The role of the Pf4 filamentous prophage in mediating Pseudomonas aeruginosa virulence and stress tolerance of biofilms.

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The role of the Pf4 filamentous prophage in mediating *Pseudomonas aeruginosa* virulence and stress tolerance of biofilms

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Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that can cause acute infections such as keratitis, burn wound infections and ventilator associated pneumonia. *P. aeruginosa* is also the main cause of chronic infections in cystic fibrosis (CF) patients, and presents symptoms such as acute exacerbations and loss of respiratory function due to damage of lung tissue and ultimately, death.

In environments such as the CF lung, *P. aeruginosa* is known to form biofilms in order to colonise, infect and persist in its host. *P. aeruginosa* biofilms are thought to be important in protecting bacteria from various environmental stresses such as protozoan grazing, surfactant stress, antibiotic stress, toxins, and the host immune response. During the cell death and dispersal phase, a bacteriophage known as Pf4 has been isolated from *P. aeruginosa* biofilms. Pf4 is a filamentous prophage that contributes to the stability of the developing biofilm and virulence of *P. aeruginosa*, but it is not clear how this occurs. The main aim of this study was to determine the role of the Pf4 phage in *P. aeruginosa* biofilm stability and virulence. This included further investigating the effect of Pf4 on the stability of *P. aeruginosa* biofilms under stress, as well as studying how the Pf4 phage influences selected *P. aeruginosa* virulence phenotypes.

Co-incubation experiments showed that the wild-type *P. aeruginosa* outcompeted the Pf4 mutant deletion strain, further supporting the hypothesis that the Pf4 phage is selected for and advantageous for *P. aeruginosa*. Experiments assessing virulence phenotypes showed that the Pf4 phage influences the invasiveness and cytotoxicity of *P. aeruginosa* in varying ways depending on the mammalian cell type, but does not contribute to the virulence of *P. aeruginosa* towards *C. elegans*. The presence of the Pf4 phage also negatively regulates the production of the pyoverdine siderophore, as determined through analysis of planktonic cultures, and determining gene expression in planktonic and late biofilm cultures. In understanding the effect of the Pf4 phage on *P. aeruginosa* biofilm stability under stress, it was observed that the phage increased resistance of *P. aeruginosa* biofilms to ciprofloxacin (fluoroquinolone) and paraquat (superoxide) stress.

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Abstract

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that can cause acute infections such as keratitis, burn wound infections and ventilator associated pneumonia. *P. aeruginosa* is also the main cause of chronic infections in cystic fibrosis (CF) patients, and presents symptoms such as acute exacerbations and loss of respiratory function due to damage of lung tissue and ultimately, death.

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Abbreviations

AHL	*N*-acylhomoserine lactone
bp	base pair
CO_2	carbon dioxide
CT	cholera toxin
COPD	chronic obstructive pulmonary disease
CFU	colony forming units
cDNA	complementary deoxyribonucleic acid
cAMP	cyclic adenosine monophosphate
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
d	day
°C	degrees Celsius
DMEM	Dulbecco's Modified Eagle's Medium
DNA	2'deoxyribonucleic acid
eDNA	extracellular DNA
EPS	extracellular polymeric substance
FBS	fetal bovine serum
g	g-force
gDNA	genomic deoxyribonucleic acid
Gm	gentamycin
g/ L	grams per litre
GAP	GTPase activating protein

h	hours
H_2O_2	hydrogen peroxide
($^{\bullet}OH$)	hydroxyl radicals
(OH^-)	hydroxyl anions
IL-8	interleukin-8
Kbp	kilobase pair
kDa	kilodalton
kHz	kilohertz
kV	kilovolts
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LB	lysogeny broth
Mbp	Megabase pair
mRNA	messenger RNA
µg	microgram
µL	microlitre
µm	micrometre
mF	millifarad
mg	milligram
mL	millilitre
mM	millimolar
MBC	minimum bactericidal concentration
MEM	Minimal Essential Medium

MIC	minimal inhibitory concentration
min	minutes
MW	molecular weight
ng	nanogram
nm	nanometre
NADPH	nicotinamide adenine dinucleotide phosphate
Ω	Ohms
ORF	open reading frame
OD	optical density
PYG	peptone yeast extract glucose broth
ΔPf4	Pf4 deletion mutant
PBS	phosphate buffered saline
pmol	picomole
PFU	plaque forming units
PCR	polymerase chain reaction
PMN	polymorphonuclear leukocyte
qPCR	quantitative reverse-transcriptase polymerase chain reaction
QS	quorum sensing
ROS	reactive oxygen species
RT	reverse transcriptase
RNA	ribonucleic acid
RPM	revolutions per minute

sec	second
SCV	small colony variant
SDS	Sodium dodecyl-sulphate
SD	standard deviation
SEM	standard error of the mean
SOD	superoxide dismutase
(O_2^-)	superoxide radical
TA system	toxin-antitoxin system
T3SS	Type three secretion system
UV	ultraviolet
VAP	ventilator-associated pneumonia
VPI	Vibrio Pathogenicity Island
w / v	weight / volume
WT	wild-type
Zot	Zonula occludens toxin

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1 Introduction and Literature Review

1.1 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative, rod-shaped bacterium with a 6 Mbp genome that contains approximately 5,770 open reading frames (ORFs) (1). It is thought that this large genetic repertoire with an extensive metabolic capacity contributes to the ecological versatility of *P. aeruginosa* (1). *P. aeruginosa* is a ubiquitous organism that exists in a variety of environments including aquatic and soil habitats, and can also cause infections in clinical settings (2). *P. aeruginosa* is an opportunistic pathogen and a primary pathogen associated with burn wounds and indwelling biomedical devices (e.g. ventilators). It is also a primary pathogen in hosts with underlying genetic defects that compromise part of the immune system, such as cystic fibrosis (CF), where the mucociliary escalator is impaired due to the thickened mucus. The versatility of *P. aeruginosa* is also apparent during the infection of a host, where it can colonise a broad spectrum of sites in the human body. In addition, *P. aeruginosa* is capable of multiple infections, which are typically categorised as being either acute or chronic.

1.2 *P. aeruginosa* acute infections

Acute infections caused by *P. aeruginosa* are a major issue in terms of healthcare costs, with ventilator-associated pneumonia (VAP) contributing to US$ 1.5 billion in healthcare costs, and septicaemia derived from catheter-associated urinary tract infections, increasing costs by approximately US$ 28,000 per patient (3). Acute infection involves a short and severe onset of symptoms resulting in clearance by the host or host death (4). *P. aeruginosa* acute infections include keratitis, burn wound infections, ventilator-associated pneumonia (VAP), otitis externa and septicaemia (5-8). The genome of *P. aeruginosa* encodes a broad range of virulence factors that can promote these acute infections. Among the best characterised virulence factors are secreted molecules such as redox-active phenazines (e.g. pyocyanin), secretion systems, pili, siderophores (e.g. pyoverdine) and enzymes (e.g. alkaline protease, LasB elastase) (reviewed in (9-12)). The expression of these virulence factors is tightly controlled by many different regulatory systems that coordinate the physiology of *P. aeruginosa* with the environmental

conditions (4). For example, the GacAS-RsmA/Z/Y network has been shown to play an important role in controlling acute virulence factors, such as expression of the type 3 secretion system and type IV pili (13-16).

1.2.1 Type 3 Secretion System (T3SS)

Secretion systems are important structures that facilitate the transport of proteins from the cytoplasmic matrix through both the plasma membrane and the cell wall (17). Bacteria encode many different secretion systems that are classified according to the apparatus used for translocation of the associated effector proteins (reviewed in (18)). One secretion system with particular importance for infections caused by *P. aeruginosa* is the type 3 secretion system (T3SS), which spans the bacterial envelope and penetrates a host cell membrane to allow the effector proteins to be directly injected in to the target cell (reviewed in (19)). The *P. aeruginosa* T3SS apparatus is encoded by thirty-six genes located across five operons (20). Three-quarters of *P. aeruginosa* clinical isolates express at least one T3SS effector protein *in vitro*, and loss of the T3SS apparatus reduces mortality in mouse models of acute pneumonia, keratitis and burn infections (21-26). Currently, there are four known T3SS effectors in *P. aeruginosa*: ExoU, ExoS, ExoT and ExoY (27-29). Through a sequence similarity search via RefSeq (NCBI), Burstein *et al.* found that of the many *P. aeruginosa* strains in the database, only *P. aeruginosa* PA14 contained homologues of *pemA* and *pemB* (28). ExoT and ExoY are found in most strains, whereas ExoS and ExoU are rarely found in the same strain (cited in (28)). The secretion of T3SS effectors can cause direct physical damage (e.g. mucosal barrier damage) or affect regulatory function (e.g. the inhibition of host immune response factors) (reviewed in (28)).

ExoU is a cytotoxin that possesses phospholipase A_2 activity and is involved in 90% of human infections by *P. aeruginosa* (27, 30). This T3SS effector is necessary for virulence in an acute pneumonia mouse model (27). ExoU exposure results in cleavage of membrane phospholipids, such as phosphatidylcholine and phosphatidylethanolamine (31, 32). This cleavage results in the production of lysophospholipid and free fatty acid, two factors involved in host signalling pathways and initiators of a potent host inflammatory response, which inevitably leads to host cell apoptosis (33). Furthermore, ExoU is also the first lipase discovered to be transported by the T3SS (32). Lipases are

enzymes that catalyse the hydrolysis of triglycerides. Previous studies have found that the activation or modification of unidentified eukaryotic factors are necessary for ExoU lipase activity (32).

The T3SS effector proteins ExoS and ExoT share 75% amino acid identity (34). They are each bifunctional proteins that have GTPase activating protein (GAP) and ADP ribosyl transferase (ADPRT) domains (28). The GAP domain is responsible for the inhibition of human cell to cell adhesion by disrupting the actin cytoskeleton, and also prevents bacterial internalisation by epithelial cells and macrophages (27, 35). Loss of GAP domain activity improves wound healing and promotes the early stages of cytokinesis (27, 36). The ADPRT activity of ExoS is cytotoxic towards mammalian cells by disrupting cellular tight junctions, and inhibiting host DNA synthesis, vesicular trafficking and endocytosis (37, 38). Expression of the ADPRT domain of ExoT leads to inhibition of focal adhesion formation which impedes cell migration, wound healing and bacterial uptake by host cells (39). When taken up by the cell, ExoT inhibits host cell division, and induces apoptosis (28).

Another T3SS effector protein, ExoY, has been shown to contribute to severe host lung damage during *P. aeruginosa* infection (40, 41). However, unlike ExoS, ExoT and ExoU, there is currently less known about the function of ExoY and its specific role in *P. aeruginosa* acute infection. It is known that once ExoY is delivered into the target cell by the *P. aeruginosa* T3SS, the effector protein is dispersed throughout the cytosolic compartment which leads to an 800-fold increase in cyclic adenosine monophosphate (cAMP) levels and consequently, induction of endothelial cell gap formation (42). From these studies, it was hypothesised that ExoY possessed adenylate cyclase activity (43). However, recent studies have shown that ExoY is in fact a nucleotidyl cyclase (44). Based on this functionality, ExoY produces high levels of the pyrimidine nucleotides, cyclic guanosine monophosphate (cGMP) and cyclic uridine monophosphate (cUMP) in epithelial cells (45). cGMP and cUMP are thought to be secondary messengers for the T3SS effector protein (44, 45).

Based on bioinformatics analysis, the genes, PA14_16720 (*pemA*) and PA14_44480 (*pemB*) have recently been proposed to be T3SS effectors in *P. aeruginosa* PA14 (29). However, it is currently unknown whether the *pemA* and *pemB* genes are located in any other *P. aeruginosa* strain, including PAO1. *pemA* is located between the *hemN* gene,

which is part of the heme pathway, and the *anr* gene, which encodes an anaerobic regulatory protein (29). The *pemB* gene has a binding site for the master transcriptional regulator of the T3SS, further suggesting its involvement in the *P. aeruginosa* PA14 T3SS response (29). While these two genes have been proposed to be part of the T3SS pathway, direct evidence showing that they are secreted by this system is yet to be reported. Neither deletion or overexpression of *pemA* and *pemB* resulted in significant differences in cytotoxicity towards *Saccharomyces cerevisiae* or HeLa (cervical cancer derived) cells (29). Therefore, this suggests that these two T3SS effectors do not contribute to *P. aeruginosa* PA14 cytotoxicity in specific human cells and yeast cells. However, whether *pemA* and *pemB* contribute to *P. aeruginosa* PA14 acute infection (and potentially *P. aeruginosa* acute infection, in general) in other ways is yet to be determined.

1.2.2 Type IV pili expression

Many proteinaceous adhesins are involved in acute infections of *P. aeruginosa*, including fimbriae, pili, and secreted proteins that are embedded in the outer membrane (46, 47). The type IV pili which have been well studied with regard to their role in acute infections, are typically composed of repeating subunits of the major fibrillar protein and may contain a variable cap of minor pilin proteins that determines antigenicity and ligand specificity (17). Pili are also involved in motility because they are retractile and can perform cycles of adhesion and retraction (reviewed in (48)). In *P. aeruginosa*, the major pilin subunit is encoded by *pilA*, and, including those encoding the nucleotide-binding proteins, *pilB* (responsible for pili extension, forming pores in the outer membrane from which the pilus is extruded), *pilT* (responsible for pili retraction and degradation) and *pilU* (enhances *pilT* activity and increases twitching motility) (reviewed in (49) and (50)). The role of type IV pili in *P. aeruginosa* acute virulence has been established in many studies. For example, *pilT* and *pilU* deficient *P. aeruginosa* PAK showed lower cytotoxicity or association with three different epithelial cell types than the wild-type *P. aeruginosa* PAK strain (46). Also, the *pilT* and *pilU* mutants colonised the liver less efficiently than the wild-type PAK in an acute pneumonia mouse model (46). It has been hypothesised that type IV pili are required for the bacterial – epithelial cell interaction, and for pili retraction to allow for full contact with the epithelial cell (46).

1.2.3 The role of proteases in *P. aeruginosa* acute infection

P. aeruginosa produces a number of proteases that are important during acute infection, including the LasA protease and the LasB elastase (51). LasA is a serine protease that assists LasB in degrading elastin by the cleavage of serine residues (11). LasB is a zinc metalloprotease that can effectively degrade several proteins, including elastin (11). Elastin is a protein found in connective tissue that helps the host tissue to return to its shape after being stretched and/or contracted (11). In the lung, elastin is responsible for keeping the lung tissue intact during lung expansion and contraction. LasB elastase is also known to degrade collagen and basement matrices, both of which are imperative for maintaining connective tissue integrity and providing a barrier against bacterial invasion (52). Therefore, LasA protease and LasB elastase can assist in dissemination of *P. aeruginosa* through the destruction of physical host barriers.

1.2.4 *P. aeruginosa* pyoverdine production

During acute infection, *P. aeruginosa* produces different types of pigments including the siderophore pyoverdine, and the phenazine pyocyanin (9, 53). Pyoverdine can chelate iron from the environment, and can also displace iron from transferrin and lactoferrin which are human glycoproteins that bind iron very tightly (54). The iron-bound form of pyoverdine, referred to as ferripyoverdine, is bound by high-affinity receptors including FpvA on the *P. aeruginosa* outer membrane and then transported into the cell (55). Once ferripyoverdine is transported to the periplasm, the iron is released and the pyoverdine is recycled to the environment via a tripartite PvdT-PvdR-OmpQ efflux pump (55).

Pyoverdine is an important factor in acute virulence due its ability to acquire iron. Acute infections must overcome iron limitation, where iron is sequestered by host proteins such as lactoferrin (56, 57). Pyoverdine has a high binding affinity which enables it scavenge iron from lactoferrin (54). In addition to iron transport, pyoverdine is necessary for regulating its own production and can regulate the synthesis of two other virulence factors, exotoxin A (ToxA) and the endoprotease PrpL (58). ToxA has been identified in 90% of clinical isolates and high ToxA levels have been associated with severe host symptoms in response to *P. aeruginosa* acute infection, such as skin necrosis, ocular damage and pulmonary infections (59-61). Furthermore, elevated levels of ToxA have been associated with more severe cases of septicaemia and otitis externa (8, 62). ToxA

appears to function by triggering the ribosylation of elongation factor-2 (EF2) and inhibiting mammalian protein synthesis (63).

The endoprotease, PrpL, is present in both clinical and environmental strains (64). It is an extracellular endoprotease that can cleave elastin, lactoferrin, casein, decorin and transferrin, and in doing so, can cause host damage (64). Cleavage of elastin by PrpL can affect tissue integrity and cause cell damage. Also, through the cleavage and degradation of decorin, a compound called dermatan sulphate is liberated, which binds to and inactivates the bactericidal activity of α-defensin, a microbicidal peptide derived from neutrophils (64, 65). Furthermore, PrpL is able to cleave and release iron from lactoferrin and transferrin, which can then be chelated by siderophores and used by the bacterium (54, 64).

1.2.5 *P. aeruginosa* pyocyanin production

Pyocyanin is a blue phenazine that has been associated with *P. aeruginosa* acute infection in the host (9). It is a redox-active molecule that can move across biological membranes, and it serves an important role in electron transport for *P. aeruginosa* (66). Pyocyanin is regulated by quorum sensing (QS); when *P. aeruginosa* cell densities are high, the QS transcriptional activators LasR and RhlR bind to N-(3-oxododecanoyl)-L-homoserine lactone and N-butyryl-1-homoserine lactone, respectively, to initiate pyocyanin production (67).

Pyocyanin has been shown to cause highly detrimental effects to host cells including the inhibition of cell respiration, ciliary function and interruption of calcium homeostasis (68, 69). Furthermore, pyocyanin has been shown to contribute to the virulence of *P. aeruginosa* in various studies (reviewed in (9)). One such study involved the examination of an acute pneumonia mouse model inflammatory response when PA14 wild-type and pyocyanin deletion mutant strains were present (70). It was noted that when the wild-type was present, neutrophil numbers decreased in response to infection after 18 h, whereas neutrophil numbers continued to increase in the presence of the pyocyanin deletion mutant for up to 48 h (70). In addition to this, the presence of pyocyanin resulted in significantly less clearance of *P. aeruginosa* from the lung, with the pyocyanin deletion mutant being cleared approximately 3-fold more than the wild-type after 72 h (70).

Pyocyanin has been linked to increased neutrophil apoptosis in the host via the activation of acid sphingomyelinase (Asm) (71). It was observed that when *P. aeruginosa* ATCC 27853 strains were inoculated in mice lacking Asm production, pyocyanin-mediated neutrophil death was inhibited, whereas neutrophils in the presence of wild-type *P. aeruginosa* ATCC 27853 strains experienced apoptosis (72). Pyocyanin interacts with the mitochondrial respiratory chain, leading to production of reactive oxygen species (ROS) and the activation of Asm (73, 74). Asm is an enzyme responsible for both the degradation of sphingomyelin to ceramide, and the release of cytochrome *c* from mitochondria, resulting in neutrophil apoptosis, impairment of the host immune response, and persistence of *P. aeruginosa* infection (73-75).

1.3 *P. aeruginosa* chronic infections

Acute infections are an important aspect of establishing bacterial colonisation within a host, and in some cases lead to chronic infections (15). A hallmark of chronic infection is the formation of resistant biofilm communities (reviewed in (76)). Most significantly, *P. aeruginosa* is the primary pathogen associated with the chronic infection of the lungs of CF patients, where it is the leading cause of morbidity and mortality (77, 78).

The definition of a chronic infection varies greatly and there is not a universally accepted definition. Johansen *et al.* defined chronic infection as the 'detection of bacteria for a period of at least 6 months and/or a serum content of two or more precipitating antibodies against *P. aeruginosa*' (79). However, an infection persisting for less than less 6 consecutive months can also be defined as chronic 'when combined with the presence of two or more *P. aeruginosa* precipitins' (80).

Chronic infections have a slower disease progression than acute infections, and in contrast to their acute infection counterparts, are often difficult or impossible to clear by antibiotic therapies or the host immune response (reviewed in (76)). The transition between *P. aeruginosa* acute to chronic infection is in part regulated by the GacAS-RsmA/Z/Y network, with RsmA functioning as a global regulator of mRNA expression (13-16). RsmA binds to mRNA encoding quorum sensing regulators and the Type 6 Secretion System to prevent their expression, leading to planktonic physiology and the expression of acute virulence traits (15, 16, 81). Alternatively, two small non-coding RNAs (RsmZ/Y) are able to sequester the RsmA protein leading to a switch from acute virulence

and production of the chronic virulence phenotype (13, 16). The chronic infection phenotype is associated with the production of extracellular polymeric substance (EPS) material and biofilm formation of *P. aeruginosa*. Since a chronic infection is not effectively cleared even after a prolonged period, a sustained immune response occurs. This involves the persistence of leukocyte recruitment, the upregulation of mononuclear leukocytes and IgG antibodies, which can result in chronic inflammation accompanied with polymorphonuclear leukocyte (PMN) recruitment and damage of the surrounding host tissue (82, 83).

1.4 Biofilms

A biofilm is a consortium of bacteria that form a community or population within a self-produced extracellular matrix (reviewed in (84)). Biofilms may occur attached to a solid surface, at an air-liquid interface (i.e. pellicles) or suspended in a solution (i.e. flocs, granules, aggregates). Biofilms are thought to have influenced life on Earth for a long time, with evidence of biofilm microcolony structures found in stromatolites dating back approximately 3.3 - 3.4 billion years (85, 86). The initial discovery of biofilms was probably in 1684 by van Leeuwenhoek, who noticed that 'animalcules' (bacteria) within 'scurf' (plaque) on teeth were not single cells but rather cell aggregates (87). However, it was not until 1978 that the term 'biofilm' was first used to describe bacterial populations or communities embedded in a self-produced matrix (88).

The biofilm matrix is comprised of extracellular polymeric substances (EPS) including, glycoproteins, glycolipids, flagella, pili and extracellular DNA (eDNA) (89). The EPS matrix confers several advantages to the biofilm. Firstly, biopolymers within the EPS are well hydrated and therefore serve to keep the biofilm intact and to retain water (89). Secondly, the EPS promotes biofilm attachment to surfaces and can sequester molecules such as nutrients to provide nutrition for organisms within the biofilm (89). The matrix also allows the biofilm to achieve high cell density, enabling cells to congregate and express cell-density specific phenotypes such as quorum sensing, which also correlates with genetic diversification of the resident cells (90, 91). Finally, the EPS protects biofilm bacteria from stresses, including shear forces and phagocytosis by host cells.

Biofilms are extremely important in industrial and medical contexts, and are also ubiquitous in nature. The use of biofilms in industry can be of great benefit. For example,

anaerobic biofilm reactors have proven useful in the treatment of wastewater systems with high organic loadings, and biofilms also increase the efficiency of microbial fuel cells (92, 93). Furthermore, biofilm-associated bacteria are more effective for bioremediation than planktonic bacteria, as the biofilm matrix provides increased tolerance of toxic compounds (94). This includes the bioremediation of heavy metals such as copper, zinc, nickel, cobalt, palladium and lead, as well as numerous chlorophenols, azo dyes and herbicides (94-102).

However, biofilms can also negatively affect industrial and medical processes. For example, biofouling is the unwanted accumulation of biological agents on surfaces that negatively impacts several industries, including aquaculture infrastructure in marine environments, biotechnological systems such as bioreactors, and fouling has even impacted space shuttle water systems (103-105). The removal of biofilms that result from biofouling is a costly and time consuming process (reviewed in (105)). Biofilms affect the quality of drinking water, by contributing to 45% of the fouling of membrane filters (103). This leads to reduced water filtration and increased costs to clean the membrane filters (103). In the aquaculture industry, 5 – 10% of costs are directly related to biofouling, which includes the removal of biofilms from cages, nets and ropes (106). This has led to production delays and a decrease in profits (106). Biofilms also grow on ship hulls, pipelines, insulation and fittings, and are difficult to clear due to their resistance to disinfectants (107-109).

Biofilms are reservoirs for infections in many different environments, including water distribution networks such as hospital plumbing (110). Drinking water has been found to serve as a long-term habitat for many biofilm forming organisms such as *Escherichia coli*, *Campylobacter* spp., *Legionella* spp. and *P. aeruginosa* (111). These biofilms can persist for long periods of time within the water systems and pose a potential health risk to humans. Opportunistic pathogens such as *Legionella pneumophila*, *Mycobacterium avium* and *P. aeruginosa* are able to persist in household plumbing due to biofilm formation in showerheads, faucets, pipes and even water heaters (112-118). Furthermore, *P. aeruginosa* is the most common pathogen found in recreational water bodies, such as pools and spas (119-121). In these environments, *P. aeruginosa* can cause infections such as hot tub folliculitis, ear and skin infections, and otitis externa or “Swimmer’s Ear”, with the latter reporting 2.4 million cases per year in the US and an approximate outpatient

cost of US$ 500 million (119, 121-124). Although these bacterial populations are mostly harmless to healthy populations, they present a major risk to the health of immunocompromised individuals (125).

Biofilms are a major medical issue as they promote host infections that are extremely difficult to clear with antimicrobials, causing between 65% and 80% of reported bacterial infections (126, 127). In particular, biofilms can grow on medical devices such as catheters, mechanical heart valves and endotracheal tubes to directly cause infection, or they may indirectly facilitate infection where bacteria enter the patient through the site of surgery (128-130). Such device related infections not only represent a significant infection risk, but also significantly contribute to increased medical costs where replacing and controlling biofilm based infections can increase treatment costs by US$ 15,000 – 50,000 per patient, depending on the severity and complications associated with the infected device (131-133). *P. aeruginosa* is one of the most frequent causes of medical device infections, causing 10% of catheter-associated urinary tract infections, 18% of intravascular-device related bloodstream infections, and 5 – 10% of peritoneal dialysis-related peritonitis infections (130, 134, 135). In addition, *P. aeruginosa* is responsible for 10 – 20% of all nosocomial infections, mainly due to its ability to form biofilms (136).

1.5 *P. aeruginosa* biofilm life cycle and associated infections

As a consequence of its broad distribution, infectivity towards representatives of most taxa, high level of intrinsic resistance to antimicrobials and its ability to form biofilms, *P. aeruginosa* is used as a model organism to study biofilm formation (137). *P. aeruginosa* biofilm formation occurs through a series of loosely defined, but highly coordinated stages (Fig. 1.1). Firstly, reversible attachment of the bacteria to the surface occurs, which may be assisted by flagellar motility and pili (138). Secondly, the cells irreversibly attach to a surface, motility stops and the *las* quorum sensing regulon is activated, which initiates the formation of cell clusters (138). Cell maturation occurs in the third stage, where the clusters create a thicker layer and the *rhl* quorum sensing system is activated (138). Late maturation occurs when the biofilm reaches a thickness of around 100 μm (138), after which time cell death in microcolonies occurs, and bacteria disperse and return to a planktonic phase (138).

Figure 1.1 The *P. aeruginosa* biofilm life cycle Biofilm formation is a multistage process that involves (i) reversible attachment of cells to a surface; (ii) secretion of adhesins and EPS, resulting in irreversible attachment of the biofilm and cell proliferation; (iii) microcolony formation and maturation of the biofilm; and (iv) cell death and dispersal occurs, completing the biofilm lifecycle. The figure is adapted from (137).

1.6 Antibiotic tolerance of *P. aeruginosa* biofilms

It has been observed that *P. aeruginosa* biofilms have a significantly higher resistance to antibiotics, which has been associated with factors including the biofilm extracellular polymeric substance, efflux pumps, persister cell formation, and slow replication and growth (139).

1.6.1 The EPS biofilm matrix

Extracellular polymeric substance (EPS) components that have been linked to *P. aeruginosa* biofilm tolerance to antibiotics include the Psl and Pel exopolysaccharides, and eDNA (140, 141). Psl is an exopolysaccharide composed of mannose and galactose, and has a role in initial attachment and biofilm formation (142). It has been observed that when *P. aeruginosa* PAO1 early-stage biofilms lacking *psl* expression were exposed to colistin and polymyxin B (polymyxin antibiotics, which disrupt the bacterial cell membrane), tobramycin (an aminoglycoside, which prevents protein synthesis) and

ciprofloxacin (a fluoroquinolone, which targets the DNA gyrase), there was a 2 – 4 fold decrease in minimal inhibitory concentration (MIC) for all antibiotics compared to the wild-type PAO1 biofilm (143). However, this effect was not observed for late-stage biofilms, indicating that Psl contributes to antibiotic resistance of *P. aeruginosa* early-stage biofilms, but not for late-stage biofilms (141).

The glucose-rich exopolysaccharide, Pel, is required for biofilm formation at the air-liquid interface, and contributes to *P. aeruginosa* biofilm antibiotic resistance (144). Previous studies indicated that Pel has no significant effect on planktonic antibiotic resistance, as mutation of *pel* in the PA14 strain did not alter the MICs during exposure to numerous antibiotics including tobramycin and gentamycin (140). However, when early stage biofilms of a PA14 *pel* mutant were exposed to tobramycin and gentamycin, it was found that there was increased susceptibility to the antibiotic in comparison to the wild-type PA14 (140). Accordingly, early biofilms grown from a Pel overproducing PA14 strain were more resistant than the PA14 wild-type and *pel* mutant towards tobramycin and gentamycin (140). However, the contribution of the Pel polysaccharide to antibiotic resistance was not observed in the PAO1 background or in the response of PA14 ciprofloxacin stress (140).

Alginate is another exopolysaccharide component that is comprised of mannuronic acid and guluronic acid monomers (145). In CF patients, *P. aeruginosa* often undergoes a transition to a mucoid phenotype, which is characterised by the overproduction of alginate (146). It has been shown that alginate overproduction in mucoid strains results in the production of biofilms that are more resistant to tobramycin than non-mucoid biofilms (147). For example, biofilms of the mucoid *P. aeruginosa* strain FRD1 were less susceptible to tobramycin and ciprofloxacin than non-mucoid cells (148). When the biofilm was resuspended, the sensitivity of the bacteria in the biofilm was restored (148). However, more recent studies have shown that alginate is not a critical component of non-mucoid PA14 and PAO1 biofilms in response to antibiotics, where no difference in susceptibility to gentamycin, tobramycin and ciprofloxacin was observed between the PA14 and PAO1 wild-type and *algD* mutants (149). This suggests that alginate may not be an important part of the *P. aeruginosa* antibiotic response in most clinical and environmental isolates (which are mainly comprised of non-mucoid *P. aeruginosa*), but plays a significant role in specific host sites, such the CF lung.

Extracellular DNA (eDNA), another component of the biofilm matrix, is made in significant quantities by *P. aeruginosa* biofilms (150, 151). It is thought that eDNA in biofilms originates from dead bacteria and / or lysed immune cells (152). eDNA is traditionally associated with maintaining the structural integrity of *P. aeruginosa* biofilms, and has also been linked to increased antibiotic resistance of *P. aeruginosa* (150, 152). eDNA is known to bind positively charged antibiotics, such as aminoglycosides (e.g. tobramycin and gentamycin) and cationic antimicrobial peptides (CAPs) (e.g. polymyxin B, colistin), delaying their penetration into the biofilm (152). It was observed that when wild-type *P. aeruginosa* PAO1 biofilms were supplemented with eDNA, the biofilms were 8-fold and 64-fold more resistant to biofilm clearance by CAPs and aminoglycosides, respectively (152). In addition to the EPS material, phenotypic diversification of the resident cells contributes to the increased resistance of the biofilm community.

1.6.2 Persister cells

Bacteria are able to survive exposure to lethal doses of antibiotics by transiently entering a metabolically dormant or inactive state known as a persister cell, which is then able to initiate growth once the antibiotic stress is removed (153-155). To regulate persister cell formation, many genes that contribute to energy production and cell maintenance are downregulated, including those involved in oxidative phosphorylation, such as nicotinamide adenine dinucleotide (NADH) dehydrogenase, adenosine triphosphate (ATP) synthase, and cytochrome *O*- ubiquinol oxidase (156). Persister cell formation has also been linked to reduced protein synthesis, where decreased levels of translation resulted in 20 fold more persister cell formation than a culture with normal protein synthesis (157). Due to the reduced metabolic activity, persister cells provide bacteria with increased resistance to lethal shock, DNA-damage and antimicrobials, including antibiotics (158).

Persister cell formation can be influenced by toxin-antitoxin (TA) systems (159). TA systems are comprised of two genes in an operon, one gene that encodes a stable toxin that is involved in the interference of host cellular processes, and another gene that encodes a labile antitoxin that inhibits the toxicity of the cognate toxin (160). The correlation between TA systems and persister cell formation has been shown for the

mqsRA TA system of *E. coli*, whereby a Δ*mqsRA* mutation resulted in the loss of persister cell formation, and the overexpression of the MqsR toxin increased persister cell formation (161). A further TA system associated with persister cells is the TisAB/IstT-1 system of *E. coli* (162). When a Δ*tisAB/istR* deletion strain was exposed to ciprofloxacin, there was a significant decrease in persister cell formation in comparison to wild-type *E. coli* (162). Conversely, when TisB toxin producing cells were exposed to ciprofloxacin, persister cell formation was increased, and these cells were tolerant to various antibiotics (162).

Persister cell formation increases significantly during the transition from the exponential growth phase to the stationary growth phase and within *P. aeruginosa* biofilms, where persister cells can reach up to approximately 1% of the total bacterial population (157, 163). Persister cells are a major issue, particularly in chronic infections, where antibiotic treatments are rendered ineffective. For example, isolates obtained from chronic CF patients contained a higher proportion of persister cells than environmental strains, and the persister phenotype was associated with high-persister (*hip*) mutations (164). It is thought that the development of persister cells within the biofilm may serve as a type of survival strategy where after the majority of the biofilm is cleared by antibiotics, this small population can regrow (165). This would mean that the persister cells are able to re-establish growth as the antibiotic dosage becomes depleted (166, 167).

1.6.3 Efflux pumps

It has also been shown that the expression of efflux pumps are induced biofilms, and that they play an important role in the removal of toxic compounds from the cytoplasm (168, 169). Efflux pumps use energy generated from proton motive force to expel antibiotics and other compounds from the bacterial cell (170). Efflux pumps associated with Gram-negative bacteria tend to be tripartite, consisting of a transporter situated in the cytoplasmic membrane, an outer membrane channel, and a periplasmic linker protein in order to cross inner and outer membranes (171-173). The resistance-nodulation-division (RND) family of efflux pumps secrete multiple ligands and are known to confer multidrug resistance (168, 169). Antibiotic efflux pumps are also major contributors of *P. aeruginosa* biofilm resistance to antibiotics (174). *P. aeruginosa* produces four distinct efflux pumps, MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprY (174). It

has been found that the MexEF-OprN efflux pump conferred the highest resistance to older quinolones (e.g. nalidixic, oxolinic and pipemedic acids), where the MexCD-OprJ efflux pump expelled new generation quinolones (e.g. ciprofloxacin, norfloxacin and sparfloxacin) better than the other *P. aeruginosa* efflux pumps (175).

1.6.4 Oxygen depletion in *P. aeruginosa* biofilms

It has been observed that increased oxygen depletion within the biofilm correlates to a decrease in antibiotic susceptibility (148). Four day old aerobic *P. aeruginosa* biofilms were more sensitive to ciprofloxacin, carbenicillin, tobramycin, tetracycline and chloramphenicol exposure than anaerobically grown *P. aeruginosa* biofilms (148). Previous studies have shown that nutrient depletion can increase antibiotic resistance of bacteria (148). Cells within the deeper parts of the biofilm can be nutrient starved due to the consumption of nutrients at the surface of the biofilm and a low diffusion of nutrients (148). It is thought that a lack of nutrients causes the bacteria within the biofilm to become slow growing or dormant (148).

1.7 Response of *P. aeruginosa* biofilms to oxidative stress

In addition to antibiotic resistance, biofilm formation is correlated with the development of chronic infections due to their resistance towards the host immune response. One of the major mechanisms involved in host innate immunity is the production of reactive oxygen species (ROS) by professional phagocytes, including neutrophils, monocytes, macrophages, mast cells and dendritic cells (176-179). ROS kill infecting bacteria by reacting with membranes, lipids and DNA.

1.7.1 Production of ROS to combat bacterial infection

When a bacterial infection is detected by the human innate immune system, professional phagocytes are recruited to the infection site (180). These professional phagocytes have surface receptors that can identify and bind to bacterial surface components to trigger phagocytosis (180). Phagocytosis involves the engulfment of bacteria by a membrane-bound vesicle known as the phagosome, which subsequently fuses with the lysosome to form an intracellular compartment called the phagolysosome where lysosomal enzymes and ROS are released that kill the bacteria (180).

In the presence of bacterial stimuli, such as lipopolysaccharide (LPS) and lipoproteins, neutrophils initiate ROS production through the induction of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase production, which is located in the surface membrane and phagosomal membrane (reviewed in (178)). NADPH oxidase is responsible for the synthesis of superoxide (O_2^-) radicals, which can be converted to hydrogen peroxide (H_2O_2) by superoxide dismutase to hydroxyl radicals ($^{\bullet}OH$) or hydroxyl anions (OH^-) (reviewed in (178)). Furthermore, H_2O_2 can be further converted to hypochlorous acid (HOCl) and chloramines by the enzyme myeloperoxidase (reviewed in (178)). In the presence of infecting bacteria, phagocytes can increase their oxygen uptake by up to 50 fold (181). This sudden intake of oxygen results in what is known as the respiratory burst, which involves the rapid release of ROS from the phagocyte triggered by pyridine nucleotide oxidase (181). This includes the reduction of oxygen to O_2^-, which is further dismutated to H_2O_2 (181).

