Characterisation of explosive cell lysis in *Pseudomonas aeruginosa*

Thesis submitted to
the University of Technology Sydney,
in fulfillment of the requirements for the degree of
Doctor of Philosophy

Amelia L. Hynen
2017

Certificate of Original Authorship

I certify that the work in this Thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the Thesis has been written by me. Any help that I have received in my research work and the preparation of the Thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the Thesis.

This research was supported by an Australian Government Research Training Program Scholarship.

Signature of Student:

Date:

Acknowledgements

Firstly I would like to thank my supervisors Cynthia Whitchurch and Lynne Turnbull. Your support, encouragement and mentorship have made this Thesis possible. Thank you for always pushing me to be a better scientist. Your knowledge, passion and drive have made the past few years an incredible experience. Thank you for all your help, advice, and patience and it has been a privilege to learn from you and work with you.

I would also like to thank Masanori Toyofuku, Scott Rice, Daniel Wozniak and Ariel Lindner for providing strains and plasmids used in this Thesis. Thanks to Nicola Petty for her wealth of knowledge on all things phage. Thanks to Raz Shimoni for performing BacFormatics analysis and for teaching me how to use the software. A massive thanks to Michael Johnson for teaching and assisting me in the Microbial Imaging Facility at UTS.

I would also like to thank the members of the Whitchurch lab group, both past and present. To Sarah, Rosy, Laura N, Erin, Jaye, Leigh, James, Laura McC, and Davide, thank you for guiding me over the years and for your unwavering support, patience, and encouragement and for always lending an ear. To my fellow students both past and present, in particular, Giulia, David M, Eby and Ibrahim, your friendship, support and understanding over the years has meant so much to me. I can always count on you to make me laugh and help me relax. Our friendship will last for many years to come. Also a special mention to Sarah, Ondine, David D and David H for providing much needed distractions over the years. You are champions.

To my family, thank you for your unconditional love and support and for everything you have done for me over the years throughout my studies and for helping me realise my ambitions. I couldn't ask for a more loving and supportive family.

And finally, Sam, thank you for all the love, support, patience, and encouragement, and for putting up with me for the past 5 years. I couldn't imagine being able to complete this PhD without you by my side. It has truly been an adventure with you and I hope that adventure continues for many years to come.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

°C	Degree Celsius
3D	Three-dimensional
3D-SIM	Three-dimensional structured illumination microscopy
α	Alpha
AHL	N-acyl-L-homo-serine lactone
Amp^R	Ampicillin resistance
ATP	Adenosine tri-phosphate
β	Beta
bp	Base pairs
BSA	Bovine serum albumin
CAMHB	Cation-adjusted Mueller Hinton broth
CAUTI	Catheter-associated urinary tract infection
CF	Cystic Fibrosis
CFP	Cyan fluorescent protein
CFU/mL	Colony forming units per millilitre
CIP	Ciprofloxacin
CL	Colistin
CSP	Competence-stimulating peptide
Δ	Delta
diH_2O	Deionised water
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
ECL	Explosive cell lysis
EDTA	Ethylenediaminetetraacetic acid
eDNA	Extracellular deoxyribonucleic acid
EHEC	Enterohaemorrhagic *Escherichia coli*
EthHD-2	Ethidium homodimer-2
exDNA	Exogenous deoxyribonucleic acid
g	Grams
g	G-force

g/mL	Grams per millilitre
g/L	Grams per litre
GFP	Green fluorescent protein
GM	Gentamicin
Gm^R	Gentamicin resistance
h	Hour/hours
IHF	Integration host factor
kb	Kilobase
kg	Kilogram
kPa	Kilo-Pascals
kV	Kilovolts
L	Litre
LB	Luria Bertani (Lennox)
LBA	Luria Bertani agar
M	Molar
MIC	Minimal inhibitory concentration
MBC	Minimal bactericidal concentration
mg	Milligram
mg/mL	Milligram per millilitre
min	Minute/minutes
min/kb	Minute per kilobase
mL	Millilitre
mM	Millimolar
mm	Millimetre
MMC	Mitomycin C
MQ	MilliQ
µF	Microfarad
µg	Micrograms
µg/µL	Micrograms per microlitre
µg/mL	Micrograms per millilitre
µL	Microlitre
µm	Micrometre
µM	Micromolar
MVs	Membrane vesicles

