatase activity compared to WT in PPM. While we cannot rule out the possibility of PAO1 strain differences, we believe the time point at which the supernatants were assayed for protein analysis and alkaline phosphatase activity in PPM (4hrs, OD_{600} 1.5) was to be too early for the maturation and extracellular secretion of LapA. This assertion is based on our observation of alkaline phosphatase activity in supernatants

recovered from PAO1-L and Δ*hxc* culture in X-Vivo^{15} which reached OD_{600} 1.5 after 7hours of incubation.

In our quest to elucidate conditions under which the *hxc* operon is expressed, we generated PAO1-L mCTX::P_{hxcT}-*lux* and PAO1-L mCTX::P_{hxcV}-*lux* promoter fusions. Our study of the two promoters showed that contrary to past report, *hxc* transcriptional activities in the P_{hxcV} orientation is approximately 10 times higher than in the P_{hxcT} orientation [79]. Additionally, our investigation of the transcriptional activities of the *hxc* operon revealed that the expression of P_{hxcT} and P_{hxcV} promoters peaks at about 10 hours and 5 hours post-incubation respectively. This observation supports our earlier assertion that PAO1-L and Δ*hxc* supernatant assayed from a 4hour culture may not contain LapA. Further, we observed that transcriptional activities from the two promoters are sustained longer in X-vivo^{15} compared to LB or PPM. However, unlike LB and PPM, addition of exogenous phosphate reduced promoter activities in X-vivo^{15}. While the factors accounting for the differences in promoter activities is unclear, we demonstrate how responsive the *hxc* secreton is to changes in the environment.

In summary, we generated Δ*hxc* and demonstrated that the secreton does not influence some key virulence factors such as biofilm formation and pyocyanin production in *P. aeruginosa.* Similar to WT,

supernatant from Δ*hxc* culture did not detectably influence *C. albicans* growth, which we propose further investigation using supernatants assayed from 7 hour culture. Data also demonstrate tight regulation and high dependency on culture conditions for the transcription of genes encoding the hxc secreton, which imply that this system may be important for the adaptation of *P. aeruginosa* to its environment. Apart from phosphate limitation, which has been shown to regulate *hxc* expression, some recent studies have reported that the secreton may be under the control of the global GacS/GacA system [258-260]. GacA is a transcriptional regulator activated through phosphorylation by GacS, a sensor kinase. Together, the GacS/GacA system regulates the expression of genes encoding RNA-binding proteins RsmA/RsmN, which have been shown to repress chronic virulence phenotypes T6SS, psl/pel polysaccharides, and biofilm formation; while promoting acute virulence phenotypes T3SS, T4 pili, virulence factor regulator (vfr) and hxc-T2SS. It has been reported that *gacA* mutation caused significant upregulation of the hxc operon. Future work could investigate the sequence of interaction between GacS/GacA system and the hxc secreton.

The third project

Previous work had shown that hxc-T2SS deficiency did not affect expression of *P. aeruginosa* virulence phenotypes. Further, transcription of the secreton was demonstrated to be tightly

regulated, dependent on environmental conditions and better sustained in eukaryotic cell media X-vivo[15]. Past transcriptomic studies of *P. aeruginosa* after exposure to primary normal human airway epithelial cells had reported the upregulation of genes involved in the acquisition and metabolism of phosphate such as *phoA*, *oprO*, *plcN*, and *glpQ* [217]. A similar study also reported upregulation of the hxc operon after *P. aeruginosa* exposure to respiratory epithelia cells [209]. These findings demonstrate that phosphate sensing and acquisition may play a key role in *P. aeruginosa* pathogenesis in the host. To determine the contribution of Hxc-T2SS to the interaction of *P. aeruginosa* with human bronchial epithelial in an acute infection setting, we infected differentiated and undifferentiated human bronchial epithelial cells with PAO1-L and its isogenic *hxc*-deficient mutant Δ*hxc* (Chapter 4). Pathogenicity of the *hxc*-deficient and WT strains and innate immune response by Calu-3 cells were evaluated by analysing bacterial CFU post infection, cellular toxicity and bacterial-induced proinflammatory cytokines. Furthermore, transcription profile of the hxc operon during infection was evaluated using *lux*-labelled PAO1-L constructs PAO1-L miniCTX::P_{hxcT}-*lux* and PAO1-L miniCTX::P_{hxcV}-*lux*. The results showed that both PAO1-L and *Δhxc* seem to grow in the presence of differentiated and undifferentiated Calu-3 cells with the mutant showing the tendency of thriving better than the WT. While it is unclear why *hxc*-deficiency will confer a higher survival rate than WT

after Calu-3 cell exposure, we speculate that this might be due to the mutant having a lower metabolic cost of exoproteins secretion in compared to WT. Since most genes associated with phosphate acquisition and metabolism are upregulated during exposure to airway epithelial cells [217], we speculate that the cost of protein production by the WT will be higher, which could reduce cell growth and fitness [261, 262]. Additionally, from our LDH assays there was a trend, though not significant, towards the *hxc* mutant being more cytotoxic than the WT. We posit that Δ*hxc* did not lose infectivity because Xcp-T2SS is also upregulated during acute infection [209, 217]. The xcp secreton is involved in the secretion of many virulent factors, including phoA which can compensate the activity of LapA, the only secretant from the hxc apparatus [79].

Our results from the assessment of bacterial-induced proinflammatory cytokines production agree with past literature that airway epithelial cells secrete diverse cytokines and chemokines that can alert, recruit and activate phagocytes in response to pathogen [206-208, 263]. Although comparison between WT and Δ*hxc*-induced cytokine production did not reveal significant differences, the results show that infected Calu-3 cells produced TNF-α, GM-CSF, G-CSF, IL-6 and IL-17C in response to *P. aeruginosa* infection. Of note, the data also corroborate with a previous finding in our lab and studies by others that Calu-3 cells constitutively express IL-8 (Yi-Chia Lui, PhD

thesis, 2014)[211, 240]. Constitutive IL-8 production is reported in cancer cells having mutation in p53 tumour suppressor gene [242] and epithelial cells with dysfunctional CFTR [241, 264, 265]. The mechanism involving constitutive IL-8 secretion in CF bronchial epithelium is unclear; however, a study has reported that downregulation of α-klotho (KL) may contribute to IL-8 production in the CF lung [266]. KL is a transmembrane or soluble protein, which functions as a co-receptor for fibroblast growth factor 23, and it is the soluble form of KL considered to exhibit anti-inflammatory, anti-fibrotic and anti-senescence effects [266, 267]. IL-8 binds to CXCR1 and CXCR2 receptors expressed on neutrophils, monocytes, endothelial cells, astrocytes, and microglia [268]. IL-8 mediated neutrophil activation and chemotaxis as well as endothelial cell proliferation has been associated with many inflammatory diseases [222, 269]. There are ongoing development and clinical trials of anti-IL-8 monoclonal antibodies which preliminary data shows protective effect against angiogenesis, and metastasis of human melanoma [269] and palmoplantar pustulosis, a chronic inflammatory skin disease [268]. The anti-IL-8 antibodies specifically bind to IL-8 thereby inhibiting neutrophil activation and endothelial cell proliferation. Owing to IL-8 major contribution to neutrophil-mediated tissue damage in CF airways, the need to study IL-8 constitutive expression in CF lungs is apparent.

Consistent with previous studies, data from our reporter study confirms that *hxc* operon is expressed during contact with bronchial epithelial cells [209]. The data additionally support our earlier observation that transcriptional activity of the hxc operon is quicker and higher in the P_{hxcV} orientation than P_{hxcT}. We cannot answer as to why the two promoters behave differently in the same environment, but speculate that this phenomenon may contribute to *P. aeruginosa* fitness and adaption to different environments.