While ROS can rapidly kill planktonic bacteria, biofilms of *P. aeruginosa* have been found to be highly recalcitrant to the activity of ROS (182). In the case of CF chronic lung infection, the presence of *P. aeruginosa* leads to chronic inflammation, with increased polymorphonuclear leukocyte (PMN) production being up to 1000 times more than an uninfected CF lung (183, 184). This leads to H_2O_2 release, although the release of ROS results in damage of the host while the bacterial biofilm remains undamaged (cited in (184)). For example, when mice were induced into a hyperoxic state, an inflammatory response related to oxidative stress was observed after 24 h, and histological damage was detected after 48 h (185).

1.7.1.1 *P. aeruginosa* biofilms and hydrogen peroxide stress

It has been shown that biofilms of *P. aeruginosa* are approximately 1000 fold more resistant to H_2O_2 stress than planktonic cells by the use of various resistance mechanisms (186, 187). For example, KatA is a major housekeeping catalase of *P. aeruginosa* that is involved in biofilm resistance towards H_2O_2 (188). KatA catalase is able to scavenge H_2O_2 in the host environment, and catalyse its breakdown into water and oxygen (189). It was shown that H_2O_2 stress could not effectively penetrate wild-type *P. aeruginosa* biofilms, which only had a minor decrease in viability (189). This was in contrast to the *katA* mutant biofilm that was rapidly inactivated by H_2O_2, leading to 90% cell death (189).

Another mechanism used by *P. aeruginosa* to combat oxidative stress is the production of alkyl hydroperoxide reductases, including the alkylhydroperoxide reductase CF (AhpCF) (186). AhpCF is comprised of AhpC, a peroxiredoxin that reduces H_2O_2 using the redox protein, thioredoxin, and AhpF, a disulphide reductase that transfers electrons from NADH via disulphides to AhpC, to reduce H_2O_2 (190). AhpCF has a high affinity for H_2O_2, and is thought to scavenge and detoxify endogenous H_2O_2 produced in aerobic conditions (191). AhpCF has been shown to provide higher protection against H_2O_2 to *P. aeruginosa* in biofilms than in the planktonic phase, with a 1.6-fold increase in *ahpCF* expression when biofilms were exposed to H_2O_2, in comparison to no significant increase in *ahpCF* expression in planktonic cultures under H_2O_2 stress (186).

To deal with H_2O_2 stress in the CF lung, *P. aeruginosa* biofilms can undergo a process called mucoid conversion (192, 193). It was observed that a non-mucoid biofilm that was exposed to H_2O_2 stress lead to the emergence of mucoid variants, leading to resistance towards PMN infiltration (193). In these mucoid variants, a mutation of the *mucA22* allele (commonly found in CF patients) was observed, which lead to a 2 – 6 fold increase in *P. aeruginosa* alginate production (193). Alginate acts as a direct barrier to phagocytosis and has also been linked to increased bacterial adherence. Alginate can also neutralise reactive oxygen intermediates and is able to inactivate hypochlorite that is produced by phagocytic cells, in order to eradicate oxidative stress placed on *P. aeruginosa* (194, 195).

1.7.1.2 *P. aeruginosa* biofilms and superoxide stress

P. aeruginosa biofilms encounter superoxide stress from the host immune response during human infection (as described in section 1.7.1). To deal with superoxide stress, *P. aeruginosa* biofilms produce superoxide dismutases (SODs), which catalyse the dismutation of O_2^- into either molecular oxygen (O_2) or hydrogen peroxide. There are two known types of SODs in *P. aeruginosa*: the manganese-cofactored superoxide dismutase (Mn-SOD) encoded by *sodA* and an iron-cofactored superoxide dismutase (Fe-SOD) encoded by *sodB* (196). It has been shown that *sodA* expression is only elevated in the presence of alginate and/or in iron limited environments, with Mn-SOD being detected

within 1 d of iron-starved biofilms compared with 6 d for biofilms replete with iron (197). Fe-SOD activity, however, is expressed in all growth conditions and is thought to be generally more important than Mn-SOD (196). It was observed that when *sodA* and *sodB* mutants were grown aerobically with paraquat, *sodA* mutants grew better than *sodB* mutants (196). Furthermore, it was found that approximately 87% of total SOD activity was lost in the *sodB* mutant, in comparison with only a 15% decrease in total SOD activity for the *sodA* mutant (196).

1.8 The role of biofilm formation in protection from protozoan grazing

Protozoa are unicellular protists that range from approximately 2 µm to more than 200 µm in size (198, 199). Protozoa graze on dissolved organic matter, detritus, bacteria and other protozoa as a food source (198). Due to their high mobility, relatively small in size, abundance in the environment, and ability to detect concentrated areas of bacteria they are a major mortality factor for bacteria in soil, freshwater and marine ecosystems (198). There are a range of different protozoa that feed on bacteria and these have classically been divided into the groups: ciliates, flagellates, and amoebae based on their mechanisms of feeding. Protozoa have developed several methods to prey on bacteria, such as phagocytosis or filter feeding. Phagocytosis involves invagination of the protozoan cell membrane to encapsulate bacteria in a protozoan food vacuole, where enzymes such as acid phosphatase aid in the digestive process (198, 200). Filter feeders do not use phagocytosis for bacterial uptake but rather use appendages to filter food from the environment, with ciliates using short hairs for feeding and flagellates using a collar of tentacles (198).

Flagellates such as *Tetrahymena pyriformis* and *Cafeteria roenbergensis* are regarded as the primary bacterial predators in aquatic environments, and a single flagellate can ingest bacteria at a rate of 2 - 300 cells h^{-1} (198, 201). They have one or more flagella, which are required for swimming and the generation of feeding currents (198, 201). In water, prey are drawn towards the protozoa via these water currents, and are ingested via temporary projections from the base of the flagellum known as pseudopodia (201, 202). The small size of flagellates (2 - 20 µm) constrains the size of particles that may be ingested to less

than 1.6 µm (198, 201, 203). Therefore, they display a grazing preference for planktonic bacteria, and only rarely target bacterial biofilms.

Ciliates are relatively larger in size compared to flagellates (20 - 1000 µm), and are able to graze on bacteria, algae, flagellates and other ciliates ref. They use cilia to swim, which are hair-like organelles that protrude from the ciliate, and membranelles, shorter hairs, for grazing (198, 204). Different ciliates such as *Uronema* spp. and *Colpoda inflata* are able to swim in suspension, and crawl on or attach to surfaces (198, 204). Most ciliates are filter feeders with the exception of suctorial ciliates (198, 204). This means they are able to draw water that contains prey into their oral cavity, cytostome, and ingest them in a single food vacuole (198, 204). Ciliate ingestion can reach rates as high as 1254 bacteria ciliate^{-1} h^{-1} (198, 204).

Amoebae are surface dwellers that move through the use of pseudopodia and by crawling along surfaces. Prior to ingesting prey into their food vacuole, amoebae enclose the prey within their pseudopodia. Amoebae such as *Acanthamoeba castellanii* are unable to graze efficiently on planktonic bacteria, and have a high preference for surface-associated bacteria, with grazing rates of 0.2 - 1465 bacteria amoeba^{-1} h^{-1} (198, 205-209).

Grazing pressure can lead to the development of mechanisms that enable bacteria to survive within protozoa, or restrict protozoan grazing (198, 210). Alternatively, biofilm formation has been linked with decreased protozoan grazing by mechanisms including microcolony formation, the T3SS, and sigma factor expression, where cell-density dependent signalling can initiate specialised antipredation mechanisms that are not seen in planktonic bacteria (210, 211).

1.8.1 Strategies used by *P. aeruginosa* to resist predation by flagellates

Biofilm formation of *P. aeruginosa* provides higher resistance to protozoan grazing in comparison to biofilm-deficient mutant strains (210). One mechanism of *P. aeruginosa* resistance towards flagellate grazing is the overproduction of alginate. Studies investigating the effect of flagellate grazing on *P. aeruginosa* non-mucoid and alginate overproducing mucoid biofilms showed that alginate provided resistance to predation by *Rhynchomonas nasuta* (212). The overproduction of alginate increased the size of

bacterial particles such that they exceeded what *R. nasuta* could ingest (210, 212). However, such a mechanism is not highly effective against amoeba that are able to ingest larger bacterial particles.

1.8.2 *P. aeruginosa* strategies to resist amoeba grazing stress

While amoeba are typically able to ingest large bacterial particles, *P. aeruginosa* biofilms are known to combat amoeba grazing by other mechanisms including the T3SS (211). Many T3SS factors have been linked to *P. aeruginosa* resistance to *A. castellanii* grazing, including the effector proteins ExoS, ExoT and ExoU (213). It was found that upon grazing of 4 d old *P. aeruginosa* PA99 biofilms, a strain that carries the *exoS, exoT and exoU* genes, but lacks *exoY, A. castellanii* were lysed at a high rate in the presence of the bacteria, whereas *A. castellanii* were able to graze on a PA99 mutant that does not produce any T3SS proteins (213). Furthermore, analysis of Δ*exoS*, Δ*exoT* and Δ*exoU* mutants showed a decrease in *A. castellanii* mortality in comparison to wild-type *P. aeruginosa* PA99, with the absence of ExoU having the greatest decrease in cytotoxicity towards the amoeba of approximately 45% less mortality than the wild-type (213). This suggests that all three T3SS effector proteins, but ExoU in particular, contribute to *P. aeruginosa* resistance towards predation by *A. castellanii*.

In addition, it was observed that the sigma factors RpoS and RpoN were involved in the *P. aeruginosa* response to *A. castellanii* grazing. RpoS is a sigma factor (σ^S) that is induced during stationary phase and when *P. aeruginosa* is under stress conditions, such as heat shock, carbon starvation and low pH (214-216). RpoN is another σ factor that is required for the transcription of a variety of *P. aeruginosa* genes, including those involved in bacterial motility, adherence and nitrogen metabolism (217). However, both RpoS and RpoN have also been shown to be involved in the regulation of *P. aeruginosa* in response to amoeba grazing stress (211). When *rpoS* mutants were exposed to *A. castellanii*, there was a significant increase in amoebal mortality, suggesting that RpoS is involved in negatively regulating *P. aeruginosa* killing of *A. castellanii* (211). In contrast, when *rpoN* mutants were exposed to *A. castellanii* grazing pressure there was a decrease in the killing of amoeba, indicating that RpoN is a positive regulator of *P. aeruginosa* cytotoxicity towards *A. castellanii* (211).

1.9 Bacteriophage and their environmental significance

Another factor that bacteria encounter in the environment that drives bacterial evolution is bacteriophage infection. Bacteriophage are viruses that infect bacteria and were initially described in 1915 and 1917 by Twort and d'Hérelle, respectively (218, 219). The diversity of bacteriophage is vast, with more than 1 x 10^{31} phage present on the planet, compared to 2 – 6 x 10^{30} bacterial cells, meaning that phage outnumber prokaryotes 10 : 1 (220, 221). Phage can be classified by their genome, which can be double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA), with most bacterial viruses having dsDNA genomes (17). Bacteriophage are typically described as being either lytic or lysogenic, depending on whether they lyse their host or become stably integrated into the host chromosome.

Phage have been extensively studied to understand their ability to infect and replicate within a host, as well as how they can be used therapeutically to combat infection in the medical, environmental, and agricultural industries (222-224). For example, a bacteriophage lysin (PlySs2) derived from *Streptococcus suis* has been shown to have broad lytic activity to various bacterial pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA) and *Streptococcus pneumoniae* (225). Phage therapy can also be used for environmental applications, including a Myoviridae-like phage (known as YC) that can inhibit *Vibrio coralliilyticus*, a bacterium associated with coral bleaching and damage in the Great Barrier Reef (226). Agriculturally, phage therapy has also been used to reduce *Campylobacter jejuni* and *Campylobacter coli* contamination in chickens (227).

In addition to being used in phage therapy, bacteriophage are important in promoting genetic diversity and providing adaptive benefits for bacteria by facilitating horizontal gene transfer (228). For example, one mechanism of horizontal gene transfer occurs when DNA from the phage-infected bacterial cell is packaged within the phage particle, which is then transported to another bacterial host which is known as generalised transduction (229) Also, genetic material from a phage attachment site may be transferred to the adjacent bacterial DNA in a process known as specialised transduction (226, 230). Whilst the amount of phage DNA that is transduced to a bacterial cell depends on the phage capsid size, the capsid is able to transfer DNA of > 100 kbp (226, 230). Both general and

specialised phage transduction has been used for genome engineering in the genomics era for model organisms including *Escherichia, Salmonella* and *Pseudomonas* (231).

When phage integrate into the host genome as a prophage, they can contribute to the pathogenicity, protection and adaptation of the host (232). For example, studies have shown that prophage in *Bacillus subtilus* increased biofilm formation, EPS production and sporulation, which enhanced survival in environments such as soil and invertebrate intestines (233). Also, studies have shown that prophage contribute to the pathogenicity of *P. aeruginosa* in a chronic rat lung infection model, and that *P. aeruginosa* prophage elements correlate with disease severity (exacerbation) in the CF lung (234).

1.9.1 Filamentous phage

Filamentous bacteriophage are characterised as long, thin filaments (6 - 7 nm in diameter, and > 500 nm in length), with circular, ssDNA genomes (235). They also have retractable pili to interact with the host surface receptors (235). The first filamentous phage to be identified were the Ff phage f1, fd and M13 of *E. coli* that were isolated from American and European sewage systems. The Ff phage are classified under the genus, *Inovirus*. These phage have been well described and used extensively in cloning and other molecular biological studies, particularly the well-known M13 phage (236, 237). There are two types of filamentous phage, phage that integrate in the host chromosome, and phage that do not integrate into the chromosome but rather replicate as extrachromosomal elements (reviewed in (235)). One interesting and distinguishing feature of filamentous phage is that in contrast to most phage, filamentous phage constantly shed viral particles without causing host death (238).

1.9.1.1 The life cycle of filamentous phage

Filamentous phage initially infect the bacterium through attachment to the host cell, which is typically mediated by pIII, an adhesive protein that interacts with pili and fimbriae on the host surface (239). After the phage is attached, the pilus retracts through the outer membrane, into the periplasm, where it interacts with a secondary receptor,

TolA (240). The phage then crosses the inner membrane and the coat protein sheath is shed, so that the phage ssDNA can be replicated (239).

Replication involves complimentary strand synthesis by host RNA and DNA polymerase and DNA gyrase. This results in the production of double-stranded supercoiled DNA known as the replicative form (RF) (241). The RF is used as a template for gene expression, which is necessary for the production of structural proteins pIII, pVI, pVII, pVIII and pIX, resulting in phage particle assembly, a process that is aided by the phage pV complex (236, 237, 242, 243). Membrane proteins pI, pXI and pIV then mediate phage particle release through the cell membrane (235, 244) (Fig. 1.2).

Figure 1.2 Infection and replication of filamentous phage. When phage DNA is injected into the host bacterial cell, the DNA is converted from ssDNA to a RF by host enzymes. The RF form serves as a template for phage DNA assembly, which occurs in the cell membrane. Once assembled, the phage particle is then released through the host cell membrane. Figure is adapted from (235).

1.9.1.2 The effect of filamentous phage on host virulence

In addition to *E. coli,* filamentous phage are prevalent in other Gram-negative bacteria including species of *Ralstonia, Xanthomonas, Vibrio* and *Pseudomonas* (236, 237, 245-254). Many of these phage have been shown to contribute to the virulence of their host bacterium through the transfer and / or integration of phage genes in the host genome.

1.9.1.2.1 Ralstonia solanacearum

R. solanacearum is an important phytopathogen that causes bacterial wilt in plant crops, including tomato, eggplant, potato and tobacco (255). Three filamentous phage that are known to infect *R. solanacearum* and effect virulence are ΦRSM1, ΦRSM3 and ΦRSS1. It has been observed that ΦRSM1 and ΦRSM3 infection of *R. solanacearum* causes a decrease or loss of virulence towards plants (256). In *R. solanacearum* infected with ΦRSM3, twitching motility, EPS production and type IV pili production decreased (256). *R. solanacearum* infected with ΦRSM3 has also been associated with a decrease in *phcA* and *phcB* expression, two genes associated with the phenotypic reversion of *R. solanacearum* from a non-pathogenic to pathogenic state (256, 257). Furthermore, tomato plants that were exposed to ΦRSM3-infected *R. solanacearum* cells did not exhibit signs of wilting (256). In contrast, when ΦRSS1 is present, the virulence of *R. solanacearum* is increased (258). It has been observed that tomato plants exposed to ΦRSS1-infected *R. solanacearum* wilt 2 - 3 days earlier than plants in the presence of wild-type *R. solanacearum* (258). ΦRSS1 infection also initiate earlier *phcA* expression, as well as increased type IV fimbriae expression, enhanced EPS production and twitching motility (258).

1.9.1.2.2 Xanthomonas axonopodis

X. axonopodis pv. *citri* is a major cause of Asiatic citrus canker disease, one of the most severe global citrus plant diseases (259, 260). A filamentous phage known as XacF1 has been shown to integrate into the *X. axonopodis* genome via the *dif* site (*att*B), facilitated by the host XerC/D recombination system (261). It was observed that XacF1-infected *X. axonopodis* had reduced motility, slower cell growth, and decreased virulence in comparison to wild-type *X. axonopodis* (261). This loss of virulence was associated with decreased EPS production, increased susceptibility to toxic plant compounds and the host immune response, and a reduced ability to invade and colonise host cells (261).

1.9.1.2.3 Vibrio cholerae

Another major pathogen, *V. cholerae*, has increased virulence due to the presence of a filamentous prophage known as CTXΦ (262). *V. cholerae* causes infection and disease by colonising the host intestine and producing virulence factors (263). These virulence factors include the exotoxin cholera toxin (CT), zonula occludens toxin (Zot) and accessory cholera enterotoxin (Ace), which were introduced from the CTXΦ genome via horizontal gene transfer into the chromosomal *dif* site of the bacterium (262, 264, 265). (266). This occurs via a series of events whereby the CT causes an increase in cAMP levels within the host epithelial cells, which affects cAMP-dependent protein kinase PKA activity resulting in an unregulated release of water and NaCl via the cystic fibrosis transmembrane conductance regulator (CFTR) (262, 267). In addition to the CT, excessive diarrhoea may also result from the Zot toxin which can increase the permeability of the small intestinal mucosa by disruption of the intracellular tight junctions known as zonula occludens (268). When CTXΦ is integrated into the host chromosome, the CTXΦ can replicate and produce infective phage particles that have the potential to be transduced to non-pathogenic *V. cholerae* environmental strains, resulting in conversion to virulent strains (269). This ability to transduce CTXΦ genes to other strains has also been observed in the more recently discovered *V. cholerae* filamentous phage, VEJΦ and VGJΦ (270, 271).

1.9.1.2.4 Pseudomonas aeruginosa

In *P. aeruginosa*, the Pf filamentous phage (named Pf1 - Pf6), are known to affect host virulence (245, 250, 251, 253, 254, 272-274). Previous studies have shown that Pf phage particles have been identified in most clinical and laboratory *P. aeruginosa* isolates tested, suggesting that Pf phage are present in most *P. aeruginosa* strains (in particular, PAO1) (reviewed in (275)). In comparison to other well-studied phage, such as the Ff phage, there is a limited understanding of the Pf phage life cycle, and the modes of infection and replication in the host.

The first Pf phage discovered was Pf1 in 1966 by Takeya and Amako (276). The Pf1 genome is thought to have a small host range, as it could only infect the PAK strain out of 50 different *P. aeruginosa* isolates (273, 276). The second identified Pf phage, Pf2, was isolated from *P. aeruginosa* P28 in the 1960s, and was identified as a filamentous phage via electron microscopy (245). It has also been shown that Pf2 can infect *P.*

aeruginosa PAK, resulting in phage producing host colonies. In contrast to the high similarity seen between Pf1 and Pf2, Pf3 phage function is significantly different to the other Pf phage in several ways (274). For example, all of the Pf phage except for Pf3 possess an integrase-encoding gene (239). This suggests that the Pf3 has a very different method of integration into the host, however this mechanism is currently unknown (239). Furthermore, Pf3 is the most phylogenetically divergent of these Pf phage, sharing a higher homology to filamentous phage of *Vibrio* spp. than to the other Pf phage of *Pseudomonas* (277). It is thought that this difference may be partly attributed to the Pf3 major coat protein being shorter than the other Pf coat proteins. The Pf5 phage is known to have high homology to the *Xanthomonas* Xf phage, as well as the Pf4 phage of *P. aeruginosa* (the latter described further in 1.9.2) (239, 251). The Pf5 phage has not been extensively studied, but through the studies that have been conducted, it is known that the phage is not involved in small colony variant (SCV) formation (described further in 1.9.2) (251). The most recently discovered Pf phage, Pf6 (also referred to as RGP42), was identified in *P. aeruginosa* MPAO1 and PAO1-DSM, but very little is currently known about its physiology (278). The Pf4 phage is the best understood of the Pf phage in PAO1 (Fig. 1.3).

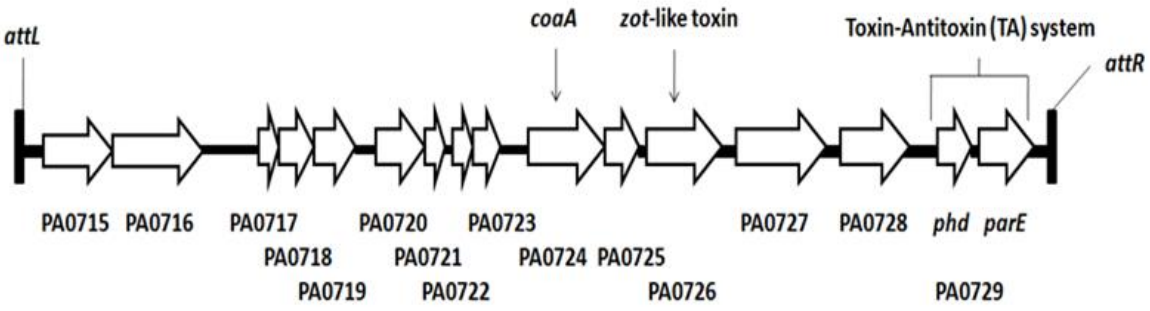

Figure 1.3 The Pf4 phage genome. The 12,437 bp phage genome appears to encode sixteen open reading frames (ORFs) of which three have homology to annotated genes, including the CoaA protein, Zot-like toxin, and ParE-Phd putative toxin-antitoxin system.

1.9.2 The Pf4 phage

This Pf4 phage was first identified in PAO1 strains and 8 of 11 *P. aeruginosa* sputum isolates from CF patients, suggesting that it is important for chronic infection (279). The Pf4 phage is a single-stranded DNA Pf1-like filamentous phage that has a 12,437 bp genome (254, 272). The Pf4 phage shares homology with other Pf phage, including the

Pf5 phage, which has homology with 12 of the 16 genes in the Pf4 phage genome (excluding the four unique genes described below), and the genes encoding the pIII attachment protein that are also present in Pf1 and Pf3 phages (239). However, there are also some unique genes associated with the Pf4 phage. For example, at the 3' end the Pf4 phage has two unique open reading frames encoding a toxin-antitoxin (TA) system, which consists of a prevent-host-death (*phd*) antitoxin and *parE* toxin (250). There are two unique genes located at the 5' end of the Pf4 phage, one that encodes a putative reverse transcriptase (RT) (PA0715), and an ATPase component of an ABC transporter (PA0716) (250).

As noted above, the presence of a filamentous phage, either as a prophage or as an epigenetic element, allows for the production of phage particles without killing the host. In addition, such host cells are protected from reinfection, analogous to the protection afforded by lambda lysogens (280). The Pf4 phage are freed from the bacterium via secretion, where cell lysis does not occur (281). The frequency of free Pf4 phage formation from wild-type PAO1 varies according the growth stage of the bacterium. For example, in *P. aeruginosa* planktonic cultures, the PFU /mL of free Pf4 phage released increases over time, and plateaus at 24 h, with the phage titre maximum being 1×10^7 PFU /mL (from 1×10^9 CFU /mL of *P. aeruginosa* cells) (282). In wild-type *P. aeruginosa* PAO1 biofilms, free Pf4 phage have been detected as early as one day of growth at 4.98×10^7 PFU /mL, and up to a maximum of 4.02×10^9 PFU /mL (after eleven days) (283).

Under natural conditions, the Pf4 phage may integrate into the genome of a naïve host and appears to do so at a conserved site in the genome. Given that the phage does not carry a readily selectable marker, this is difficult to observe in the laboratory without explicitly designing experiments to test for the frequency of insertion. However, it should be noted that for the Pf4 deletion strain used here, the insertion site and flanking DNA have been excised and thus, it is highly unlikely that the Pf4 phage will reintegrate into the genome of the Pf4 mutant. The emergence of superinfective Pf4 was linked to the cell death and dispersal stage of the biofilm life cycle (279). In addition, the development of the superinfection form of the Pf4 also correlated with the appearance of SCVs in the biofilm effluent (253). Furthermore, when planktonic *P. aeruginosa* PAO1 was exposed to superinfective Pf4, approximately 20% of the culture was shown to have a SCV

morphology compared to no SCVs without phage infection. Similarly, biofilms of the phage mutant showed a significant reduction in the formation of SCVs (250).

Interestingly, it has been shown that particular stresses can induce the SI form of the Pf4 phage. For example, it was observed that DNA damage induced by H_2O_2 and mitomycin C increased the prevalence of SI Pf4 phage (284). Hypermutation is typically associated with inactivation of the methyl directed mismatch repair (MMR) system and when a *mutS* mutant was tested, it showed early and elevated production of SI phage relative to the wild type strain (284). In addition, the results showed that the reactive oxygen or nitrogen species, and mutations that caused an increase in the number of superinfective Pf4 phage also resulted in the proliferation of morphotypic variants, such as SCVs (284). SCVs are commonly isolated from CF patient sputum and are associated with poor lung function, increased biofilm adherence and accelerated biofilm formation (250, 285). These CF patient sputum isolates have also been shown to contain Pf4 phage, which are thought to be associated with the emergence of the SCVs. Hypermutators are also frequently isolated from chronically infected CF patients and similarly, the appearance of hypermutators in the sputum of patients is linked with poor prognoses (286). Therefore, it can be speculated that SI phage may play an important role in the conversion of wild type isolates into morphotypic variants, which would provide added resistance to *P. aeruginosa* biofilms under stress.

Variant formation has previously been linked to increasing the resilience of biofilm populations (287). For example, a study of variant formation in a *P. aeruginosa* PAO1 biofilm indicated that after five days, there was an emergence of wrinkly and mini colony variants making up 48% of the biofilm population (287). Upon analysis of mini and wrinkly variant biofilms, significant phenotypic differences were observed compared to the wild-type biofilm (287). The mini variant biofilm attached to a surface at a 4 fold higher rate than the wild-type biofilm, but was also a hyperdetacher, dispersing after 2 d of growth (287). The wrinkly-variant biofilm however, had increased initial attachment and earlier cell cluster formation in comparison to the wild-type biofilm (287). Furthermore, the wrinkly-variant biofilm had 100 fold more bacteria after 5 d, was 9 fold more resistant to detachment, and was more resistant to antimicrobials such as H_2O_2, hypochlorite and tobramycin, than the wild-type biofilm (287).

More recently, the physical effects of the Pf4 phage on the physical properties of the biofilm have been demonstrated (288). It was observed that the exogenous addition of the Pf4 phage to *P. aeruginosa* infected CF sputum samples increased liquid crystal formation of the biofilm matrix compared to sputum samples without the Pf4 phage added (288). It is thought that the Pf4 phage can interact with DNA, mucin and other polymers in the CF sputum to form more stable liquid crystalline biofilm matrices (288). It is suggested that these liquid crystalline biofilm matrices could be increasing *P. aeruginosa* biofilm adhesion in the CF lung, and providing greater protection against stresses such as desiccation and aminoglycoside antibiotics (288).

When the Pf4 phage was deleted from the genome of PAO1, biofilms of the variant-deficient mutant were also more sensitive to surfactant stress than the wild-type (253). Thus, the phage contributes to biofilm dispersal, variant formation, and stress adaptation, and therefore may play an important role in the development of chronic infection phenotypes. Indeed, when the ΔPf4 mutant was used to infect mice, it was less virulent than the WT PAO1 (253). This suggests that the Pf4 phage is involved in increased virulence of *P. aeruginosa*.

1.10 Aims of this study

It is known that the Pf4 phage contributes to the virulence and biofilm stability of *P. aeruginosa* PAO1 during surfactant stress. Furthermore, it is known that the presence of the Pf4 phage triggers the formation of SCV and microcolony formation, phenotypes associated with bacterial persistence in the host. Genetic analysis of the Pf4 phage has shown that it encodes for potential virulence factors, including a Zot-like toxin and *parE-phd* toxin-antitoxin system. However, it is currently unclear how the Pf4 phage specifically contributes to these processes.

It was hypothesised that in addition to surfactant stress, the Pf4 increases the ability for *P. aeruginosa* PAO1 to resist antibiotic and oxidative stress. Furthermore, it was hypothesised that the *parE-phd* toxin-antitoxin system of the Pf4 phage was contributing significantly to virulence of *P. aeruginosa* PAO1; in particular, cytotoxicity of tissue culture cells and nematodes.

Therefore, the aims were:

Aim 1: To investigate the role of the Pf4 phage in *P. aeruginosa* PAO1 in modulating virulence using *in vitro* assays.

Aim 2: To determine the effect of the Pf4 phage on the stress tolerance of *P. aeruginosa* PAO1.

Aim 3: To test how the Pf4 phage specifically affects *P. aeruginosa* PAO1 through quantitative gene expression analysis and complementation studies, both genetic and functional.

2 The effect of the Pf4 phage on the virulence of *Pseudomonas aeruginosa*

2.1 Introduction

Pseudomonas aeruginosa is an important opportunistic human pathogen that is often highly resistant to antibiotic therapy (289). In addition to the intrinsic resistance of this pathogen, extensively drug resistant (XDR) *P. aeruginosa* strains have recently been isolated from human infections, including urinary and respiratory tracts (290, 291). It is able to survive in health-care facilities and is one of the most prominent bacteria associated with nosocomial infections (292). *P. aeruginosa* is known to play a role in a number of acute infections, including those associated with ventilator-associated pneumonia, keratitis, wounds and burns (289). Moreover, it can establish several chronic infections including chronic obstructive pulmonary disease and lifelong infection of the lungs of cystic fibrosis patients. *In vitro* studies using lung, intestinal, ovarian and corneal cells have found that *P. aeruginosa* can invade and damage these different cell lines (51, 293-295).

P. aeruginosa infections are typically described as acute or chronic, depending on the longevity and aggressiveness of the infection (296). Detailed descriptions of an acute infection and chronic infection are provided in sections 1.2 and 1.3, respectively. Chronic infection is associated with biofilm formation and a reduction in virulence factor expression, which leads to increased inflammation, higher neutrophil recruitment and serine protease release (76, 297, 298). This host immune response results in lung damage and death and has been hypothesised to be the primary mechanism of host tissue damage during chronic infection by *P. aeruginosa* (76, 297, 298). In order to establish an infection, *P. aeruginosa* must survive the innate and adaptive immune systems of the host. The innate immune response largely involves the influx of white blood cells such as neutrophils and macrophages, which phagocytose and kill pathogens, and induce the expression of inflammatory mediators and production of cytokines (299, 300). When the macrophage kills the pathogen and it is degraded within the maturing phagosome, parts of the pathogen are transported to T cells, which results in the activation of the adaptive immune response (299, 300). *P. aeruginosa* also stimulates an adaptive immune response

by the activation of dendritic cells, primary epithelia and macrophages, by lipopolysaccharides (LPS) and flagella, which leads to neutrophil influx. The increased infiltration by neutrophils subsequently damages the host tissues as described above.

In addition to the role of the biofilm in chronic infection, *P. aeruginosa* also encodes a range of specific virulence factors. These include Type 3 Secretion System (T3SS) factors, protease production and siderophore production. Several mechanisms contribute to the protection of bacteria from the host's innate immunity. One such mechanism is the direct intoxication of mammalian cells by the T3SS. Initial infection is strongly influenced by the expression of T3SS effector proteins, with a reduction in T3SS secretion significantly reducing the severity of the bacterial infection in murine lungs (28, 301). The three T3SS effector proteins (ExoS, ExoT and ExoU) were previously described in detail (Section 1.2.1).

Proteases, such as the LasB (elastase) are also important virulence factors of *P. aeruginosa*, and are associated with degradation of the host innate and adaptive immune systems such as surfactant proteins, antibacterial peptides, cytokines, chemokines and immunoglobulins (see section 1.2.3 for more information on proteases). Elastase expression has also been linked to the increased invasion of mammalian cells by *P. aeruginosa* through degradation of ExoS (302). Since the ADPRT domain of ExoS disrupts phagocytic uptake of *P. aeruginosa*, ExoS degradation results in increased bacterial invasion (303). Furthermore, elastase is known to be involved in the degradation of transferrin, a protein that binds and transports iron throughout the body (304). This degradation results in iron release from transferrin to assist *P. aeruginosa* in obtaining iron from the host environment (304).

Iron is sequestered by host iron chelators to prevent bacterial growth and this serves as part of the innate immune system. However, bacteria can secrete their own iron chelators to compete for iron in the host. *P. aeruginosa* primarily produces two siderophores, pyoverdine and pyochelin, that bind iron (305). More information for the roles of pyoverdine and pyochelin in *P. aeruginosa* virulence is provided in sections 1.2.4 and 1.2.5, respectively.

It has recently been shown that the presence of a Pf1-like filamentous prophage known as Pf4, contributes to the virulence of *P. aeruginosa* (more detail provided in section

1.9.2). Despite Pf4 having been shown to increase the virulence of its host, the mechanism(s) involved this increased virulence remain unknown. While no obvious Pf4 virulence factors have been identified, it is possible that some of the Pf4 genes play roles in modifying host virulence, with some of these genes being described in section 1.9.2.

Therefore, the present study was conducted to investigate the role of the Pf4 phage in mediating the virulence of *P. aeruginosa*. Changes in virulence factor expression were studied and the impact of the Pf4 phage in different virulence models was also tested. The production and expression of virulence factors, including pyoverdine production, ExoS/ExoU expression and proteolytic activity was compared for the ΔPf4 mutant and the wild-type. Additionally, virulence phenotypes such as invasion and cytotoxicity of mammalian cells as well as virulence in a *Caenorhabditis elegans* model were also tested.

These studies demonstrated that the Pf4 phage has cell-line specific effects on the invasion and cytotoxicity of *P. aeruginosa*. Furthermore, pyoverdine activity was increased by the absence of the Pf4 phage. Finally, the Pf4 phage did not impact virulence of *P. aeruginosa* in a nematode infection model.

2.2 Materials and Methods

2.2.1 Media and growth conditions

Bacteria (Table 2.1) were maintained on lysogeny broth (LB) solid medium (10 g/ L tryptone, 10 g/ L NaCl, 5 g/ L yeast extract, 15 g/ L agar), unless otherwise stated. To maintain particular strains, antibiotics (50 µg/ mL carbenicillin, 100 µg/ mL ampicillin or 40 µg/ mL gentamicin) were incorporated into the LB agar as needed. Bacterial liquid cultures were grown in M9 minimal medium (4.76 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 0.86 mM NaCl, 1.85 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 11 mM glucose), pH 7, and incubated overnight at 37 °C with shaking at 200 rpm.

HepG2 tissue cells were cultivated in HepG2 complete medium (10% (v / v) fetal bovine serum (FBS) (Gibco, U.S.A.), 89% (v / v) Dulbecco's Modified Eagles Serum F-12 Ham (Sigma-Aldrich, U.S.A.), 1% (v / v) 100 x antimycotic-antibiotic solution (Sigma-Aldrich, U.S.A)) and grown in T75 cm^2 tissue culture flasks (Sarstedt, Germany) at 37 °C and 5% CO_2. CFTE29o- tissue cells were maintained in DMEM complete medium (10% (v / v) FBS, 98% (v / v) Minimal Essential Medium w/ Earle's Salt (MEM) (Sigma-Aldrich, U.S.A.), 2 mM L-glutamine (Sigma-Aldrich, U.S.A.), 1 x antimycotic-antibiotic solution and grown in T75 cm^2 tissue culture flasks at 37 °C and 4 – 5% CO_2.