NA	Numerical aperture
Na_2EDTA	EDTA disodium salt dehydrate
ng	Nanogram
OD	Optical density
P	P-value
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCD	Programmed cell death
PFU/mL	Plaque forming units per millilitre
pH	Potential of hydrogen
PQS	Pseudomonas quinolone signalling molecule
QS	Quorum sensing
RNA	Ribonucleic acid
RO	Reverse osmosis
ROS	Reactive oxygen species
rpm	Revolutions per min
s	Second/s
SDS	Sodium dodecyl sulphate
sec	Second/s
s.e.m.	Standard error of the mean
SOB	Super optimal broth
SOS	‘Save-our-souls’
TBE	Tris Borate EDTA
TE	Tris-EDTA buffer
TMGG	Twitching motility gellan gum
U	Units
U/mL	Units per millilitre
UV	Ultra violet
v	Version
VBM	Vogel-Bonner media
VBMA	Vogel-Bonner media agar
v/v	Volume per volume
w/v	Weight per volume
YFP	Yellow fluorescent protein

Abstract

Pseudomonas aeruginosa is a Gram-negative pathogen commonly associated with nosocomial infections and implanted medical devices. It causes both acute and chronic infections, which are often associated with the formation of biofilms. Extracellular DNA (eDNA) is a major component of these biofilms and is involved in adhesion, intercellular connectivity and facilitating cell migration. eDNA is also essential for the formation of biofilms, however the mechanism of its production at the early stages of development is unknown.

At the initiation of this Thesis, it was observed that during the early stages of submerged and interstitial biofilm development, a small proportion of cells spontaneously transform into round cells before rapidly lysing and releasing eDNA and cellular content into the extracellular milieu. This phenomenon is termed 'explosive cell lysis'. This process accounts for all eDNA produced in these biofilms as well as a number of other 'public goods' including cytosolic proteins and membrane vesicles (MVs) that may benefit the biofilm community. As eDNA, MVs and extracellular proteins contribute to biofilm development and virulence, explosive cell lysis may be a critical process for *P. aeruginosa*. Therefore, it is important to identify the mechanism mediating this process.

The aim of this Thesis was to identify and characterise the genes and cellular processes that lead to explosive cell lysis and eDNA release, and to better understand the role of eDNA in early stages of biofilm development. This Thesis identifies an endolysin of a cryptic bacteriophage as the enzyme responsible for this phenomenon and reports that explosive cell lysis is also under the control of the SOS response regulator RecA. Investigations into the induction of explosive cell lysis determined that intrinsic stressors like the formation of inclusion bodies and exogenous stressors that cause DNA damage induce explosive cell lysis and eDNA release. This Thesis also identifies that explosive cell lysis is mediated by the action of three independent holins that work in coordination to translocate the endolysin responsible for explosive cell lysis. Exploration into the role of explosive cell lysis in submerged biofilm formation determined that explosive cell lysis is essential for the formation of microcolonies, microcolony formation is spatially and temporally correlated to

explosive cell lysis, and sequential explosive events are required for building microcolonies.

Overall the results presented in this Thesis add to our understanding of the complex nature of eDNA production during biofilm development and have identified a novel role for cryptic bacteriophage.

Chapter One:

General Introduction

1 General Introduction

1.1 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative, facultative, anaerobic, rod-shaped bacterium that is ubiquitous in soil and marine environments and infects many organisms including plants, nematodes, insects and animals (1). *P. aeruginosa* is an opportunistic pathogen in that it favours compromised tissue and predominantly causes nosocomial infections (2). These include individuals with open wounds, burns, Cystic Fibrosis (CF), cancer and other immune-compromised individuals (3-7). *P. aeruginosa* is also a common cause of infections in individuals with implanted medical devices such as catheters and ventilators, causing both acute and chronic infections (8-11). Health care-associated infections in developed countries are the fourth leading cause of disease, half of these resulting from implanted medical devices (12, 13).

Many strains of *P. aeruginosa* are multidrug resistant and it is predicted that the number of these multidrug resistant strains will increase in the future, making treatment of *P. aeruginosa* acute and chronic infections extremely challenging (14). The success of *P. aeruginosa* in various niches and in causing different infections is attributed to its broad metabolic versatility, production of numerous virulence factors, production of efflux pumps, ability to change membrane permeability and cell wall structure, production of antibiotic-degrading enzymes, and its ability to grow as part of multi-cellular communities, known as biofilms (4, 15-18).

1.2 Biofilms

Most bacteria are capable of growing either as free-living planktonic cells or as a complex aggregate of cells known as a biofilm (19). Biofilms are typically described as a sessile aggregate of cells encased in a matrix of extracellular polymeric substances, produced by the cells, allowing them to adhere to each other and/or a surface (4). Most bacteria have a natural propensity to form biofilms as a protective shield against the external environment (20). Consequently the formation of a biofilm impedes antibiotic action and phagocytosis by the host, making them nearly impossible to eradicate once

established (21). Cells growing in biofilms can be up to one thousand times more resistant to antimicrobial treatment than planktonic cells due to limited diffusion, expression of efflux pumps and reduced growth rate in the biofilm (4, 22, 23). The increased persistence and resistance of cells within biofilms has important ramifications in both industry and healthcare (24).