In summary, we have demonstrated that exposure of *P. aeruginosa* to bronchial epithelial cells lead to pro-inflammatory immune response and that the hxc operon is among bacterial genes activated during acute infection. We also show that deletion of hxc operon does not significantly affect bacterial fitness. Neutrophil and macrophage accumulation often characterise bacterial infection in CF, thus future work can examine if *hxc* upregulation occurs in the presence of these cell types and how beneficial this could be for the host or the bacterium.

References

1. Hibbing, M.E., et al., *Bacterial competition: surviving and thriving in the microbial jungle.* Nature reviews. Microbiology, 2010. **8**(1): p. 15-25.
2. Adamberg, S., et al., *Survival and synergistic growth of mixed cultures of bifidobacteria and lactobacilli combined with prebiotic oligosaccharides in a gastrointestinal tract simulator.* Microbial Ecology in Health and Disease, 2014. **25**(1): p. 23062.
3. Hogan, D.A. and R. Kolter, *Pseudomonas-Candida interactions: an ecological role for virulence factors.* Science, 2002. **296**(5576): p. 2229-32.
4. Fourie, R., et al., *Candida albicans and Pseudomonas aeruginosa Interaction, with Focus on the Role of Eicosanoids.* Frontiers in Physiology, 2016. **7**: p. 64.
5. Gomila, M., et al., *Phylogenomics and systematics in Pseudomonas.* Frontiers in Microbiology, 2015. **6**(214).
6. Gupta, R.S., *The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes.* FEMS Microbiol Rev, 2000. **24**(4): p. 367-402.
7. Parte, A.C., *LPSN—list of prokaryotic names with standing in nomenclature.* Nucleic Acids Research, 2014. **42**(D1): p. D613-D616.
8. Peix, A., M.-H. Ramírez-Bahena, and E. Velázquez, *Historical evolution and current status of the taxonomy of genus Pseudomonas.* Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases, 2009. **9**(6): p. 1132-1147.
9. Silby, M.W., et al., *Pseudomonas genomes: diverse and adaptable.* FEMS Microbiology Reviews, 2011. **35**(4): p. 652-680.
10. Block, A. and J.R. Alfano, *Plant targets for Pseudomonas syringae type III effectors: virulence targets or guarded decoys?* Current Opinion in Microbiology, 2011. **14**(1): p. 39-46.
11. Silby, M.W., et al., *Genomic and genetic analyses of diversity and plant interactions of Pseudomonas fluorescens.* Genome Biology, 2009. **10**(5): p. R51.
12. Wixon, J., *Featured Organism: Pathogen special: Vibrio cholerae, Pseudomonas aeruginosa and Xylella fastidiosa.* Yeast, 2000. **17**(4): p. 307-313.

13. Lister, P.D., D.J. Wolter, and N.D. Hanson, *Antibacterial-Resistant Pseudomonas aeruginosa: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms.* Clinical Microbiology Reviews, 2009. **22**(4): p. 582-610.

14. LaBauve, A.E. and M.J. Wargo, *Growth and Laboratory Maintenance of Pseudomonas aeruginosa.* Current Protocols in Microbiology, 2012. **25**(1): p. 6E.1.1-6E.1.8.

15. Stover, C.K., et al., *Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen.* Nature, 2000. **406**(6799): p. 959-964.

16. Klockgether, J., et al., *Pseudomonas aeruginosa Genomic Structure and Diversity.* Frontiers in Microbiology, 2011. **2**(150).

17. Rahme, L., et al., *Common virulence factors for bacterial pathogenicity in plants and animals.* Science, 1995. **268**(5219): p. 1899-1902.

18. Roy, P.H., et al., *Complete Genome Sequence of the Multiresistant Taxonomic Outlier Pseudomonas aeruginosa PA7.* PLoS ONE, 2010. **5**(1): p. e8842.

19. Mathee, K., et al., *Dynamics of Pseudomonas aeruginosa genome evolution.* Proceedings of the National Academy of Sciences, 2008. **105**(8): p. 3100-3105.

20. Cheng, K., et al., *Spread of beta-lactam-resistant Pseudomonas aeruginosa in a cystic fibrosis clinic.* The Lancet. **348**(9028): p. 639-642.

21. Lee, D., et al., *Genomic analysis reveals that Pseudomonas aeruginosa virulence is combinatorial.* Genome Biology, 2006. **7**(10): p. R90.

22. Imberty, A., et al., *Structures of the lectins from Pseudomonas aeruginosa: insight into the molecular basis for host glycan recognition.* Microbes Infect, 2004. **6**(2): p. 221-8.

23. Gellatly, S.L. and R.E.W. Hancock, *Pseudomonas aeruginosa : new insights into pathogenesis and host defenses.* Pathogens and Disease, 2013. **67**(3): p. 159-173.

24. Branski, L.K., et al., *Emerging Infections in Burns.* Surgical Infections, 2009. **10**(5): p. 389-397.

25. Sadikot, R.T., et al., *Pathogen-host interactions in Pseudomonas aeruginosa pneumonia.* Am J Respir Crit Care Med, 2005. **171**(11): p. 1209-23.

26. Kerr, K.G. and A.M. Snelling, *Pseudomonas aeruginosa: a formidable and ever-present adversary.* J Hosp Infect, 2009. **73**(4): p. 338-44.
27. Lyczak, J.B., C.L. Cannon, and G.B. Pier, *Establishment of Pseudomonas aeruginosa infection: lessons from a versatile opportunist.* Microbes Infect, 2000. **2**(9): p. 1051-60.
28. Roux, D., et al., *Candida albicans impairs macrophage function and facilitates Pseudomonas aeruginosa pneumonia in rat.* Crit Care Med, 2009. **37**(3): p. 1062-7.
29. Kollef, M.H., et al., *Epidemiology and outcomes of health-care-associated pneumonia: results from a large US database of culture-positive pneumonia.* Chest, 2005. **128**(6): p. 3854-62.
30. Ala'Aldeen, D.A.A. and K.G. Wooldridge, *13 - Bacterial pathogenicity A2 - Greenwood, David*, in *Medical Microbiology (Eighteenth Edition)*, M. Barer, R. Slack, and W. Irving, Editors. 2012, Churchill Livingstone: Edinburgh. p. 156-167.
31. Lau, P.C.Y., et al., *Differential Lipopolysaccharide Core Capping Leads to Quantitative and Correlated Modifications of Mechanical and Structural Properties in Pseudomonas aeruginosa Biofilms.* Journal of Bacteriology, 2009. **191**(21): p. 6618-6631.
32. Maldonado, R.F., I. Sá-Correia, and M.A. Valvano, *Lipopolysaccharide modification in Gram-negative bacteria during chronic infection.* FEMS Microbiology Reviews, 2016. **40**(4): p. 480-493.
33. Lam, J., et al., *Genetic and Functional Diversity of Pseudomonas aeruginosa Lipopolysaccharide.* Frontiers in Microbiology, 2011. **2**(118).
34. King, J.D., et al., *Review: Lipopolysaccharide biosynthesis in Pseudomonas aeruginosa.* Innate Immunity, 2009. **15**(5): p. 261-312.
35. Cigana, C., et al., *Pseudomonas aeruginosa exploits lipid A and muropeptides modification as a strategy to lower innate immunity during cystic fibrosis lung infection.* PLoS One, 2009. **4**(12): p. e8439.
36. Feldman, M., et al., *Role of Flagella in Pathogenesis of Pseudomonas aeruginosa Pulmonary Infection.* Infection and Immunity, 1998. **66**(1): p. 43-51.
37. Kipnis, E., T. Sawa, and J. Wiener-Kronish, *Targeting mechanisms of Pseudomonas aeruginosa pathogenesis.* Médecine et Maladies Infectieuses, 2006. **36**(2): p. 78-91.

38. Church, D., et al., *Burn Wound Infections.* Clinical Microbiology Reviews, 2006. **19**(2): p. 403-434.

39. Adamo, R., et al., *Pseudomonas aeruginosa Flagella Activate Airway Epithelial Cells through asialoGM1 and Toll-Like Receptor 2 as well as Toll-Like Receptor 5.* American Journal of Respiratory Cell and Molecular Biology, 2004. **30**(5): p. 627-634.