2.2.2 Virulence of ΔPf4 mutant and wild-type *P. aeruginosa* in the *Caenorhabditis elegans* infection model

The nematodes were synchronised to an L4 stage, or when the nematodes have developed to adult hermaphrodites, as previously described (306). Once the L4 stage was reached, the plate was washed with 2 mL of sterile H_2O to collect the nematodes into a 2 mL microfuge tube. The tube was placed on ice until the nematodes settled to the bottom of the tube and 1 mL of the liquid was removed. The nematodes were used to seed slow killing (NGM plates modified with 3.5 g/ L instead of 2.5 g/ L peptone) and fast killing (PGS plates; 10 g/ L peptone, 10 g/ L glucose, 27.3 g/ L sorbitol and 17 g/ L agar) plates, with 20 - 40 nematodes per plate, in duplicate, for each strain (307, 308). The slow killing and fast killing plates were prepared as described by an experiment conducted by Tan *et al.* (1998), involving the killing of *C. elegans* by *Pseudomonas* (308). The plates were

previously inoculated with either ΔPf4 or WT PAO1 and incubated at 37 °C overnight prior to the addition of the nematodes.

Table 2.1 Bacteria, tissue cell lines and nematodes used in this study

Strain	Genotype/Description	Source
Bacteria		
P. aeruginosa WT PAO1	Wild-type	(309)
P. aeruginosa ΔPf4 PAO1	PAO1 with the Pf4 prophage deleted via chromosomal deletion and loss of the RF of the phage. Gm^R	(253)
E. coli OP50	*E. coli* OP50 strain	(310)
Tissue cell lines		
HepG2*	Hepatocellular carcinoma derived from a human liver	ATCC® HB-8065™
CFTE29o-	Lung cell line derived from a cystic fibrosis with a ΔF508 in the CFTR	(11)
Nematode strains		
C. elegans	Wild-type *C. elegans*, Bristol N2 strain	(311)

*Provided by A. Brown, UNSW

The nematodes were incubated at 24 °C on the bacterial lawns and the number of live nematodes was counted at 24 h intervals using a light microscope. When eggs were observed, the nematodes were transferred to fresh slow or fast killing plates with the corresponding bacterial strain, to avoid confounding effects of the hatching worms on the worm counts. Results represent the average of four biological replicates. Survival curves were calculated according to the Kaplan-Meier method (312), using the equation:

$$\hat{S}(t)=\prod_{i=1}^{j}\left(1-\frac{d_i}{n_i}\right)$$

Where *t* = time (days), *n* = the initial number of worms, and *d* = number of deaths. Data represent the cumulative percentage survival of the worms over time.

2.2.3 Mammalian cell assays with *P. aeruginosa* ΔPf4 and WT PAO1

2.2.3.1 Growth of HepG2 and CFTE29o- cells

HepG2 cells and CFTE 29o- cells were grown from cryogenic stocks at 2 - 2.5 x 10^6 cells/ mL and 2 x 10^5 cells/ mL respectively. The HepG2 cells were thawed and added to 19 mL of complete DMEM in a T75 cm^2 vented tissue culture flask. The CFTE29o- cells were resuspended in 10 mL of complete MEM and centrifuged (Eppendorf) at 206*g* for 3 min. The supernatant was removed, the pellet was resuspended in 9 mL of complete MEM medium and pipetted into coated [1% (v / v) bovine collagen I (Becton Dickinson, U.S.A.); 1% (v / v) human fibronectin (Becton Dickinson, U.S.A.); 10% (v / v) bovine serum albumin; 88% (v / v) LHC basal medium (Invitrogen, U.S.A.)] T25cm^2 vented tissue culture flasks. The cells were incubated for 4 d at 37 °C with 5% CO_2. For both invasion and cytotoxicity assays, three independent experiments were performed, with three biological replicates in duplicate for each condition per independent experiment.

2.2.3.2 Preparation of HepG2 and CFTE29o- cells for invasion and cytotoxicity assays

At approximately 75 - 80% confluence, the tissue culture cells were split to maintain the cell line and to allow for optimum growth and proliferation. Spent medium was removed from the T75 cm^2 flask. Two millilitres of 0.25% (v / v) Trypsin-EDTA (Sigma-Aldrich, U.S.A.) were added to the flask to wash away any excess medium from the cells and removed. Another 2 mL of Trypsin-EDTA was added to the T75 cm^2 flask and incubated with the tissue culture cells for 10 min at 37 °C and with 5% CO_2 to detach the cells. Four millilitres of complete DMEM (for HepG2) or MEM (for CFTE29o-) was added to the flask to detach any remaining cells. The HepG2 or CFTE29o- cells were transferred to a 15 mL centrifuge tube (Becton Dickinson, U.S.A.) and centrifuged at 322 g (Eppendorf) for 4 min, resuspended with 4 mL of complete DMEM or MEM, respectively. One millilitre of the resuspended cell pellet was added to two T75 cm^2 tissue culture flasks/ cell line (the flasks for the CFTE29o- cells were pre-coated with the fibronectin coating

mix described above in 2.2.3.1), with 19 mL of complete DMEM or MEM per flask, and incubated at 37 °C and 5% CO_2 for 3 - 4 d, or when cells reached at least 80% confluence.

The seeding of the HepG2 and CFTE29o- cells into tissue culture flasks was conducted as described in section 2.2.3.1 above, with the following modifications. Cells were detached and harvested from a T75cm^2 tissue culture flask with approximately 80% confluence. Cell numbers were determined by Trypan Blue staining and counting using a haemocytometer (Neubauer, Germany) via light microscopy (Leica Camera AG, Germany). The number of cells/ mL was determined by using the calculation: (total number of live cells in counted quadrants / number of quadrants) x dilution factor x 10^4. Cells were inoculated into 24 well, flat-bottom tissue culture plates (Becton-Dickinson, U.S.A.). For the CFTE29o- cells, the wells were pre-coated with the fibronectin coating mix, as described in section 2.2.3.1. For invasion assays, the HepG2 and CFTE29o- cells were seeded at 3 x 10^5 cells/ mL and 1.5 x 10^5 cells/ mL respectively, and complete DMEM or MEM without antibiotic-antimycotic solution was added to the cells at a total volume of 1 mL per well. For cytotoxicity assays, the cells were seeded at 1.5 x 10^5 cells/ mL for both the HepG2 and CFTE29o- cells with complete DMEM (and no antibiotic-antimycotic solution), to a total volume of 1 mL/ well. Seeded plates were incubated at 37 °C and 4 - 5% CO_2 for 2 d. The medium was changed after 1 d of incubation.

2.2.3.3 Invasion of HepG2 and CFTE29o- cells by ΔPf4 mutant and wild-type P. aeruginosa PAO1

After 2 d incubation, spent medium was removed from the wells inoculated with the mammalian cells and the monolayers were washed three times with DMEM or MEM without FBS and antibiotic-antimycotic solution. Overnight cultures of *P. aeruginosa* ΔPf4 and WT PAO1 (a total of 10 mL each) grown in complete M9 medium at 37 °C and 200 rpm, were centrifuged at 10,000*g* for 2 min. The supernatant was removed and the pellets were resuspended in 2 mL 1 x phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4). The OD_{600} of the resuspended cells was adjusted to 1 and 100 µL of the cells were added to 900 µL of complete DMEM or MEM without antibiotic-antimycotic solution in each well. The CFU/ mL of the resuspended cells for both *P. aeruginosa* ΔPf4 and WT PAO1 were determined by plate counts. The tissue culture plates were centrifuged at 323*g* for 4 min (Sorvall) to enhance

contact of *P. aeruginosa* with the mammalian cells. The cells were then incubated for 4 h at 37 °C and 5% CO_2 to allow for invasion of the mammalian cells by *P. aeruginosa*.

After the 4 h incubation, the mammalian cell monolayers were washed three times with 1 mL of DMEM or MEM (without FBS and antibiotic-antimycotic solution) to remove unattached bacteria, immersed in 1 mL complete DMEM / MEM without antibiotic-antimycotic solution and tobramycin (20 μg/ mL) was added to kill the extracellular bacteria after incubation for 1 h at 37 °C and 5% CO_2. The monolayers were subsequently washed with DMEM or MEM (without FBS and antibiotic-antimycotic solution) three times and the CFU was determined to quantify extracellular bacteria that were not killed by the tobramycin treatment.

The washed samples were then exposed to 1 mL of 1% (v / v) Triton-X (Sigma-Aldrich, U.S.A.), and incubated for 5 min at room temperature to lyse the mammalian cells and release the intracellular *P. aeruginosa*. The supernatant was used to determine the amount of invasion through serial dilution and drop plate / CFU analysis. The percentage of bacterial invasion of the HepG2 / CFTE29o- cells was determined as follows:

$$\mathbf{[(}n_{bac} - n_{res}\mathbf{) / }i\mathbf{] \times 100}$$

Where n_{bac} = total number of bacterial colonies harvested from mammalian cell wells, n_{res}= total number of resistant extracellular bacteria after tobramycin treatment and i =initial bacterial cell number in the inoculum.

All statistical analyses of the replicates for the invasion assay were conducted using Prism 5 (GraphPad, U.S.A). Six biological replicates with six technical replicates each were tested for both *P. aeruginosa* strains.

2.2.4 Cytotoxicity of ΔPf4 mutant and wild-type *P. aeruginosa* PAO1 on HepG2 / CFTE29o- cells

The mammalian cells were infected with the *P. aeruginosa* as described in 2.2.3.3, with the modification that the FBS in the DMEM / MEM was heat-inactivated, to minimise background LDH interference. The *In Vitro* Toxicology Assay Kit (Sigma-Aldrich, U.S.A.) was used to determine lactate dehydrogenase release, as an indicator of cytotoxicity. Forty-five minutes prior to the completion of the 4 h incubation, 100 μL of the LDH Assay Lysis Solution was added to the total LDH release control wells that

contained tissue culture cells and no bacteria, at a 1:10 volume to determine total cell LDH release and incubated for the remaining 45 min at 37 °C and 5% CO_2. Control wells with tissue culture cells only and no lysis solution, were used to determine spontaneous lysis of the tissue culture cells. The total LDH released from the control wells was used to quantify the maximum amount of LDH released from the mammalian cells.

The plate was then centrifuged at 250 *g* for 4 min. After 4 h incubation, 75 µL of each cell supernatant was transferred to a fresh 96 well plate (Corning, U.S.A.). One hundred and fifty microlitres of Lactate Dehydrogenase Assay Mixture (1 part of LDH Assay Substrate Solution, 1 part of LDH Assay Dye Solution and 1 part of 1 x LDH Assay Cofactor Preparation) was added to each well and incubated for 30 min at room temperature in the dark. After the incubation, 25 µL of 1 N HCl was added to each well to stop the reaction. The absorbance of the LDH activity was measured at 490 nm and the background absorbance was measured at 690 nm.

The percentage of LDH released per sample after exposure to bacteria was determined as =

$$\frac{i_{490} - i_{690}}{l_{490} - l_{690}}$$

Where i_{490} and i_{690} = absorbance at 490 nm and 690 nm of LDH released from HepG2 cells incubated with bacteria, respectively and $l_{490}$and l_{690}= absorbance at 490 nm and 690 nm of LDH released from HepG2 cells with lysis solution, respectively. The experiment was conducted in three independent experiments, with three biological replicates in triplicate analysed per independent experiment.

2.2.5 Expression of *exoS* and *exoT* in the presence of tissue culture cells

2.2.5.1 mRNA extraction for quantitative PCR

To quantify the expression of *exoS* and *exoT* when the ΔPf4 mutant and wild-type strains were cultivated with and without HepG2 cells, bacteria were added at an OD_{600} of 0.1 to 2 d grown tissue culture cells seeded in 24 well plates (as described in 2.2.2.2), or to wells

without HepG2 cells. Each condition was tested with three biological replicates in triplicate. The plates were incubated for 4 h at 37 °C and 5 % CO_2. After incubation, 1 mL of the supernatant from each well was added to 1 mL of RNAprotect Bacterial Reagent (Qiagen) according to the manufacturer's instructions.

The RNA extraction was conducted according to the manufacturer's instructions using the Qiagen RNAprotect Bacteria Reagent. The optional on-column DNAse digest was performed according to the manufacturer's instructions (Qiagen), and an additional DNAse digestion was performed following elution of the total RNA with a Turbo DNAse Kit (Ambion) to remove any leftover gDNA. The concentration of total RNA was determined spectrophotometrically using the NanoDrop ND-1000, with the absorbance ratios at 260 and 230 nm used to determine RNA purity. RNA extraction was performed with the help of Dr B. Colley, Centre for Marine Bio-Innovation, UNSW Australia, Sydney, Australia.

cDNA was synthesised from 500 ng of total RNA template using iScript reverse transcriptase (RT), as per the manufacturer's instructions (Bio-Rad). qPCR was performed in black-shell/white-well, low-profile, thin-walled, 96 well PCR plates (Bio-Rad) in a CFX-1000 thermocycler (Bio-Rad) using the SsoFast™EvaGreen®Supermix (Biorad), 0.5 µL cDNA, 300 nM of each primer (Table 2.2) in a final reaction volume of 25 µL. A negative control where the RT enzyme was omitted from the cDNA synthesis was included to test for gDNA contamination. The expression data was normalised to the *proC* housekeeping gene (313). Relative mRNA expression was determined using the $2^{-(\Delta\Delta)Ct}$ method (314). The data was analysed using Prism 5 (GraphPad, Inc., U.S.A).

Table 2.2 Primers used in this study

Primer	Sequence (5'-3')	Annealing temperature (°C)
proC qPCR F*	CAGGCCGGGCAGTTGCTGTC	60
proC qPCR R*	GGTCAGGCGCGAGGCTGTCT	60
exoS qPCR F	CGGGGCGGATGCGGAAAAGT	60
exoS qPCR R	CCCTGACGCAGAGCGCGATT	60
exoT qPCR F	CGGACGGGCCGTACAGCGAT	60
exoT qPCR R	CGCATCCAGCTCCCGTCCCT	60

*Housekeeping gene primers

2.2.6 Determination of protease activity

Cultures of *P. aeruginosa* ΔPf4 and WT PAO1 (10 mL each) were grown in M9 medium with 0.2% (w / v) glucose for 22 h at 37 °C and shaking at 200 rpm. After incubation, the cell density of each culture was determined at OD_{600}. The cultures were then centrifuged at 10,000 g for 5 min and the supernatant was filtered through a 0.22 μm filter (Millipore, Ireland) to obtain cell-free supernatant. To determine total protease and elastase activity, 1 mg/ mL of Hide-Remazol Brilliant Blue R (Sigma-Aldrich) or 20 mg/ mL Elastin Congo-Red (Sigma-Aldrich) were used, respectively. Both were added to 1 mL of Tris-buffer, (30 mM stock, pH 7.2). One millilitre of *P. aeruginosa* ΔPf4 and WT PAO1 supernatant were added to the solutions and incubated for 3 h at 37 °C with shaking at 200 rpm (Ratek Instruments). Samples were then centrifuged at 10,000*g* for 5 min. Total protease activity was measured at OD_{595} and elastase activity was measured at OD_{495}. Both the protease and elastase activity were normalised by dividing the protease activity (OD_{595} or OD_{495}) by the optical cell density (OD_{610}). Both the total protease and elastase activity assays were analysed in three independent experiments, with three biological replicates in triplicate for each condition per independent experiment.

2.2.7 Quantification of pyoverdine

Overnight cultures of ΔPf4 mutant and wild-type *P. aeruginosa* PAO1 were grown in M9 medium with 0.2% (w / v) glucose at 37 °C and shaking at 200 rpm (Ratek Instruments) for 16 h. The cultures were then diluted in fresh M9 medium at an OD_{600} of 0.05 in flat-

bottomed, 96 well plates and grown statically at 37 °C. Absorbance of the cultures were read at OD_{600} to quantify cell density and fluorometrically read at an excitation of 398 nm and emission of 460 nm to quantify pyoverdine over a period of 18 h at 0.5 h intervals using a WallacVictor^2 (Perkin-Elmer, U.S.A.) spectrophotometer. Three independent pyoverdine experiments were conducted, with three biological replicates in triplicate for each condition per independent experiment.

2.3 Results

2.3.1 *C. elegans* fast and slow killing virulence assays

The Pf4 phage has previously been shown to influence the virulence of *P. aeruginosa* in a mouse model of acute lung infection, and this study sought to establish an *in vivo* system suitable to further analyse the impact of the Pf4 on virulence traits and whether altered biofilm formation plays a role in the observed reduction in virulence of the ΔPf4 mutant. There are two established *C. elegans* models: fast killing or acute infection mediated by the release of bacterial toxins and slow killing or chronic infection mediated by biofilm formation in the worm gut (307, 308). In the slow killing assays (Fig. 2.1A), it was observed that there was an overall higher *C. elegans* survival when they were exposed to the ΔPf4 mutant relative to the wild-type, however this was not statistically significant. In the fast killing assays (Fig. 2.1B), the trend suggested that there was a higher survival of the nematodes exposed to the ΔPf4 mutant between 0 – 4 d than the WT PAO1, although this trend was not statistically significant. Between 4 – 6 d, the nematode survival was equivalent for both strains in the fast killing assay.

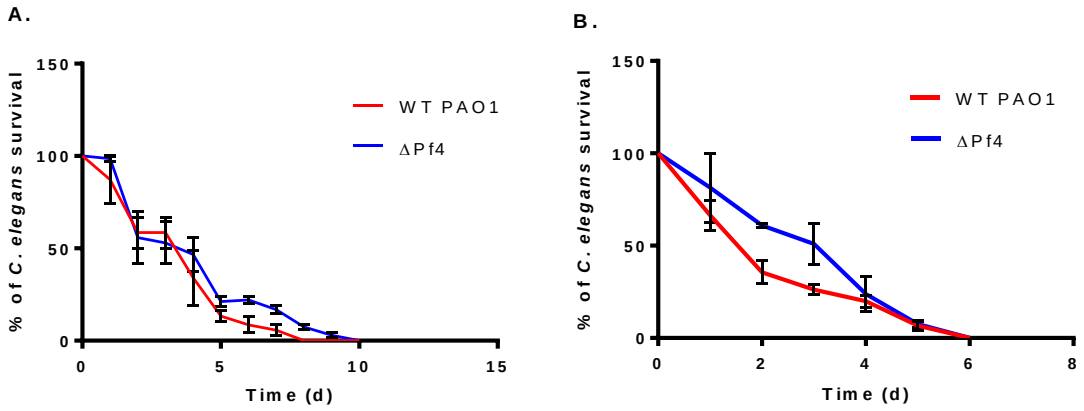

Figure 2.1 Virulence of *P. aeruginosa* and the ΔPf4 mutant in the *C. elegans* infection model. Twenty to thirty *C. elegans* were propagated on lawns of *P. aeruginosa* in the slow (A) and fast (B) killing assays and incubated at room temperature until all of the nematodes were killed (up to 9 d). Worm survival was scored at 24 h intervals and survival curves were calculated according to the Kaplan-Meier method (312). The data was analysed using Prism 5 (GraphPad, U.S.A.) with an unpaired, two-tailed Student's t-test, $n = 4$, $p < 0.01$. Error bars represent standard deviations (SD).

2.3.2 Invasion of HepG2 and CFTE29o- cells by ΔPf4 mutant and wild-type *P. aeruginosa* PAO1

One aspect of acute *P. aeruginosa* virulence is the invasion of corneal epithelial cells, contributing to acute keratitis infections (315). To determine if the Pf4 phage contributed to the invasion of other mammalian cells by *P. aeruginosa*, lung epithelial (CFTE29o-) and liver (HepG2) cells were exposed to either the ΔPf4 or the WT PAO1. In the HepG2 cells, WT PAO1 showed significantly higher invasion than the ΔPf4 strain (1.9 fold, $p = 0.04$) when incubated with the HepG2 cells. In contrast, the opposite trend was observed in experiments using the CFTE29o- cells, where the wild-type invasion was found to be significantly less than the ΔPf4 mutant (2.7 fold, $p < 0.0001$) (Fig. 2.2)

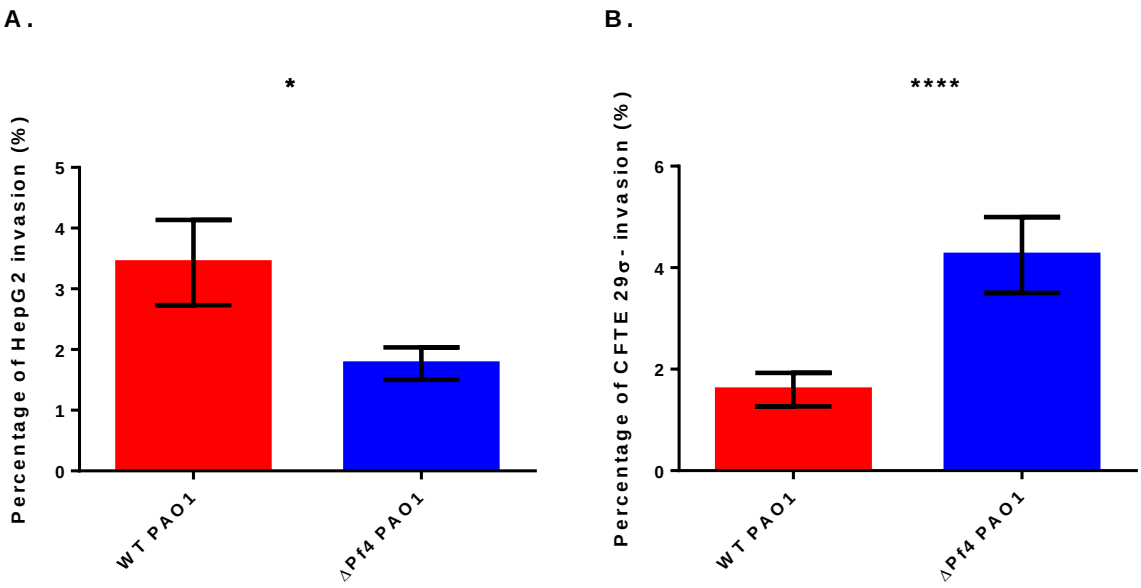

Figure 2.2 Invasion of HepG2 and CFTE 29o- cells by ΔPf4 mutant and wild-type *P. aeruginosa*. ΔPf4 mutant and wild-type *P. aeruginosa* were incubated with (A) HepG2 and (B) CFTE29o- mammalian cells in 24 well plates at 37 °C for 4 h to promote internalisation of bacteria in eukaryotic cells. Invasion is expressed as the percentage of intracellular bacteria compared to the initial bacterial inoculum. Data were analysed using GraphPad Prism 5 (GraphPad Software Inc., U.S.A.) and statistical significance was determined via an unpaired, two-tailed, Student's t-test; n = 6. Data represent the mean values of 3 independent experiments with the corresponding SD. Statistical significance, $p < 0.05$ and $p < 0.0001$, is indicated by either * or ****, respectively).

2.3.3 Cytotoxicity of ΔPf4 mutant and wild-type *P. aeruginosa* PAO1 on HepG2 and CFTE29o- cells

To determine if the cytotoxicity of PAO1 is associated with Pf4 phage production, HepG2 and CFTE29o- cells were incubated with either ΔPf4 mutant or wild-type *P. aeruginosa*. When using the HepG2 cells, the ΔPf4 mutant strain was more cytotoxic than the WT PAO1 although this was not statistically significant (1.4 fold, p = 0.32). The ΔPf4 mutant and wild-type strains both showed similar levels of cytotoxicity against the CFTE29o- cells (Fig. 2.3).

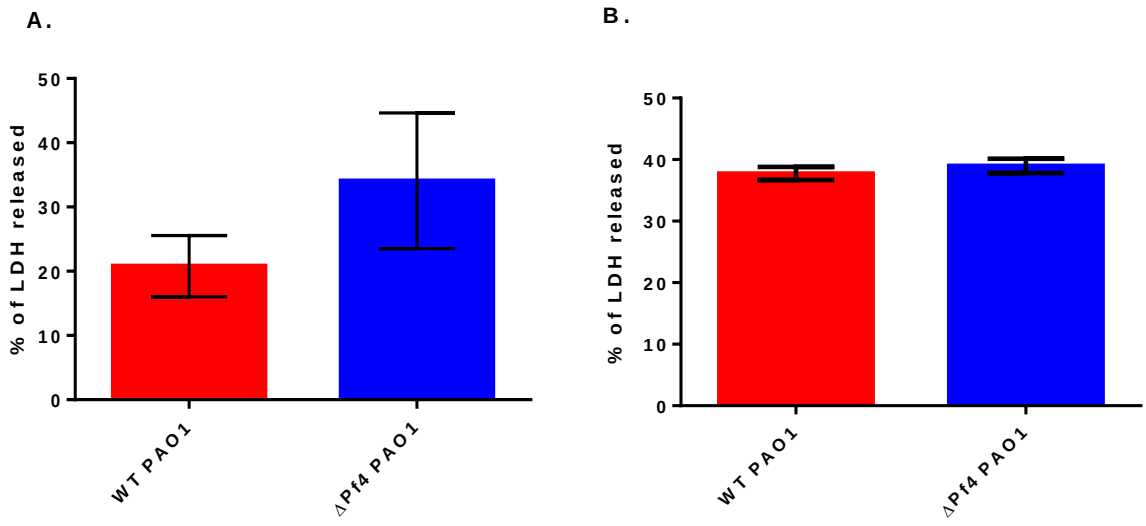

Figure 2.3 Cytotoxicity of ΔPf4 mutant and wild-type *P. aeruginosa* towards HepG2 and CFTE29o- cells. HepG2 (A) and CFTE 29o- (B) mammalian cells were exposed to the ΔPf4 mutant and wild-type *P. aeruginosa* in 24 well plates for 4 h at 37 °C and analysed for LDH activity to quantify mammalian cell cytotoxicity. Data were analysed using GraphPad Prism 5 (GraphPad Software Inc., U.S.A.) and statistical significance was determined via an unpaired, two-tailed, Student's t-test; n = 9. Error bars represent the SEM.

2.3.4 *exoS* and *exoT* expression in the presence and absence of HepG2 cells

Invasion and cytotoxicity are closely linked to the secretion of T3SS linked virulence factors. Therefore, qPCR was used to compare the expression of two of the key T3SS genes, *exoS* and *exoT*, in the presence of HepG2 cells. From these experiments, it was observed that *exoS* and *exoT* expression was significantly higher for both the ΔPf4 mutant

(5 fold and 4 fold, respectively) and WT PAO1 strains (6 fold and 5 fold respectively) when planktonic bacteria were incubated with HepG2 cells compared to controls with no mammalian cells. However, the expression of *exoS* / *exoT* were not significantly different between the ΔPf4 and WT PAO1 strains in the presence or absence of the HepG2 cells (Fig. 2.4).

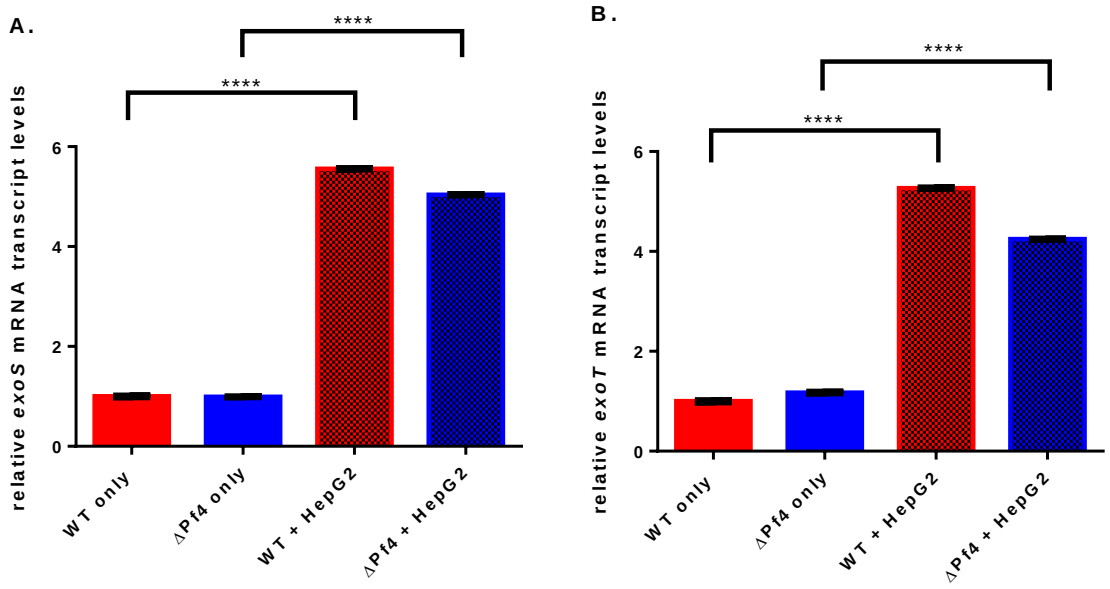

Figure 2.4 Relative expression of *exoS* and *exoT* in the presence and absence of HepG2 tissue culture cells. The ΔPf4 and WT PAO1 strains were incubated in 24 well plates for 4 h at 37 °C in the presence and absence of mammalian cells (HepG2). The supernatant was analysed for expression of *exoS* (A) and *exoT* (B) was determined by qPCR. Relative gene expression was determined by the $2^{\Delta\Delta Ct}$ method using the *proC* housekeeping gene as an internal control (314). Data were analysed using GraphPad Prism 5 (GraphPad Software Inc., U.S.A.) and statistical significance was determined via an unpaired, two-tailed, Student's t-test; n = 3. Data represent the mean value of 3 independent experiments with SD. Statistical significance, $p < 0.0001$, is indicated by ****.

2.3.5 Total protease and elastase activity of ΔPf4 mutant and wild-type *P. aeruginosa*

Protease production is an important virulence factor for many bacteria, including *P. aeruginosa*. Total protease and elastase activity was determined through incubation of the ΔPf4 mutant and wild-type *P. aeruginosa* supernatants with Hide-Remazol Brilliant Blue R and Elastin-Congo Red, respectively. The total protease activity of the wild-type *P. aeruginosa* was equal to that of the ΔPf4 mutant (Fig. 2.5 A). In contrast, the elastase activity of the ΔPf4 was slightly higher than that of the WT PAO1 (8 fold, $p = 0.01$) (Fig. 2.5 B).

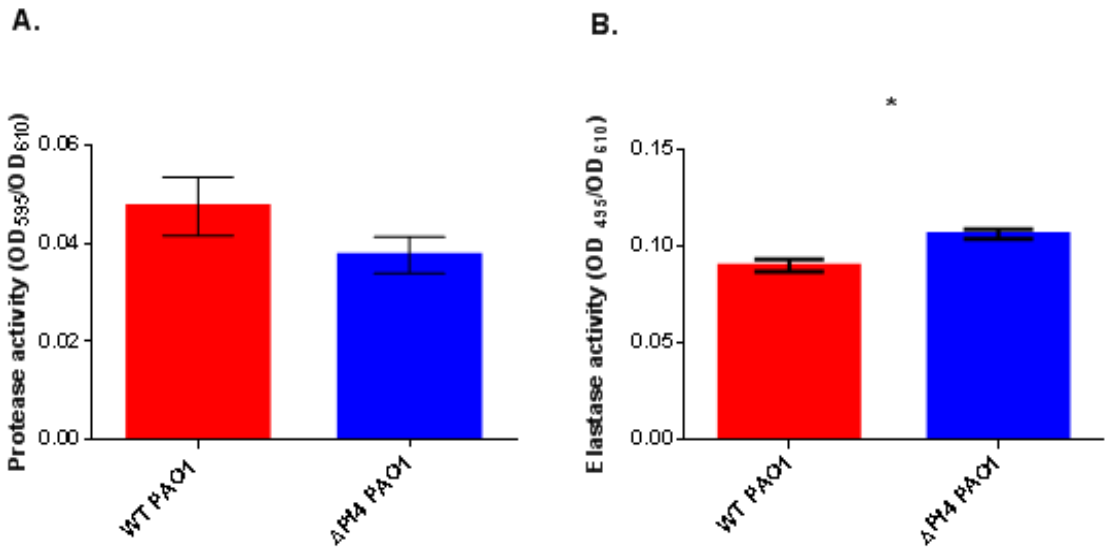

Figure 2.5 Total protease and elastase activity in the ΔPf4 mutant and wild-type *P. aeruginosa* strains. Cell-free supernatant was harvested from 22 h overnight cultures of ΔPf4 mutant and wild-type *P. aeruginosa* PAO1 for quantification of (A) total protease activity and (B) elastase activity. Total protease and elastase activity were quantified spectrophotometrically as protease and elastase per cell (OD_{595} / OD_{600} and OD_{495} / OD_{600}, respectively). Data was analysed using GraphPad Prism 5 and statistical significance was determined via an unpaired, two-tailed, Student's t-test; $n = 9$, $p < 0.05$ (indicated by *). Error bars represent SD.

2.3.6 The ΔPf4 mutant overexpresses pyoverdine

The siderophore pyoverdine is another key virulence trait of *P. aeruginosa* that was studied to determine the impact of the Pf4 phage on host virulence. Pyoverdine was observed to increase by approximately 10 fold for both strains. Interestingly, at 6 h,

corresponding to the early log stage of growth (OD_{600} = 0.1 – 0.15), pyoverdine production increased exponentially for both strains. During the mid-log growth stage (OD_{600} = 0.2 – 0.3), pyoverdine production by the wild-type ceased to increase further, whereas it continued to increase for the ΔPf4 strain until approximately 14 h. Further, the ΔPf4 mutant was observed to produce higher total levels of pyoverdine (OD_{450} = 2.69 x 10^6/ cell) than the wild-type (OD_{450} = 2.04 x 10^6/ cell), although this was not statistically significant (p = 0.29) (Fig. 2.6).

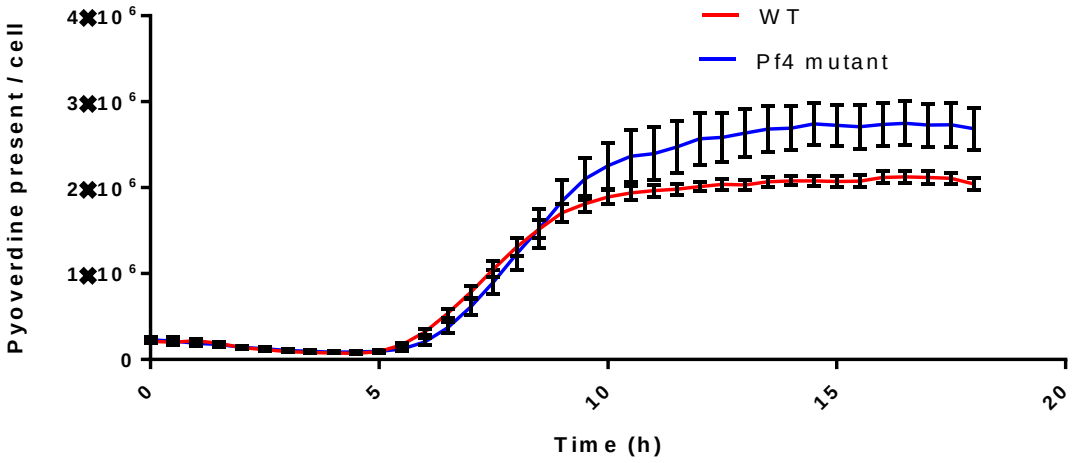

Figure 2.6 Pyoverdine production in the ΔPf4 mutant and wild-type *P. aeruginosa* strains. The ΔPf4 mutant and wild-type *P. aeruginosa* PAO1 strains were monitored over 18 h statically at room temperature in 96 well plates for the production of pyoverdine (excitation 390 nm, emission 460 nm) and cell density readings (OD_{600}). Data is presented as the pyoverdine normalised to cell density. Data was analysed using GraphPad Prism 5 and statistical significance was determined via an unpaired, two-tailed, Student's t-test; n = 9, $p < 0.01$. Error bars represent standard deviations.

2.4 Discussion

The Pf4 phage has been previously shown to be important for the acute virulence of *P. aeruginosa* PAO1 (253). However, the molecular basis for how the Pf4 phage contributes to acute virulence is currently unknown. The phage may mediate this effect either directly by encoding explicit virulence genes, or indirectly by altering the expression of host encoded virulence factors. An additional indirect mechanism could be attributed to changes in biofilm development observed for the ΔPf4 mutant relative to the wild-type strain. Therefore, experiments were undertaken to investigate the role of the Pf4 phage in biofilm related infections as well as its role in mediating the expression of key virulence factors of *P. aeruginosa*.

2.4.1 The effect of *P. aeruginosa* on slow and fast killing *Caenorhabditis elegans* models

C. elegans is a simple model that has been used extensively to study pathogenicity of bacteria including *P. aeruginosa* (306, 316, 317). Various mechanisms by which *P. aeruginosa* strains kill *C. elegans* have been identified. For example, it has been observed that *P. aeruginosa* PA14 can kill *C. elegans* via 'slow killing' or 'fast killing' (306). 'Slow killing' involves the accumulation (and hence, infection) of live PA14 in the *C. elegans* gut over several days, and 'fast killing' involves the exposure of *C. elegans* to diffusible toxins, such as phenazines, produced by the PA14 (306). In the case of *P. aeruginosa* PAO1, there is increasing evidence showing that fast killing correlates with an acute infection phenotype while slow killing mimics chronic, biofilm-associated infection phenotypes (308, 318, 319).