1.2.1 Biofilms in natural settings

Bacterial biofilms form in a range of natural environments including hydrothermal hot springs, freshwater rivers and areas of the ocean (25, 26). Naturally occurring biofilms play a significant role in degradation of organic matter and the balance of mineral and metal levels in ecosystems (19, 27). In industry, biofilms are utilised for the breakdown of organic material in bioremediation, fermentation and management of nitrogen and carbon levels in wastewater treatment (28). As biofilm formation is ubiquitous in the environment, it also presents a massive cost to industry. Biofouling is the formation of biofilms in undesirable locations that impact upon performance or processes of industry (29). Biofouling of surfaces like the hulls of ships and other surfaces in the marine environment can lead to corrosion, inefficient heat transfer and increased energy consumption (27, 30).

1.2.2 Biofilms in clinical settings

In humans, bacterial biofilms exist on almost all mucosal surfaces (31). These biofilms are comprised of commensal bacteria or of opportunistic pathogens, which colonise the host (32). These pathogenic biofilms can increase the morbidity and mortality of effected individuals and can develop into chronic and persistent infections, impairing bodily functions (4, 5). Chronic lung biofilm infections lead to airway obstruction, urinary tract infections can obstruct urine flow causing inflammation and recurring infections, and infective endocarditis disrupts heart valve function (33). Bacterial biofilms are also problematic when they form on implanted medical devices such as catheters, prosthetics, cardiac pacemakers and endotracheal tubes (26). Formation of biofilms on abiotic or biotic surfaces within a host commonly leads to localised tissue damage and/or inflammation. Detachment of bacterial cells or aggregates, or migration of the biofilm can spread the infection (34). As

implanted medical devices are being increasingly used in modern medicine, it can be expected that the number of these types of infections will continue to rise (35).

1.2.3 Laboratory models of biofilms

1.2.3.1 **Submerged biofilms**

Despite the wide range of environments in which bacterial biofilms form, laboratory models for the most part are able to closely reflect biofilms formed in natural and clinical settings (36, 37). Submerged biofilms are also termed hydrated or sessile biofilms as they form in well-hydrated environments. Submerged biofilms are the most extensively studied type of bacterial biofilm, of which *P. aeruginosa* is the model organism. These biofilms form whilst immersed in liquid media either under static or flow conditions. To form 'flow' biofilms, fresh media is continually pumped through the system for a number of days. The current literature presents *P. aeruginosa* submerged biofilm development as a multi-stage process initiated by the reversible attachment of bacterial cells to a surface (Figure 1.1) (30, 38, 39). Following attachment to the surface or other cells, extracellular matrix materials are produced, resulting in stronger adhesion to the surface and irreversible attachment. Cells aggregate at these sites of extracellular matrix and form microcolonies that then develop into mature biofilms. Under certain conditions, mature biofilms of *P. aeruginosa* have a characteristic mushroom-like structure comprised of a cap and stalk (38, 40, 41). Single cells or bacterial aggregates encased in the matrix can detach from the mushroom-like structure and go on to seed another biofilm (38). Submerged biofilms are typically associated with biofouling in industry and marine environments (42, 43).

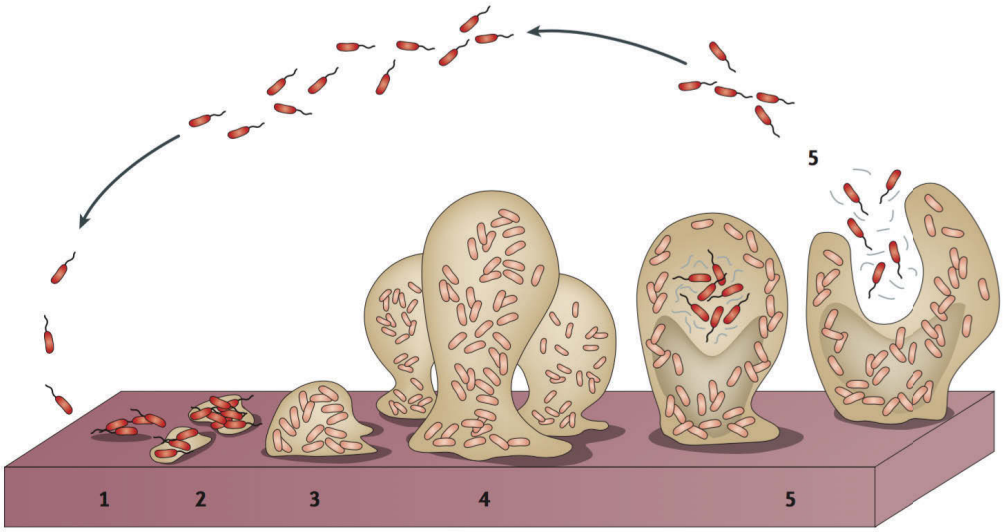


Figure 1.1: Biofilm formation of *P. aeruginosa* in submerged systems.
Biofilm formation in submerged systems is a multi-stage process. 1) Initial attachment of cells to the substratum. 2) Production of matrix components leads to irreversible attachment. 3) Microcolony formation. 4) Development of mature architecture showing the characteristic mushroom structure. 5) Dispersal of cells. Adapted from (36, 38)