40. Döring, G., C. Meisner, and M. Stern, *A double-blind randomized placebo-controlled phase III study of a Pseudomonas aeruginosa flagella vaccine in cystic fibrosis patients.* Proceedings of the National Academy of Sciences, 2007. **104**(26): p. 11020-11025.

41. Van Delden, C. and B.H. Iglewski, *Cell-to-cell signaling and Pseudomonas aeruginosa infections.* Emerging Infectious Diseases, 1998. **4**(4): p. 551-560.

42. Sopena, N., et al., *Prospective study of community-acquired pneumonia of bacterial etiology in adults.* Eur J Clin Microbiol Infect Dis, 1999. **18**(12): p. 852-8.

43. Yahr, T.L., et al., *Identification of type III secreted products of the Pseudomonas aeruginosa exoenzyme S regulon.* Journal of Bacteriology, 1997. **179**(22): p. 7165-7168.

44. Yahr, T.L., et al., *ExoY, an adenylate cyclase secreted by the Pseudomonas aeruginosa type III system.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13899-904.

45. Iglewski, B.H., et al., *Pseudomonas aeruginosa exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from toxin A.* Proceedings of the National Academy of Sciences, 1978. **75**(7): p. 3211-3215.

46. Vance, R.E., A. Rietsch, and J.J. Mekalanos, *Role of the Type III Secreted Exoenzymes S, T, and Y in Systemic Spread of Pseudomonas aeruginosa PAO1 In Vivo.* Infection and Immunity, 2005. **73**(3): p. 1706-1713.

47. Michalska, M. and P. Wolf, *Pseudomonas Exotoxin A: optimized by evolution for effective killing.* Frontiers in Microbiology, 2015. **6**: p. 963.

48. Woods, D.E. and B.H. Iglewski, *Toxins of Pseudomonas aeruginosa: new perspectives.* Rev Infect Dis, 1983. **5 Suppl 4**: p. S715-22.

49. Hoffmann, N., et al., *Novel Mouse Model of Chronic Pseudomonas aeruginosa Lung Infection Mimicking Cystic Fibrosis.* Infection and Immunity, 2005. **73**(4): p. 2504-2514.

50. Williams, P., *Quorum sensing, communication and cross-kingdom signalling in the bacterial world.* Microbiology, 2007. **153**(12): p. 3923-3938.
51. Pesci, E.C., et al., *Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa.* Journal of Bacteriology, 1997. **179**(10): p. 3127-3132.
52. Papenfort, K. and B.L. Bassler, *Quorum sensing signal–response systems in Gram-negative bacteria.* Nature Reviews Microbiology, 2016. **14**: p. 576.
53. Lee, J., et al., *A cell-cell communication signal integrates quorum sensing and stress response.* Nat Chem Biol, 2013. **9**(5): p. 339-43.
54. Lee, J. and L. Zhang, *The hierarchy quorum sensing network in Pseudomonas aeruginosa.* Protein & Cell, 2015. **6**(1): p. 26-41.
55. Wolz, C., et al., *Iron release from transferrin by pyoverdin and elastase from Pseudomonas aeruginosa.* Infection and Immunity, 1994. **62**(9): p. 4021-4027.
56. Yanagihara, K., et al., *Role of elastase in a mouse model of chronic respiratory Pseudomonas aeruginosa infection that mimics diffuse panbronchiolitis.* J Med Microbiol, 2003. **52**(Pt 6): p. 531-5.
57. Park, P.W., et al., *Syndecan-1 shedding is enhanced by LasA, a secreted virulence factor of Pseudomonas aeruginosa.* J Biol Chem, 2000. **275**(5): p. 3057-64.
58. Daddaoua, A., et al., *Genes for Carbon Metabolism and the ToxA Virulence Factor in Pseudomonas aeruginosa Are Regulated through Molecular Interactions of PtxR and PtxS.* PLoS ONE, 2012. **7**(7): p. e39390.
59. McEwan, D.L., N.V. Kirienko, and F.M. Ausubel, *Host Translational Inhibition by Pseudomonas aeruginosa Exotoxin A Triggers an Immune Response in Caenorhabditis elegans.* Cell host & microbe, 2012. **11**(4): p. 364-374.
60. Laarman, A.J., et al., *Pseudomonas aeruginosa alkaline protease blocks complement activation via the classical and lectin pathways.* J Immunol, 2012. **188**(1): p. 386-93.
61. Jensen, V., et al., *RhlR Expression in Pseudomonas aeruginosa Is Modulated by the Pseudomonas Quinolone Signal via PhoB-Dependent and -Independent Pathways.* Journal of Bacteriology, 2006. **188**(24): p. 8601-8606.
62. Adam, E.C., et al., *Pseudomonas aeruginosa II lectin stops human ciliary beating: therapeutic implications of fucose.* Am J Respir Crit Care Med, 1997. **155**(6): p. 2102-4.

63. Ryall, B., et al., *Pseudomonas aeruginosa, cyanide accumulation and lung function in CF and non-CF bronchiectasis patients.* Eur Respir J, 2008. **32**(3): p. 740-7.

64. Denning, G.M., et al., *Pseudomonas Pyocyanin Increases Interleukin-8 Expression by Human Airway Epithelial Cells.* Infection and Immunity, 1998. **66**(12): p. 5777-5784.

65. Singh, P.K., et al., *Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms.* Nature, 2000. **407**(6805): p. 762-4.

66. Williams, B.J., J. Dehnbostel, and T.S. Blackwell, *Pseudomonas aeruginosa: host defence in lung diseases.* Respirology, 2010. **15**(7): p. 1037-56.

67. Tart, A.H. and D.J. Wozniak, *Shifting paradigms in Pseudomonas aeruginosa biofilm research.* Curr Top Microbiol Immunol, 2008. **322**: p. 193-206.

68. Trust, C.F. FAQs 2018 [cited 2018 11/12/2018]; Available from: https://www.cysticfibrosis.org.uk/what-is-cystic-fibrosis/faqs#How%20common%20is%20cystic%20fibrosis?

69. Kreda, S.M., C.W. Davis, and M.C. Rose, *CFTR, Mucins, and Mucus Obstruction in Cystic Fibrosis.* Cold Spring Harbor Perspectives in Medicine, 2012. **2**(9).

70. Rowe, S.M., S. Miller, and E.J. Sorscher, *Cystic Fibrosis.* New England Journal of Medicine, 2005. **352**(19): p. 1992-2001.

71. Hauser, A.R., et al., *Clinical Significance of Microbial Infection and Adaptation in Cystic Fibrosis.* Clinical Microbiology Reviews, 2011. **24**(1): p. 29-70.

72. Burns, J.L., et al., *Longitudinal Assessment of Pseudomonas aeruginosa in Young Children with Cystic Fibrosis.* The Journal of Infectious Diseases, 2001. **183**(3): p. 444-452.

73. Ute, R., et al., *Worldwide distribution of Pseudomonas aeruginosa clone C strains in the aquatic environment and cystic fibrosis patients.* Environmental Microbiology, 2005. **7**(7): p. 1029-1038.

74. Bryan, R., et al., *Overproduction of the CFTR R Domain Leads to Increased Levels of AsialoGM1 and Increased Pseudomonas aeruginosa Binding by Epithelial Cells.* American Journal of Respiratory Cell and Molecular Biology, 1998. **19**(2): p. 269-277.

75. Bjarnsholt, T., et al., *Pseudomonas aeruginosa biofilms in the respiratory tract of cystic fibrosis patients.* Pediatric Pulmonology, 2009. **44**(6): p. 547-558.

76. Pier, G.B., *Role of the cystic fibrosis transmembrane conductance regulator in innate immunity to*

Pseudomonas aeruginosa infections.* Proceedings of the National Academy of Sciences, 2000. **97**(16): p. 8822-8828.