Surprisingly, there was no effect of the Pf4 phage on *P. aeruginosa* toxicity towards *C. elegans* in the slow or fast killing models. There was a slightly higher toxicity towards *C. elegans* in the slow killing model when the Pf4 phage was present in the wild type strain, but this was not a significant difference. This may suggest that the phage plays some role in this model, but is not the dominant factor involved in *C. elegans* killing.

2.4.2 Interaction of *P. aeruginosa* with tissue culture cell lines

The infectivity of *P. aeruginosa* has also been linked to its ability to invade or cause cytotoxicity to host cells and *P. aeruginosa* is known to infect a variety of cell types including corneal, lung, liver and kidney cells (11, 320-322). This study revealed that the Pf4 phage altered the invasiveness of *P. aeruginosa* for lung epithelial and liver cells. It has been shown that *P. aeruginosa* is able to infect the livers of female BALB/c mice after 6 h of the bacteria being intranasally introduced into the host and the role of *P. aeruginosa* in the colonisation and infection of lungs is well documented, especially in the case of infection of cystic fibrosis patients (323, 324).

In this study, it was observed that when the Pf4 phage was present, invasion of HepG2 cells by *P. aeruginosa* was significantly increased (Fig. 2.2A). In contrast, the wild-type was less invasive when the cystic fibrosis, CFTE29o- cells were used as a target. The results may indicate some degree of tissue tropism, although the overall invasion of the wild-type *P. aeruginosa* was similar for both mammalian cell types. It has been shown that some bacteria, such as *E. coli* and *Yersinia*, display altered cell tropism depending on host conditions. In *E. coli*, the FimH lectin of Type 1 fimbriae is an adhesive factor involved in the binding to host cells (322). FimH is known to undergo genetic variation to increase *E. coli* tissue tropism, assisting in the transition from a commensal to virulent phenotype (322). Additionally, in *Yersinia* the invasion of immune cells is temporally regulated (325, 326). *Yersinia* is able to cause apoptosis in naïve macrophages via the translocated effector, YopJ, which triggers a non-inflammatory response and enables the bacterium to avoid host phagocytes (327). However, in activated macrophages, *Yersinia* is able to cause cell death via the T3SS (and not YopJ) through inflammatory pyroptosis (325).

Therefore, it is possible that the presence of the Pf4 phage alters the tissue tropism of *P. aeruginosa*, thus affecting the ability of the bacterium to invade the particular cell type. Since the Pf4 phage invasion of HepG2 is higher relative to CFTE29o-, this could be due to the Pf4 phage regulating an adhesion factor or receptor that is required for the liver but not the lung. Therefore, it would be of interest to determine if the presence of the phage alters surface protein production, in particular those proteins that are involved in the invasion process, such as pili.

Another possible mechanism could involve the putative Zot-like toxin of the Pf4 phage. It is known that the Zot of *V. cholerae* opens intracellular tight junctions of the small intestine, increasing the permeability of the small intestinal mucosa (328). Since tight junctions join epithelial cells together and form a boundary between the apical and basolateral surfaces of the cells, compromised tight junctions result in the exposure of these basolateral surfaces (328). It has been shown that *P. aeruginosa* invasiveness is increased at exposed basolateral surfaces (328, 329). Therefore, the Zot-like toxin could be important for increasing basolateral cell surface vulnerability of HepG2 cells, thus increasing the ability of *P. aeruginosa* to invade host cells.

To further investigate the effect of the Pf4 on *P. aeruginosa*-host interaction, the cytotoxic effect of the ΔPf4 mutant and wild-type on HepG2 and CFTE29o- cells was studied. The study showed that there was no statistically significant effect of the Pf4 on cytotoxicity towards the HepG2 and CFTE29o- cells by *P. aeruginosa*. However, there was an overall trend of higher cytotoxicity in the HepG2 cells when the Pf4 was absent. If the Pf4 is truly more cytotoxic, the results would suggest that loss of the phage results in the upregulation of factors involved in mammalian cell lysis.

2.4.3 Influence of T3SS effectors on *P. aeruginosa* virulence in mammalian cells

Given that the Pf4 phage plays a role in virulence of *P. aeruginosa* in a murine pneumonia model, it was hypothesised that the Pf4 phage could possibly influence the expression of T3SS effectors, such as *exoT* and *exoS.* Since *exoT* and *exoS* are known to inhibit invasion of *P. aeruginosa* into mammalian cells, it was postulated that the Pf4 phage affects *exoT* and/or *exoS* gene expression. qPCR revealed that the levels of *exoS* and *exoT* mRNA were increased upon exposure to mammalian cells. This is in agreement with earlier studies that have detected an increased expression of T3SS effectors in the presence of Chinese hamster ovary (CHO) cells (330). However, the levels of *exoS* and *exoT* mRNA were not dependent on the Pf4 phage. The results suggest that any impact of the Pf4 phage on the expression of *exo*S and *exoT* does not involve transcriptional or post-transcriptional regulation. It remains possible that the phage impacts T3SS at a translational or posttranslational level and this could be tested through a proteomics or Western blotting based approach.

2.4.4 Protease production

Proteases are important virulence factors for *P. aeruginosa*, wherein they can damage host leukocytes, inhibit the function of different immune cells and destroy cytokines (331, 332). The overall protease production for both strains was similar and suggests that the phage does not impact total protease production. The *lasB* encoded elastase is known to degrade many components in the innate and adaptive immune systems (51). It was observed in this study that the elastolytic activity of the wild-type supernatant was significantly lower than that of the ΔPf4 mutant. Further experiments, such as qPCR on planktonic and biofilm ΔPf4 mutant and wild-type bacteria could help to clarify the influence of the Pf4 phage on *lasB* gene expression.

2.4.5 Pyoverdine production

It has been observed that, on solid agar, the ΔPf4 mutant appears to be more green than the wild-type (data not shown), which is a colour typically associated with pyoverdine production. Pyoverdine is known to be an important virulence factor for *P. aeruginosa*. In a further attempt to identify how the Pf4 phage is affecting *P. aeruginosa* virulence, pyoverdine (PVD) production in the ΔPf4 mutant and wild-type was studied. In this study, the ΔPf4 mutant produced higher levels of pyoverdine than the wild-type. Since PVD is a virulence factor, the results would suggest that the ΔPf4 mutant is more virulent than the wild-type, however the ΔPf4 mutant has previously been shown to be less virulent than the wild-type in an acute mouse infection model (253). It has been proposed that the early production of virulence factors during infection can elicit an immune response that results in the effective clearance of the pathogen before it can cause disease. For example, in *Pantoea stewartii* the quorum sensing receptor protein, EsaR, represses expression of an extracellular polysaccharide that functions as a virulence factor (333). The *esaR* mutant, which over produces the exopolysaccharide, was less virulent than the wild-type (333). This was thought to be due to early activation of the host innate immune response, which resulted in clearance of the *esaR* mutant (333). It has been suggested that pyoverdine acts as a signalling molecule and has the ability to control the expression of other virulence factors. It is possible therefore, that the higher production of pyoverdine could result in an early induction of the host immune response *in vivo*, to help clear the ΔPf4 mutant during infection. It would be particularly interesting to test the effect of

overexpressing pyoverdine in the wild-type *P. aeruginosa* to determine if this strain shows reduced virulence *in vivo* as was observed for the ΔPf4 mutant.

2.4.6 Conclusions

Based on the results presented here, there were no differences between the wild-type *P. aeruginosa* and the ΔPf4 mutant during infection of nematodes or mammalian tissue culture cells, despite there being a clear increase in pyoverdine production in the ΔPf4 mutant. These results suggest that overproduction of pyoverdine is not linked to reduced virulence of the ΔPf4 mutant observed during infection of the mouse lung (253). It is therefore possible that the host immune response plays an important role in the observation of differential pathogenicity of the wild-type and ΔPf4 mutant. For example, *C. elegans* lacks an adaptive immune system and relies solely on its innate immune function. Similarly, the host tissue culture cells lack an adaptive immune response. Therefore, it is possible that the ΔPf4 mutant, through increased production of pyoverdine, stimulates the adaptive immune response of the host, triggering the production of phagocytes, natural killer cells and basophils, as well as antibodies. Stimulation of this immune response in turn results in the more effective clearance of the ΔPf4 *in vivo*, resulting in the increased survival of infected mice.

3 The role of the Pf4 phage in mediating stress tolerance in biofilms of *Pseudomonas aeruginosa*

3.1 Introduction

Bacterial biofilms can increase the survival of the resident cells by up to 1000 times compared to their free living planktonic counterparts when exposed to a stress (76, 334). The increased protection of bacteria within a biofilm relative to planktonic cells has been attributed to a number of factors associated with biofilm development. One of these characteristics is the production of an exopolymeric substance (EPS) matrix, which surrounds the biofilm and is comprised of polysaccharides, proteins and extracelluar DNA (141, 335). The EPS matrix may increase antibiotic resistance by acting as a diffusion barrier or by specifically binding to antibiotics or sequestering cations (336).

Additionally, bacteria within a biofilm may be in a different physiological state to that of free-living bacteria (76, 337). Biofilm bacteria, especially those deep within microcolonies, have been shown to have reduced metabolism and may be in a starved or stationary phase (338). Some antibiotics, such as β-lactams, are only effective against rapidly dividing cells (339, 340). Thus, metabolic inactivity can contribute to the overall high level of antibiotic tolerance of biofilms. Another factor that plays an important role in stress adaptation of biofilms is the formation of morphotypic variants, which are commonly observed in the biofilm dispersal population of many bacterial species (341, 342). For example, it has been shown that biofilms of a *P. aeruginosa recA* mutant failed to produce morphotypic variants and this biofilm was highly sensitive to H_2O_2 stress (287). Thus, the genetic diversity of the morphotypic variants is thought to increase the resistance of the biofilm population.

The increased resistance of biofilms severely impacts human health by providing a reservoir for organisms to cause infections and by promoting bacterial persistence in spite of host immunity or antibiotic therapy (343, 344). The impact of biofilms in healthcare settings is detailed in Section 1.4. Most environmental isolates are as virulent as clinical isolates, and the majority of infections come from environmental sources (345). As such, it is of great interest to understand the ecological drivers for clinically relevant phenotypes such as the formation of resistant biofilms. For example, the clinically relevant phenotype

of biofilm formation is an important ecological adaptation that protects bacteria from a range of natural stresses such as UV exposure, oxidative stress and predation by protozoa (reviewed in (84)).

The grazing pressure of protozoa is thought to have driven the development of highly specific antipredator strategies such as biofilm formation and the production of secreted toxins that kill or deter predators (346). For example, biofilm formation has been shown to increase persistence of *P. aeruginosa* and *V. cholerae* when challenged by protozoan grazing in the environment (211). It seems possible that predation defence strategies could impact immune evasion because of the similarities between protozoa (e.g. amoeba) and professional phagocytes of the mammalian cellular immune system (e.g. macrophages) (347-349). As an example, some bacteria (e.g. *Salmonella* spp., *Mycobacterium avium*, *Listeria monocytogenes* and *Legionella pneumophilia*) have developed the ability to survive within protozoa following phagocytosis, and this also conferred the capability of surviving within macrophages (347, 350-352). Another factor involved in both protozoan grazing and mammalian cellular immunity is that bacteria must also be able to cope with high concentrations of reactive oxygen species (ROS) within phagolysosomes or released by and also within phagocytes (353).

When a professional phagocyte of the mammalian immune system encounters and phagocytoses a foreign substance, respiratory burst ensues (354). More detail of respiratory burst is provided in section 1.7.1. The formation of biofilms has been shown to be protective against the effects of oxidative stress. For example, the rate of PMN oxidative burst is reduced by 30 – 80% in response to biofilms compared to planktonic bacteria (355). It has also been shown that hydrogen peroxide is unable to fully penetrate a *P. aeruginosa* biofilm (188). Thus, the biofilm is generally protective from the host immune response as well as oxidative stress.

The Pf4 filamentous phage has been shown to play an important role in the entire biofilm development life-cycle in *P. aeruginosa*, where the phage deletion mutant is impaired in hollow colony formation, cell death, dispersal and variant formation (250, 253, 279). Additionally, it has been shown that the Pf4 phage increased *P. aeruginosa* biofilm stability in the presence of surfactant (sodium dodecyl sulphate) stress (253). While the phage clearly contributed to surfactant stress protection, it is unclear whether the Pf4 contributes specifically to surfactant stress, or has a role in the protection of *P. aeruginosa*

biofilms against other stresses. Given the general importance of the phage in biofilm development, it is likely that the Pf4 phage also plays an important role in a range of stress responses. Therefore, the present study aimed to determine the role of the Pf4 phage in providing stability to *P. aeruginosa* biofilms in the presence of environmental stresses. This was achieved by exposing the *P. aeruginosa* wild-type and Pf4 phage deletion strains to protozoan grazing, oxidative and antibiotic stress, and assessing biofilm biomass and bacterial survival before and after stress.

3.2 Materials and Methods

3.2.1 General Materials and Reagents

Unless otherwise stated, lysogeny broth (LB) agar plates (10 g/ L tryptone, 10 g/ L NaCl, 5 g/ L yeast extract, 15 g/ L agar) were used to maintain bacterial strains. To select for particular strains, various antibiotics were incorporated into the LB agar as needed. The bacterial strains used for the study were: *P. aeruginosa* wild-type PAO1 and *P. aeruginosa* PAO1 with the Pf4 prophage deleted (Gm^R) (ΔPf4) (253, 309). Bacterial cultures were grown in M9 minimal medium (4.76 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 0.86 mM NaCl, 1.85 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 11 mM glucose), pH 7, or LB agar and incubated overnight at 37 °C and shaking at 200 rpm (Ratek Instruments)

The protozoan strains used for this study were: *Tetrahymena pyriformis* (flagellate grazer) and *Acanthamoeba castellanii* (surface grazer) (351) (ATCC 30234). All protozoan cultures used were maintained in peptone yeast glucose (PYG) media and T25 cm^2 tissue culture flasks (Sarstedt, Germany). *A. castellanii* and *T. pyriformis* were grown up under static conditions at 30 °C and 22 °C respectively, prior to use.

Table 3.1 DNA primer sequences used in this study

Primer name	Sequence (5'- 3')	Reference
PA3064 F	CCTTCAGCCATCCGTTCTTCT	(356)
PA3064 R	TCGCGTACGAAGTCGACCTT	(356)
PA2231 F	AAGATCAAGAAACGCGTGGAAT	(357)
PA2231 R	TGTAGAGGTCGAACCACACCG	(357)

3.2.2 Selective grazing of *A. castellanii* on *P. aeruginosa* ΔPf4 mutant and wild-type PAO1

To compare the grazing resistance of ΔPf4 mutant and wild-type, and whether *A. castellanii* has a grazing preference for the wild-type or the ΔPf4 mutant, the selective grazing assay was conducted as previously described (351) with modifications. Cultures of ΔPf4 mutant and wild-type were grown for 18 h in complete M9 medium at 37 °C and 200 rpm (Ratek Instruments). After incubation, the cultures were adjusted to a final OD_{600} of 0.001 and were inoculated on 1% (w / v) PYG agar plates, 1 cm from the centre of the

agar plate outward. Experiments were replicated twice with three technical replicates per experiment (a total of six replicates per strain). Two millilitres of three-day grown *A. castellanii* was then centrifuged at 800*g*, the supernatant was removed and the pellet was resuspended in 2 mL PYG medium. Fifteen microlitres of the resuspended *A. castellanii* was then spotted at the centre of each plate (Fig. 3.1). The plates were then incubated at 23 °C for 16 d. Grazing was determined by measuring the distance of the grazing front of the bacterial streaks every 4 d. Grazing analysis was analysed in three independent experiments, with three biological replicates in triplicate for each condition per independent experiment.

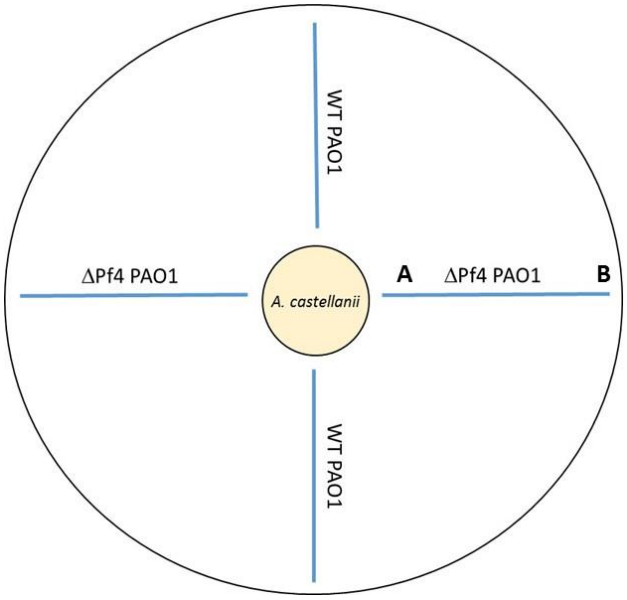

Figure 3.1 Selective grazing of *A. castellanii* on *P. aeruginosa* ΔPf4 mutant and wild-type PAO1. The bacteria (ΔPf4 mutant and wild-type) were streaked equidistant from the middle of the agar plate in duplicate and a small drop of *A. castellanii* was placed in the centre of the streaks. The grazing distance (from A to B) was determined at 4 d intervals for 16 d.

3.2.3 Grazing of *P. aeruginosa* ΔPf4 and WT PAO1 by protozoa

3.2.3.1 Preparation and cell counting of protozoa for experiment

Two days prior to inoculation with the bacteria, the protozoa were prepared as follows. Two and a half millilitres of *T. pyriformis* was transferred from the stock T25 cm^2 vented tissue culture flask (Sarstedt, Germany) containing PYG medium (maintained as

described in 3.2.1) to a T25 cm^2 vented tissue culture flask (Sarstedt, Germany) containing 7.5 mL of complete M9 medium and 1 mL heat killed *P. aeruginosa*. Heat killed *P. aeruginosa* were prepared by incubating the bacteria, grown to an OD_{600} of 1 at 65 °C for 1 h, with inversion of the tube containing the cells every 10 min to ensure even heat distribution. *T. pyriformis* was incubated for 2 d at 23 °C statically in preparation for the experiment. To determine the number of *T. pyriformis* present, a 300 µL aliquot was taken from the culture flask and mixed with Lugol's solution (Sigma-Aldrich, U.S.A.) to immobilise the protozoa for counting. The protozoa were counted using a haemocytometer (Neubauer, Germany) via light microscopy (Leica Camera AG, Germany).

A. castellanii was prepared by placing the stock T25 cm^2 vented tissue flask (Sarstedt, Germany) (which was maintained as described in 3.2.1) onto ice for 10 min to detach all of the protozoa from the monolayer formed into the PYG supernatant. The protozoa were then resuspended in the flask and a 2 mL aliquot was transferred from the stock flask into a new T25 cm^2 vented tissue culture flask containing 8 mL of fresh PYG medium. The flask was incubated for 2 d at 30 °C until ready to be used in the grazing experiments.

3.2.3.2 Grazing of early stage biofilms of **P. aeruginosa** *ΔPf4 and WT PAO1 by* **Acanthamoeba castellanii** *and* **Tetrahymena pyriformis**

P. aeruginosa ΔPf4 and WT PAO1 were grown overnight in complete M9 medium at 37 °C and 200 rpm. After 18 h, overnight cultures were adjusted to an OD_{600} of 0.01, as determined by a Novaspec III spectrophotometer (GE Healthcare Life Sciences, U.S.A). Protozoa numbers were determined (as described in 3.2.3i.) and adjusted to 1 x 10^4 cells/ mL. The bacteria and protozoa were added at a 1:1 volume ratio into 24 well, flat bottomed, non-tissue culture treated plates (Becton-Dickinson, U.S.A.) and co-incubated at 23 °C and 70 rpm for 3 d. After the 3 d incubation, the supernatant was removed from the biofilm and used to determine planktonic bacterial numbers. Bacterial numbers were determined via serial dilution and drop plating onto LB_{10} agar. The wells were then washed once with 1 mL of 1 x PBS and 1 mL of 0.2% (v / v) crystal violet (Becton-Dickinson) was added to each of the wells and the plate was incubated at 23 °C statically for 15 min. After incubation, the crystal violet was removed from the wells, and the wells were washed twice with 1 mL of 1 x PBS. After removal of the last 1 x PBS wash, 1 mL

of absolute ethanol was added to each of the wells. The plate was incubated at 23 °C and 100 rpm for 10 min. Biofilm biomass was determined by quantifying the amount of crystal violet spectrophotometrically at OD_{550} using a Wallac Victor^2 spectrophotometer (Perkin-Elmer, U.S.A). Analysis was conducted via three independent experiments, with three biological replicates in triplicate for each condition per independent experiment.

3.2.3.3 Grazing by Acanthamoeba castellanii *or* Tetrahymena pyriformis *of late stage biofilms formed by* P. aeruginosa

P. aeruginosa ΔPf4 and WT PAO1 were grown overnight in complete M9 medium at 37 °C and 200 rpm. After 18 h, 1 mL of the overnight cultures (diluted initially to an OD_{600} of 0.001) were inoculated into 24 well, non-tissue culture treated, flat-bottomed plates. The cells were inoculated for 3 d at 23 °C and 70 rpm, with exchange of medium each day for the duration of the experiment. Protozoa numbers were determined (as described in Section 3.2.3i.) and adjusted to 1 x 10^4 cells/ mL. After the spent medium was removed from the *P. aeruginosa* wells, 1 mL of protozoa was added to each well. The plates were incubated for a further 3 d at 23 °C and 70 rpm. The number of planktonic bacteria and biofilm biomass were determined as described in Section 3.2.3ii. Analysis was conducted via three independent experiments, with three biological replicates in triplicate for each condition per independent experiment.

3.2.4 Competition of *P. aeruginosa* ΔPf4 and WT PAO1 under *T. pyriformis* predation pressure

To determine whether the ΔPf4 mutant or wild-type strains have a competitive advantage over each other when exposed to predation pressure, a competition assay was conducted. *P. aeruginosa* ΔPf4 and WT PAO1 were grown overnight in complete M9 medium at 37 °C and shaking at 200 rpm. After 18 h, the overnight cultures were adjusted to a final OD_{600} of 0.01, as determined by a Novaspec II spectrophotometer (GE Healthcare Life Sciences, U.S.A). *T. pyriformis* numbers were determined (as described in 3.2.3i.) and adjusted to 1 x 10^4 cells/ mL. The ΔPf4 mutant, wild-type and *T. pyriformis* were added at a 1:1:1 volume ratio into 24 well, flat bottomed, non-tissue culture treated plates (Becton-Dickinson, U.S.A.) and co-incubated at 23 °C and 70 rpm for 3 d. After 3 d incubation, the supernatant was removed from the biofilm and bacterial numbers in the

planktonic and biofilm phases were quantified. Total ΔPf4 mutant and wild-type numbers were determined via serial dilution and drop plating onto LB_{10} agar to analyse CFU counts, and ΔPf4 mutant CFU counts were determined via plating on LB_{10} and Gm (40 µg/ mL) agar plates. The wild-type CFU counts were calculated by subtracting the ΔPf4 mutant CFUs from the total cell counts. Analysis was conducted via three independent experiments, with three biological replicates in triplicate for each condition per independent experiment.

3.2.5 Comparison of the effect of oxidative stress on *P. aeruginosa* ΔPf4 and WT PAO1 biofilms

P. aeruginosa ΔPf4 and WT PAO1 were grown overnight in LB_{10} medium at 37 °C and 200 rpm. After 18 h, the cultures were washed twice in 1 x PBS and added into 24 well, non-tissue culture treated, flat-bottomed plates (Corning) at a 1:200 dilution in a total of 1 mL complete M9/ well. The plates were incubated with shaking at 200 rpm and 37 °C for 6 h. After 6 h incubation, the spent medium was removed and fresh M9 medium was added to the wells with various concentrations of hydrogen peroxide or paraquat (0 mM, 0.3 mM, 0.5 mM, 0.7 mM and 1 mM). The plates were incubated for a further 1 h at which time, the spent medium was removed from the wells and the biofilms were washed once with 1 x PBS. The plates were then sonicated in a water bath 100 W, 40 kHz (Unisonics Australia, Australia) for 10 min to break up the biofilm. After sonication, the supernatant was resuspended in each well to determine CFUs using the drop plate method (358). The percentage of viability was determined by:

$$((\text{CFU / mL}_{\text{treated}}) / (\text{CFU / mL}_{\text{untreated}}) \times 100).$$

Analysis was conducted via three independent experiments, with three biological replicates in triplicate for each condition per independent experiment.

3.2.6 Effect of antibiotic stress on *P. aeruginosa* ΔPf4 and WT PAO1

P. aeruginosa ΔPf4 and WT PAO1 were grown and inoculated as described in Section 3.2.5. After the 6 h incubation, either ciprofloxacin, colistin or tobramycin was added to the wells (at a final concentration of 0, 10, 20 or 50 µg/ mL) and the plates were incubated for a further 1 h. The spent media was subsequently removed, the wells were washed once

with 1 x PBS and the plates were sonicated in a water bath at 100 W, 40 kHz (Unisonics Australia, Australia) for 10 min. The resuspended biofilms were serially diluted to quantify CFUs using the drop plate method (358) on LB_{10} agar plates 37 °C overnight. Three independent experiments were conducted with all strain, with three biological replicates in triplicate for each condition per independent experiment.

3.3 Results

3.3.1 Selective grazing resistance of *P. aeruginosa* ΔPf4 and WT PAO1 by *A. castellanii*

Predation by protozoa is a key driving factor for the diversification of bacterial characteristics and is thought to select for anti-predation bacterial traits (359). To determine the effect of the Pf4 phage on the grazing resistance of *P. aeruginosa* and if the protozoa had a feeding preference, a selective grazing assay with *A. castellanii* and *P. aeruginosa* ΔPf4 mutant and wild-type was performed (Fig. 3.1). The grazing zone for both *P. aeruginosa* strains steadily increased by 20 – 30% every 4 d over the 16 d of the experiment. However, there was no significant difference between the grazing resistance and preference for the two bacterial strains (p = 0.99) (Fig. 3.2).

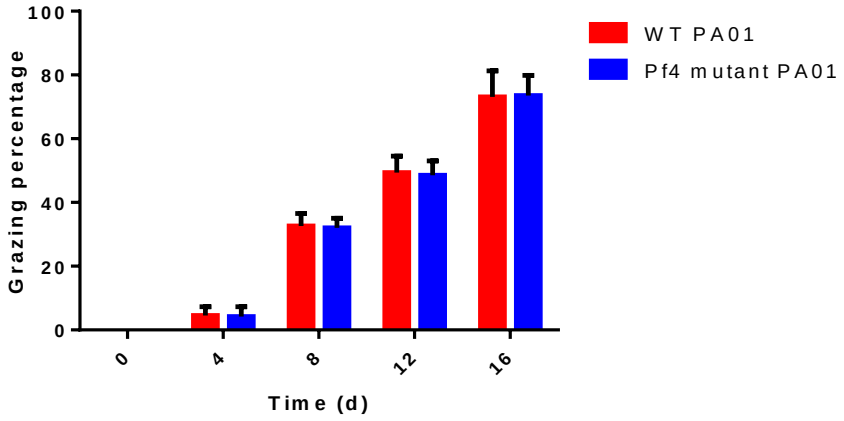

Figure 3.2 The impact of grazing of *A. castellanii* on *P. aeruginosa* ΔPf4 and WT PAO1. The grazing of *P. aeruginosa* strains by *A. castellanii* was observed over the course of 16 d on LB_{10} agar plates. This was determined by measuring the grazing distance from the start of the line where the protozoa was placed to the nearest edges of the bacterial streak. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc.), with a Student's t-test (n = 9, $p < 0.01$). Error bars represent SD.

3.3.2 The effect of *T. pyriformis* and *A. castellanii* grazing on *P. aeruginosa* early stage biofilms

To determine the effect of the Pf4 phage on *P. aeruginosa* viability and early biofilm formation in the presence of grazing stress, the wild-type and ΔPf4 mutant cultures were inoculated with the flagellate grazer, *T. pyriformis* or the surface grazer, *A. castellanii* for 3 d, and planktonic bacterial cell numbers and biofilm biomass were quantified.

After exposure to *T. pyriformis*, both strains had significantly increased biofilm biomass (Fig. 3.2A) ($p = 0.0003$ and $p = 0.0001$ for wild-type and ΔPf4 mutant, respectively), with the wild-type and ΔPf4 mutant biomass being 1.54 fold and 1.38 fold more after grazing, respectively. With respect to the planktonic cells (Fig. 3.2B), both strains had a significant decrease in CFU/ mL after *T. pyriformis* grazing (by approximately 1×10^4 cells/ mL for both strains). However, there was no significant difference for the biomass between the two strains after grazing stress.

When the ΔPf4 mutant and wild-type were exposed to *A. castellanii*, a significant decrease in the biofilm biomass was observed for both strains ($p = 0.003$ and $p = 0.008$ for wild-type and ΔPf4 mutant, respectively) (Fig. 3.2C). However, both strains were reduced approximately equally, suggesting there was no difference in sensitivity between the two strains under grazing pressure (78% and 79% of the biomass retained for the wild-type and ΔPf4 mutant, respectively). After *A. castellanii* grazing, the planktonic bacterial numbers significantly increased for both the WT and ΔPf4 PAO1 (3.08 times, $p = 0.001$ and 5.01 times, $p = 0.0001$, for wild-type and ΔPf4 mutant, respectively) (Fig. 3.2D). However, there was no significant difference between the two strains.

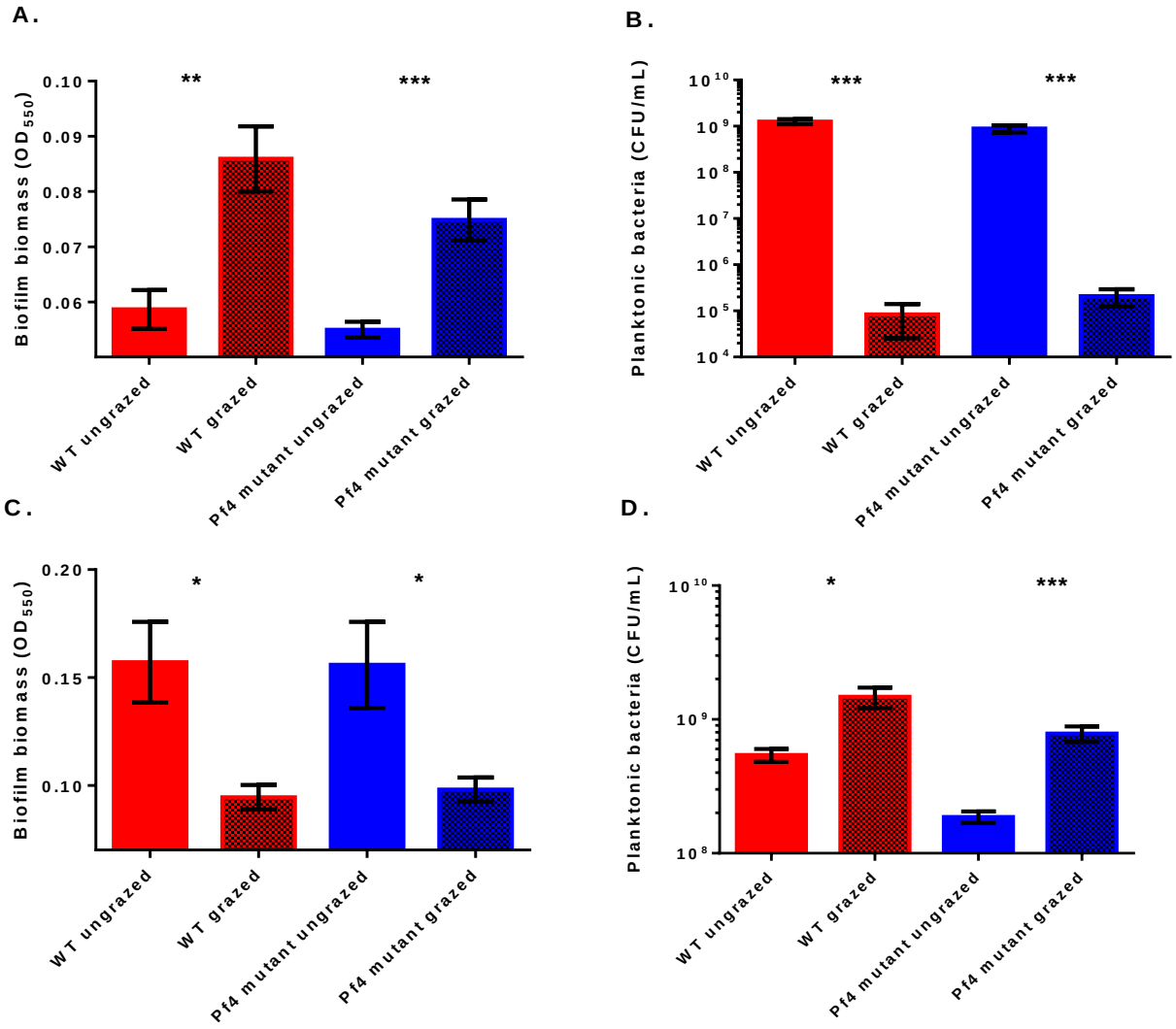

Figure 3.3 Effect of predation by *T. pyriformis* or *A. castellanii* on early biofilms of the ΔPf4 mutant and wild-type. Bacteria were co-incubated with *T. pyriformis* (A and B) and *A. castellanii* (C and D) for 3 d at room temperature. The data are from three separate experiments, with four biological replicates and eight technical replicates per experiment. After incubation, the biofilm biomass was quantified by crystal violet staining (A and C) and bacterial CFUs in the planktonic phase were also enumerated (B and D). Data were analysed using GraphPad Prism 5 and statistical significance was determined via an unpaired, two-tailed, Student's t-test; n = 9, $p < 0.001$, $p < 0.0005$ and $p < 0.0001$ are indicated by *, ** or ***, respectively. Error bars represent the SD.

3.3.3 *T. pyriformis* and *A. castellanii* grazing on mature biofilms of *P. aeruginosa*

To determine the influence of the Pf4 phage on *P. aeruginosa* viability and biofilm biomass in the presence of grazing stress for established biofilms, preformed, three day old biofilms of ΔPf4 and WT *P. aeruginosa* PAO1 were inoculated with *T. pyriformis* or *A. castellanii* for a further three days, and the biofilm biomass and planktonic CFU counts after grazing exposure were quantified.

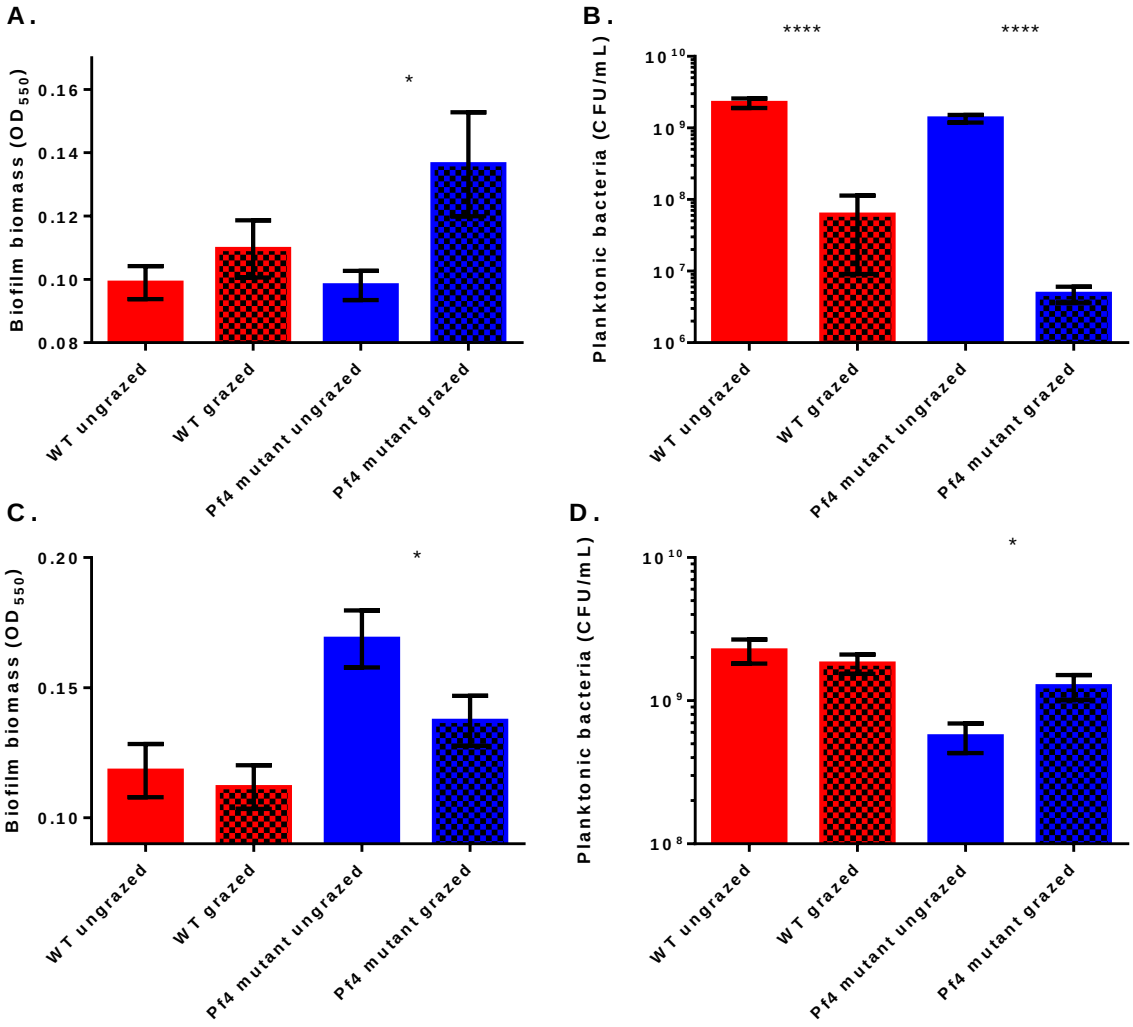

Figure 3.4 Predation by *T. pyriformis* or *A. castellanii* on late biofilms of the ΔPf4 and WT PAO1. Three day old *P. aeruginosa* biofilms were incubated for a further 3 d with *T. pyriformis* (A and B) or *A. castellanii* (C and D) and evaluated for biofilm biomass (A and C) and planktonic bacterial numbers (B and D) with CV staining and CFU counts, respectively. Data were analysed using GraphPad Prism 5 and statistical significance was determined via an unpaired, two-tailed, Student's t-test; n = 9, * and **** represent $p < 0.05$ and $p < 0.0001$, respectively. Error bars represent the SD.