1.2.3.2 Interstitial biofilms

Within a semi-hydrated environment, *P. aeruginosa* is able to form biofilms on the surface of solidified nutrient media and at the interstitial space between solidified nutrient media and another surface, such as a coverslip or petri dish base (44). In this Thesis, these biofilms will be referred to as interstitial biofilms. These interstitial biofilms undergo active expansion by flagellum-independent translocation termed twitching motility. Twitching motility is dependent upon the extension and retraction of long surface filaments called type-IV pili (45, 46). Detailed analyses of these biofilms at the interstitial space between a solidified nutrient media-coated microscope slide and a coverslip revealed that this active expansion results in the development of a vast, intricate lattice-like network of interconnected trails (Figure 1.2) (44, 47, 48). Semmler *et al*. (44) demonstrated that this highly organised, multicellular behaviour arises through the collective behaviour of individual cells within the interstitial biofilm, initiated by migration of rafts at the leading edge of the biofilm that appear to lay down a trail along which cells preferentially migrate (44). Recent work has revealed that this preferential migration occurs along a network of furrows, which are created by the leading edge rafts (49). Additionally, it has been shown that extracellular DNA (eDNA) facilitates the

efficient flow of cells through this furrow network by maintaining coherent cell alignments, ensuring that cells continue to actively migrate away from the main colony (Figure 1.2B – D) (49).

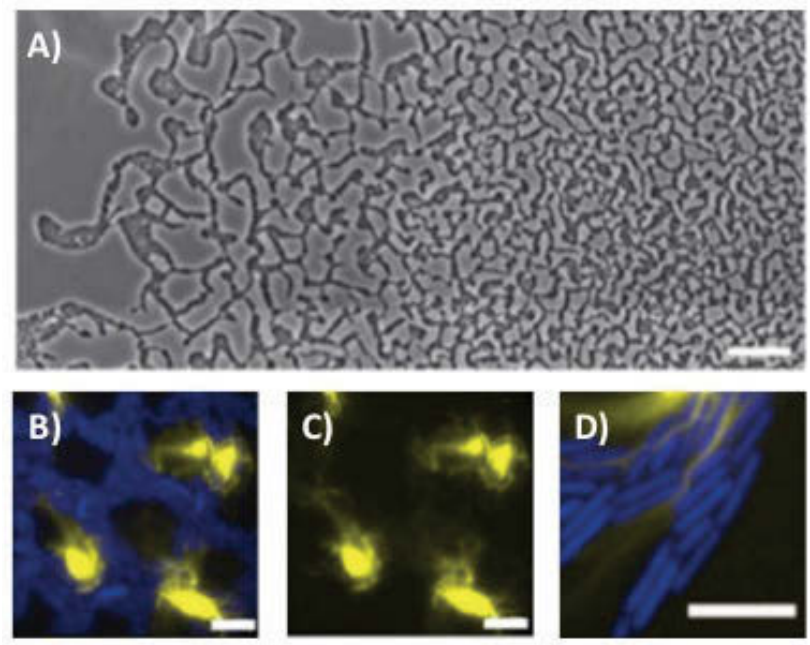


Figure 1.2: Interstitial biofilms of *P. aeruginosa.*

A) Phase-contrast images of PAK interstitial biofilm showing the intricate network of cells as the biofilm expands; scale 30 μm. eDNA is produced throughout the interstitial biofilm in a punctate pattern, facilitating biofilm expansion (B-D). PAK containing pUCP*cfp* (blue) cultured in the presence of eDNA stain TOTO-1 (yellow); scale 5 μm. Adapted from (49).

Interstitial biofilms are not as well studied as submerged biofilms but are still clinically relevant. Interstitial biofilms are commonly associated with medical device-associated infections whereby the bacteria are able to travel across the implanted device between the outer abiotic surface of the device and the epithelial layer of the tissue, using twitching motility (50). One common device-associated infection is catheter-associated urinary tract infections (CAUTIs) (51). In this type of infection, the bacteria are able to traverse the catheter, allowing for the spread of the infection to other organs including the bladder and kidneys (10, 33). From there, the disease can spread to distal organs, leading to polynephritis and bacteraemia (26, 30). The wide spread use of catheters for a large number of surgeries and hospital stays increases the incidence of catheter-associated *P. aeruginosa* infections (12).