77. Bragonzi, A., et al., *Nonmucoid Pseudomonas aeruginosa Expresses Alginate in the Lungs of Patients with Cystic Fibrosis and in a Mouse Model.* The Journal of infectious diseases, 2005. **192**(3): p. 410-419.
78. Hauser, A.R., *The Type III Secretion System of Pseudomonas aeruginosa: Infection by Injection.* Nature reviews. Microbiology, 2009. **7**(9): p. 654-665.
79. Ball, G., et al., *A novel type II secretion system in Pseudomonas aeruginosa.* Mol Microbiol, 2002. **43**(2): p. 475-85.
80. Tseng, T.-T., B.M. Tyler, and J.C. Setubal, *Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology.* BMC Microbiology, 2009. **9**(Suppl 1): p. S2-S2.
81. Bleves, S., et al., *Protein secretion systems in Pseudomonas aeruginosa: A wealth of pathogenic weapons.* Int J Med Microbiol, 2010. **300**(8): p. 534-43.
82. Bardoel, B.W., et al., *Pseudomonas evades immune recognition of flagellin in both mammals and plants.* PLoS Pathog, 2011. **7**(8): p. e1002206.
83. Korotkov, K.V., M. Sandkvist, and W.G.J. Hol, *The type II secretion system: biogenesis, molecular architecture and mechanism.* Nature reviews. Microbiology, 2012. **10**(5): p. 336-351.
84. Shi, L., et al., *Direct involvement of type II secretion system in extracellular translocation of Shewanella oneidensis outer membrane cytochromes MtrC and OmcA.* J Bacteriol, 2008. **190**(15): p. 5512-6.
85. Mudrak, B. and M.J. Kuehn, *Specificity of the Type II Secretion Systems of Enterotoxigenic Escherichia coli and Vibrio cholerae for Heat-Labile Enterotoxin and Cholera Toxin.* Journal of Bacteriology, 2010. **192**(7): p. 1902-1911.
86. Pugsley, A.P., *The complete general secretory pathway in gram-negative bacteria.* Microbiol Rev, 1993. **57**(1): p. 50-108.
87. Cornelis, G.R., *The type III secretion injectisome.* Nat Rev Microbiol, 2006. **4**(11): p. 811-25.
88. Anantharajah, A., M.-P. Mingeot-Leclercq, and F. Van Bambeke, *Targeting the Type Three Secretion System in*

Pseudomonas aeruginosa. Trends in Pharmacological Sciences, 2016. **37**(9): p. 734-749.

89. Milla, C.E., et al., *Anti-PcrV antibody in cystic fibrosis: a novel approach targeting Pseudomonas aeruginosa airway infection.* Pediatr Pulmonol, 2014. **49**(7): p. 650-8.
90. Goure, J., et al., *The V antigen of Pseudomonas aeruginosa is required for assembly of the functional PopB/PopD translocation pore in host cell membranes.* Infect Immun, 2004. **72**(8): p. 4741-50.
91. Botha, P., et al., *Pseudomonas aeruginosa colonization of the allograft after lung transplantation and the risk of bronchiolitis obliterans syndrome.* Transplantation, 2008. **85**(5): p. 771-4.
92. Faure, K., et al., *Effects of monoclonal anti-PcrV antibody on Pseudomonas aeruginosa-induced acute lung injury in a rat model.* Journal of Immune Based Therapies and Vaccines, 2003. **1**(1): p. 1-9.
93. Wu, W., et al., *MucA-mediated coordination of type III secretion and alginate synthesis in Pseudomonas aeruginosa.* J Bacteriol, 2004. **186**(22): p. 7575-85.
94. Filloux, A., *Protein Secretion Systems in Pseudomonas aeruginosa: An Essay on Diversity, Evolution, and Function.* Frontiers in Microbiology, 2011. **2**: p. 155.
95. Henderson, I.R., et al., *Type V Protein Secretion Pathway: the Autotransporter Story.* Microbiology and Molecular Biology Reviews, 2004. **68**(4): p. 692-744.
96. Costa, T.R.D., et al., *Secretion systems in Gram-negative bacteria: structural and mechanistic insights.* Nature Reviews Microbiology, 2015. **13**: p. 343.
97. van den Berg, B., *Crystal Structure of a Full-Length Autotransporter.* Journal of Molecular Biology, 2010. **396**(3): p. 627-633.
98. Wilhelm, S., et al., *The Autotransporter Esterase EstA of Pseudomonas aeruginosa Is Required for Rhamnolipid Production, Cell Motility, and Biofilm Formation.* Journal of Bacteriology, 2007. **189**(18): p. 6695-6703.
99. Pukatzki, S., et al., *Identification of a conserved bacterial protein secretion system in Vibrio cholerae using the Dictyostelium host model system.* Proc Natl Acad Sci U S A, 2006. **103**(5): p. 1528-33.
100. Mougous, J.D., et al., *A virulence locus of Pseudomonas aeruginosa encodes a protein secretion apparatus.* Science, 2006. **312**(5779): p. 1526-30.

101. Bingle, L.E., C.M. Bailey, and M.J. Pallen, *Type VI secretion: a beginner's guide.* Curr Opin Microbiol, 2008. **11**(1): p. 3-8.
102. Shrivastava, S. and S.S. Mande, *Identification and Functional Characterization of Gene Components of Type VI Secretion System in Bacterial Genomes.* PLoS ONE, 2008. **3**(8): p. e2955.
103. Boyer, F., et al., *Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources?* BMC Genomics, 2009. **10**: p. 104-104.
104. Henderson, I.R., R. Cappello, and J.P. Nataro, *Autotransporter proteins, evolution and redefining protein secretion.* Trends in Microbiology, 2000. **8**(12): p. 529-532.
105. Ho, Brian T., Tao G. Dong, and John J. Mekalanos, *A View to a Kill: The Bacterial Type VI Secretion System.* Cell Host & Microbe, 2014. **15**(1): p. 9-21.
106. Chotirmall, S.H., C.M. Greene, and N.G. McElvaney, *Candida species in cystic fibrosis: A road less travelled.* Med Mycol, 2010. **48 Suppl 1**: p. S114-24.
107. Deveau, A., et al., *Bacterial-fungal interactions: ecology, mechanisms and challenges.* FEMS Microbiol Rev, 2018. **42**(3): p. 335-352.
108. Peleg, A.Y., D.A. Hogan, and E. Mylonakis, *Medically important bacterial-fungal interactions.* Nat Rev Microbiol, 2010. **8**(5): p. 340-9.
109. Morales, D.K., et al., *Control of Candida albicans metabolism and biofilm formation by Pseudomonas aeruginosa phenazines.* MBio, 2013. **4**(1): p. e00526-12.
110. Azoulay, E., et al., *Candida colonization of the respiratory tract and subsequent pseudomonas ventilator-associated pneumonia.* Chest, 2006. **129**(1): p. 110-7.
111. Nseir, S., et al., *Impact of antifungal treatment on Candida-Pseudomonas interaction: a preliminary retrospective case-control study.* Intensive Care Med, 2007. **33**(1): p. 137-42.
112. Blaschke-Hellmessen, R., et al., *[Mycological surveillance of children with cystic fibrosis].* Mycoses, 1991. **34 Suppl 1**: p. 43-7.
113. Neely, A.N., E.J. Law, and I.A. Holder, *Increased susceptibility to lethal Candida infections in burned mice preinfected with Pseudomonas aeruginosa or pretreated with proteolytic enzymes.* Infection and Immunity, 1986. **52**(1): p. 200-204.

114. Jones, T., et al., *The diploid genome sequence of Candida albicans.* Proc Natl Acad Sci U S A, 2004. **101**(19): p. 7329-34.

115. Moran, G.P., D.C. Coleman, and D.J. Sullivan, *Candida albicans versus Candida dubliniensis: Why Is C. albicans More Pathogenic?* International Journal of Microbiology, 2012. **2012**: p. 7.