After *T. pyriformis* grazing, the biofilm biomass for the wild-type showed no significant change (p = 0.31). However, the ΔPf4 mutant biofilm biomass significantly increased after grazing (1.37 fold, p = 0.03). The ΔPf4 mutant biomass was 1.24 times higher than the wild-type biomass after *T. pyriformis* grazing, but this was not a statistically significant difference (Fig. 3.4A). However, *T. pyriformis* grazing stress resulted in a significant decrease of planktonic numbers for both the ΔPf4 mutant and wild-type (0.004 fold, p = 0.0001 and 0.02, p = 0.0001 for the ΔPf4 mutant and wild-type, respectively) (Fig. 3.4B). In contrast, predation by *A. castellanii* resulted in a significant decrease of the ΔPf4 biofilm biomass (0.81 fold, p = 0.04), whereas the wild-type biomass remained unchanged after grazing (Fig. 3.4C). The planktonic bacterial numbers for the ΔPf4 PAO1 significantly increased after grazing (2.24 fold, p = 0.02), whereas the wild-type numbers slightly decreased (0.81 fold) (Fig. 3.4D).

3.3.4 Competition between *P. aeruginosa* ΔPf4 and WT PAO1 during biofilm formation under *T. pyriformis* grazing stress

In order to observe how the Pf4 phage affects the competitiveness of *P. aeruginosa* during predation by the flagellate *T. pyriformis*, the ΔPf4 mutant and wild-type strains were co-incubated with *T. pyriformis* for 3 d, and analysed for planktonic (Fig. 3.5A) and biofilm (Fig. 3.5B) cell numbers. It was found that after co-incubation of the two strains for 3 d, the wild-type planktonic and biofilm (ungrazed) cell numbers were significantly higher (p = 0.0001) than the ΔPf4 mutant cell numbers (approximately 95% for wild-type and 5% for ΔPf4 mutant for both planktonic and biofilm cells, respectively) as a percentage of the total mixed culture (ΔPf4 mutant and wild-type). However, when the two strains were grown separately for 3 d, the cell numbers were similar (0.2 fold difference) (data not shown). After grazing, it was observed in both the planktonic phase and biofilm that the wild-type retained nearly 100% cell viability (approximately 3% and 5% decrease for planktonic and biofilm cells, respectively), where the ΔPf4 mutant had a slight increase in cell viability after protozoan grazing (approximately 3% and 8% for the planktonic and biofilm cells, respectively). The differences observed between the strains for both ungrazed and grazed planktonic and biofilm cell numbers were statistically significant (p = 0.0001 and p = 0.0017 for the ungrazed and grazed planktonic numbers, respectively and p = 0.0001 for both the ungrazed and grazed biofilm numbers). However, the cell

number differences before and after grazing for each strain were not of statistical significance.

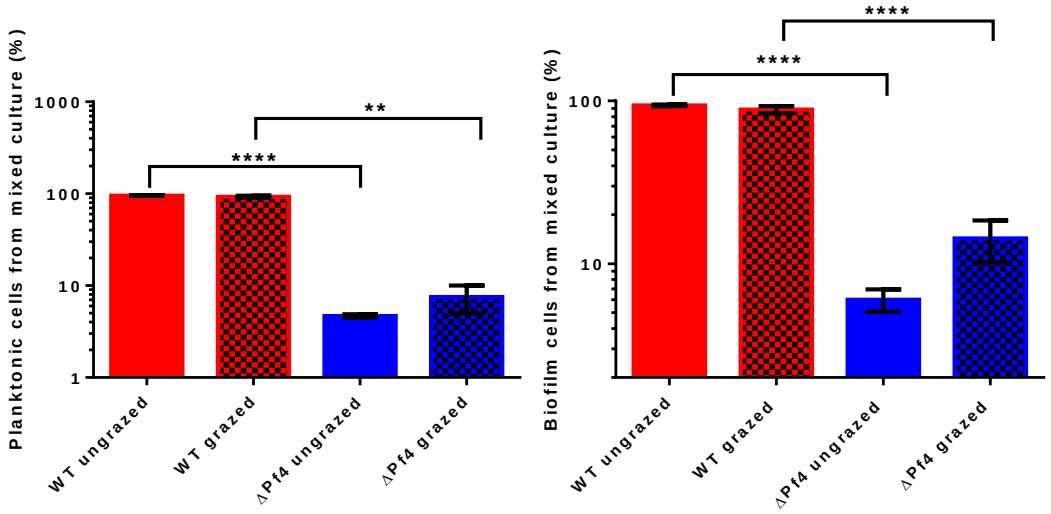

Figure 3.5 Competition of wild-type *P. aeruginosa* and the ΔPf4 mutant in the presence of *T. pyriformis*. ΔPf4 mutant and wild-type *P. aeruginosa* biofilms were co-incubated with *T. pyriformis* in 24 well plates for 3 d at room temperature and the cell numbers for planktonic (A) and biofilm (B) cultures in relation to total mixed culture (ΔPf4 mutant / wild-type) were determined by CFU counts and CV staining, respectively. Data were analysed using GraphPad Prism 5 and statistical significance was determined via an unpaired, two-tailed, Student's t-test; n = 9, ** and **** represent $p < 0.05$ and $p < 0.0001$, respectively. Error bars represent the SD.

3.3.5 Comparison of the effect of oxidative stress on *P. aeruginosa* ΔPf4 and WT PAO1 biofilms

Oxidative stress, such as reactive oxygen species produced by the host immune system has the potential to reduce the viability of *P. aeruginosa* biofilms. To determine if the Pf4 phage contributes to the oxidative stress resistance of *P. aeruginosa* biofilms, both strains were exposed to a range of concentrations of H_2O_2 and paraquat. It was found that biofilms of both strains were significantly reduced upon H_2O_2 exposure (31% and 30% for ΔPf4 mutant and wild-type, respectively), but there was no significant difference between the response of the two strains to H_2O_2 ($p = 0.79$) (Fig. 3.6A). The reduction in biofilm biomass, as determined by crystal violet staining, was the same irrespective of the concentration of H_2O_2 used. While there was only a small effect on the total biofilm biomass, H_2O_2 treatment resulted in 80% - 90% reduction in CFUs for the wild type strain.

Similarly, the total CFUs were also reduced for the ΔPf4 mutant strain upon H_2O_2 exposure, although the ΔPf4 mutant strain was significantly less sensitive to 0.5 mM and 0.7 mM than the wild-type (P = 0.003 and P = 0.011, respectively), showing approximately 60% and 80% reduction at both 0.5 mM and 0.7 mM H_2O_2, respectively (Fig. 3.6B). Interestingly, there was a 3 fold decrease in cell numbers when the ΔPf4 mutant was exposed to 0.7 mM H_2O_2 compared to 0.5 mM H_2O_2 which was similar to the effect on the wild-type strain for all concentrations of H_2O_2 tested.

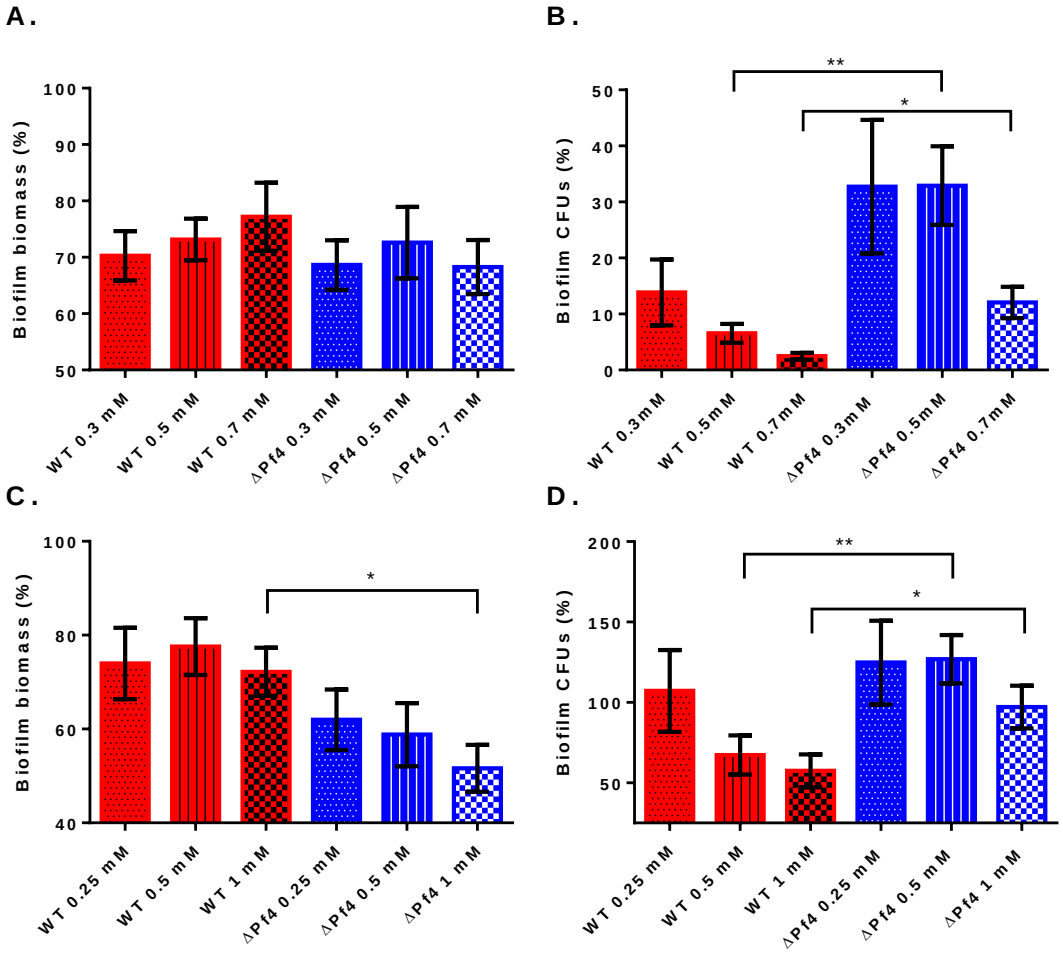

Figure 3.6 Comparison of the effect of oxidative stress on *P. aeruginosa* ΔPf4 and WT PAO1 biofilms. Six-hour grown ΔPf4 mutant and wild-type *P. aeruginosa* biofilms were exposed to varying concentrations of hydrogen peroxide (A and B) and paraquat (C and D) for 1 h at 37 °C. Post treatment, the strains were quantified for the reduction of biofilm biomass (A and C) and cell numbers (B and D) by CFU counts and CV staining, respectively. Data were analysed using GraphPad Prism 5 and statistical significance was determined via an unpaired, two-tailed, Student's t-test; n = 9, * and ** represent $p < 0.05$ and $p < 0.01$, respectively. Error bars represent the SD.

When exposed to paraquat stress, the wild-type biofilm was reduced by approximately 20 – 25% for all concentrations tested and there was no apparent effect of increasing concentration. In contrast, the ΔPf4 mutant showed a 35% reduction in biofilm biomass at 0.25 mM paraquat and was significantly more sensitive to 1 mM paraquat in comparison to the wild-type (P = 0.011) with a 65% reduction in biomass (Fig. 3.6C). Overall, there was no reduction in viable cell counts (CFU/ mL) for the ΔPf4 mutant when exposed to 0.25 mM – 1 mM paraquat treatment, whereas the wild-type showed significantly higher sensitivity to 0.5 mM and 1 mM paraquat than the wild-type (P = 0.008 and P = 0.035, respectively), with reductions of 40% and 45% in CFUs for 0.5 mM and 1 mM paraquat exposure, respectively (Fig. 3.6D).

3.3.6 Comparison of the effect of antibiotics on *P. aeruginosa* ΔPf4 and WT PAO1 biofilms

Antibiotic therapies have been widely used in an attempt to eliminate *P. aeruginosa* biofilm infections in human hosts. To assess if the Pf4 phage has an effect on mediating the antibiotic resistance of *P. aeruginosa* biofilms, 6 h pre-grown biofilms were exposed to varying concentrations of ciprofloxacin, tobramycin and colistin. The biofilms were then assessed for total cell numbers before and after antibiotic treatment to determine the percentage survival.

Upon ciprofloxacin treatment, biofilm cell numbers for both strains decreased proportionately as the concentration of ciprofloxacin increased (Fig. 3.7A). Interestingly, at 50 µg/ mL ciprofloxacin, viable wild type cells could still be cultured (approximately 0.00001% of untreated cell total). The ΔPf4 mutant showed similar reductions for 10 and 20 µg/ mL ciprofloxacin, while there were no detectable viable cells for the mutant at 50 µg/ mL (p = 0.03). When the strains were exposed to tobramycin, the cell numbers for both the ΔPf4 mutant and wild-type showed similar decreases in viable cell counts as the tobramycin concentration increased (Fig. 3.7 B). When exposed to colistin stress up to 50 µg/ mL, both the ΔPf4 mutant and wild-type cell numbers were relatively unchanged compared to the untreated controls and there was no difference in response to colistin between the two strains (Fig. 3.7 C).

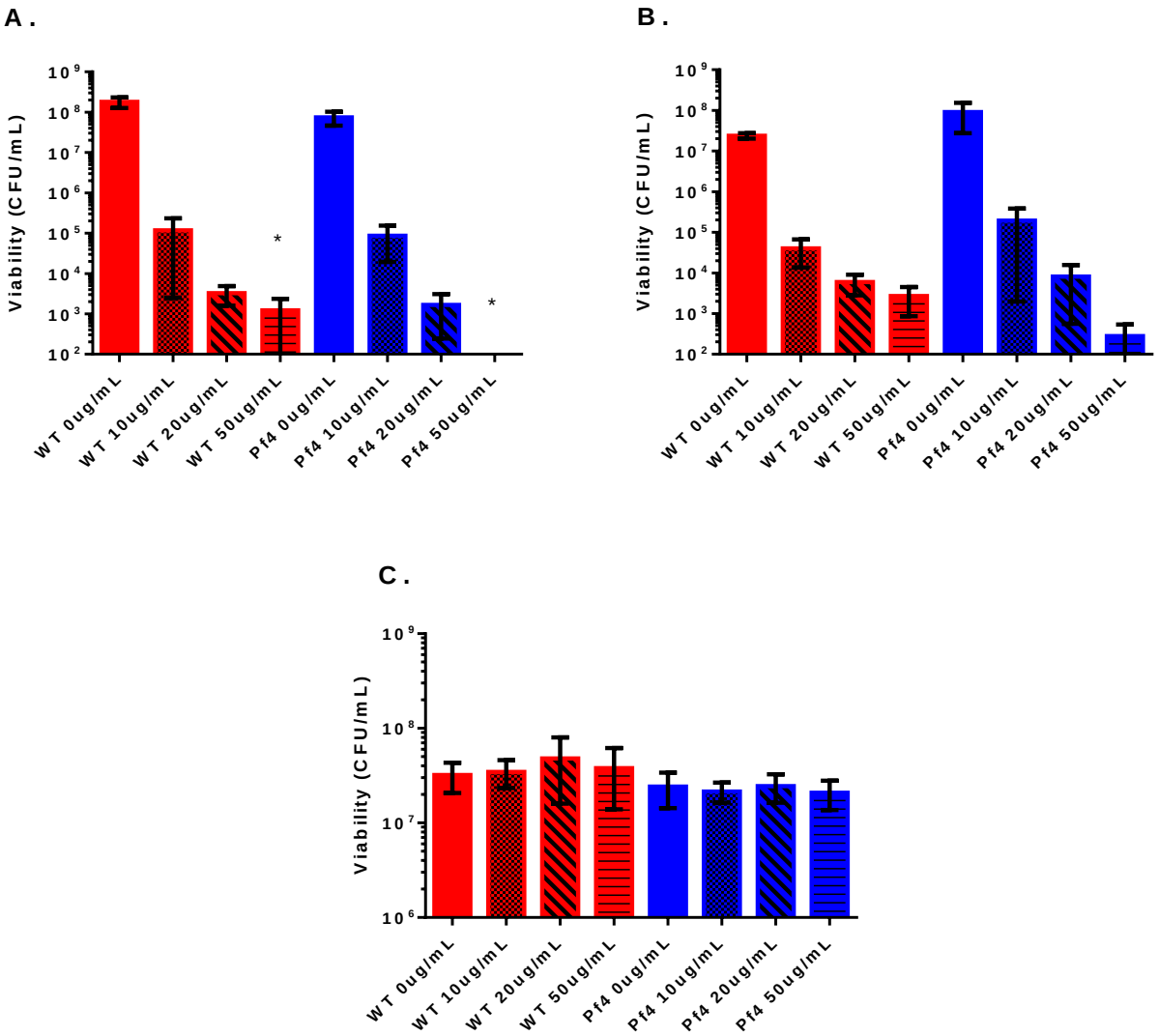

Figure 3.7 Comparison of the effect of antibiotics on *P. aeruginosa* ΔPf4 and WT PAO1 biofilms. Biofilms of the ΔPf4 mutant and wild-type strains of *P. aeruginosa* were pre-grown for 6 h before exposure to varying concentrations of ciprofloxacin (A), tobramycin (B) and colistin (C) for 1 h at 37 °C. Cell numbers for the exposed and control biofilms were compared to determine the percentage survival. Data were analysed using GraphPad Prism 5 and statistical significance was determined via an unpaired, two-tailed, Student's t-test; n = 9, * represents $p < 0.05$. Error bars represent the SD.

3.4 Discussion

Previously, the Pf4 phage has been found to increase the stability of *P. aeruginosa* biofilms in the presence of sodium dodecyl sulphate (surfactant) stress (253). Given the overall role of the Pf4 in biofilm development of *P. aeruginosa*, it is likely that the phage contributes more generally to the stress adaptation and tolerance of *P. aeruginosa* biofilms. Therefore, studies were undertaken to determine the role of the Pf4 phage in the protection of *P. aeruginosa* biofilms when exposed to predation pressure as well as oxidative and antibiotic stresses.

3.4.1 *T. pyriformis* and *A. castellanii* grazing on *P. aeruginosa* early and late stage biofilm stability

Grazing by heterotrophic protists, commonly known as protozoa, is a driving force for bacterial evolution and adaptation (360), since predation by protozoa is a major cause of bacterial mortality in the environment (361). As a consequence, such selection pressure would be expected to result in the development of anti-predator defences that enable bacteria to survive such intense grazing pressure. Given the similarities between some predators and human immune cells, it is also possible that mechanisms associated with virulence towards mammalian hosts are derived from predation defences (359). The Pf4 phage has been shown to contribute to virulence factor expression (Chapter 2), as well as biofilm stability (253). Therefore, the role of the phage in general stress adaptation was tested here. To determine the effect of grazing pressure on *P. aeruginosa*, two protozoa were used in this study; *Tetrahymena pyriformis*, a free-living ciliate protozoa that feeds on planktonic and biofilm bacterial cells and *Acanthamoeba castellanii*, a surface grazer that specialises in grazing on biofilm or surface-attached bacterial cells (362).

The response of the two bacterial strains to predation pressure was consistent with the feeding type of the predators used. For example, when exposed to the planktonic predator, *T. pyriformis*, there was a clear reduction in planktonic cells and a concomitant increase in biofilm biomass (Figs. 3.3 and 3.4 B). Similarly, when exposure to the biofilm feeding specialist, *A. castellanii*, the biofilm biomass was strongly reduced for both the wild type and the ΔPf4 mutant, while there was a corresponding increase in the number of planktonic cells. The grazing behaviour of *T. pyriformis* and *A. castellanii* in this study correlates with the previous observations that filter feeders such as *T. pyriformis* prefer to

graze on planktonic bacteria and are generally unable to ingest large aggregates of bacteria (198, 361, 363). *A. castellanii* is able to graze upon larger bacteria and biofilms due to its large size and ability to graze surfaces using its pseudopodia (198).

When the grazing responses for the individual *P. aeruginosa* strains were compared, there was no obvious effect of the Pf4 phage on grazing resistance or feeding preference for early-stage *P. aeruginosa* biofilms and planktonic cells when exposed to either *T. pyriformis* or *A. castellanii*. However, while there was no difference between the wild type *P. aeruginosa* and the ΔPf4 mutant for late stage biofilms in the presence of *T. pyriformis*, it was observed that the presence of the Pf4 prophage increased the predation resistance of the wild type *P. aeruginosa* for late stage biofilms when subjected to *A. castellanii* predation pressure. It has previously been observed that *P. aeruginosa* biofilms can show increased resistance as the biofilms mature, when exposed to biofilm specialists such as *Rhynchomonas nasuta* and *Acanthamoeba polyphaga*, and this resistance was linked to the formation of microcolonies (210, 362). Interestingly, the presence of the Pf4 phage in the wild-type has previously been linked to increased microcolony size and frequency in *P. aeruginosa* biofilms (250). Since microcolony formation is associated with EPS production, it would be interesting to determine if the Pf4 phage regulates the production of EPS components, such as alginate, Psl or Pel, as well as the incorporation of eDNA into the biofilm matrix (364).

Alternatively, it is possible that the Pf4 phage increases toxicity of *P. aeruginosa* late biofilms towards *A. castellanii*. It has been shown previously that the quorum sensing regulators *rhlR* and *lasR* are upregulated in late biofilms, resulting in increased toxicity towards protozoa and higher grazing resistance (210). However, this quorum sensing induced toxicity was not observed in *P. aeruginosa* early biofilms (210). This could explain why the effect of the Pf4 phage on grazing resistance is only observed in late biofilms and experiments designed to determine if the Pf4 phage plays a role in controlling the quorum sensing behaviour of *P. aeruginosa* could be undertaken to clarify this potential mechanism.

3.4.2 The competition of ΔPf4 mutant and wild-type *P. aeruginosa* early stage biofilms under stress

To determine whether the wild-type *P. aeruginosa* had an increased fitness relative to the ΔPf4 mutant, the two strains were co-cultivated during biofilm development in the presence or absence of the predator, *T. pyriformis*. The results indicated that there was no selective grazing (Fig. 3.5). Interestingly, even in the absence of the predator, the relative abundance of the ΔPf4 mutant was significantly reduced. Since both strains form similar amounts of biofilm biomass when cultivated as monospecies biofilms (data not shown), the differences in cell numbers observed here is most likely due to direct competition between the two strains. This competition could be the consequence of the change in the biofilm life-cycle observed for the ΔPf4 mutant, which does not make large microcolonies in late stage biofilms. Furthermore, the biofilms of the ΔPf4 mutant are less stable than the wild type. An alternative explanation could be related to the sensitivity of the ΔPf4 mutant to infection by the Pf4 phage, which could result in lysis of the mutant. Therefore, the wild type may outcompete the ΔPf4 mutant by killing the phage mutant through reinfection by the superinfective phage, which occurs during late biofilm development and has been shown to result in killing of biofilm cells via superinfection. In this way, the presence of the prophage would be a selective advantage when grown in the presence of *P. aeruginosa* strains that lack the phage.

3.4.3 The effect of oxidative stress on *P. aeruginosa* biofilms

Another major environmental stressor encountered by *P. aeruginosa* is oxidative stress. *P. aeruginosa* grows optimally under aerobic conditions, using oxygen to produce energy via oxidative phosphorylation, resulting in the production of reactive oxygen species (ROS). In addition, the host immune response is a key contributor of oxidative stress, which, upon contact with bacteria, upregulates macrophage and leukocyte production, which ultimately results in respiratory burst and release of antimicrobial agents such as superoxide, hydrogen peroxide, hydroxyl radicals and hypochlorous acid into the environment (365). To counter oxidative stress, bacteria can grow as a biofilm, which has been shown to increase their resistance to oxidative stress relative to planktonic bacteria (188). Interestingly, it has been shown that bacteriophage can provide additional defense against oxidative stress. For example, in *Salmonella enterica*, it has been observed that the *Gifsy*-1 and *Gifsy*-2 phage encode a superoxide dismutase gene that is involved in

protecting the bacterium from macrophage oxidative burst (366-368). The results presented here indicated that the wild-type *P. aeruginosa* was more resistant to paraquat-induced oxidative stress than the ΔPf4. When exposed to H_2O_2, the Pf4 phage seemed to have no effect on biofilm biomass. However, the decreased biofilm cell viability observed when the Pf4 phage was present in the presence of H_2O_2, suggested that the cells could still be attached as a biofilm, but experiencing sub-lethal stress, whereby the bacteria were unable to grow post treatment.

Paraquat catalyses the formation of the superoxide (O_2^-) free radical and subsequently, H_2O_2 or a hydroxyl radical ($OH^\bullet$) is formed as part of the O_2^- detoxification pathway (369). Therefore, the difference in protection between paraquat and H_2O_2 may indicate that the phage is important for protection from O_2^- or hydroxyl radicals and does not play a role in H_2O_2 protection. This specificity in oxidative stress response could be mediated through several pathways. *P. aeruginosa* has two superoxide dismutases (SODs) known as iron-cofactored superoxide dismutase (Fe-SOD) and manganese-cofactored superoxide dismutase (Mn-SOD), which serve to catalyse the dismutation of superoxide into either molecular oxygen or hydrogen peroxide (196). In particular, Fe-SOD is thought to be more important for aerobic growth and survival and protection of the biofilm against paraquat stress (196). When *P. aeruginosa* wild-type biofilms are exposed to superoxide, *sodB* gene (encodes for Fe-SOD) expression increases (196). This results in the superoxide being dismutated to O_2^-, which induces *P. aeruginosa* catalase activity (through *katA* expression) (196). However, since the results suggested that the Pf4 phage contributed to biofilm resistance in response to paraquat, but not hydrogen peroxide stress, it is thought that the phage is most likely involved in protection against superoxide, but not hydroxyl radical stress. Therefore, the Pf4 phage could contribute to increased *sodB* expression and thus, Fe-SOD activity as a means to protect the *P. aeruginosa* biofilm from oxidase expression.

3.4.4 The effect of antibiotic stress on *P. aeruginosa* biofilms

One of the best recognised biofilm phenotypes is increased antibiotic resistance. This is typically attributed to biofilm specific physiology that involves changes in gene expression such as efflux pumps, and the physical effects of the biofilm matrix (reviewed in (370)). To determine if the Pf4 phage also contributed to the antibiotic resistance

phenotype, biofilms of *P. aeruginosa* and the ΔPf4 mutant were tested for sensitivity to the antibiotics tobramycin, ciprofloxacin and colistin. These three antibiotics are clinically relevant and are known to have significantly higher minimal bactericidal concentrations (MBC) for biofilms compared to planktonic cells (371, 372). There was no effect of the Pf4 on biofilm resistance to colistin or tobramycin. However, the Pf4 phage significantly increased *P. aeruginosa* biofilm resistance in the presence of ciprofloxacin.

Although ciprofloxacin is used regularly in combination with other antibiotics such as inhaled tobramycin or colistin for the treatment of *P. aeruginosa* infections, ciprofloxacin as a sole treatment for *P. aeruginosa* biofilms is not highly effective. For example, mucoid *P. aeruginosa* isolates in bronchiectasis patients have MICs as high as 64 μg/mL (373). Furthermore, another study was conducted with 304 children aged 1 to 12 years old suffering from CF to determine the effect of ciprofloxacin in preventing *P. aeruginosa* lung infection (374). The patients received either a treatment of tobramycin, or a combination of tobramycin and ciprofloxacin every 3 months, or for 18 months when *P. aeruginosa* was detected in the patient (374). It was observed at 70 months that there was no difference in the prevention of *P. aeruginosa* infection or incidence of pulmonary exacerbations in the patients, regardless of whether they were administered with tobramycin alone, or with tobramycin and ciprofloxacin (374). Thus, it is suggested that wild-type *P. aeruginosa* PAO1 has a mechanism of resistance against ciprofloxacin stress that could be potentially influenced by the Pf4 phage.

Ciprofloxacin binds to and inhibits bacterial DNA gyrase, and the Pf4 encodes a toxin with homology to the ParE protein that also targets DNA gyrase (375-377). Furthermore, ParE is predicted to form part of a toxin-antitoxin (TA) system, and it has been previously found that ciprofloxacin can activate the expression of TA systems in *E. coli* (i.e. HipAB and TisB-IstR) (162, 377, 378). Therefore, a similar phenomenon could be occurring in *P. aeruginosa*, where the presence of ciprofloxacin could activate the expression of *parE-phd*, which could lead to the binding of the ParE toxin to DNA gyrase and potentially persister cell formation (378, 379). However, since it is known that some *P. aeruginosa* strains, such as *P. aeruginosa*-NC do not possess the *parE* toxin, it is hypothesised that this potential mechanism would not explain ciprofloxacin resistance for all *P. aeruginosa* strains (380).

3.4.5 Summary and conclusions

Based on the results described here, the Pf4 phage contributes to *P. aeruginosa* biofilm tolerance to antibiotic and oxidative stress. Furthermore, the inclusion of the Pf4 phage in the *P. aeruginosa* genome provides a competitive edge over *P. aeruginosa* strains lacking the phage when the wild-type and Pf4 mutant were co-cultured under planktonic or biofilm conditions (Fig. 3.5). Indeed, despite being inoculated in a 1:1 ratio, the wild-type outnumbered the phage mutant by 10:1. Since there is no difference in growth rate for the two strains (data not shown), this must be due to competition between the two strains. Therefore, the increased resistance against stress observed in wild-type *P. aeruginosa* could be due to the selective advantage provided by the phage. However, the mechanisms for how the phage contributes to *P. aeruginosa* stress resistance are unclear and provide an interesting topic for future studies.

There are several potential mechanisms for the increased resistance of *P. aeruginosa* provided by the phage. One such mechanism could involve the ParE-Phd TA system of the Pf4 phage promoting persister cell formation in response to ciprofloxacin stress (which is further described in Section 5.2 of this thesis), based on the observations of the RelE-RelB TA system of *E. coli* (381, 382). This hypothesis could be explored in future through studying the prevalence of persister cell formation by a ParE-Phd complementation or deletion strain compared to the wild-type and Pf4 deletion mutant strains, in the presence of ciprofloxacin stress.

Another possible way in which the Pf4 phage could contribute to *P. aeruginosa* antibiotic resistance is through its involvement in *P. aeruginosa* EPS production and biofilm stability. Recently, Secor *et al.* showed that the Pf4 phage is directly involved in the formation of the *P. aeruginosa* crystalline biofilm matrix, and as a result, increases bacterial resistance to antibiotic stress, and desiccation (288) (more detail is provided in Section 1.9.2). Although that study showed that the Pf4 did not contribute to protection against ciprofloxacin, the study was conducted with *E. coli* rather than *P. aeruginosa.* Therefore, in order to validate these hypotheses, further experimentation would have to be undertaken. This could include testing the effect of Pf4 liquid crystalline mixtures on *P. aeruginosa* biofilms during ciprofloxacin stress.'

4 Analysing the effect of the Pf4 phage on *P. aeruginosa* PAO1 through quantitative gene expression analysis and complementation studies

4.1 Introduction

Bacteriophage can expand the genetic capacity of their host bacterium through lysogenic conversion. In some cases, the genes provided by the bacteriophage directly contribute to the virulence of the host. This was first documented by Frobisher and Brown (reviewed in (383)) who observed that when a non-toxigenic *Streptococcus pyogenes* culture was exposed to filtered supernatants from a toxigenic scarlatinal toxin-producing (a toxin associated with scarlet fever) *S. pyogenes* isolate, the nontoxigenic culture was subsequently able to produce the scarlatinal toxin. It has since been shown that the toxigenic supernatant contained a bacteriophage that transferred the toxin genes to the non-toxigenic *S. pyogenes* via transduction (383).

Since then, many more phage-encoded toxins have been discovered. For example, the CTXΦ phage of *V. cholerae* encodes the *ctxAB* genes, responsible for production of cholera toxin, as well as two other proteins, Ace and Zot, which also play a role in virulence (262). These three phage-encoded products increase the virulence of *V. cholerae* and are responsible for the severe symptoms in the host such as excessive diarrhoea and dehydration. In Shiga toxin-encoding *Escherichia coli* (STEC), the Shiga toxin gene, *stx*1, is carried in the genome of bacteriophage phage H-19B and this phage is responsible for the conversion of non-pathogenic strains of *E. coli* into deadly pathogens. (384).

In addition to toxin production, bacteriophage can increase bacterial virulence through effector proteins, which assist in the invasion of host tissues. For example, SopE is a T3SS effector protein that was horizontally transferred to the *Salmonella* pathogenicity island-1 (SPI-1) of *S. enterica* serovar Typhimurium via the SopEΦ phage (385). When injected into the mammalian host, SopE activates the host Rho GTPases, CDC42 and Rac1, which stimulates bacterial internalisation in tissue cells that is essential for this intracellular pathogen (386). Another bacteriophage of *S. enterica* serovar Typhimurium, Gifsy-1, also contributes a T3SS effector protein known as GogB, which is located on the *Salmonella*

pathogenicity islands 1 and 2 (SPI-1 and SPI-2) (387, 388). This particular protein is translocated by the SPI-2 complex into the host cytoplasm during infection, where it interferes with ubiquitination and reduces host inflammatory effects (387, 389). Therefore, the bacterium can reach high cell densities in the host, due to the evasion of the host immune response and reduction of host tissue damage (389).

Bacteriophage can also influence host virulence by altering bacterial host gene expression, such as in the cases of ΦRSS1 and *Ralstonia solanacearum*, and XacF1 and *Xanthomonas axonopodis* (258) (261). More detail on these examples is provided in sections 1.9.1.2.1 and 1.9.1.2.2, respectively. Another example is the Pf4 phage, which has been previously shown to increase the virulence of *Pseudomonas aeruginosa* in an acute virulence model, and increase biofilm stability in the presence of stress factors (253). In addition, the results from this study have shown that the Pf4 phage influences the production of virulence factors such as pyoverdine, and affects invasion of mammalian hosts. Furthermore, the study has shown that, in addition to surfactant stress, the Pf4 phage also contributes to the resistance of *P. aeruginosa* biofilms when challenged with ciprofloxacin and oxidative stress. However, it is unknown as to how the Pf4 phage specifically influences these phenotypes.

Therefore, the aim of the present study was to determine how the Pf4 phage contributes to the virulence and stress tolerance of biofilms formed by *P. aeruginosa*. This was approached through a combination of targeted qPCR to determine gene expression levels for key virulence and stress tolerance genes. The studies demonstrated that direct contact of the Pf4 phage particles did not have a significant effect on the mammalian host nor did the infection of mammalian cells alter phage production by *P. aeruginosa*. Interestingly, the addition of inactive phage particles to biofilms restored resistance to ciprofloxacin in the Pf4 mutant. In the absence of the Pf4 phage, changes in gene expression were observed for genes involved in siderophore production (*pchF* and *pvdO*), transcriptional regulation (*ptxS*) and the activation of a multidrug efflux pumps (*mexE*, *mexF* and *oprN*).

4.2 Materials and Methods

4.2.1 General Materials and Reagents

Unless otherwise stated, lysogeny broth (LB) agar plates (10 g/ L tryptone, 10 g/ L NaCl, 5 g/ L yeast extract, 15 g/ L agar) were used to maintain bacterial strains (Table 4.1). To select for particular strains, antibiotics were incorporated into the LB agar as needed. Bacterial cultures were grown in M9 minimal medium (4.76 mM Na_2HPO_4, 2.2 mM KH_2PO_4, 0.86 mM NaCl, 1.85 mM NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% (w / v) glucose), pH 7, or LB agar and incubated overnight at 37 °C and shaking at 200 rpm (Ratek Instruments Pty Ltd., Australia).