1.2.3.3 **Colony biofilms**

Colony morphology was found to reflect biofilm-forming capabilities of *P. aeruginosa* with the production of extracellular polymeric substances effecting colony appearance and architecture (28, 41). Colony biofilms also provide insight into motility driven biofilm expansion. Twitching motility can be visualised in colony biofilm expansion and results in the characteristic 'ground glass' edge, comprising of highly active twitching cells (44, 47). In more hydrated environments, colony expansion can occur through flagella-mediated swarming motility which can be observed as the formation of a dendritic pattern (52).

1.3 Biofilms matrix components

Along with the bacterial cells, the biofilm contains an extracellular matrix that is produced by the bacteria in the biofilm. The biofilm matrix is often considered to be the dark matter of biofilms due to its complex make up and difficulty in profiling each individual component (53). The matrix of many biofilm-forming bacteria includes polysaccharides, eDNA, proteins, lipids and membrane vesicles (MVs) and constitutes up to 90% of the biofilm biomass (54). Together these components give the biofilm structure, stability, protection and a microenvironment allowing for stable metabolic processes and gene expression in a coordinated fashion. These extracellular polymeric substances (EPS) components of the matrix form the scaffold for the three-dimensional bacterial community and are mainly responsible for adhesion to surfaces and cohesion within the community.

Many of these components are produced only once the bacteria have attached to a surface and initiated microcolony formation to provide structural integrity to the developing biofilm (38). The most commonly researched structural components of the *P. aeruginosa* biofilm matrix are the exopolysaccharides alginate, Pel and Psl, and eDNA.

1.3.1 Alginate

Alginate was the first exopolysaccharide identified in the biofilm matrix. It is a high-molecular-mass unbranched heteropolymer consisting of 1,4-linked uronic residues of β-D-mannuronate and α-L-guluronate. Alginate production arises from a mutation in the negative regulator *mucA*, typically occurring *in*

vivo, and results in a mucoid phenotype (55). Alginate production is a common phenotype in chronic lung infections in CF patients and may be an indicator of lung disease progression and increased virulence (3). Alginate production confers a selective advantage for *P. aeruginosa* as the polysaccharide protects the bacteria from reactive oxygen species (ROS) produced by macrophages at the site of infection and protects against phagocytic clearance (56, 57).

In mucoid strains of *P. aeruginosa,* alginate is involved in the mechanical stabilisation of the mature biofilms (58). Interestingly, studies on the non-mucoid strains PAO1 and PA14 of *P. aeruginosa* demonstrated that alginate was not the only exopolysaccharide in the matrix (59) and is not essential in biofilm development or structure, although it does confer an advantage (60, 61). Strains of PAO1 and PA14 with alginate synthesis genes disrupted were capable of forming biofilms with a polysaccharide matrix, suggesting other polysaccharides may be required for the formation of non-mucoid biofilms (59). These other polysaccharides were identified as Psl and Pel and are involved in biofilm architecture.

1.3.2 Psl

Psl (polysaccharide synthesis locus) is a repeating pentasaccharide containing D-mannose, D-glucose and L-rhamnose and is essential in the biofilm development of mucoid and non-mucoid strains. It was first identified to be involved in biofilm formation in PAO1 (62-65). It is constitutively expressed in planktonic cultures but is limited to the centre of microcolonies in developing biofilms (66). Disruption of *psl* gene cluster results in cell-surface and cell-cell interaction defects and Psl is required for adherence to epithelial tissue in the lung of CF patients (62).

Psl is observed to coat the surface of *P. aeruginosa* cells in a helical pattern and forms fibre-like strands that interconnect, forming a Psl mesh in biofilms (67). This spider web-like fibre network of Psl is thought to aid in the recruitment of bacteria for the formation of microcolonies and is associated with increased biomass (68). Single cells migrating on a surface lay down Psl trails and other cells preferentially migrate on these trails (69). This Psl trail network is thought to aid in the recruitment of bacteria, which results in the production of more Psl and the formation of microcolonies in a positive feedback loop (69).

Psl also interacts with other matrix components within the biofilm. Psl and eDNA are thought to interact and bind through hydrogen bonds in the matrix of pellicle and submerged biofilms (70). Psl is able to interact with eDNA from *P. aeruginosa* and from other sources such as neutrophils and *S. aureus* to form Psl-eDNA fibres that extend in a radial pattern throughout the biofilm (70).