116. Jenkinson, H.F. and L.J. Douglas, *Interactions between Candida species and bacteria in mixed infections.* 2002.

117. Chibana, H., et al., *Sequence Finishing and Gene Mapping for Candida albicans Chromosome 7 and Syntenic Analysis Against the Saccharomyces cerevisiae Genome.* Genetics, 2005. **170**(4): p. 1525-37.

118. Sudbery, P.E., *Growth of Candida albicans hyphae.* Nat Rev Microbiol, 2011. **9**(10): p. 737-48.

119. Mallick, E.M. and R.J. Bennett, *Sensing of the Microbial Neighborhood by Candida albicans.* PLoS Pathog, 2013. **9**(10).

120. Lindsay, A.K. and D.A. Hogan, *Candida albicans: Molecular interactions with Pseudomonas aeruginosa and Staphylococcus aureus.* Fungal Biology Reviews, 2014. **28**(4): p. 85-96.

121. Ramage, G., et al., *Candida Biofilms: an Update.* Eukaryot Cell, 2005. **4**(4): p. 633-8.

122. Sudbery, P., N. Gow, and J. Berman, *The distinct morphogenic states of Candida albicans.* Trends Microbiol, 2004. **12**(7): p. 317-24.

123. Berman, J. and P.E. Sudbery, *Candida Albicans: a molecular revolution built on lessons from budding yeast.* Nat Rev Genet, 2002. **3**(12): p. 918-30.

124. Kadosh, D. and A.D. Johnson, *Induction of the Candida albicans Filamentous Growth Program by Relief of Transcriptional Repression: A Genome-wide Analysis.* Mol Biol Cell, 2005. **16**(6): p. 2903-12.

125. Wightman, R., et al., *In Candida albicans, the Nim1 kinases Gin4 and Hsl1 negatively regulate pseudohypha formation and Gin4 also controls septin organization.* J Cell Biol, 2004. **164**(4): p. 581-91.

126. Dalle, F., et al., *Cellular interactions of Candida albicans with human oral epithelial cells and enterocytes.* Cell Microbiol, 2010. **12**(2): p. 248-71.

127. Scherwitz, C., *Ultrastructure of Human Cutaneous Candidosis.* J Investig Dermatol, 1982. **78**(3): p. 200-205.

128. Lorenz, M.C., J.A. Bender, and G.R. Fink, *Transcriptional response of Candida albicans upon internalization by macrophages.* Eukaryot Cell, 2004. **3**(5): p. 1076-87.

129. Gow, N.A.R., A.J.P. Brown, and F.C. Odds, *Fungal morphogenesis and host invasion.* Current Opinion in Microbiology, 2002. **5**(4): p. 366-371.

130. Ernst, J.F., *Transcription factors in Candida albicans – environmental control of morphogenesis.* Microbiology, 2000. **146**(8): p. 1763-1774.

131. Feng, Q., et al., *Ras Signaling Is Required for Serum-Induced Hyphal Differentiation in Candida albicans.* Journal of Bacteriology, 1999. **181**(20): p. 6339-6346.

132. Kadosh, D. and A.D. Johnson, *Rfg1, a Protein Related to the Saccharomyces cerevisiae Hypoxic Regulator Rox1, Controls Filamentous Growth and Virulence in Candida albicans.* Molecular and Cellular Biology, 2001. **21**(7): p. 2496-2505.

133. Lo, H.J., et al., *Nonfilamentous C. albicans mutants are avirulent.* Cell, 1997. **90**(5): p. 939-49.

134. Stoldt, V.R., et al., *Efg1p, an essential regulator of morphogenesis of the human pathogen Candida albicans, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi.* The EMBO Journal, 1997. **16**(8): p. 1982-1991.

135. Braun, B.R. and A.D. Johnson, *TUP1, CPH1 and EFG1 make independent contributions to filamentation in candida albicans.* Genetics, 2000. **155**(1): p. 57-67.

136. Zou, H., et al., *Candida albicans Cyr1, Cap1 and G-actin form a sensor/effector apparatus for activating cAMP synthesis in hyphal growth.* Mol Microbiol, 2010. **75**(3): p. 579-91.

137. Hornby, J.M., et al., *Quorum sensing in the dimorphic fungus Candida albicans is mediated by farnesol.* Appl Environ Microbiol, 2001. **67**(7): p. 2982-92.

138. Marakalala, M.J., et al., *Differential adaptation of Candida albicans in vivo modulates immune recognition by dectin-1.* PLoS Pathog, 2013. **9**(4): p. e1003315.

139. Brown, G.D., et al., *Hidden killers: human fungal infections.* Sci Transl Med, 2012. **4**(165): p. 165rv13.

140. Chandra, J., et al., *Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug resistance.* J Bacteriol, 2001. **183**(18): p. 5385-94.

141. Naglik, J.R., S.J. Challacombe, and B. Hube, *Candida albicans Secreted Aspartyl Proteinases in Virulence and Pathogenesis.*

Microbiology and Molecular Biology Reviews, 2003. **67**(3): p. 400-428.

142. Ibrahim, A.S., et al., *Evidence implicating phospholipase as a virulence factor of Candida albicans.* Infect Immun, 1995. **63**(5): p. 1993-8.
143. Lilic, D., *New perspectives on the immunology of chronic mucocutaneous candidiasis.* Curr Opin Infect Dis, 2002. **15**(2): p. 143-7.
144. Hernandez-Santos, N. and S.L. Gaffen, *Th17 cells in immunity to Candida albicans.* Cell Host Microbe, 2012. **11**(5): p. 425-35.
145. Morales, D.K. and D.A. Hogan, *Candida albicans interactions with bacteria in the context of human health and disease.* PLoS Pathog, 2010. **6**(4): p. e1000886.
146. Peters, B.M., et al., *Polymicrobial Interactions: Impact on Pathogenesis and Human Disease.* Clinical Microbiology Reviews, 2012. **25**(1): p. 193-213.
147. Levison, M.E. and P.G. Pitsakis, *Susceptibility to Experimental Candida albicans Urinary Tract Infection in the Rat.* The Journal of Infectious Diseases, 1987. **155**(5): p. 841-846.
148. Bamford, C.V., et al., *Streptococcus gordonii modulates Candida albicans biofilm formation through intergeneric communication.* Infect Immun, 2009. **77**(9): p. 3696-704.
149. Klotz, S.A., et al., *Candida albicans Als proteins mediate aggregation with bacteria and yeasts.* Med Mycol, 2007. **45**(4): p. 363-70.
150. O'Sullivan, J.M., H.F. Jenkinson, and R.D. Cannon, *Adhesion of Candida albicans to oral streptococci is promoted by selective adsorption of salivary proteins to the streptococcal cell surface.* Microbiology, 2000. **146 (Pt 1)**: p. 41-8.
151. Purschke, F.G., et al., *Flexible Survival Strategies of Pseudomonas aeruginosa in Biofilms Result in Increased Fitness Compared with Candida albicans.* Molecular & Cellular Proteomics : MCP, 2012. **11**(12): p. 1652-1669.
152. Pinto, M., M. Morange, and O. Bensaude, *Denaturation of proteins during heat shock. In vivo recovery of solubility and activity of reporter enzymes.* Journal of Biological Chemistry, 1991. **266**(21): p. 13941-6.
153. Casadevall, A. and F.C. Fang, *Reproducible Science.* Infection and Immunity, 2010. **78**(12): p. 4972-4975.
154. Stover, C.K., et al., *Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen.* Nature, 2000. **406**(6799): p. 959-64.

155. Ben Abdeljelil, J., et al., *Molecular typing of Candida albicans isolates from patients and health care workers in a neonatal intensive care unit.* Journal of Applied Microbiology, 2011. **111**(5): p. 1235-1249.

156. Hua, X., et al., *Morphogenic and genetic differences between Candida albicans strains are associated with keratomycosis virulence.* Molecular Vision, 2009. **15**: p. 1476-1484.