HepG2 tissue cells were cultivated in HepG2 complete medium (10% (v / v) fetal bovine serum (FBS) (Gibco, U.S.A.), 89% (v / v) Dulbecco's Modified Eagles Serum F-12 Ham (Sigma-Aldrich, U.S.A.), 1% (v / v)100 x antimycotic-antibiotic solution (Sigma-Aldrich, U.S.A)) and grown in T75 cm^2 tissue culture flasks (Sarstedt, Germany) at 37 °C and 5% CO_2 CFTE29o- tissue cells were maintained in DMEM complete medium (10% (v / v) FBS, 98% (v / v) Minimal Essential Medium w/ Earle's Salt (MEM) (Sigma-Aldrich, U.S.A.), 2 mM L-glutamine (Sigma-Aldrich, U.S.A.), 1 x antimycotic-antibiotic solution and grown in T75 cm^2 tissue culture flasks at 37 °C and 4 – 5% CO_2.

Table 4.1 Bacterial strains used in this study

Strain	Genotype/Description	Source
P. aeruginosa WT PAO1	Wild-type	(309)
P. aeruginosa ΔPf4 PAO1	PAO1 with the Pf4 prophage deleted: Gm^R	(253)
P. aeruginosa ΔPf4 [*phd*] PAO1	*P. aeruginosa* ΔPf4 PAO1 with the *phd* (antitoxin) gene complemented: Amp^R	This study
P. aeruginosa WT SCV2	WT that produces small colony variants	(390)
P. aeruginosa [pHERD20T] PAO1	WT with the pHERD20T plasmid inserted: Amp^R	(391)
One Shot® TOP10 Electrocomp™ *E. coli*	Electrocompetent *E. coli* cells	Life Technologies

4.2.2 Construction of the ΔPf4 pHERD20T [*phd*] complementation strain

4.2.2.1 Polymerase Chain Reaction (PCR)

A 50 µL PCR reaction with 1 µL of *P. aeruginosa* WT PAO1 genomic DNA, 5 µL of 10 x AccuPrime™ Pfx DNA polymerase reaction mix (Life Technologies, U.S.A), 10 pmol of both *phd* F with *Eco*RI and *phd* R with *Kpn*I primers (Table 4.1) (IDT Technologies, U.S.A.) and 1 µL of AccuPrime™ Pfx DNA polymerase (Life Technologies, U.S.A.), and DNase-free water was added to a final volume of 50 µL. The conditions were as follows: a denaturing step at 95 °C for 3 min, 30 cycles of 95 °C for 15 s, annealing at 55 °C for 30 s and extension at 68 °C for 30 s, with a final extension step at 68 °C for 10 min.

4.2.2.2 Extraction of pHERD20T plasmid from **P. aeruginosa** *WT PAO1*

P. aeruginosa [pHERD20T] PAO1 was inoculated in LB_{10} broth supplemented with 50 µg/ mL carbenicillin, and grown overnight at 37 °C with shaking at 200 rpm (Ratek Instruments Pty Ltd., Australia). The overnight culture was then sub-cultured into 10 mL of fresh LB_{10} broth supplemented with 50 µg/ mL carbenicillin, and grown at 37 °C and 200 rpm until the OD_{600} reached 0.5 (Ratek Instruments Pty Ltd., Australia). The pHERD20T plasmid was then extracted using the sub-cultured cells and the PureLink® Quick Plasmid Miniprep Kit (Life Technologies, U.S.A.), according to the manufacturer's instructions (391).

4.2.2.3 Restriction endonuclease digestion

Both the amplified DNA and pHERD20T plasmid were digested with the restriction enzymes, *Kpn*I and *Eco*RI-HF (New England BioLabs, U.S.A.) as follows. One microgram of DNA (either from amplified DNA or pHERD20T) was mixed with 5 µL of 10 x CutSmart® buffer, 1 µL of both *Kpn*I and *Eco*RI restriction enzymes and DNase-free water was added to a final volume of 50 µL. The reaction was incubated at 37 °C for 1 h and then heat-inactivated at 65 °C for 20 min.

Table 4.2 DNA primer sequences used in this study

Primer name	Sequence (5'- 3')	Reference
phd F with *EcoR*I	GAATTCATGCGAGTCGAGACAATTA GTTATT	This study
phd R with *Kpn*I	GGTACCTTCTGGCTGAGCGAACCT	This study
phd F	CATGCGGCTGACCTGGATTTA	This study
phd R	GAGCGATGCTTGCCTTCTGC	This study
PA0106 F (*coxA*)	CCTGCCGCGGATGAACAACT	This study
PA0106 R (*coxA*)	TCGACAGCGGCGCATAGAAGG	This study
PA1148 F (*toxA*)	GATGCCACCTTCTTCGTCAG	(392)
PA1148 R (*toxA*)	GCTGGGCGAGGTAGTTGTAG	(392)
PA2231 F (*pslA*)	CCTTCAGCCATCCGTTCTTCT	This study
PA2231 F (*pslA*)	TCGCGTACGAAGTCGACCTT	This study
PA2259 F (*ptxS*)	CCGCGACGACGAGCAGGAG	This study
PA2259 R (*ptxS*)	ACCAGCACCATCGGCAGTTCG	This study
PA2395 F (*pvdO*)	CCAAGCACGCCAACACCTACG	This study
PA2395 R (*pvdO*)	CAGCAATCGGCGACCCACTC	This study
PA2493 F (*mexE*)	TCCTGGATGGCGACAACAAGAC	This study
PA2493 R (*mexE*)	CCGCTGCAGGCCATTCACG	This study
PA2494 F (*mexF*)	GTGATGCACTACAACGGCTT	This study
PA2494 R (*mexF*)	GTAGGTCAGCTCGGTCCACT	This study
PA2495 F (*oprN*)	TACGACCTTGGCCTGGATA	This study
PA2495 R (*oprN*)	GTAGGCGTCCACCAGCTC	This study
PA3064 F (*pelA*)	AAGATCAAGAAACGCGTGGAAT	This study
PA3064 R (*pelA*)	TGTAGAGGTCGAACCACACCG	This study
PA3516 F (probable lyase)	AAATGCTTGCCCGTTCCTCGTG	This study
PA3516 R (probable lyase)	CCGCCGCCATGGGTTTTAGA	This study
PA3617 F (*recA*)*	CTGCCTGGTCATCTTCATCA	(393)
PA3617 R (*recA*)*	ACCGAGGCGAGAACTTCAG	(393)
PA4225 F (*pchF*)	CCGGGCAATGGCTGGAG	This study
PA4225 R (*pchF*)	GGGCGCGGTCGGTCTTG	This study

* Housekeeping gene primers

4.2.2.4 Confirmation and clean-up of amplified DNA

The amplified DNA from the PCR reaction was analysed on a 1% (v / v) agarose gel (TAE buffer 19.98 mM Tris base, 32.76 mM glacial acetic acid and 0.64 mM EDTA plus 1% agarose w / v). The gel was stained for 15 min in ethidium bromide (0.0001% w / v), and the size of the amplified product was estimated by comparison to a 100 bp molecular marker (Generuler, Fermentas). The PCR product was cleaned up using the PureLink® PCR Purification Kit (Life Technologies, U.S.A.), according to the manufacturer's instructions.

4.2.2.5 Dephosphorylation of pHERD20T plasmid

The pHERD20T plasmid was dephosphorylated using Antarctic phosphatase (New-England BioLabs, U.S.A.). Antarctica phosphatase reaction buffer (10 x) was added in a 1:10 ratio with three micrograms of cut plasmid and 1 µL of Antarctic phosphatase. The reaction mixture was incubated at 37 °C for 1 h, and then heat-inactivated at 70 °C for 5 min.

4.2.2.6 Phosphorylation of amplified DNA

The amplified DNA was phosphorylated using T4 polynucleotide kinase (PNK) (New England BioLabs, U.S.A.). One hundred picomoles of amplified DNA was mixed with 5 µL of 10 x T4 PNK reaction buffer, 5 µL of 10 mM ATP, 1 µL of T4 PNK, and DNase-free water was to a total volume of 50 µL. The reaction was incubated at 37 °C for 30 min, and then heat-inactivated at 65 °C for 20 min.

4.2.2.7 Ligation of pHERD20T plasmid to amplified DNA insert

The amplified DNA insert was ligated into the pHERD20T plasmid using T4 DNA ligase (New England BioLabs) using a 1:3 plasmid to insert ratio, where 50 ng of plasmid DNA was mixed with 7.53 ng of insert DNA, 2 µL of 10 x T4 DNA ligase buffer and 1 µL of T4 DNA ligase. DNA-free water was added to a total volume of 20 µL. The reaction mixture was then incubated overnight at 16 °C, before heat-inactivation at 65 °C for 10 min. The ligation mixture was then drop dialysed on 0.025 µm DNA filter paper (Millipore, Ireland) prior to transformation.

4.2.2.8 Transformation and confirmation of plasmid with **phd** *insert into TOP10* **E. coli** *cells*

The insert/vector was transformed into One Shot® TOP10 Electrocomp™ *E. coli* cells via electroporation, where cells and pre-chilled disposable cuvettes were kept on ice (0.1 cm width; Biorad). Plasmid DNA was mixed with electrocompetent *E. coli* cells by gentle pipetting and electroporation was performed by a single pulse of 1.8 kV at 200 Ω and 25 mF in a BioRad Micropulser. The cells were then promptly recovered using 1 mL of LB_{10} broth and transferred to a fresh 15 mL tube (Corning, U.S.A.), where the cells were incubated at 37 °C for 1 h with 200 rpm shaking (Ratek Instruments Pty Ltd., Australia). The transformed cells were then screened by blue/white selection on LB agar plates containing 40 µg/ mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 100 µg/ mL carbenicillin. Clones were confirmed for correct insert size and orientation by PCR, using a primer located on the plasmid and another primer located on the 3' end of the insert. The PCR product was the visualised on a 1% (v / v) agarose gel, via gel electrophoresis, ethidium bromide staining and UV illumination (see section 4.2.2.2).

Once the pHERD20T plasmid with the *phd* insert was confirmed (designated as pHERD20T [*phd*]) to be in the *E. coli* TOP10 cells, an overnight culture was made from one of the confirmed colonies; the colony was inoculated in LB_{10} broth with 100 µg / mL carbenicillin and grown overnight at 37 °C and 200 rpm shaking. The overnight culture was then used to purify the plasmid, as described in section 4.2.2.3.

4.2.2.9 Preparation of **P. aeruginosa** *ΔPf4 PAO1 electrocompetent cells for transformation of the pHERD20T* **[phd]** *plasmid*

P. aeruginosa electrocompetent cells were prepared as described by Choi *et al.* (394). In brief, *P. aeruginosa* ΔPf4 PAO1 cells were grown overnight in 10 mL of SOB broth (Tryptone 20 g/ L, yeast extract 5 g/ L, NaCl 0.56 g/ L, KCl 0.17 g/ L) at 37 °C and 200 rpm shaking (Ratek Instruments Pty Ltd., Australia). The cells were centrifuged for 2 min at 10, 000*g* and 2 °C (Hettich, Germany), washed twice in 15 mL of 300 mM sucrose solution, then once in 2 mL of 500 mM sucrose solution, and finally resuspended in 1 mL of 30 mM sucrose solution for immediate use as 200 µL for electroporation.

The purified pHERD20T [*phd*] plasmid was then electroporated into *P. aeruginosa* ΔPf4 PAO1 using cuvettes with 0.2 cm width at 2.5 kV at 200 Ω and 25 mF. The transformed cells were then selected via blue/white selection on LB agar plates containing 40 µg/ mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 200 µg/ mL carbenicillin.

4.2.3 Determination of mRNA expression in ΔPf4 pHERD20T [*phd*] complementation strain

4.2.3.1 Determination of mRNA in planktonic samples

Cultures of *P. aeruginosa* ΔPf4 mutant, wild-type, and ΔPf4 pHERD20T [*phd*] complementation strain were grown overnight in complete M9 at 37 °C and 200 rpm shaking (Ratek Instruments Pty Ltd., Australia). The overnight cultures were sub-cultured into 6 mL of fresh, complete M9 medium and grown at 37 °C and 200 rpm shaking to an OD_{600} of 0.8. The sub-culture was then split into 1 mL aliquots and half of the aliquots were exposed to 2% (w / v) L-arabinose while the other half were left untreated. The samples were then incubated at 37 °C and 200 rpm shaking for a further 3 h (Ratek Instruments Pty Ltd., Australia). The supernatant in each well was then harvested, stored in RNAprotect Bacterial Reagent, and used to extract RNA, convert RNA to cDNA, and perform qPCR (as described in 2.2.5i) using primers specific for selected genes (Table 4.2). The experiment was performed with three biological replicates in triplicate.

4.2.3.2 Quantification of mRNA expression during biofilm development by qPCR

4.2.3.3 Continuous flow biofilms

P. aeruginosa wild-type, ΔPf4 mutant, and ΔPf4 pHERD20T [*phd*] complementation strain biofilms were grown in silicon tubing (inner diameter 2.64 ± 0.28 mm and outer diameter 4.88 ± 0.28 mm) (Silastic® laboratory tubing and Tygon® XL-60 pump tubing) (Fig. 4.1), as described (395), with modifications (253), and further modifications here. Prior to inoculation of the tubing, complete M9 medium was passed through the system at 24 mL/ h for 1 h using a peristaltic pump (Spirax-Sarco Engineering plc, U.K.) to introduce medium into the system. The peristaltic pump was then turned off to stop

medium flow and the tubing was clamped at the inlet of the continuous-flow system to prevent backflow of bacteria during inoculation. Three millilitres of overnight *P. aeruginosa* wild-type, ΔPf4 mutant, and ΔPf4 pHERD20T [*phd*] complementation strain cultures were slowly inoculated into the inlet side of the biofilm tubing using a syringe and a 26G needle (Becton-Dickinson Precision Glide Needle). The site of injection in the tubing was sealed using silicon to prevent leakage. The outlet end of the tubing was then clamped and the system was incubated for 2 h at room temperature.

After incubation, the system was unclamped and the peristaltic pump was turned on, with the medium flowing at 6 mL/ h for 7 d. On day 7, complete M9 medium supplemented with 2% (w / v) L-arabinose was flowed through half of the ΔPf4 pHERD20T [*phd*] complementation strain replicates in the system at 6 mL/ h for 3 h at room temperature, with the other replicates (including *P. aeruginosa* wild-type and ΔPf4 mutant) remaining untreated. After the 3 h incubation, the tubing was clamped on the inlet end and the peristaltic pump was stopped. The samples were then harvested by squeezing the tubing to fully dislodge the biofilm and effluent into 4 mL of RNAprotect Bacterial Reagent (Qiagen). RNA extraction, cDNA conversion and qPCR were then performed (as detailed in Chapter 2), using gene specific primers (Table 4.2). The house-keeping gene *recA* was used in place of *proC* in this study due to *recA* having demonstrated consistently similar expression levels in both the wild-type and ΔPf4 mutant. The experiment was performed with three biological replicates in triplicate.

4.2.4 Protein determination of ΔPf4 pHERD20T *[phd]* complementation strain

Overnight cultures of the *E. coli* TOP10 and *P. aeruginosa* ΔPf4 PAO1 strains harbouring either the empty pHERD20T plasmid as a control or the pHERD20T [*phd*] plasmids were grown in 10 mL LB_{10} (Corning, U.S.A.) with 100 µg/ mL Cb for 16 h at 37 ^{o}C with shaking at 200 rpm (Ratek Instruments Pty Ltd., Australia). These cultures were used to inoculate 100 mL fresh LB_{10} medium with 100 µg/ mL in a 1:100 dilution, and were grown in 500 mL Erlenmeyer flasks at 37 ^{o}C with shaking at 200 rpm. Samples were grown to an OD_{600} of 0.5, at which point arabinose (0.2% or 2% w / v) was added to induce protein expression. The cultures were then further incubated for 3 h. To extract soluble proteins, 50 mL cell culture was collected in a Falcon tube (Corning, U.S.A) and harvested by centrifugation at 9,500*g* at 2 ^{o}C for 3 min (Hettich, Germany). The

supernatant was discarded and the cell pellet was lysed with 1 mL of Bacterial Protein Extraction Reagent (B-PER), according to the manufacturer's instructions (Life Technologies, U.S.A.). Soluble proteins were quantified using the Pierce bicinchoninic acid (BCA) protein assay (Life Technologies, U.S.A.) and 10 mg of each sample was resolved on an 8 – 12% (v / v) SDS-PAGE gel with Laemmli running buffer, according to the manufacturer's instructions (Biorad, U.S.A.). Precision Plus Protein™ WesternC™ protein standard (Bio-Rad, U.S.A.) was used for protein size comparison. The gel was then stained with Coomassie blue for 1 h, washed twice in destaining solution (50% methanol (v / v), 10% glacial acetic acid (v / v), 40% (v / v) H_2O) and finally washed in destaining solution overnight. The gel was imaged at 1200 dpi using an Umax Powerlook 1000 flatbed scanner with V4.71 software.

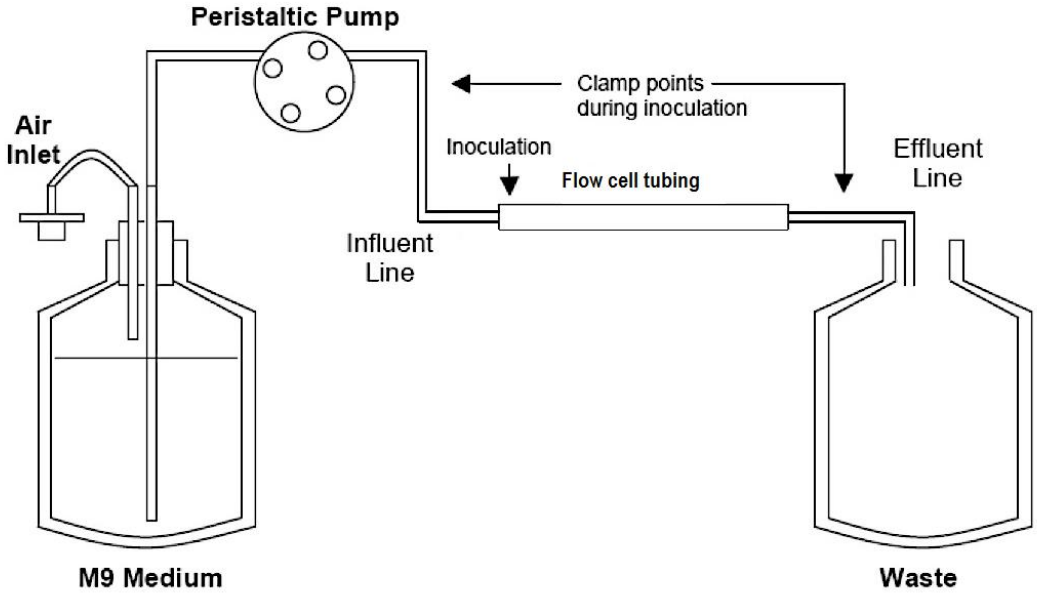

Figure 4.1 Diagram of a continuous-flow biofilm system. M9 medium supplemented with 0.2% (w / v) glucose was fed into the system via a peristaltic pump at a continuous flow of 6 mL/ h to constantly supply fresh nutrients to the growing biofilms within the flow cell tubing. The effluent was collected in a waste bottle at the end of the system. Before inoculation, the system was clamped on the influent side to prevent backflow and contamination of the medium. Post inoculation, the system was clamped on the effluent side to concentrate the bacteria in the flow cell tubing to promote colonisation and adhesion to the tubing. This diagram was adapted from (390, 396).

4.2.5 Analysis of *P. aeruginosa* WT PAO1 Pf4 phage production in the presence of HepG2 cells

HepG2 cells were seeded at 2.5 x 10^5 cells/ mL with complete DMEM (no antibiotic-antimycotic solution) in 24 well flat bottomed culture plates and grown for 2 d, as described in Chapter 2 (section 2.2.3). After 1 d, the supernatant was changed with antibiotic-free complete DMEM. On the day of experimentation, the cells were washed twice with antibiotic-free complete DMEM. Triplicate WT PAO1 overnights (in complete M9 with 0.2% (w / v) glucose, 200 rpm, 37 °C) were washed once with 1 x PBS and then adjusted in 1 x PBS to an OD_{600} of 1. The PAO1 cells were added to the HepG2 cells at a dilution of 1:10, with three biological replicates analysed in duplicate (with three independent experiments conducted). The plates were centrifuged at 320*g* for 4 min, and then incubated at 37 °C and 4 - 5% CO_2 for 4 h.

After incubation, the supernatant was used to perform a phage analysis. This was performed by serial dilutions on the samples and drop plating the dilutions onto prepared LB_{10} plates with a layer of 0.8% (w / v) soft LB_{10} agar that was inoculated with an overnight culture of *P. aeruginosa* ΔPf4 PAO1. The plates were incubated statically at 37 °C overnight and PFU/ mL was then determined. The cytotoxicity of Pf4 phage particles on HepG2 and CFTE29o- cells

4.2.5.1 Extraction of phage particles

Biofilms were grown in the continuous flow system for 2 d, as described in 4.2.3.2i. After 2 d, the biofilm effluent was collected from the tubing, and cells were harvested via centrifugation at 15,000*g* for 20 min at room temperature. The supernatant was collected and passed through a 0.22 µm filter (Millipore, Ireland). Phage precipitation buffer (2 M NaCl, 30% (w / v) polyethylene glycerol (MW 8000)) was then added at a 1:1 ratio and the phage mixture was incubated statically at 4°C overnight to increase phage yield. After the overnight incubation, the mixture was centrifuged at 15,000*g* for 20 min. The supernatant was removed and the pellet containing the phage particles was resuspended in 3 mL of SM buffer (100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris HCl (pH 7.5)). The phage titre of the supernatant was determined through serial dilutions, drop plating the dilutions onto LB_{10} agar with a pre-prepared 0.8% (w / v) soft agar layer inoculated with *P. aeruginosa* WT or ΔPf4 PAO1 and determining PFU/ mL.

4.2.5.2 Effect of phage particles on tissue culture cell viability

HepG2 cells and CFTE29o- cells were both seeded at 1.5 x 10^5 cells/ mL with complete DMEM or MEM, respectively (and no antibiotic-antimycotic solution), to a total volume of 1 mL/ well and grown for 2 d, as described in section 2.2.3. After 1 d, the supernatant was changed with antibiotic-free complete DMEM. On the day of experimentation, the cells were washed twice with antibiotic-free complete DMEM or MEM and phage particles were added at 1 x 10^8 PFU/ mL in antibiotic-free complete DMEM or MEM to a total volume of 1 mL. The cultures were then incubated for 4 h at 37°C and 4 – 5% CO_2. After the 4 h incubation, cytotoxicity of the phage particles towards the HepG2 and CFTE29o- cells was determine through the use of an *In Vitro* Toxicology Assay Kit (Sigma-Aldrich) (as described in section 2.2.4).

4.2.6 The effect of inactive Pf4 phage on ΔPf4 mutant and wild-type *P. aeruginosa* PAO1 biofilm stability and cell viability in response to ciprofloxacin stress

Prior to the assay being conducted, Pf4 phage particles were extracted from the *P. aeruginosa*, according to the protocol detailed in Section 4.2.5.1. Pf4 phage were inactivated via gamma irradiation (75 Gy) and UV irradiation (10,000 mJ/ cm^2). *P. aeruginosa* ΔPf4 and WT PAO1 were grown and inoculated as described in Section 3.2.5 with or without 1 x 10^9 of inactive Pf4 phage. After the 6 h incubation, ciprofloxacin was added to the wells (at a final concentration of 0, 10, or 50 µg/ mL). For a selected number of control wells, 1 x 10^9 of Pf4 inactive phage were added at the 6 h incubation time point instead of at the start of the experiment. All of the plates were incubated for a further 1 h. The spent media was subsequently removed, the wells were washed once with 1 x PBS and the plates were sonicated in a water bath at 100 W, 40 kHz (Unisonics Australia, Australia) for 10 min. The resuspended biofilms were serially diluted to quantify CFUs using the drop plate method (358) on LB_{10} agar plates 37°C overnight. Three independent experiments were conducted with all strains, with three biological replicates in triplicate for each condition per independent experiment.

4.2.7 Effect of the Pf4 phage on mRNA expression in selected *P. aeruginosa* wild-type PAO1 genes

P. aeruginosa WT and ΔPf4 PAO1 strains were grown planktonically, and in 2 d and 7 d biofilms, to be used for RNA extraction and qPCR (as described in 4.2.3). qPCR was performed using the primers for PA0106 (*coxA*; encoding for the cytochrome c oxidase, PA1148 (*toxA*; expressing exotoxin A), PA2231 (*pslA*; responsible for the expression of the major polysaccharide, Psl), PA2259 (*ptxS*; a transcriptional regulator), PA2395 (*pvdO*; an enzyme involved in pyoverdine synthesis), PA2493, PA2494 and PA2495 (*mexE*, *mexF* and *oprN*, respectively; all three genes contributing to the expression of a multidrug efflux membrane fusion protein), PA2494 (*mexF*; expressing a multidrug efflux membrane fusion protein), PA2495 (*oprN*; expressing a multidrug efflux membrane fusion protein), PA3064 (*pelA*; responsible for the expression of the major polysaccharide, Pel), PA3516 (a putative lyase), PA4225 (*pchF*; responsible for pyochelin production) and *recA* (housekeeping gene) (see Table 4.2). Three biological replicates were analysed in triplicate for each strain and condition.

4.3 Results

4.3.1 The effect of mammalian cells on Pf4 phage production by *P. aeruginosa* WT PAO1

It was observed previously (Chapter 2) that there was a difference in cytotoxicity between the wild-type and ΔPf4 mutant towards HepG2 cells. Further, recent data suggests that phage can directly interact with mammalian cells (397). Therefore, to determine whether cultivation with the tissue cells altered phage production, WT PAO1 bacteria were grown with and without HepG2 cells for 4 h. In the absence of HepG2 cells *P. aeruginosa* produced approximately 8 x 10^6 PFU/ mL (Fig. 4.2), while there was a slight, but not significant decrease in phage production when grown in the presence of HepG2 cells (approximately 5 x 10^6 PFU/ mL), which is 1.6 fold less than when the wild-type was grown without mammalian cells (Fig. 4.2). When the wild-type cells were incubated with and without HepG2 cells the CFUs varied for the wild-type with HepG2, and without HepG2, being 9.5 x 10^7 and 8.2 x 10^7 CFU/ mL, respectively. However, the difference in CFU did not alter the results significantly (data not shown). That is, when the PFUs were normalised to the CFU numbers, the same trend was observed (3.69 x 10^6 PFU/ CFU and 2.67 x 10^6 PFU/ CFU for the wild-type without, and with HepG2, respectively).

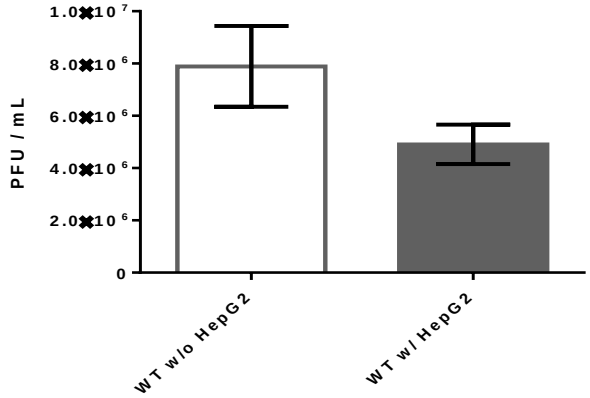

Figure 4.2 Production of the Pf4 phage by *P. aeruginosa* WT PAO1 in the presence of HepG2 cells. *P. aeruginosa* WT PAO1 cells were exposed to HepG2 cells and analysed for Pf4 production through the observation of plaque forming units (PFU) / mL on ΔPf4 PAO1 soft agar lawns. Data were analysed using GraphPad Prism 5 (GraphPad Software Inc., U.S.A.) and statistical significance was determined via an unpaired, two-tailed, Student's t-test; n = 9. Error bars represent the SEM.

4.3.2 The Pf4 phage is directly cytotoxic to HepG2 and CFTE29o- cells, but does not account for all of the toxicity of *P. aeruginosa*

To determine whether Pf4 phage particles contribute to the cytotoxicity of *P. aeruginosa* towards mammalian cells, HepG2 and CFTE29o- cells were incubated with purified Pf4 phage particles and toxicity was measured by lactate dehydrogenase (LDH) release. HepG2 and CFTE29o- cells incubated without Pf4 phage particles were used as a negative control. There was an approximate 7 fold increase in cytotoxicity when the HepG2 cells were incubated with the Pf4 phage particles (5% LDH release), in comparison to HepG2 cells grown without phage particles (0.7% LDH release). When CFTE29o- cells were incubated with Pf4 phage particles (2.5% LDH release), there was a 3.5 fold increase in comparison to CFTE29o- grown without phage particles. Therefore, the Pf4 phage appears to be directly toxic to the tissue culture cells and there appears to be a tissue specific difference in response, where the HepG2 cells were more sensitive to the phage (2.5 fold, $p = 0.006$) than CFTE29o- (Fig. 4.3).

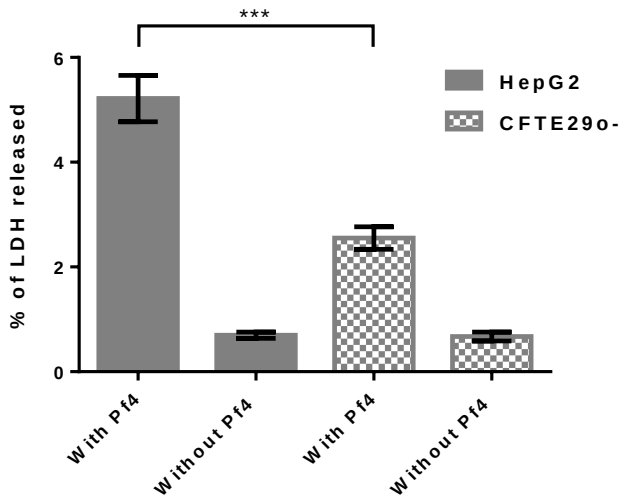

Figure 4.3 Cytotoxicity of HepG2 and CFTE29o- cells in the presence of Pf4 phage particles. HepG2 and CFTE29o- cells were exposed to Pf4 phage particles in 24 well plates for 4 h at 37 °C and analysed for LDH activity to determine mammalian cell cytotoxicity. The negative controls were HepG2 and CFTE29o- cells incubated without Pf4 phage particles. Data were analysed using GraphPad Prism 5 (GraphPad Software Inc., U.S.A.) and statistical significance was determined via an unpaired, two-tailed, Student's t-test; $n = 9$. Error bars represent the SEM. Statistical significance, $p < 0.01$ is represented by *.

4.3.3 The effect of inactive Pf4 phage on ΔPf4 mutant and wild-type *P. aeruginosa* PAO1 biofilm stability and cell viability in response to ciprofloxacin stress

Recently, Secor *et al.* (2015) showed that the Pf4 phage can physically contribute to *P. aeruginosa* biofilm stability. Therefore, inactive Pf4 phage were added back to ΔPf4 mutant and wild-type *P. aeruginosa* PAO1 biofilms to determine whether the phage increased biofilm cell viability and stability (Fig. 4.4 and 4.5, respectively). The results showed that exposure to the inactive Pf4 phage during biofilm development increased resistance of both the ΔPf4 mutant and wild-type PAO1 biofilm cells when exposed to 10 µg/ mL ciprofloxacin (Fig. 4.4 A and 4.4 B). For example, the wild type and Pf4 mutant PAO1 biofilms only decreased 3.8 fold and 10 fold ($P = 0.018$ and $P = 0.0006$, respectively) respectively in cell viability, when exposed to 10 µg/ mL ciprofloxacin and pre-exposed to the inactive Pf4 phage prior to ciprofloxacin exposure, in comparison to 189 fold and 17 fold ($P = 0.005$ and $P = 0.010$, respectively), respectively when exposed to 10 µg/ mL ciprofloxacin without exposure to inactive phage

The greatest effect was observed for the Pf4 mutant when exposed to 50 µg/ mL ciprofloxacin where the mutant only decreased 2.4 fold in cell viability ($P = 0.0001$), in comparison to a 4,100 fold decrease in viability when the Pf4 mutant when exposed to 50 µg/ mL ciprofloxacin without exposure to the inactive phage. Similar results were observed when the phage was added at the same time as ciprofloxacin (Fig. 4.4 C), although the degree of protection in the presence of the phage was lower for the Pf4 mutant (42 fold decrease in viability, $P = 0.007$).

When the biofilm biomass was quantified by crystal violet staining, it was observed that ciprofloxacin had no significant impact on the biofilm for the wild-type or the mutant (Fig. 4.5). Further, addition of the phage to the biofilm, either for 6 h during development, or 1 h during ciprofloxacin exposure, also had no impact on the total amount of biofilm present. It should be noted that only inactive phage were tested here to avoid confounding issues of infection by the active phage, which will potentially cause lysis of the Pf4 mutant and hence make interpretation of the data, based on viable cell counts, difficult.

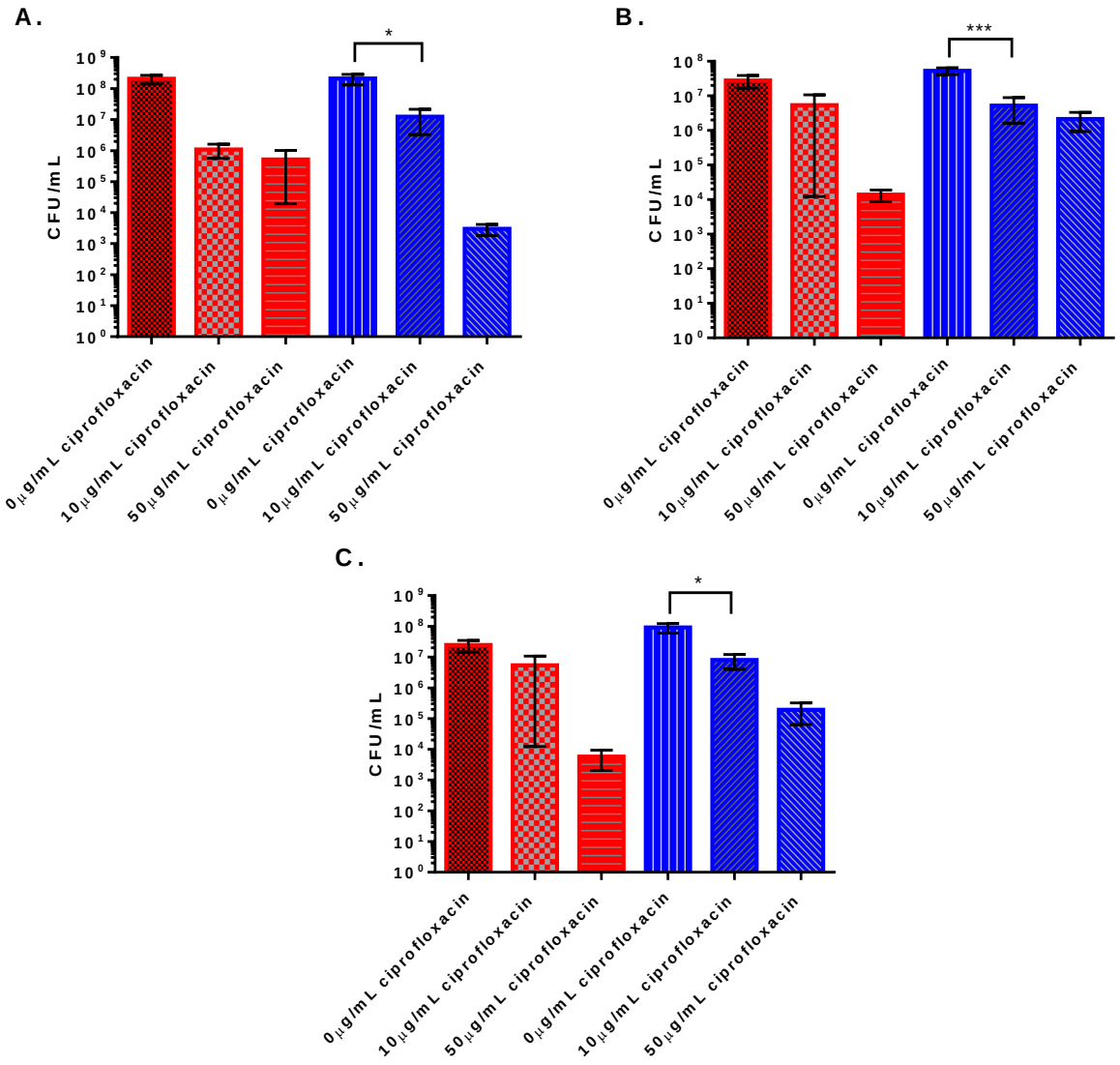

Figure 4.4 Comparison of the effect of antibiotics on *P. aeruginosa* ΔPf4 and WT PAO1 biofilm cell viability in the presence of inactive Pf4 phage. Biofilms of the ΔPf4 mutant (blue bars) and wild-type strains (red bars) of *P. aeruginosa* were pre-grown for 6 h with (A) no treatment for the 6 h period, (B) inactive Pf4 phage for the 6 h, and (C) no treatment for the 6 h period for the 6 h period, before exposure to varying concentrations of ciprofloxacin for 1 h at 37°C. At the point of ciprofloxacin addition, inactive phage were added to (C) for 1 h. Cell numbers for the exposed and control biofilms were compared to determine the percentage survival. Data were analysed using GraphPad Prism 5 and statistical significance was determined via an unpaired, two-tailed, Student's t-test; n = 3, *, ** and *** represents $p < 0.05$, $p < 0.01$ and $p < 0.001$. Error bars represent the SD.