1.3.3 Pel

Pel is a glucose-rich exopolysaccharide, partially comprised of acetylated 1,4-glycosidic linkages of *N*-acetylgalactosamine and *N*-acetylglucosamine (71). Pel is involved in the formation of pellicle biofilms that form at the air-liquid interface of static cultures. It was first identified in a PA14 transposon mutant library screen for pellicle-deficient mutants (72). Pel is involved in biofilm formation at the early and later stages of development, associated with initiating and maintaining cell-cell interactions (73, 74). However, its requirement for adherence to a surface and microcolony formation is strain-dependent (73, 75, 76). Pel tethers the cap of the mature mushroom-shaped biofilm structure to the substratum and cross-links with eDNA through ionic-binding to form a structural core in mature biofilms (71). Pel also plays a role in enhancing resistance to aminoglycoside antibiotics in biofilms by possibly binding or sequestering the antibiotic or by influencing the biofilm structure and therefore permeability to the drug (73, 74).

Although both Psl and Pel contribute to biofilm formation, they also provide a structural redundancy. A range of environmental and clinical isolates of *P. aeruginosa* were examined for their dependence on Psl and Pel for biofilm development and it was reported that they can serve as redundant structural scaffolds (75). This was proposed to serve as a preservation measure in the event of mutations disrupting the production of either exopolysaccharide (75).

Although these exopolysaccharides are involved in the formation of biofilms *in vitro*, the production of alginate, Psl and Pel are not required for biofilm formation in a murine model of CAUTIs (77). These biofilms require eDNA as the major interconnecting component.

1.3.4 Extracellular DNA

eDNA is a major biofilm matrix component for a multitude of bacterial and fungal species and is an incredibly versatile component of the matrix (78). It was first identified as essential for *P. aeruginosa* saturated biofilm development in 2002 in a pioneering study by Whitchurch *et al.* (79). In this study Whitchurch *et al.* (79) examined the importance of eDNA over the course of submerged biofilm development by using the DNA-degrading enzyme Deoxyribonuclease I (DNase I) to remove eDNA. Young biofilms (up to 60 h old) are easily removed and biofilms are inhibited from forming initially in the presence of DNase I, whilst established mature biofilms are resistant to DNase I treatment. This suggests that eDNA is involved in attachment and initial microcolony formation of submerged biofilms. As the biofilm matures, removal of eDNA has no effect on the biofilm structure suggesting eDNA appears less important in mature biofilms. This temporal association with DNase I efficacy suggests that as the biofilm matures, other matrix components may have a greater role in the structural integrity of biofilm and may protect, interact or replace eDNA over time, or DNase I is no longer able to access the DNA in the matrix (70, 80).

eDNA is the most abundant polymer of the *P. aeruginosa* biofilm matrix and its production levels vary between strains, including clinical isolates (63, 81). Its main roles include mediating bacterial aggregation, coordinating migration, assembling mature biofilms, horizontal gene transfer, interacting with other matrix components and virulence factors, effecting antibiotic tolerance and can also be utilised as a nutrient source under starvation conditions (82). As it is an integral part of the biofilm matrix and development, it is imperative to understand how it is produced throughout biofilm development and the implications of its production. This Thesis will focus on eDNA production and its role in early stage biofilm development.

1.4 Roles of eDNA in *P. aeruginosa* biofilms

eDNA is most notably described as having ‘glue’-like properties, facilitating cell-cell and cell-substratum interactions and thus serving as a major inter-connecting component of the biofilm matrix. eDNA can be adsorbed onto the surface of bacterial cells and form loop-like structures extending from the

cell, up to 300 nm (83). These eDNA loops have been identified to promote attractive acid-base and Lifahitz-Van der Waals interactions between cells and the substratum, which is hypothesised to facilitate initial bacterial attachment (84-86). Interestingly, in rare cases eDNA can act as a barrier to bacterial adhesion (87) and even inhibit growth when provided in high enough concentrations (88). In *P. aeruginosa,* an increase in eDNA adsorption is associated with an increase in bacterial aggregation, until a threshold is reached where it then hinders aggregation (83). This may be due to eDNA saturating areas or features on the cell that are required for cell-cell binding interactions. eDNA can bind to and chelate divalent ions, such as calcium, which promotes bacterial aggregation (89). Calcium ions are present on the cell surface and play an important role in maintaining the integrity of the entire cell wall and the outer lipopolysaccharide layer (90, 91). eDNA and Ca^{2+} interactions mediate cationic bridging between bacterial cells, leading to aggregation (89).

In *P. aeruginosa* biofilms, eDNA has been implicated in coordinating bacterial migration during biofilm formation, by providing a conditioning film on the substratum and coating microcolonies (40, 92, 93). In the typical 3-D mature mushroom-shaped structure, eDNA forms a border between motile and non-motile cell populations of the cap and stalk of the mushroom, respectively (Figure 1.3) (40, 92, 93). Over time, eDNA extends throughout the microcolony, possibly playing a greater role in stability (92).