157. Fleming, A., *On the Antibacterial Action of Cultures of a Penicillium, with Special Reference to their Use in the Isolation of B. influenzæ.* British journal of experimental pathology, 1929. **10**(3): p. 226-236.

158. Hogan, D.A., A. Vik, and R. Kolter, *A Pseudomonas aeruginosa quorum-sensing molecule influences Candida albicans morphology.* Mol Microbiol, 2004. **54**(5): p. 1212-23.

159. Kerr, J.R., et al., *Pseudomonas aeruginosa pyocyanin and 1-hydroxyphenazine inhibit fungal growth.* J Clin Pathol, 1999. **52**(5): p. 385-7.

160. Brand, A., et al., *Cell wall glycans and soluble factors determine the interactions between the hyphae of Candida albicans and Pseudomonas aeruginosa.* Fems Microbiology Letters, 2008. **287**(1): p. 48-55.

161. Blier, A.-S., et al., *C-type natriuretic peptide modulates quorum sensing molecule and toxin production in Pseudomonas aeruginosa.* Microbiology, 2011. **157**(Pt 7): p. 1929-1944.

162. Melstrom, K.A., et al., *Cytotoxicity of Pseudomonas Secreted Exotoxins Requires OxyR Expression.* The Journal of surgical research, 2007. **143**(1): p. 50-57.

163. Yang, J., et al., *Bacterial Secretant from Pseudomonas aeruginosa Dampens Inflammasome Activation in a Quorum Sensing-Dependent Manner.* Frontiers in Immunology, 2017. **8**: p. 333.

164. Klockgether, J., et al., *Genome Diversity of Pseudomonas aeruginosa PAO1 Laboratory Strains.* Journal of Bacteriology, 2010. **192**(4): p. 1113-1121.

165. Durand, E., et al., *The Assembly Mode of the Pseudopilus: A HALLMARK TO DISTINGUISH A NOVEL SECRETION SYSTEM SUBTYPE.* Journal of Biological Chemistry, 2011. **286**(27): p. 24407-24416.

166. Douzi, B., A. Filloux, and R. Voulhoux, *On the path to uncover the bacterial type II secretion system.* Philosophical Transactions of the Royal Society B: Biological Sciences, 2012. **367**(1592): p. 1059-1072.

167. Green, E.R. and J. Mecsas, *Bacterial Secretion Systems – An overview.* Microbiology spectrum, 2016. **4**(1): p. 10.1128/microbiolspec.VMBF-0012-2015.

168. Sandkvist, M., *Type II Secretion and Pathogenesis.* Infection and Immunity, 2001. **69**(6): p. 3523-3535.

169. Viarre, V., et al., *HxcQ liposecretin is self-piloted to the outer membrane by its N-terminal lipid anchor.* J Biol Chem, 2009. **284**(49): p. 33815-23.

170. Nunn, D.N. and S. Lory, *Product of the Pseudomonas aeruginosa gene pilD is a prepilin leader peptidase.* Proceedings of the National Academy of Sciences, 1991. **88**(8): p. 3281-3285.

171. DeShazer, D., et al., *Molecular characterization of genetic loci required for secretion of exoproducts in Burkholderia pseudomallei.* J Bacteriol, 1999. **181**(15): p. 4661-4.

172. Llamas, M.A., et al., *A Novel extracytoplasmic function (ECF) sigma factor regulates virulence in Pseudomonas aeruginosa.* PLoS Pathog, 2009. **5**(9): p. e1000572.

173. Bomberger, J.M., et al., *Long-Distance Delivery of Bacterial Virulence Factors by Pseudomonas aeruginosa Outer Membrane Vesicles.* PLoS Pathogens, 2009. **5**(4): p. e1000382.

174. Engel, J. and Y. Eran, *Subversion of Mucosal Barrier Polarity by Pseudomonas Aeruginosa.* Frontiers in Microbiology, 2011. **2**(114).

175. Chekabab, S.M., J. Harel, and C.M. Dozois, *Interplay between genetic regulation of phosphate homeostasis and bacterial virulence.* Virulence, 2014. **5**(8): p. 786-793.

176. Mulcahy, H., L. Charron-Mazenod, and S. Lewenza, *Pseudomonas aeruginosa produces an extracellular deoxyribonuclease that is required for utilization of DNA as a nutrient source.* Environmental Microbiology, 2010. **12**(6): p. 1621-1629.

177. Buchanan, J.T., et al., *DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps.* Curr Biol, 2006. **16**(4): p. 396-400.

178. Bazire, A., et al., *Osmotic stress and phosphate limitation alter production of cell-to-cell signal molecules and rhamnolipid biosurfactant by Pseudomonas aeruginosa.* FEMS Microbiol Lett, 2005. **253**(1): p. 125-31.

179. Ramalho, R., et al., *Modified Pseudomonas agar: new differential medium for the detection/enumeration of*

Pseudomonas aeruginosa in mineral water. Journal of Microbiological Methods, 2002. **49**(1): p. 69-74.

180. Ikeh, M.A., et al., *Pho4 mediates phosphate acquisition in Candida albicans and is vital for stress resistance and metal homeostasis.* Mol Biol Cell, 2016.
181. Kuchma, S.L., et al., *BifA, a cyclic-Di-GMP phosphodiesterase, inversely regulates biofilm formation and swarming motility by Pseudomonas aeruginosa PA14.* J Bacteriol, 2007. **189**(22): p. 8165-78.
182. Holloway, B.W., V. Krishnapillai, and A.F. Morgan, *Chromosomal genetics of Pseudomonas.* Microbiological Reviews, 1979. **43**(1): p. 73-102.
183. Hickman, J.W., D.F. Tifrea, and C.S. Harwood, *A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels.* Proc Natl Acad Sci U S A, 2005. **102**(40): p. 14422-7.
184. Grant, S.G., et al., *Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants.* Proc Natl Acad Sci U S A, 1990. **87**(12): p. 4645-9.
185. de Lorenzo, V., et al., *Early and late responses of TOL promoters to pathway inducers: identification of postexponential promoters in Pseudomonas putida with lacZ-tet bicistronic reporters.* J Bacteriol, 1993. **175**(21): p. 6902-7.
186. Schnider-Keel, U., et al., *The sigma factor AlgU (AlgT) controls exopolysaccharide production and tolerance towards desiccation and osmotic stress in the biocontrol agent Pseudomonas fluorescens CHA0.* Appl Environ Microbiol, 2001. **67**(12): p. 5683-93.
187. Sambrook, J.M., Tom & Fritsch, E. F & Cold Spring Harbor Laboratory, *Molecular cloning : a laboratory manual* 1987 (2nd ed).
188. Essar, D., et al., *Identification and characterization of genes for a second anthranilate synthase in Pseudomonas aeruginosa: interchangeability of the two anthranilate synthases and evolutionary implications.* Journal of bacteriology, 1990. **172**(2): p. 884-900.
189. O'Toole, G.A., *Microtiter Dish Biofilm Formation Assay.* Journal of Visualized Experiments : JoVE, 2011(47): p. 2437.
190. Palmer, K.L., L.M. Aye, and M. Whiteley, *Nutritional Cues Control Pseudomonas aeruginosa Multicellular Behavior in Cystic Fibrosis Sputum.* Journal of Bacteriology, 2007. **189**(22): p. 8079-8087.

191. Singh, S., et al., *Granulocyte-Macrophage Colony Stimulatory Factor Enhances the Pro-Inflammatory Response of Interferon-γ-Treated Macrophages to Pseudomonas aeruginosa Infection.* PLOS ONE, 2015. **10**(2): p. e0117447.

192. Huang, C.-T., et al., *Spatial Patterns of Alkaline Phosphatase Expression within Bacterial Colonies and Biofilms in Response to Phosphate Starvation.* Applied and Environmental Microbiology, 1998. **64**(4): p. 1526-1531.

193. Deligianni, E., et al., *Pseudomonas aeruginosa Cystic Fibrosis isolates of similar RAPD genotype exhibit diversity in biofilm forming ability in vitro.* BMC Microbiology, 2010. **10**(1): p. 1-13.