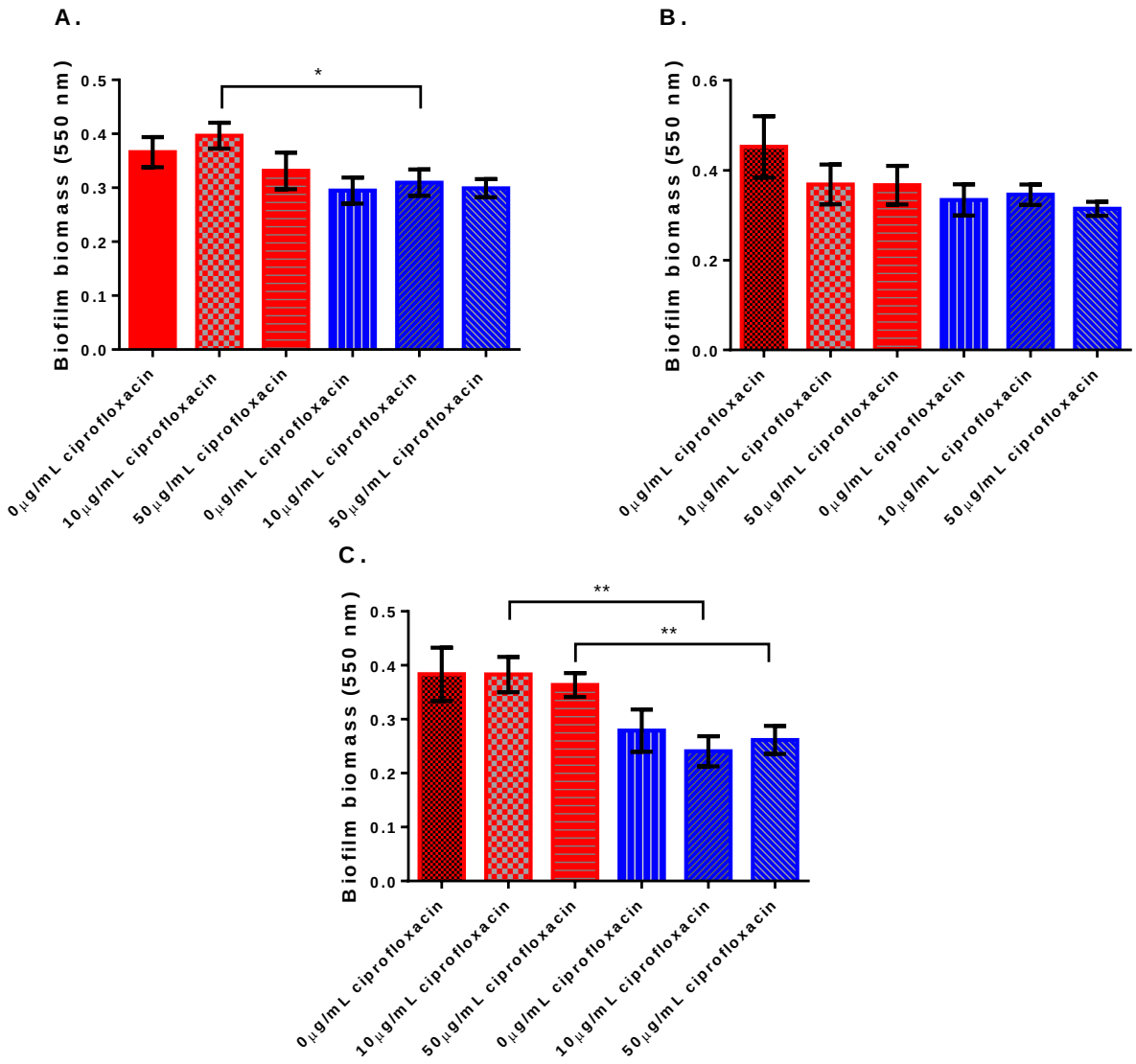

Figure 4.5 Comparison of the effect of antibiotics on *P. aeruginosa* ΔPf4 and WT PAO1 biofilm biomass in the presence of inactive Pf4 phage. Biofilms of the ΔPf4 mutant (blue bars) and wild-type strains (red bars) of *P. aeruginosa* were pre-grown for 6 h with (A) no treatment for the 6 h period, (B) inactive Pf4 phage for the 6 h, and (C) no treatment for the 6 h period before exposure to varying concentrations of ciprofloxacin for 1 h at 37°C. At the point of ciprofloxacin addition, inactive phage were added to (C) for 1 h. Biofilm biomass was determined via crystal violet staining and analysis with spectrophotometry at 550 nm. Data were analysed using GraphPad Prism 5 and statistical significance was determined via an unpaired, two-tailed, Student's t-test; n = 3, * and ** represents $p < 0.05$ and $p < 0.01$, respectively. Error bars represent the SD.

4.3.4 The Pf4 phage changes expression of *pvdO, ptxS, mexE, pchF, coxA* and PA3516

Genes were selected based on previous transcriptomic data, and mRNA expression was determined via qPCR, to determine whether the Pf4 phage is involved in the regulation of specific genes in *P. aeruginosa* PAO1 (278). In planktonic cultures, it was observed that there was significantly higher expression of *pvdO* (8 fold), *ptxS* (5 fold), *mexE* (5 fold) and *pchF* (7 fold) expression in the ΔPf4 mutant compared to the wild-type (Fig. 4.4A). There was no significant difference in expression observed for *coxA* and the probable lyase PA3516 between the wild-type and the ΔPf4 mutant. In the 2 d early biofilms, there was a slight decrease in the expression of *pvdO*, *coxA* and *pchF* (approximately 1.5 fold) for the ΔPf4 mutant in comparison to the wild-type (Fig. 4.4B). For *pvdO* and *coxA* the difference in expression was not significantly different (P = 0.39, P = 0.33, respectively), while the difference was significantly different for *pchF* (P = 0.05).

There was a slight increase of *mexE* and the probable lyase PA3516 expression in the ΔPf4 mutant compared to the wild-type (approximately 1.5 fold increase for both), but this was not statistically significant (P = 0.73 and P = 0.40, respectively). *ptxS* expression remained relatively unchanged between the ΔPf4 mutant and wild-type, and the difference was not statistically significant (P = 0.90). There was an overall trend of increased gene expression for the ΔPf4 mutant in comparison to the wild-type for the 7 d biofilm samples (Fig. 4.4C). In particular, there was a significant increase in *pvdO* and *pchF* gene expression observed for the ΔPf4 mutant in comparison to the wild-type (approximately 8 and 5 fold, respectively).

4.3.5 The Pf4 phage alters expression of *exoA*

The gene *exoA* is responsible for expression of exotoxin A, which is indirectly regulated by the transcriptional regular, *ptxS*. In this study, the Pf4 phage was shown to negatively regulate *ptxS* expression (Fig 4.6). Therefore, qPCR was used to determine whether the phage also affects *P. aeruginosa* exotoxin A expression (Fig. 4.7). The results from this experiment showed that the ΔPf4 mutant had higher *exoA* expression in both planktonic and 7 d biofilms than the wild-type (approximately 2 fold and 7 fold, respectively).

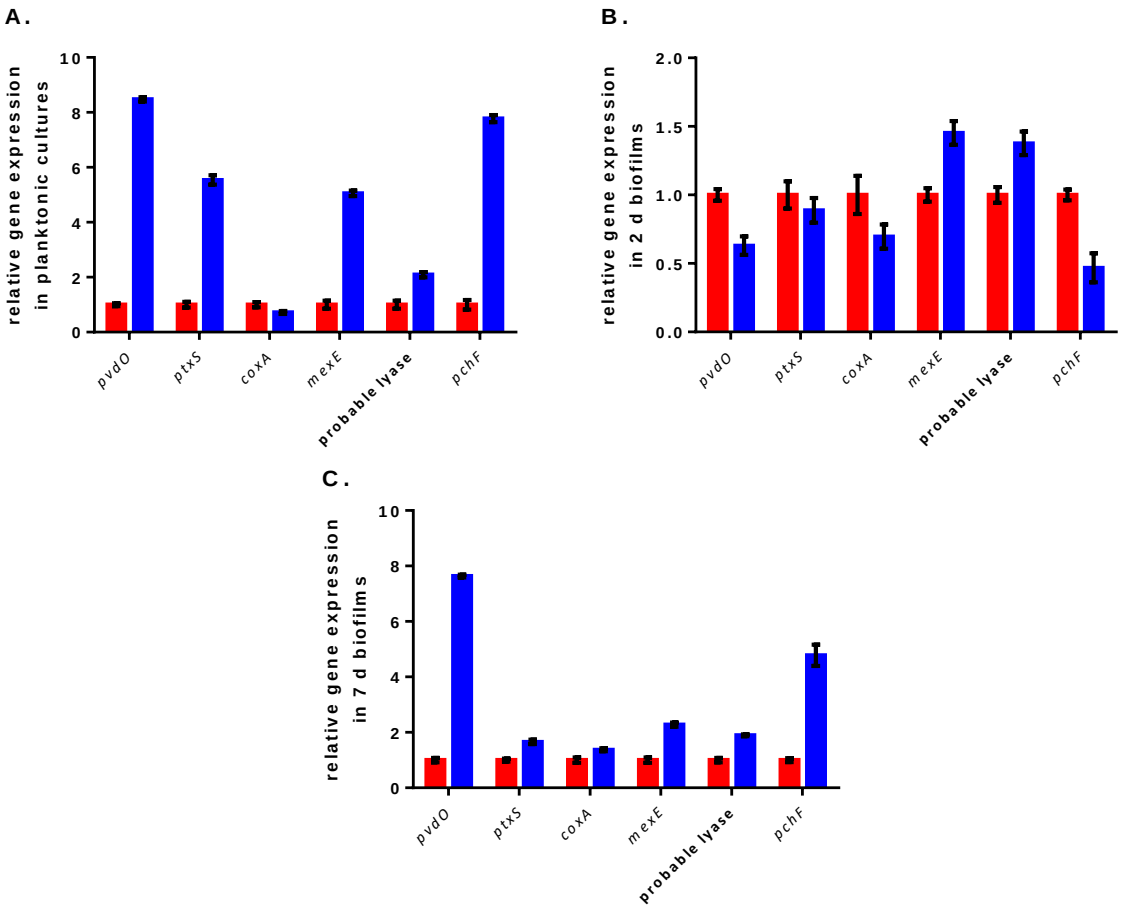

Figure 4.6 Relative expression of selected *P. aeruginosa* genes in planktonic and biofilm cultures. *P. aeruginosa* WT and ΔPf4 PAO1 planktonic cultures (A), 2 d biofilms (B) and 7 d biofilms (C) were grown and the harvested supernatant was analysed for expression of *pvdO*, *ptxS*, *coxA*, *mexE*, *pchF* and a probable lyase (PA3516) as determined via qPCR. Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method using the *recA* housekeeping gene as an internal control, due to it having similar Ct values for all samples (where *proC* Ct values varied considerably between samples) (314). Red and blue bars represent the WT and ΔPf4 PAO1 strains, respectively. Data were analysed using GraphPad Prism 5 (GraphPad Software Inc., U.S.A.) and statistical significance was determined via an unpaired, two-tailed, Student's t-test. Data represent the mean value of 3 independent experiments with SD.

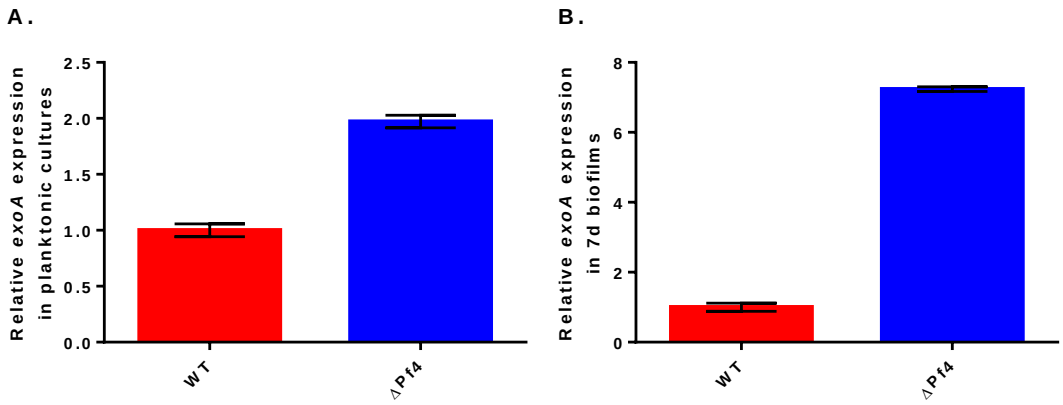

Figure 4.7 Relative expression of *exoA* by *P. aeruginosa* in planktonic and biofilm cultures. *P. aeruginosa* WT and ΔPf4 PAO1 planktonic cultures (A), and 7 d biofilms (B) were grown and the harvested supernatant was analysed for expression of *exoA* as determined via qPCR. Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method using the *recA* housekeeping gene as an internal control (314). Red and blue bars represent the WT and ΔPf4 PAO1 strains, respectively. Data were analysed using GraphPad Prism 5 (GraphPad Software Inc., U.S.A.) and statistical significance was determined via an unpaired, two-tailed, Student's t-test. Data represent the mean value of 3 independent experiments with SD.

4.3.6 The Pf4 phage affects expression of *mexF* and *oprN*

The gene *mexE* is part of an operon, which also includes the genes *mexF* and *oprN*. The three genes encode a *P. aeruginosa* efflux pump known as MexEF-OprN. Since it was shown that the Pf4 phage was associated with decreased *mexE* expression in planktonic cultures and biofilms, it was hypothesised that *mexF* and *oprN* expression would also be decreased when the Pf4 phage was present. It was observed that in planktonic, and both 2 d and 7 d biofilm cultures, *mexF* and *oprN* expression was lower in the wild-type compared to the ΔPf4 mutant (approximately 1.4 and 1.5 fold for *mexF and oprN* in planktonic cultures, approximately 2.3 fold and 4 fold for *mexF* and *oprN* in 2 d biofilms, and approximately 3.7 and 3.8 fold for *mexF* and *oprN* in 7 d biofilms) (Fig. 4.8). The differences were significantly different in the planktonic cultures (P = 0.0001 and P = 0.005 for *mexF* and *oprN*, respectively), the 2 d biofilms (P = 0.0001 for both *mexF* and

oprN), and the 7 d biofilms (P = 0.0001 for both *mexF* and *oprN*).

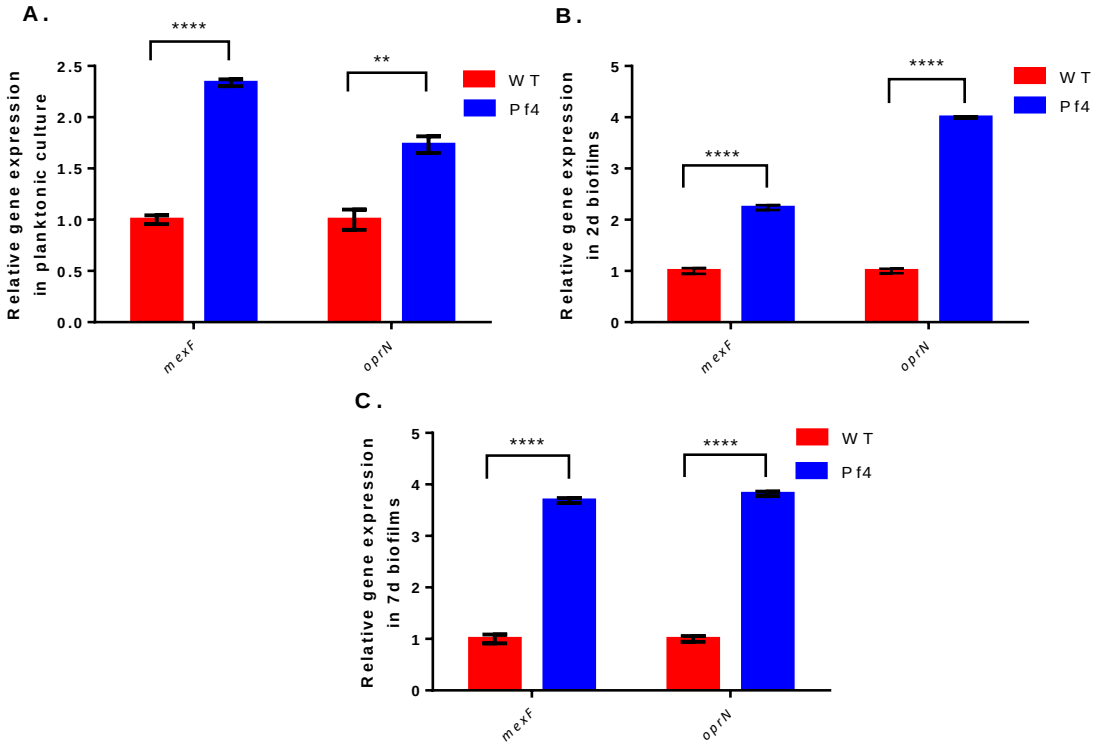

Figure 4.8 Relative expression of *mexF* and *oprN* *P. aeruginosa* genes in planktonic and biofilm cultures. *P. aeruginosa* WT and ΔPf4 PAO1 planktonic cultures (A), 2 d biofilms (B), and 7 d biofilms (C) were grown and the harvested supernatant was analysed for expression of *mexF and oprN* as determined via qPCR. Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method using the *recA* housekeeping gene as an internal control (314). Red and blue bars represent the WT and ΔPf4 PAO1 strains, respectively. Data were analysed using GraphPad Prism 5 (GraphPad Software Inc., U.S.A.) and statistical significance was determined via an unpaired, two-tailed, Student's t-test. Data represent the mean value of 3 independent experiments with SD, ** and **** represent $p < 0.05$ and $p < 0.0001$, respectively.

4.3.7 The Pf4 phage plays a role in *pslA* and *pelA* expression

In order to further understand the role of the Pf4 phage in contributing to *P. aeruginosa* EPS synthesis and stability, expression of the two major components, *pelA* and *pslA* in the presence and absence of the Pf4 phage was investigated. It was observed that in planktonic cultures, both *pelA* and *pslA* expression was lower in the Pf4 mutant than the wild-type (approximately 1.2 and 1.8 fold for *pelA* and *pslA*, respectively) (Fig. 4.9). The difference for *pelA* expression between the wild-type and Pf4 mutant was not statistically

significant (P = 0.30). However, *pslA* expression in the wild-type and Pf4 mutant was significantly different (P = 0.03).

There was an opposite trend observed for *pelA* and *pslA* expression in 2 d and 7 d biofilms, compared to planktonic cultures. After 2 d of biofilm growth, the expression of *pelA* and *pslA* in the wild-type was significantly lower than in the Pf4 mutant (approximately 4.6 fold; P < 0.0001, and approximately 3.8 fold; P < 0.0001 for *pelA* and *pslA*, respectively). The same trend was observed for *pelA* and *pslA* expression in 7 d biofilms, where both *pelA* and *pslA* expression was lower in the wild-type than the Pf4 mutant. The difference in *pelA* expression was not statistically significant (approximately 1.2 fold; P < 0.26). However, the different in *pslA* expression was significant (and 2.2 fold, P < 0.0001).

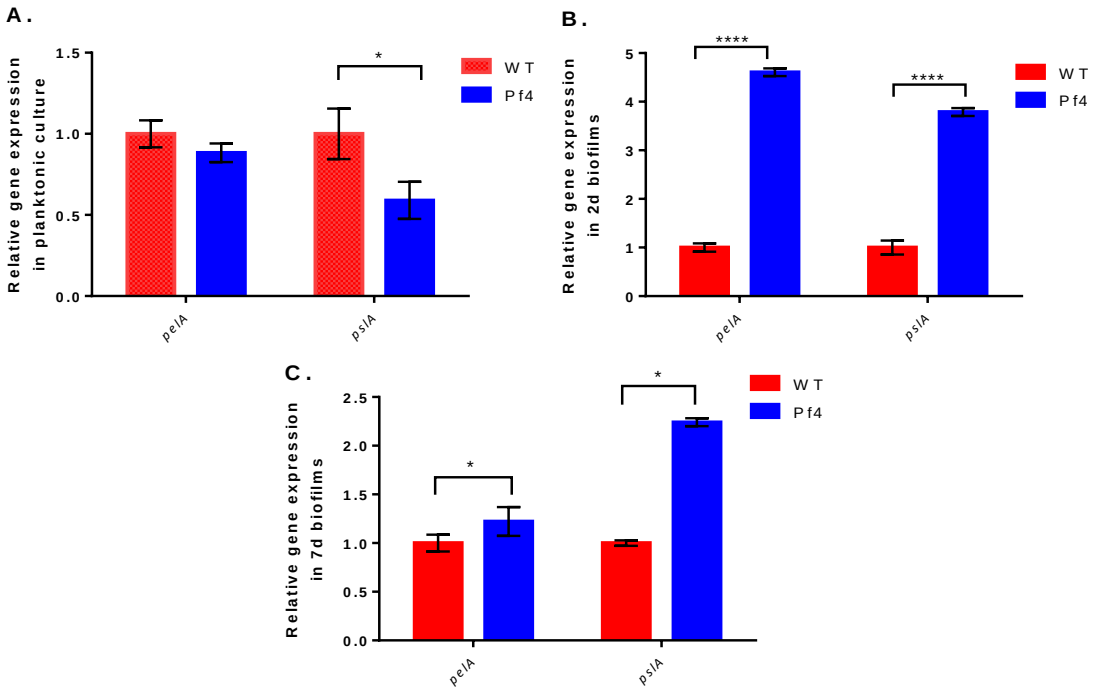

Figure 4.9 Relative expression of *pelA* and *pslA* *P. aeruginosa* genes in planktonic and biofilm cultures. *P. aeruginosa* WT and ΔPf4 PAO1 planktonic cultures (A), 2 d biofilms (B) and 7 d biofilms (C) were grown and the harvested supernatant was analysed for expression of *pelA* and *pslA* as determined via qPCR. Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method using the *recA* housekeeping gene as an internal control (314). Red and blue bars represent the WT and ΔPf4 PAO1 strains, respectively. Data were analysed using GraphPad Prism 5 (GraphPad Software Inc., U.S.A.) and statistical significance was determined via an unpaired, two-tailed, Student's t-test. Data represent the mean value of 3 independent experiments with SD, * and **** represent p < 0.05 and p < 0.0001, respectively.

4.3.8 *phd* mRNA expression in *P. aeruginosa*

The Pf4 prophage encodes a putative TA system comprised of the *parE* toxin gene which forms a complex with the *phd* antitoxin. TA systems have been associated with the alteration of host cell processes such as the disruption of DNA gyrase activity and cell wall synthesis, and the induction of persister cell formation, involving slowed or inhibited cell growth, and sometimes cell damage or death (161, 162, 398-400). An attempt was made to make complementation strains of all of the TA genes (*phd*, *parE*, and combined *phd-parE*). However, construction of these complementation strains was unsuccessful and this may be due to the activity of the ParE toxin. However, the *phd* antitoxin was successfully cloned into pHERD20T (Fig. 4.10), which allows for controlled expression of the *phd* gene. The cloned gene was verified by Sanger sequencing, and growth of *P. aeruginosa* transformed with the complementation vector in the absence of antibiotics for several days indicated that the plasmid was stably maintained (data not shown).

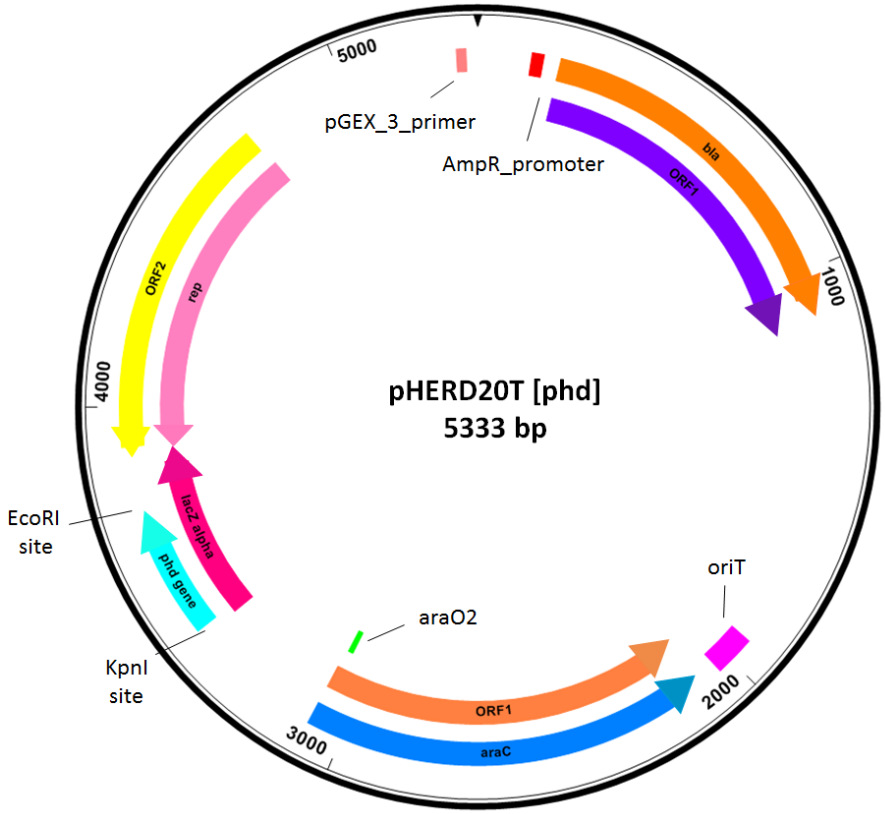

Figure 4.10 Plasmid map of the pHERD20T [*phd*] complementation vector. The *phd* gene was inserted into the pHERD20T plasmid, at the *Eco*RI and *Kpn*I restriction enzyme sites. Cloning into this position allows for arabinose inducible control of gene expresssion and the plasmid codes for ampicillin resistance (due to the *bla* gene of the plasmid) and allows for blue/white screening to identify clones with inserts (391).

This construct was used to determine the role of the Phd protein in controlling the various phenotypes associated with the Pf4 prophage. Therefore, ΔPf4 pHERD20T [*phd*] complementation strain was transformed into *P. aeruginosa* and the effect of *phd* expression on *pvdO, ptxS, coxA, mexE, pchF* and the probable alginate lyase was determined by qPCR with and without arabinose induction (Fig. 4.10). Arabinose induction of *phd* expression in the ΔPf4 mutant resulted in repression of gene expression for *pvdO*, *ptxS* and *pchF*, and in the case of *pchF* complementation appeared to restore the wild-type level of expression (Fig. 4.10). However, this was not observed for *coxA*, *mexE* and the probable lyase.

The results showed that upon induction *phd* expression, the wild-type expression levels were not restored. Closer inspection of the data showed that in some cases, e.g. *mexE*, the uninduced complementation vector resulted in wild-type levels of gene expression, while arabinose induction was associated with gene expression levels that were indistinguishable from the ΔPf4 mutant. However, the results showed that upon induction of the ΔPf4 pHERD20T [*phd*] complementation strain, the wild-type phenotype was not restored.

For example, *pvdO* and *mexE* expression in the ΔPf4 mutant were 8.5 and 5 fold, respectively, while the uninduced ΔPf4 pHERD20T [*phd*] complementation strain was 4.1 and 1.9 fold, respectively (Fig. 4.11). Induction of the ΔPf4 pHERD20T [*phd*] complementation strain with arabinose also yielded ambiguous results, with *mexE* expression in the absence of induction being 1.9 fold higher than the wild-type, and 4.5 fold higher than the wild-type when induced. This expression was more reflective of the ΔPf4 mutant, when it was expected to be more reflective of wild-type expression.

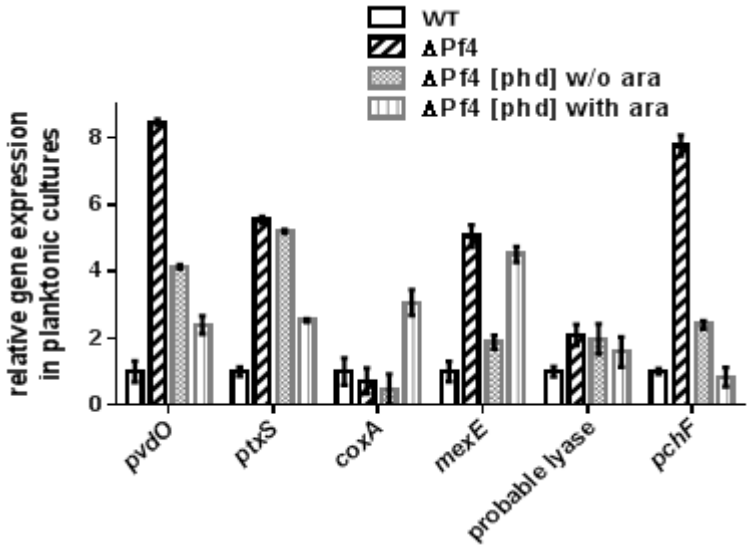

Figure 4.11 Relative expression of selected *P. aeruginosa* genes in planktonic cultures. Planktonic cultures of the *P. aeruginosa* ΔPf4 pHERD20T [*phd*] complementation strain were incubated with 2% (w / v) arabinose for 3 h (and compared to ΔPf4 pHERD20T [*phd*] grown with no arabinose, as well as planktonically grown *P. aeruginosa* WT and ΔPf4). The cells were harvested to determine the expression of the *pvdO*, *ptxS*, *coxA*, *mexE*, *pchF* and a probable lyase (PA3516) genes via qPCR. Relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method using the *recA* housekeeping gene as an internal control (314). Data were analysed using GraphPad Prism 5 (GraphPad Software Inc., U.S.A.) and statistical significance was determined via an unpaired, two-tailed, Student's t-test. Data represent the mean value of 3 independent experiments with SD.

The results suggest that either the *phd* gene does not play a role in regulating those genes or that the complementation vector was not functional. To determine whether the *phd* gene was being expressed upon induction, qPCR was conducted for samples from planktonic cultures and 7 d continuous-flow biofilms in the presence and absence of L-arabinose. In the presence of 2% (w / v) arabinose, there was a statistically significant increase in the levels of *phd* mRNA for both planktonic and 7 d biofilms (approximately 200 fold and 60 fold, respectively) compared to the wild-type and the uninduced control (Fig. 4.12).

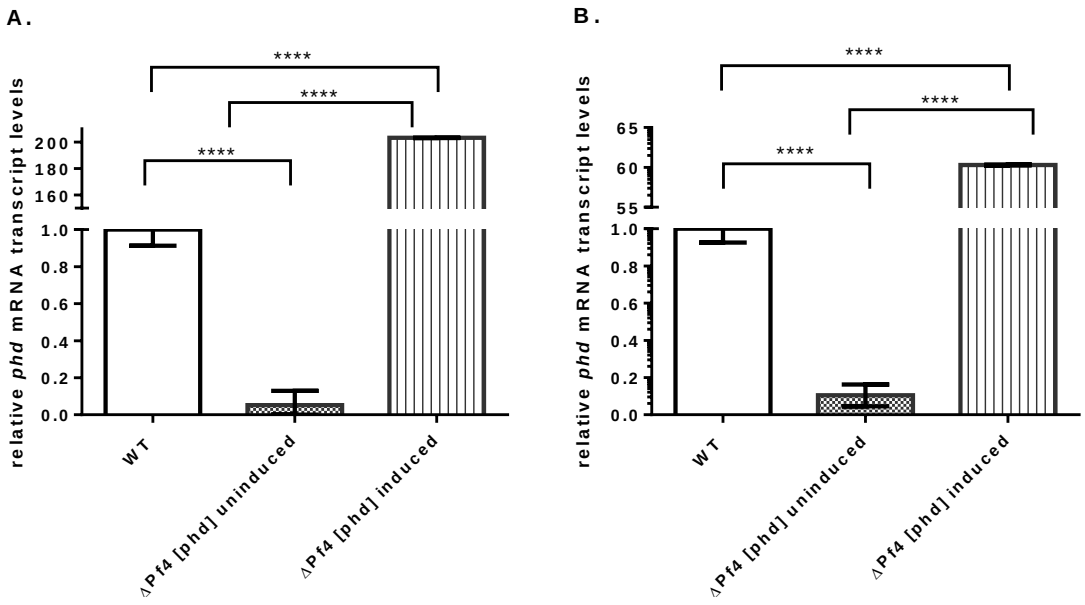

Figure 4.12 Relative expression of *phd* in *P. aeruginosa* wild-type and ΔPf4 pHERD20T [*phd*] planktonic and 7 d biofilm cultures. The *P. aeruginosa* wild-type and ΔPf4 pHERD20T [*phd*] strains were grown planktonically (A) or as 7 d biofilms (B). After growth, the ΔPf4 pHERD20T [*phd*] planktonic cells and 7 d biofilms were incubated with 2% (w / v) arabinose for 3 h (and compared to ΔPf4 pHERD20T [*phd*] grown with no arabinose). The cells were harvested and used to determine the expression of *phd* by qPCR and relative gene expression was determined by the $2^{-\Delta\Delta Ct}$ method using the *recA* housekeeping gene as an internal control (314). Data were analysed using GraphPad Prism 5 (GraphPad Software Inc., U.S.A.) and represent the mean value of 3 independent experiments with SD. Statistical significance, $p < 0.0001$ is represented by ****.

4.3.9 Determination of Phd protein production in *E. coli* and *P. aeruginosa*

In addition to mRNA expression, protein production was also investigated. Soluble protein was extracted from both *E. coli* [pHERD20T] TOP10 and *P. aeruginosa* ΔPf4 [pHERD20T] PAO1 in the presence and absence of arabinose and visualised by polyacrylamide gel electrophoresis. The Phd protein is predicted to be 252 amino acids in length and to be approximately 8.56 kDa. Despite induction of *phd* mRNA upon the addition of arabinose, no proteins of the expected size were observed for either *E. coli* or *P. aeruginosa* (Fig. 4.13).

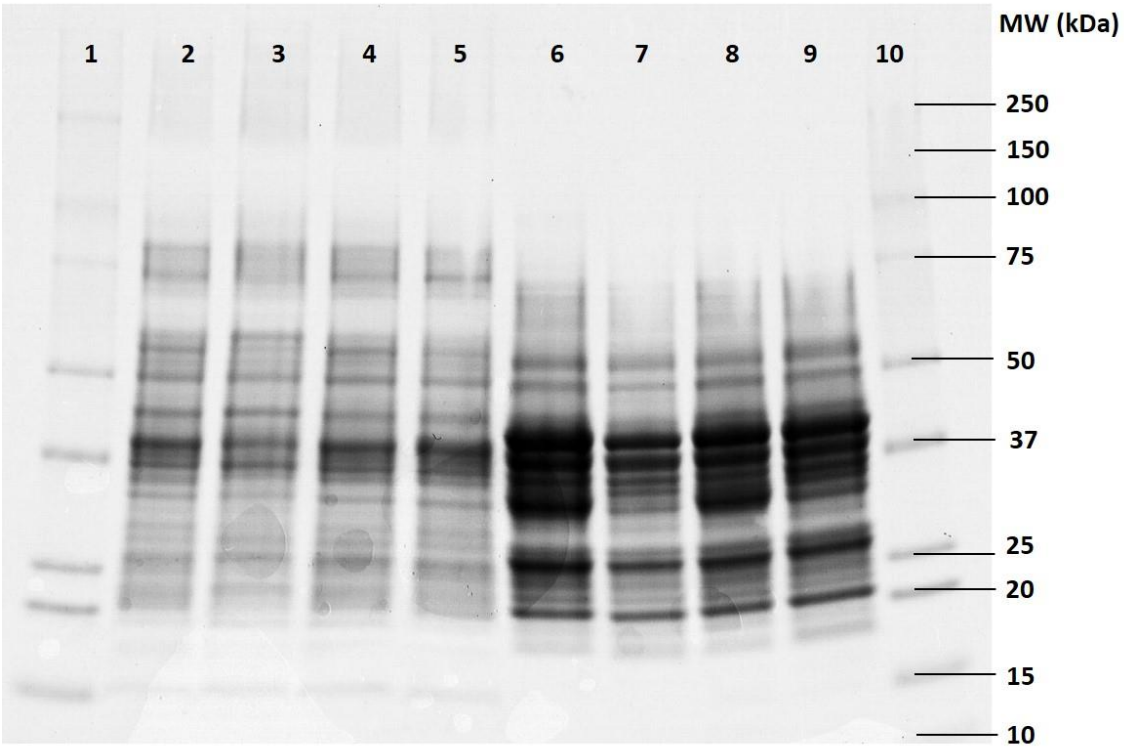

Figure 4.13 Protein expression of the Phd antitoxin in the *E. coli* TOP10 and *P. aeruginosa* PAO1 cells. Proteins were separated by SDS-PAGE to determine the presence or absence of the Phd protein (expected size of 8.53 kDa). The samples on the gel were (by lane): 1 and 10) Precision Plus Protein^{TM} WesternC^{TM} protein standard, 2) *E. coli* TOP10 [pHERD20T], 3) uninduced *E. coli* TOP10 [pHERD20T] [*phd*], 4) *E. coli* TOP10 [pHERD20T] [*phd*] induced with 0.2% (w / v) arabinose, 5) *E. coli* TOP10 [pHERD20T] [*phd*] induced with 2% (w / v) arabinose, 6) *P. aeruginosa* ΔPf4 [pHERD20T], 7) uninduced *P. aeruginosa* ΔPf4 [pHERD20T] [*phd*], 8) *P. aeruginosa* ΔPf4 [pHERD20T] [*phd*] with 0.2% (w / v) arabinose and 9) *P. aeruginosa* ΔPf4 [pHERD20T] [*phd*] induced with 2% (w / v) arabinose.