The type IV pili of *P. aeruginosa* are able to bind to eDNA present in submerged and interstitial biofilms through electrostatic interactions (94). In the mushroom structure of *P. aeruginosa* biofilms the type IV pili function as adhesins through their interaction with eDNA (92, 95). *P. aeruginosa* cells can spontaneously orient along aligned eDNA molecules independent of type IV pili or flagella (96). eDNA has also been implicated in the self-organisation of *P. aeruginosa* interstitial biofilms, allowing the cells to migrate as a cohesive unit through furrows and aid in efficient traffic flow of cells to the leading edge of the biofilm (see Figure 1.2) (49).

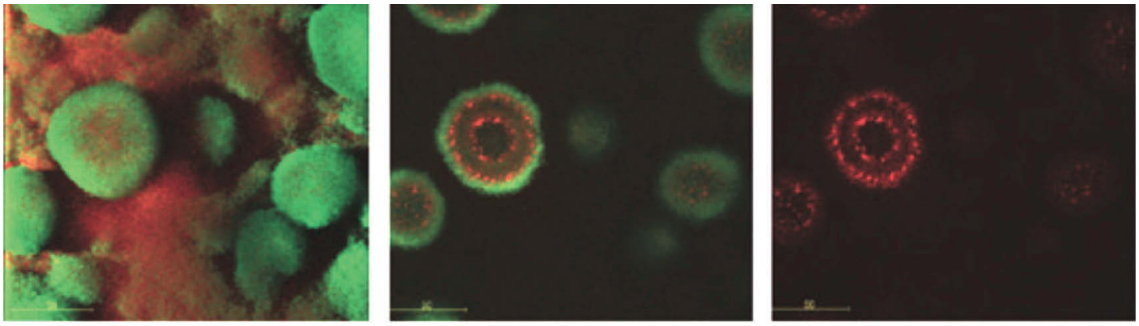


Figure 1.3: eDNA in mature biofilm mushroom structures.

GFP-tagged PAO1 biofilms (green) at 6 days with eDNA stained with DDAO (red) imaged with confocal scanning laser microscopy, with top-down view (left) and horizontal optical section of bacteria and eDNA (middle) or eDNA alone (right). Adapted from (92).

eDNA can also be incorporated from external sources throughout biofilm formation. eDNA can accumulate from heavy recruitment of host immune cells and production of neutrophil extracellular traps *in vivo* (97). Sputum in the lungs of CF patients has a high DNA content (98, 99) and almost all eDNA found in these biofilms is of human origin (100). Neutrophils are some of the first cells to be recruited to the site of infection and most eDNA from the lungs comes from these neutrophils (97). Interestingly, exposing *P. aeruginosa* to high concentrations of eDNA also induces cell death by perturbing the cell's membrane through cation chelation similar to the mechanism of EDTA, which in turn releases more eDNA into the milieu (88).

eDNA interacts with exopolysaccharides in different bacterial biofilms. In *P. aeruginosa*, the exopolysaccharide Psl interacts with eDNA in the matrix of pellicle and flow biofilms (70). This is thought to occur through hydrogen bonding and results in the formation of Psl-eDNA fibres extending throughout the microcolony. DNA from *P. aeruginosa*, human neutrophils and *S. aureus* can interact with Psl and aid in the protection against DNase I treatment (70).

Another integral component of bacterial biofilms that eDNA interacts with are membrane vesicles (MVs). MVs are bi-layered spherical membrane structures, ranging from 50-300nm in diameter and play a role in several biological functions such as cell-to-cell signalling, secretion of virulence determinants and insoluble molecules as well as stimulation of host immune responses (24, 101-105). MV surface bound DNA appears to be an electrostatic bridging component that is central to MV-bacterium-biofilm matrix interactions

and enhances structural integrity of the biofilm (106). MVs will be further discussed in section 1.10.

Pyocyanin is a redox-reactive phenazine molecule that is produced by 90 – 95% of *P. aeruginosa* strains (107) and is present in high concentrations in CF lung infections (108). Pyocyanin is a recognised virulence factor, impacting gene expression, colony size and biofilm thickness (109). Pyocyanin intercalates with eDNA, altering cell surface properties such as hydrophobicity and attractive surface energies, which promotes aggregation (110).

P. aeruginosa biofilms are inherently resistant to antibiotics due to production of antibiotic-degrading enzymes, efflux pumps, limited diffusion, slow growth rate, presence of 'persister' cells and the bacteria's ability to change membrane permeability and antibiotic target sites (22, 111-113). eDNA also plays a major role in the resistance or tolerance to antibiotic treatment. The negative charge of eDNA contributes to its ability to bind and sequester divalent cations like Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}, cationic antibiotics and antimicrobial peptides (114, 115). Removal of eDNA makes the biofilm more susceptible to antibiotic stresses (116, 117).