194. Wolfgang, M.C., et al., *Pseudomonas aeruginosa regulates flagellin expression as part of a global response to airway fluid from cystic fibrosis patients.* Proc Natl Acad Sci U S A, 2004. **101**(17): p. 6664-8.

195. Colvin, K.M., et al., *The Pel and Psl polysaccharides provide Pseudomonas aeruginosa structural redundancy within the biofilm matrix.* Environmental microbiology, 2012. **14**(8): p. 10.1111/j.1462-2920.2011.02657.x.

196. Das, T., et al., *Pyocyanin Facilitates Extracellular DNA Binding to <italic>Pseudomonas aeruginosa</italic> Influencing Cell Surface Properties and Aggregation.* PLoS ONE, 2013. **8**(3): p. e58299.

197. Hassett, D.J., et al., *Response of Pseudomonas aeruginosa to pyocyanin: mechanisms of resistance, antioxidant defenses, and demonstration of a manganese-cofactored superoxide dismutase.* Infection and Immunity, 1992. **60**(2): p. 328-336.

198. Lamarche, M.G., et al., *The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis.* FEMS Microbiology Reviews, 2008. **32**(3): p. 461-473.

199. Bhatti, A.R., I.W. DeVoe, and J.M. Ingram, *Cell division in Pseudomonas aeruginosa: participation of alkaline phosphatase.* J Bacteriol, 1976. **126**(1): p. 400-9.

200. Zaborin, A., et al., *<italic>Pseudomonas aeruginosa</italic> Overrides the Virulence Inducing Effect of Opioids When It Senses an Abundance of Phosphate.* PLoS ONE, 2012. **7**(4): p. e34883.

201. Romano, S., et al., *Phosphate Limitation Induces Drastic Physiological Changes, Virulence-Related Gene Expression, and Secondary Metabolite Production in Pseudovibrio sp. Strain FO-BEG1.* Applied and Environmental Microbiology, 2015. **81**(10): p. 3518-3528.

202. Long, J., et al., *Depletion of Intestinal Phosphate following Surgical Injury Activates the Virulence of P. aeruginosa causing Lethal Gut-Derived Sepsis.* Surgery, 2008. **144**(2): p. 189-197.

203. Zaborina, O., et al., *Structure–Function Aspects of PstS in Multi-Drug–Resistant Pseudomonas aeruginosa.* PLOS Pathogens, 2008. **4**(2): p. e43.

204. Rackley, C.R. and B.R. Stripp, *Building and maintaining the epithelium of the lung.* The Journal of Clinical Investigation, 2012. **122**(8): p. 2724-2730.

205. Ichikawa, J.K., et al., *Interaction of Pseudomonas aeruginosa with epithelial cells: Identification of differentially regulated genes by expression microarray analysis of human cDNAs.* Proceedings of the National Academy of Sciences, 2000. **97**(17): p. 9659-9664.

206. Tam, A., et al., *The airway epithelium: more than just a structural barrier.* Therapeutic Advances in Respiratory Disease, 2011. **5**(4): p. 255-273.

207. Cigana, C., et al., *Dampening Host Sensing and Avoiding Recognition in Pseudomonas aeruginosa Pneumonia.* Journal of Biomedicine and Biotechnology, 2011. **2011**: p. 10.

208. Parker, D. and A. Prince, *Innate immunity in the respiratory epithelium.* Am J Respir Cell Mol Biol, 2011. **45**(2): p. 189-201.

209. Chugani, S. and E.P. Greenberg, *The Influence of Human Respiratory Epithelia on Pseudomonas aeruginosa Gene Expression.* Microbial pathogenesis, 2007. **42**(1): p. 29-35.

210. Fogh, J., J.M. Fogh, and T. Orfeo, *One Hundred and Twenty-Seven Cultured Human Tumor Cell Lines Producing Tumors in Nude Mice23.* JNCI: Journal of the National Cancer Institute, 1977. **59**(1): p. 221-226.

211. Marshall, L.J., et al., *IL-8 Released Constitutively by Primary Bronchial Epithelial Cells in Culture Forms an Inactive Complex with Secretory Component.* The Journal of Immunology, 2001. **167**(5): p. 2816-2823.

212. Ballinger, M.N., et al., *Role of granulocyte macrophage colony-stimulating factor during gram-negative lung infection with Pseudomonas aeruginosa.* Am J Respir Cell Mol Biol, 2006. **34**(6): p. 766-74.

213. Kube, D., et al., *Proinflammatory cytokine responses to P. aeruginosa infection in human airway epithelial cell lines.* Am J Physiol Lung Cell Mol Physiol, 2001. **280**(3): p. L493-502.

214. Chan, F.K.-M., K. Moriwaki, and M.J. De Rosa, *Detection of Necrosis by Release of Lactate Dehydrogenase (LDH) Activity.* Methods in molecular biology (Clifton, N.J.), 2013. **979**: p. 65-70.

215. Parker, D. and A. Prince, *Epithelial Uptake of Flagella Initiates Proinflammatory Signaling.* PLOS ONE, 2013. **8**(3): p. e59932.

216. Azghani, A.O., *Pseudomonas aeruginosa and epithelial permeability: role of virulence factors elastase and exotoxin A.* Am J Respir Cell Mol Biol, 1996. **15**(1): p. 132-40.

217. Frisk, A., et al., *Transcriptome Analysis of Pseudomonas aeruginosa after Interaction with Human Airway Epithelial Cells.* Infection and Immunity, 2004. **72**(9): p. 5433-5438.

218. Jyot, J., et al., *Type II Secretion System of Pseudomonas aeruginosa: In Vivo Evidence of a Significant Role in Death Due to Lung Infection.* The Journal of Infectious Diseases, 2011. **203**(10): p. 1369-1377.

219. Ball, G., et al., *Type II-dependent secretion of a Pseudomonas aeruginosa DING protein.* Research in Microbiology, 2012. **163**(6): p. 457-469.

220. Wolfgang, M.C., et al., *Conservation of genome content and virulence determinants among clinical and environmental isolates of Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8484-9.

221. Winsor, G.L., et al., *Pseudomonas Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes.* Nucleic Acids Research, 2011. **39**(Database issue): p. D596-D600.

222. Cohen-Cymberknoh, M., et al., *Airway inflammation in cystic fibrosis: molecular mechanisms and clinical implications.* Thorax, 2013. **68**(12): p. 1157-62.

223. Duffield, J.S., et al., *Host responses in tissue repair and fibrosis.* Annu Rev Pathol, 2013. **8**: p. 241-76.

224. Hams, E., et al., *IL-25 and type 2 innate lymphoid cells induce pulmonary fibrosis.* Proc Natl Acad Sci U S A, 2014. **111**(1): p. 367-72.

225. Bedard, M., et al., *Release of interleukin-8, interleukin-6, and colony-stimulating factors by upper airway epithelial cells: implications for cystic fibrosis.* Am J Respir Cell Mol Biol, 1993. **9**(4): p. 455-62.

226. Cole, N., et al., *TNF-α production in the cornea in response to Pseudomonas aeruginosa challenge.* Immunology & Cell Biology, 1999. **77**(2): p. 164-166.

227. Ye, D., I. Ma, and T.Y. Ma, *Molecular mechanism of tumor necrosis factor-α modulation of intestinal epithelial tight junction barrier.* American Journal of Physiology-Gastrointestinal and Liver Physiology, 2006. **290**(3): p. G496-G504.

228. Lee, J.-H., et al., *Modulation of Bacterial Growth by Tumor Necrosis Factor-α In Vitro and In Vivo.* American Journal of Respiratory and Critical Care Medicine, 2003. **168**(12): p. 1462-1470.

229. Martin, L.D., et al., *Airway epithelium as an effector of inflammation: molecular regulation of secondary mediators.* The European respiratory journal, 1997. **10**(9): p. 2139-2146.