4.4 Discussion

In this study (Chapters 2 and 3), the Pf4 phage was shown to contribute to the virulence and biofilm stability of *P. aeruginosa* PAO1, which supports previous work (253). Virulence phenotypes that the Pf4 phage influences include invasion of mammalian cells and pyoverdine production. Furthermore, it was observed that, in *P. aeruginosa* PAO1 biofilms, the Pf4 phage significantly contributes to increased biofilm viability in the presence of ciprofloxacin. Therefore, to determine how the Pf4 phage contributes to these phenotypes, differential virulence gene expression as well as genetic and functional complementation. For the latter, the effects of phage particles on mammalian cells as well as sensitivity of biofilms to ciprofloxacin were tested here.

4.4.1 The interaction of the Pf4 phage with mammalian cells

In this study, it was shown that the presence of the Pf4 prophage contributes to *P. aeruginosa* PAO1 invasion of HepG2 and CFTE29o- mammalian cells (Fig. 2.2). However, it is unknown as to how the Pf4 contributes to this virulence factor. It has been previously observed that phage particles can bind to and interact with mammalian host cells. For example, fd filamentous phage particles harvested from *E. coli* were able to facilitate binding of *E. coli* to human epithelial type 2 (HEp-2) cells due to a peptide present on the capsid surface (401).

It was observed here that the presence of Pf4 phage particles induced cytotoxicity in both the HepG2 and CFTE29o- cells and that the toxicity was between 1.8 – 4.5% higher than the negative control. This would suggest that the phage particles contribute to virulence of the Pf4 encoding *P. aeruginosa*. However, when the results are compared with the cytotoxicty of PAO1 and the ΔPf4 mutant, the effect of the phage particles observed here accounts for only 8% and 25% respectively of the total cytotoxicity observed when CFTE29o- or HepG2 cells were exposed to *P. aeruginosa* wild-type PAO1 (Fig. 2.3B). Thus, the phage particles do not account for all of the toxicity of *P. aeruginosa*.

This may not be surprising since *P. aeruginosa* encodes a range of virulence factors independent of the phage. This suggests that Pf4 phage particles may contribute to the cytotoxicity of *P. aeruginosa* on mammalian cells, but is not the main factor involved. It has been previously shown in other bacterial systems that the presence of mammalian

cells increases phage production within the bacterial host. For example, when *S. pyogenes* was incubated in the presence of human pharyngeal cells, phage particle production increased by approximately 1 x 10^2 PFU/ mL (a total of approximately 1 x 10^5 PFU/ mL), in comparison to when *S. pyogenes* was grown in the absence of pharyngeal cells (397). Therefore, the effect of the presence of HepG2 mammalian cells on Pf4 phage production in *P. aeruginosa* PAO1 was determined. There was a slight decrease in phage production in the presence of the HepG2 cells, but this was not significantly different.

Collectively, these data indicate that Pf4 phage production influences *P. aeruginosa* PAO1 contact with and toxicity towards mammalian cells, as the phage particles alone do not account for the differences in cytotoxicity observed for the wild-type and ΔPf4 mutant. This suggests that the Pf4 phage and its products may be involved in the regulation of *P. aeruginosa* PAO1 virulence, rather than primarily acting upon mammalian cells.

The contribution of virulence genes by phage has previously been investigated in different systems. For example, both *V. cholerae* and *Campylobacter concisus* encode a Zot toxin, which is part of the filamentous phage genome, increases the intestinal permeability of the host through disruption of tight junctions (402). Since *P. aeruginosa* also possesses a gene that encodes a Zot-like toxin, it is possible that this gene could contribute to the toxicity towards mammalian cells. Future experiments designed to test whether the Zot-like toxin of *P. aeruginosa* can disrupt tight gap junctions would help to clarify this possible mechanism of virulence.

4.4.2 The effect of inactive Pf4 phage on ciprofloxacin sensitivity of the ΔPf4 mutant and wild-type *P. aeruginosa* PAO1 during biofilm growth

In this study, it was observed that the presence of the Pf4 phage increased resistance the tolerance of *P. aeruginosa* biofilms to ciprofloxacin stress (Chapter 3). However, the mechanism by which this occurred was unknown. Recently, Secor *et al.* showed that the Pf4 phage physically contributes to a liquid crystalline matrix, which increased the resistance of *P. aeruginosa* biofilms to aminoglycoside antibiotics (288). However, the physical effect of the Pf4 on resistance of *P. aeruginosa* to ciprofloxacin was not explored. To investigate this concept, inactive Pf4 phage were added to *P. aeruginosa*

biofilms to determine cell viability and biofilm stability with and without ciprofloxacin stress (Fig. 4.4 and Fig. 4.5).

The results suggested that the presence of inactive Pf4 phage increased resistance of *P. aeruginosa* biofilms for both the wild-type and the phage mutant. However, the greatest increase in resistance was observed when the ΔPf4 mutant was exposed to 50 µg/ mL ciprofloxacin stress after being pre-exposed to inactive Pf4 phage. These results would also suggest that the presence of Pf4 phage particles contributes to the higher ciprofloxacin resistance observed in the wild-type compared to the ΔPf4 mutant (Fig. 3.7).

Since the use of inactive Pf4 phage resulted in increased ciprofloxacin resistance, it is likely that the resistance is not caused by the Pf4 phage genetically conferring this phenotype, but rather through a physical effect. One possibility is that, as was shown for aminoglycoside resistance by Secor *et al.*, the Pf4 phage alters the physical structure of the biofilm matrix. It is possible that the phage particles directly bind to the ciprofloxacin to sequester the antibiotic, although this would require further work to test this hypothesis. Interestingly, the addition of the phage particles did not alter the amount of biofilm biomass and thus differences in the amount of biofilm cannot account for the enhanced stress tolerance of biofilms formed in the presence of the phage particles. None the less, the data clearly indicate a physical role of the phage particles in mediating stress tolerance for biofilms of *P. aeruginosa*.

4.4.3 The effect of the Pf4 phage on *pvdO, ptxS, mexE, mexF, oprN, pchF, coxA, psl, pel* and PA3516 expression

The results suggested that the Pf4 phage did not significantly contribute to *P. aeruginosa* PAO1 virulence through direct interaction of phage particles with the host or an increase in phage production. However, bacteriophage are known to influence bacterial virulence and persistence in the environment through the alteration of host gene expression. Therefore, it was further hypothesised that the Pf4 phage was influencing PAO1 host virulence and biofilm stability through the increase and decrease of specific host gene expression. The *P. aeruginosa* PAO1 genes tested were chosen based on previous transcriptomic data conducted by Tay *et al.* (278). These genes were *pvdO*, an enzyme involved in pyoverdine synthesis; *ptxS*, a transcriptional regulator; *mexE*, expressing a

multidrug efflux membrane fusion protein; *pchF*, responsible for pyochelin production, *coxA*, encoding for the cytochrome c oxidase, and *PA3516*, a putative lyase.

When the Pf4 prophage is present in the *P. aeruginosa* genome, it was observed that *pvdO*, *ptxS*, *mexE* and *pchF* expression decreased in planktonic cultures relative to the ΔPf4 mutant. In 7 d late biofilms, the presence of the Pf4 phage also resulted in decreased *pvdO* and *pchF* expression. Interestingly, however, in 2 d early biofilms, the Pf4 phage had no significant effect on the expression of the tested PAO1 genes. The two time points differ in that the early stage biofilm, day 2, occurs after irreversible attachment and involves the congregation of bacteria to form small clusters (138, 279). In contrast, the 7 d biofilm represents a population that is transitioning into the dispersal phase, and hence the two stages would be characterised by marked changes in gene expression (403). For example, *lasI*, a quorum sensing gene involved in biofilm adhesion and microcolony formation, is increased in early biofilm development, with *lasI* expression decreasing over the course of biofilm development (404). Dispersed biofilms, however, have been associated with gene expression such as increased flagella (*fliC*) and decreased pilus (*pilA*) expression (405).

Interestingly, in the presence of the Pf4 phage, *pvdO* and *pchF* were both downregulated in planktonic and dispersal stage biofilms. The two genes are responsible for the production of siderophores pyoverdine and pyochelin, respectively. These siderophores can sequester iron from the environment for use by the host bacterium for metabolic processes. However, pyoverdine and pyochelin are also known to control the expression of genes involved in the production of particular *P. aeruginosa* virulence factors. For example, (ferri)pyoverdine acts as a signalling molecule for the regulation of its own production (via the *pvd* locus), as well as the synthesis of exotoxin A and the PrpL protease (58). The siderophore, pyochelin has been shown to cause endothelial cell injury, including in the pulmonary arteries, through catalysis of hydroxyl radical formation (406). Ferripyochelin is also known to act as a signalling molecule for the upregulation of pyochelin biosynthesis in the bacterial cell (407).

The production of pyoverdine and pyochelin *in vivo* induces the host immune response (408, 409). In the case of free-swimming bacteria, there is a higher chance of clearance by the immune response than bacteria residing within a biofilm. Therefore, the results suggest that when the Pf4 phage downregulates *pvdO* and *pchF* gene expression,

pyoverdine and pyochelin production is reduced. Indeed, it has been previously shown that *pvdO* is necessary for pyoverdine production (410). The *pchF* gene encodes a peptide synthetase responsible for pyochelin formation from dihydroaeruginoate, an iron chelator (411). Therefore, the results suggest that when *pvdO* and *pchF* gene expression is reduced in the Pf4 mutant, pyoverdine and pyochelin production will be reduced accordingly.

Therefore, based on its role in modifying gene expression, the decrease in siderophore production observed when the Pf4 phage was present in planktonic cultures and 7 d biofilms (this study; Fig. 2.6 and Fig. 4.6), particularly in the case of pyoverdine, could decrease virulence factor expression, and thus increase the immune evasion for planktonic and dispersed cells. However, in the 2 d early biofilm, the bacteria may be protected from the host immune response through a number of mechanisms, including changes in gene expression as well as the production of the biofilm matrix. Therefore, increased expression of *pvdO* and *pchF* would not result in clearance of the biofilm encased cells.

Another interesting aspect of these results is that they indicate that the Pf4 phage is involved in the regulation of *P. aeruginosa* iron sequestration encoding genes that can scavenge divalent cations from the environment, and play a role in iron-mediated virulence. In *E. coli*, the bacteriophage encoded Shiga-like toxin-1 (SLT-I) operon is regulated by the host bacterial *fur* gene and the presence of iron (412). Furthermore, the diphtheria toxin (Dtx) of *Corynebacterium diphtheriae*, which is encoded by the corynebacteriophage *β*, is also regulated by iron, with high levels of iron inhibiting toxin production (413). Therefore, it is possible that the Pf4 phage could regulate *pchF* and *pvdO* expression to control specific phage-encoded virulence factors. However, further explore this hypothesis, further studies would need to be conducted, which could include testing the sensitivity of the ΔPf4 mutant to different iron levels. The effect of *pchF* and *pvdO* expression on *P. aeruginosa* PAO1 gene expression could also be determined, particularly *zot*-like toxin expression, in low and high iron conditions, through qPCR. This could give insight as to whether siderophore expression (influenced by the Pf4) could be regulating *P. aeruginosa* toxin production.

ptxS expression was also decreased in wild-type *P. aeruginosa* planktonic cells compared to the ΔPf4 mutant, a trend which was also observed in 7 d biofilm cultures. However, this trend was not observed in 2 d biofilms. The *ptxS* gene is a transcriptional regulator, which has been shown to negatively regulate *ptxR* expression, a gene which positively

regulates exotoxin A expression (414). Therefore, this would suggest that the Pf4 phage alleviates the negative regulation of *ptxR* resulting in increased exotoxin A production in planktonic cells, and to some degree, 7 d biofilms. However, this contradicts the hypothesis of decreased exotoxin A production in planktonic cells, which is caused by lowered pyoverdine production (as a result of decreased *pvdO* expression) in the presence of the Pf4 phage.

It was then hypothesised that when pyoverdine production, and hence exotoxin A production, is lowered, there may not be sufficient levels of exotoxin A required for negative regulation of *ptxR* by *ptxS*. In order to test this hypothesis, qPCR was performed to determine the effect of the Pf4 phage on *exoA* expression (Fig. 4.7). It was found that the presence of the Pf4 phage decreased *exoA* expression in planktonic cultures and 2 d biofilms, which supported this hypothesis. More importantly, these results further suggest that the Pf4 phage contributes to *P. aeruginosa* persistence in the host through the regulation of virulence factors such as pyoverdine and exotoxin A.

There was an overall trend of decreased *mexE* expression in the wild-type *P. aeruginosa* relative to the ΔPf4 mutant for planktonic, and 2 d and 7 d biofilm cultures. MexE is part of a multidrug efflux pump that also includes MexF, a cytoplasmic membrane efflux pump and OprN, an outer membrane protein (415). The three proteins are thought to form a channel across the inner and outer membranes to transport various antibiotics including fluoroquinolones, trimethoprim, imipenem and chloramphenicol out of the cell, increasing bacterial antibiotic resistance (415). Interestingly, it has been shown that while overexpression of the MexEF-OprN efflux pump does not affect the metabolic fitness of the bacterial cell, it does affect the *P. aeruginosa* transcriptome. Overexpression of MexEF-OprN decreases the production of *rhl* controlled extracellular virulence factors, including pyocyanin, elastase and rhamnolipids (416). Furthermore, overexpression results in impaired production of 2-heptyl-3-hydroxy-4-quinolone (PQS), a signalling molecule involved in iron scavenging, and decreased QS-regulated gene expression (417, 418).

Therefore, it was hypothesised that a Pf4 phage mediated reduction in *mexE* expression would also be associated with a reduction in *mexF* and *oprN* expression and could therefore increase virulence of the wild-type *P. aeruginosa*. Through qPCR analysis, this hypothesis was confirmed; that is, the presence of the Pf4 phage in the wild-type strain

decreased *mexF* and *oprN* expression in planktonic, and 2 d and 7 d biofilm cultures. This suggested that the presence of the Pf4 phage has a significant effect on the expression of all the genes in the MexE-OprN efflux pump operon. This might explain the increased virulence of the wild-type *P. aeruginosa* relative to the phage mutant, when compared in an acute mouse infection model (253).

Recent studies by Secor *et al.* (2015) have highlighted the role of the Pf4 phage in increasing *P. aeruginosa* EPS synthesis and biofilm stability (288). Therefore, it was of interest to determine the effect of the Pf4 phage on expression of the two main genes associated with *P. aeruginosa* EPS synthesis, *pelA* and *pslA* (140). It was observed that the Pf4 phage decreased both *pelA* and *pslA* expression in planktonic cultures, but increased *pelA* and *pslA* expression in biofilm cultures. This data supports the work of Secor *et al.* as it suggests that the Pf4 phage promotes the expression of EPS associated genes.

Another observation was that *pslA* and *pelA* expression was lower in 2 d biofilms when the Pf4 phage was present. Similarly, the expression of *pslA* was also lower in the 7 d biofilm for the wild type relative to the phage mutant. In contrast, the 7 d biofilm qPCR data suggested that the Pf4 phage has no significant effect on Pel production. These observations were unexpected since the Pf4 phage, present in the wild type strain, is associated with increased biofilm formation (253). It remains to be determined if these changes in gene expression result in changes in the biopolymers of the biofilm matrix and its physical properties. Further work should either use lectin stains specific for Pel and Psl to determine their relative proportions in the two strains. Alternatively, imaging techniques such as atomic force microscopy (AFM) or video particle tracking could be used to determine the physical properties of the biofilm, e.g. viscoelasticity, which might explain differences in the biofilm formed by the two strains. Thus, while it is clear that the Pf4 phage has a significant impact on the expression of genes involved production of polysaccharides associated with the biofilm matrix, it is currently unknown how this effect is mediated or the impact on biofilm development and response to stressors such as antibiotics or surfactants.

Another observation made was that *pslA* and *pelA* expression was lower in 2 d biofilms when the Pf4 phage was present. However, it is known that the Pf4 phage increases biofilm development. Since *pelA* and *pslA* are involved in expression of Pel and Psl, two

polysaccharides involved in EPS production, it was hypothesised that the Pf4 phage would increase *pelA* and *pslA* expression in *P. aeruginosa* PAO1 biofilms. However, the 7 d biofilm data suggested that the Pf4 phage has no significant effect on Pel production, as evidenced by similar *pelA* expression levels in the wild-type and ΔPf4 mutant. There was still higher expression of *pslA* in the 7 d biofilm when the Pf4 mutant was absent. It is unclear as to why the Pf4 mutant downregulates *pelA* and *pslA* expression in *P. aeruginosa* PAO1 biofilms, and how this contributes to biofilm development and stability.

4.4.4 Construction of a prevent-host-death (*phd*) antitoxin complementation strain in *P. aeruginosa* ΔPf4

To determine if any of the specific Pf4 genes were involved in the differences in either gene expression or phenotypes observed, the various Pf4 genes were to be cloned and expressed in the ΔPf4 mutant. This approach would help to determine if the specific effects on gene expression or phenotypes were attributable to a single gene or not. Of the 17 genes encoded by the Pf4 phage, only the *phd* gene was successfully cloned despite trying multiple cloning strategies. This may be due to the overall toxic effects of individual phage genes. Conversely, there is a possibility that cell death occurred due to a particular single gene being inactivated in the phage genome (e.g. the *parE* toxin). This phenomenon has been previously observed in bacteria infected with Ff phage, where 'abortive infection' occurs. This process involves the bacterial cell being killed due to the inhibition of progeny virion release, as a result of any single phage gene being inactivated, with the exception of gII (419). This inhibition can be triggered by the inactivation of single phage genes, such as those involved in viral DNA replication (419).

In the Pf4 phage, the *phd* antitoxin gene is transcriptionally coupled with a putative toxin gene, *parE* and appear to constitute a type II toxin-antitoxin (TA) system (250). The *phd* and *parE* genes are arranged in the same operon, where upon binding, the Phd antitoxin controls ParE toxin expression by inhibiting translation of the toxin; when active, the ParE toxin inhibits DNA gyrase activity of the host cell, therefore inhibiting cell growth and replication (250). It has been previously hypothesised that the TA system could be involved in regulating *P. aeruginosa* PAO1 cell death during biofilm development (239). Unfortunately, the induction of *phd* mRNA expression from the pHERD20T plasmid construct did not result in increased levels of the Phd protein. Artificially increased levels

of proteins is known to result in some cases in insoluble complex formation due to interaction between hydrophobic interfaces (420). These protein aggregates are most often rapidly degraded by cellular protein quality control systems. Indeed, in *P. aeruginosa*, the overexpression of quorum sensing receptors (in the absence of the cognate HSL) is known to result in protein aggregation and degradation (420, 421). Therefore, it is possible that artificially high levels of the Phd protein (and in the absence of the ParE toxin) lead to rapid degradation of Phd protein complexes.

Therefore, testing whether complementation by the ectopic expression of the *phd* gene has an effect on *P. aeruginosa* PAO1 biofilm cell death could indicate whether the ParE toxin is responsible for cell death, or whether the process is independent of the TA system. The ΔPf4 pHERD20T [*phd*] complementation strain was successfully constructed and upon induction of the plasmid by arabinose, there was clear expression of the *phd* gene as determined by qPCR (Fig 4.11). However, when the protein production of the induced *phd* complementation strain in both *E. coli* and *P. aeruginosa* cells was examined via SDS-PAGE, no Phd product was observed (Fig. 4.13). It is hypothesised that the lack of protein production was due to rapid degradation of the Phd protein, as the antitoxin components of TA systems are generally more unstable in comparison to their toxin counterparts and are prone to degradation by proteases (160). For example, in TA systems like RelE - RelB of *E. coli*, the Lon protease degrades the RelB antitoxin, so that the RelE toxin can be translated and disrupt mRNA translation of the host cell (381, 382). Since the ParE toxin is not present in the *phd* complementation strain, the Phd antitoxin could be rapidly degraded for this reason. In either case, due to the instability of Phd protein, future studies may include complementation of the entire *parE-phd* toxin-antitoxin (TA) system for analysis of the system on *P. aeruginosa* PAO1 biofilm cell death.

4.4.5 Conclusions

Based on the results presented here, the direct interaction of Pf4 phage particles significantly impacted cytotoxicity towards mammalian cells, yet only contributed a small percentage of the levels of cytotoxicity observed in the presence of WT PAO1. Furthermore, the presence of mammalian cells did not induce a significant change in Pf4 phage production. This suggests that direct interaction of Pf4 phage particles is not the main contributing factor to the invasion and cytotoxicity phenotype observed in Chapter

2. Therefore, it is more likely the Pf4 mediates its effects by modifying gene expression in *P. aeruginosa*. Indeed, it was observed that expression of *pvdO*, *pchF*, *mexE* and *ptxS* were lower in the wild-type *P. aeruginosa* relative to the ΔPf4 mutant. Therefore, there is a possibility that the Pf4 phage decreases specific host gene expression to increase particular host virulence phenotypes. Further experimentation is required to gain a better understanding of the influence of specific phage genes on the virulence and biofilm stability of *P. aeruginosa*. This includes phenotypic studies involving *P. aeruginosa* phage gene complementation strains. Construction of a *P. aeruginosa* complementation strain with the *phd* antitoxin was attempted. The *phd* sequence was successfully introduced into the ΔPf4 *P. aeruginosa* strain and an increase of *phd* mRNA expression was detected upon induction of the *phd* gene. However, further optimisation and troubleshooting is required to understand why no protein was observed via SDS-PAGE after induction of the *phd* gene. The role of the Pf4 phage in mediating biofilm phenotypes in *P. aeruginosa* may extend beyond effects on gene expression, where the phage particles may also physically alter the biofilm matrix in some way to afford protection to the host bacterium. Thus, the phage may interact with, and increase the fitness of, its host through multiple mechanisms.

5 General Discussion

Numerous studies have suggested that *P. aeruginosa* undergoes adaptive evolution during chronic infection of the CF lung, which is indicative of allopatric speciation (422). However, chronic infection sites are not the primary growth environment for *P. aeruginosa*. This organism is found in a wide variety of habitats, ranging from fresh and salt water to soils, and it is likely that the broad metabolic capabilities and high intrinsic resistance have contributed to its emergence as an opportunistic pathogen, while the CF lung is yet another habitat that *P. aeruginosa* can successfully colonise (1, 2). Understanding the evolutionary origins of bacterial traits that lead to human infections is of great interest and may aid in the development of novel therapeutic approaches (e.g. anti-virulence drugs) (423). One theory to explain the emergence of bacterial opportunistic pathogens is the coevolution of the pathogen with the natural antagonists present in their primary growth environment such as protozoa and phage (examples include (344, 359, 424-426)).

For *P. aeruginosa*, it has previously been shown that the filamentous prophage Pf4 has a significant impact on the development and function of *P. aeruginosa* biofilms. For example, isolates of the *P. aeruginosa* AES-1 clonal strain (also called P1) has been shown to form significantly larger biofilms compared to isolates of P. aeruginosa non-clonal (NC) strains and even *P. aeruginosa* PAO1 (380). This increased biofilm development has been linked to the presence of, and higher expression of, the *parE* toxin gene (PA0729) in AES-1 than in *P. aeruginosa* PAO1, which is located in the Pf4 prophage (380). Furthermore, biofilm development is even greater in *P. aeruginosa* AES-1 compared to *P. aeruginosa* NC, and this is likely to be due to the lack of the *parE* toxin gene in most NC isolates (415).

In an acute mouse virulence model, the presence of the Pf4 phage has been associated with increased virulence in *P. aeruginosa* PAO1 (253). Also, the Pf4 phage significantly increased *P. aeruginosa* PAO1 biofilm stability when subjected to sodium dodecyl sulphate surfactant stress (253). Thus, the Pf4 phage contributes significantly to the virulence and biofilm developmental program of *P. aeruginosa* PAO1.

In the case of *R. solanacearum*, the filamentous phage ΦRSS1 contributes to host virulence through increased twitching motility and type IV pili production, and increased EPS production, leading to early wilting of tomato plants (258). It has also been observed that some ΦRSS1 ORFs do not contribute to *R. solanacearum* virulence, but instead promote filamentous phage assembly on the host cell surface, which is thought to alter cell surface nature and promote cell-to-cell interactions, and as a consequence, lead to increased cell densities (258). This observation is similar to what has been observed in SCVs, where there were high numbers of Pf4 filaments observed on the bacterial cell surface that were not seen in the wild-type (250). Therefore, like ΦRSS1, the Pf4 phage may contribute to *P. aeruginosa* virulence directly or indirectly by stabilising the biofilm. Based on these results, it is tempting to speculate that the phage particles physically contribute to the biofilm matrix and may be the proximal reason why the wild-type PAO1 biofilm is more resistant to SDS stress than the Pf4 phage mutant.

Similarly, the CTXΦ of *V. cholerae*, encodes the CTX cholera toxin and zonula occludens toxin (Zot) (262). Interestingly, the Pf4 phage contains a Zot-like toxin, which indicates some homology of the Pf4 phage to other filamentous phage, as well as the potential for the Pf4 phage to be involved in toxin production. However, this study did not indicate whether the Pf4 phage contributed to toxicity of *P. aeruginosa* (as seen in the cytotoxicity assays performed in mammalian cells, and the toxicity assay performed with *C. elegans*; Chapter 2). One significant point of difference between the human host, and these tissue culture or *C. elegans* models is the adaptive immune response of mammals, which is not present in nematodes or the tissue culture cells. Therefore, it may be of interest to investigate the effect of wild-type versus Zot-deficient *P. aeruginosa* strains in an acute infection mouse model. Beyond these factors, the filamentous phage may also impact their bacterial host through the regulation of specific virulence factors, antibiotic resistance markers or nutrient scavenging mechanisms to increase their fitness.

5.1 Bacterial competitiveness and survival

The Pf4 phage has been identified in many medical and laboratory strains, where it has been shown to be present in 73% of CF lung sputum isolates, suggesting that the phage confers a selective advantage to its host. It is possible that the phage is selected for based on the positive effects relating to biofilm development during infection or it may be

retained due to the presence of an addiction module (i.e. the ParE-Phd TA system). At least one study has suggested that there is positive selection within the Pf4 phage. This was based on the observation that, in stark contrast to the host genome, the phage genome accumulates non-synonymous mutations at an increased rate of 2.43×10^{-3} substitutions per site per day in *P. aeruginosa* biofilms grown for 12 d (281) and that the mutations are confined to specific genes within the Pf4 genome (281). In the co-incubation experiments performed in this study, it was observed that the Pf4 deletion mutant was less competitive than the wild-type strain, suggesting that the phage increased the fitness of its host (Chapter 3). This effect was presumably caused by infection of the Pf4 deletion mutant by phage particles that were produced by the wild-type strain, resulting in the death of the newly infected cells. It is also possible that under natural conditions, where a phage positive and phage negative strain of *P. aeruginosa* co-occur, that the phage negative strain can become lysogenised by the Pf phage and in this way, the Pf4 phage would spread throughout the population.

5.2 The Pf4 phage and antibiotic stress

Biofilm formation of *P. aeruginosa* in the cystic fibrosis lung leads to a chronic and persistent infection that involves repeated host inflammatory responses that cause lung tissue injury, decreased lung function, and eventually patient mortality (427). *P. aeruginosa* biofilm infections are extremely difficult to eradicate because they are often recalcitrant to antibiotics, reactive oxygen species, surfactant proteins and amoebal grazing (reviewed in (362, 428)). Current therapeutic options focus on aggressive antimicrobial regimes that aim to eradicate the initial infection and limit the effects of acute exacerbations (429). The use of non-antibiotic strategies such as gallium, antimicrobial peptides such as lactoferrin, and garlic compounds such as allicin and ajoene have been explored for the eradication of *P. aeruginosa* CF lung infection (430). However, the current therapeutic approach for the treatment of early stage and acute *P. aeruginosa* CF lung infection is based on the use of antibiotics, such as ciprofloxacin; although, as noted above, ciprofloxacin is not effective on its own against chronic infections (431, 432). Despite the lack of efficacy with ciprofloxacin, other antibiotic therapies have shown to eradicate between 63 - 100% (mean of 81.2%) of infections (reviewed in (429)).

In this study, it was observed that the Pf4 phage contributes to increased resistance of *P. aeruginosa* biofilms to ciprofloxacin exposure (Chapter 3). Furthermore, it was shown that inactive Pf4 phage contribute to the tolerance of *P. aeruginosa* under ciprofloxacin stress (Chapter 4). While the specific mechanism by which the Pf4 phage contributes to the increased resistance of *P. aeruginosa* to ciprofloxacin is still to be determined, there are several possible mechanisms that could be involved.

The involvement of inactive Pf4 phage particles suggests that the phage has a physical effect on the stress tolerance of *P. aeruginosa* to ciprofloxacin. It has been shown that the Pf4 phage increases resistance to aminoglycosides through the formation of a liquid crystalline matrix for *P. aeruginosa* and as a result, binding and sequestration of aminoglycosides (288). Therefore, it is possible the phage increases *P. aeruginosa* resistance to ciprofloxacin through binding and sequestration of the fluoroquinolone, although this is yet to be experimentally demonstrated. Thus, the phage may influence the function of *P. aeruginosa* biofilms through a combination of physical effects as well as specific changes in gene expression that influence virulence and stress tolerance.

It is also possible that the increased resistance is, at least in part, mediated through oxidative stress responses (Chapter 3). It has been suggested that ciprofloxacin kills bacteria through the production of toxic oxygen free radicals in conjunction with DNA gyrase inhibition, although further work is needed to confirm this hypothesis (433-436). For example, it has been shown that in the presence of ciprofloxacin stress, *P. aeruginosa* biofilms produced cytotoxic levels of hydroxyl radicals, partly due to the formation of superoxide, and scavenging of these radicals can reduce ciprofloxacin toxicity (433). Interestingly, the results presented here (Chapter 3) showed that the Pf4 phage contributed to biofilm stability in the presence of superoxide (paraquat) stress. Therefore, it is possible that the Pf4 phage could contribute to the resistance of *P. aeruginosa* biofilms from hydroxyl radicals produced in the presence of ciprofloxacin.

Another hypothesis based on recently published work is that OxyR could be involved in conversion of the Pf4 phage to a superinfective (SI) form, thus leading to increased small colony variant (SCV) formation which is a phenotype associated with increased antimicrobial resistance (284, 285). It has been shown that the OxyR gene, which can activate the production of catalases, alkyl hydroperoxide reductases and superoxide dismutases in response to oxidative stress, binds within the ORF of the repressor *c* gene

in the Pf4 phage (437). However, it has been shown that *oxyR* mutants in the presence of reactive oxygen and nitrogen species, have an earlier onset of SI infection and also had more SCV formation in late stage biofilm development (284). It is thought that the OxyR could bind to the repressor C promoter and prevent its expression in the absence of oxidative stress (284). However, in the presence of oxidative stress, the repressor C promoter region could acquire mutations that inhibit the binding of OxyR to this region, and hence increase SI infection and SCV formation (284). Therefore, hydroxyl radicals generated as a result of ciprofloxacin stress could increase the production of SCVs that confer increased resistance to *P. aeruginosa* by a mechanism involving the Pf4 phage. This could be assessed by comparing resistance to ciprofloxacin wild-type and *oxyR* deletion mutant strains.

An additional mechanism to explain the Pf4 dependent ciprofloxacin resistance could involve the ParE-PhD toxin-antitoxin system inducing persister cell or SCV formation. Persister cell formation is the temporary inhibition of growth that enables survival of bacterial cells in otherwise lethal growing conditions, and allows for subsequent growth once the conditions improve (reviewed in (167)). One of the best characterised mechanisms for persister cell formation involves the expression of a toxin-antitoxin (TA) system (reviewed in (159)). In *E. coli*, it has been shown that ciprofloxacin exposure induced persister cell formation in a TA dependent fashion. Specifically, the TisB toxin (which is part of the TisB-IstR TA system) is involved in persister cell formation as a result of ciprofloxacin exposure (162). By promoting a dormant state in the bacterium, the TisB toxin affords resistance to the metabolically dependent activities of ciprofloxacin (gyrase inhibition and the production of reactive oxygen species) (162). The Pf4 phage encodes a TA system comprising the putative toxin ParE and putative antitoxin Phd (239). It is known that persister cells have been associated with increased levels of TA transcripts, where the presence of a DNA-damaging antibiotic can induce an SOS response in the host, resulting in induction of various TA genes, which promote persister cell formation (162). Therefore, it is possible that, in the presence of ciprofloxacin, *P. aeruginosa* may undergo an SOS response, resulting in increased *parE-phd* expression that in turn promotes persister cell formation and population survival. It would be of interest to compare persister cell formation by wild-type *P. aeruginosa* after ciprofloxacin stress in comparison to persister cells formed from a *parE-phd* deletion mutant.

Another important aspect of the ParE-Phd TA system is that the ParE toxin binds gyrase in a complex formation similar to how ciprofloxacin binds gyrase. In fact, overexpression of the ParE toxin led to an increase in SCV formation, a characteristic associated with antibiotic resistance ((253), Lau and Kjelleberg, unpublished data). This suggests that the phage could impact bacterial resistance because the action of ciprofloxacin binding to the bacterial DNA gyrase is lethal, whereas binding of the ParE toxin to the DNA gyrase is not lethal, and transiently inhibits gyrase activity to promote persister cell formation and resistance to ciprofloxacin (239, 376).

5.3 The Pf4 phage affects pyoverdine production

The siderophore pyoverdine is involved in the chelation of iron from the environment and acts as a virulence factor during *P. aeruginosa* infections (58). Since it was observed that the Pf4 phage increases the virulence of its bacterial host in an acute mouse infection model, it was hypothesised that pyoverdine production would increase in the presence of the Pf4 phage (253). Surprisingly, this study revealed that the Pf4 phage decreased expression of *pvdO* (Chapter 4), and also reduced the production of pyoverdine in planktonic cultures (Chapter 2). It should be noted that iron regulation by *P. aeruginosa* is extremely complex, involving multiple systems that differ in their metabolic requirements and affinity for iron (438). Accordingly, the use of specific uptake systems would likely be optimised according to environmental conditions experienced by the cell (438). Given the complexity of iron regulation, it is not clear what effects altering PvdO regulation may have on bacterial fitness, as the role of pyoverdine may be specific for particular environmental conditions not seen here.

Previous studies have suggested that the production of individual iron acquisition systems can be maladaptive to *P. aeruginosa* populations depending on the levels of available iron. An experiment conducted in King's broth, a low iron medium, showed that the pyoverdine defective mutant had higher cell density, and therefore increased fitness when compared with the pyoverdine producing wild-type strain (439). Although it was not a significant difference, it is interesting to note that there was a negative correlation between pyoverdine production and cell growth in *P. aeruginosa* planktonic cultures (this study; data not shown). This trend was similar to that observed by Zhang and Rainey (439).

The role for individual iron regulatory systems in specific infection settings remains unclear. Although the siderophore can provide the bacterium with iron, a necessary component for the bacterium to survive, it can also upregulate other virulence factors and as a consequence, the host immune response (58). This could also indicate that pyoverdine overproduction may not be beneficial or necessary in an acute mouse infection model. It has been shown that *P. aeruginosa* uses other iron acquisition pathways in a mouse model, including pyochelin and a high affinity heme uptake system (440, 441). To determine whether *P. aeruginosa* is using pyoverdine or potentially using another iron acquisition system in the absence of pyoverdine, it would be of interest in the future to expose the wild-type and a pyoverdine mutant to varied levels of iron through the addition of varying amounts of iron chelator (e.g. 2,2'- dipyridyl), and determine whether the bacteria can survive in the presence of those iron levels.

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