230. Broug-Holub, E., et al., *Alveolar macrophages are required for protective pulmonary defenses in murine Klebsiella pneumonia: elimination of alveolar macrophages increases neutrophil recruitment but decreases bacterial clearance and survival.* Infection and Immunity, 1997. **65**(4): p. 1139-1146.

231. and, B.C.T. and J.A. Whitsett, *GM-CSF Regulates Pulmonary Surfactant Homeostasis and Alveolar Macrophage-Mediated Innate Host Defense.* Annual Review of Physiology, 2002. **64**(1): p. 775-802.

232. Ramirez-Carrozzi, V., et al., *IL-17C regulates the innate immune function of epithelial cells in an autocrine manner.* Nature Immunology, 2011. **12**: p. 1159.

233. Gregory, A.D., et al., *Regulation of systemic and local neutrophil responses by G-CSF during pulmonary Pseudomonas aeruginosa infection.* Blood, 2007. **109**(8): p. 3235-43.

234. Raoust, E., et al., *Pseudomonas aeruginosa LPS or Flagellin Are Sufficient to Activate TLR-Dependent Signaling in Murine Alveolar Macrophages and Airway Epithelial Cells.* PLOS ONE, 2009. **4**(10): p. e7259.

235. Cole, N., et al., *Pseudomonas aeruginosa keratitis in IL-6-deficient mice.* Int Arch Allergy Immunol, 2003. **130**(2): p. 165-72.

236. Pappu, R., S. Rutz, and W. Ouyang, *Regulation of epithelial immunity by IL-17 family cytokines.* Trends in Immunology, 2012. **33**(7): p. 343-349.

237. Iwakura, Y., et al., *Functional Specialization of Interleukin-17 Family Members.* Immunity, 2011. **34**(2): p. 149-162.

238. Zheng, Y., et al., *Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens.* Nature Medicine, 2008. **14**: p. 282.

239. Chmiel, J.F., M. Berger, and M.W. Konstan, *The role of inflammation in the pathophysiology of CF lung disease.* Clin Rev Allergy Immunol, 2002. **23**(1): p. 5-27.

240. Shanks, K.K., et al., *Interleukin-8 Production by Human Airway Epithelial Cells in Response to Pseudomonas aeruginosa Clinical Isolates Expressing Type a or Type b Flagellins.* Clinical and Vaccine Immunology, 2010. **17**(8): p. 1196-1202.

241. Cohen, T.S. and A. Prince, *Cystic fibrosis: a mucosal immunodeficiency syndrome.* Nature medicine, 2012. **18**(4): p. 509-519.

242. Hidaka, H., et al., *Constitutive IL-8 expression in cancer cells is associated with mutation of p53.* J Exp Clin Cancer Res, 2005. **24**(1): p. 127-33.

243. Coggan, K.A. and M.C. Wolfgang, *Global regulatory pathways and cross-talk control pseudomonas aeruginosa environmental lifestyle and virulence phenotype.* Curr Issues Mol Biol, 2012. **14**(2): p. 47-70.

244. Williams, P. and M. Cámara, *Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules.* Current Opinion in Microbiology, 2009. **12**(2): p. 182-191.

245. Short, F.L., S.L. Murdoch, and R.P. Ryan, *Polybacterial human disease: the ills of social networking.* Trends in Microbiology, 2014. **22**(9): p. 508-516.

246. Brogden, K.A., J.M. Guthmiller, and C.E. Taylor, *Human polymicrobial infections.* The Lancet, 2005. **365**(9455): p. 253-255.

247. Tuft, S., *Polymicrobial infection and the eye.* The British Journal of Ophthalmology, 2006. **90**(3): p. 257-258.

248. He, X.-s. and W.-y. Shi, *Oral Microbiology: Past, Present and Future.* International Journal of Oral Science, 2009. **1**(2): p. 47-58.

249. Bogaert, D., et al., *Colonisation by Streptococcus pneumoniae and Staphylococcus aureus in healthy children.* The Lancet, 2004. **363**(9424): p. 1871-1872.

250. Regev-Yochay, G., et al., *Association between carriage of Streptococcus pneumoniae and Staphylococcus aureus in Children.* Jama, 2004. **292**(6): p. 716-20.

251. Lawn, S.D., *AIDS in Africa: the impact of coinfections on the pathogenesis of HIV-1 infection.* J Infect, 2004. **48**(1): p. 1-12.

252. Almand, E.A., M.D. Moore, and L.-A. Jaykus, *Virus-Bacteria Interactions: An Emerging Topic in Human Infection.* Viruses, 2017. **9**(3): p. 58.

253. Slifka, M.K., et al., *Measles virus infection results in suppression of both innate and adaptive immune responses to secondary bacterial infection.* J Clin Invest, 2003. **111**(6): p. 805-10.

254. Kean, R., et al., *Candida albicans Mycofilms Support Staphylococcus aureus Colonization and Enhances Miconazole Resistance in Dual-Species Interactions.* Front Microbiol, 2017. **8**: p. 258.

255. Terada, L.S., et al., *Pseudomonas aeruginosa Hemolytic Phospholipase C Suppresses Neutrophil Respiratory Burst Activity.* Infection and Immunity, 1999. **67**(5): p. 2371-2376.

256. Lamarche, M.G., et al., *The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis.* FEMS Microbiol Rev, 2008. **32**(3): p. 461-73.

257. Rashid, M.H., et al., *Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of Pseudomonas aeruginosa.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(17): p. 9636-9641.

258. Wei, X., et al., *Global Control of GacA in Secondary Metabolism, Primary Metabolism, Secretion Systems, and Motility in the Rhizobacterium Pseudomonas aeruginosa M18.* Journal of Bacteriology, 2013. **195**(15): p. 3387-3400.

259. Marden, J.N., et al., *An unusual CsrA family member operates in series with RsmA to amplify posttranscriptional responses in Pseudomonas aeruginosa.* Proc Natl Acad Sci U S A, 2013. **110**(37): p. 15055-60.

260. Chua, S.L., et al., *Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles.* Nature Communications, 2014. **5**: p. 4462.

261. Kaleta, C., et al., *Metabolic costs of amino acid and protein production in Escherichia coli.* Biotechnology Journal, 2013. **8**(9): p. 1105-1114.

262. Kafri, M., et al., *The Cost of Protein Production.* Cell Rep, 2016. **14**(1): p. 22-31.

263. Pfeifer, P., et al., *IL-17C is a mediator of respiratory epithelial innate immune response.* Am J Respir Cell Mol Biol, 2013. **48**(4): p. 415-21.

264. Khan, T.Z., et al., *Early pulmonary inflammation in infants with cystic fibrosis.* American Journal of Respiratory and Critical Care Medicine, 1995. **151**(4): p. 1075-1082.

265. Muhlebach, M.S. and T.L. Noah, *Endotoxin Activity and Inflammatory Markers in the Airways of Young Patients with Cystic Fibrosis.* American Journal of Respiratory and Critical Care Medicine, 2002. **165**(7): p. 911-915.

266. Krick, S., et al., *Klotho Inhibits Interleukin-8 Secretion from Cystic Fibrosis Airway Epithelia.* Scientific Reports, 2017. **7**(1): p. 14388.

267. Zhao, Y., et al., *Klotho Depletion Contributes to Increased Inflammation in Kidney of the Mouse Model of Diabetes via RelA (Serine) 536 Phosphorylation.* Diabetes, 2011. **60**(7): p. 1907-1916.

268. Skov, L., et al., *IL-8 as Antibody Therapeutic Target in Inflammatory Diseases: Reduction of Clinical Activity in Palmoplantar Pustulosis.* The Journal of Immunology, 2008. **181**(1): p. 669-679.

269. Huang, S., et al., *Fully Humanized Neutralizing Antibodies to Interleukin-8 (ABX-IL8) Inhibit Angiogenesis, Tumor Growth, and Metastasis of Human Melanoma.* The American Journal of Pathology, 2002. **161**(1): p. 125-134.