Horizontal gene transfer plays an important role in the dissemination of genetic material for bacterial evolution and as a result, considerable proportions of most bacterial genomes were acquired through horizontal gene transfer (118). Besides mobile genetic elements and bacteriophage-induced gene transfer, eDNA serves as a natural material for horizontal gene transfer (119).

1.5 Mechanisms of eDNA production in bacteria

There is no universal mechanism for eDNA production across different species. However, it is apparent that in the majority of cases eDNA is derived from genomic DNA released into the extracellular milieu as a consequence of cell death. In many bacterial species comparative PCR and Southern analysis have shown eDNA is similar to genomic DNA of the producing bacteria, consistent with lysis (92, 120, 121). The release of cytoplasmic enzymes such as β-galactosidase into the biofilm milieu (of strains with *lacZ* inserted into the chromosome) have also been employed as reporters to show that eDNA release is concomitant with cell lysis (92). This section explores the different

mechanisms through which eDNA has been found to be produced in different bacterial species.

1.5.1 Lysis-independent eDNA release

Oral commensal species of *Streptococcus* are able to specifically release DNA without lysing the cell, retaining the cell wall functionally intact and preventing the release of other molecules like RNA and ATP (122). If lysis were to occur in commensal bacteria, it may leave open spaces that could allow competing bacteria to colonise (122).

Pathogenic bacteria are also capable of releasing eDNA without lysis mediating the process. *Neisseria gonorrhoeae* was the first organism identified to produce eDNA for natural transformation without the requirement of lysis (123). *N. gonorrhoeae* utilises the type IV secretion system to secrete single stranded DNA and this was demonstrated by the reduction in eDNA release when homologues of the type IV secretion were interrupted (123), resulting in the reduction of biofilm formation and delayed initiation (124). Unlike most type IV secretion systems, no cell-cell contact is required for eDNA to be extruded. Interestingly, a thermonuclease, which is also secreted, is involved in biofilm formation and remodelling by degrading DNA in the biofilm matrix (125).

1.5.2 Autolysin-mediated eDNA release

The two main mechanisms of autolysin-mediated eDNA release are through fratricidal killing or altruistic suicide of a sub-population in the biofilm community. In the case of fratricidal killing, the biofilm community differentiates into separate populations where one produces killing factors that target the remaining population and are, in turn, protected from the killing factors by immunity proteins (126). Altruistic suicide is a process similar to programmed cell death (PCD) in eukaryotic cells whereby a cell will die for the benefit of the community, providing public goods, based on internal signals (127).

Enterococcus faecalis, Staphylococcus aureus and *Staphylococcus epidermidis* have been shown to use autolysin-mediated DNA release during biofilm formation. eDNA release in *E. faecalis* occurs in a fratricidal manner whilst staphylococcal species do so in an altruistic manner, however they all use autolysins to mediate the process. Autolysins are peptidoglycan hydrolases

capable of breaking down the cell wall of the producing bacterium (128). During autolysis, the peptidoglycan layer progressively thins as enzymatic activities of the autolysins, which can be hydrolytic transglycosylases, endopeptidases and N-acetylmuramoyl-L-alanine amidases, act on components of the peptidoglycan layer of the cell wall (129, 130). When the major autolysins of *E. faecalis, S. aureus* and *S. epidermidis* are knocked-out, levels of eDNA dramatically decrease and their biofilm forming capabilities are effected as seen by a decrease in biofilm biomass (121, 131, 132).

1.5.2.1 ***Enterococcus faecalis***

E. faecalis depends on autolysin-triggered lysis of pockets of cells in biofilms to release DNA as an important constituent in biofilm maturation and the autolysin Atn is responsible for this eDNA release (120, 133). When the autolysin is deleted, the biofilms formed are more resistant to continuous DNase I treatment, and DNase I treatment after 48 h of growth. Deletion of *atn* also delays biofilm formation compared to the wild-type parent (131), suggesting another mechanism may be responsible for eDNA release at later stages. This autolysin-mediated eDNA release is said to be regulated by the gelatinase GelE and the serine protease SprE whereby GelE induces lysis of a subset of the population in discrete foci during the initiation of biofilm formation and SprE acts as a negative regulator for this activity (133). The biofilm is separated into two sub-populations, one which is susceptible to the fratricidal action controlled by GelE, whilst the other portion that co-transcribes the SprE protein is immune to fratricide. When SprE is inactivated, biofilm biomass increases with increased lysis and eDNA production (120). Interestingly, Thomas *et al.* (106) showed that the GelE-mediated eDNA release is involved in microcolony differentiation 96 h. They also demonstrated that eDNA is essential at initial attachment where DNase I treatment after six h biofilm growth severely effected attachment and consequently no biofilms formed after 26 h (120). Epidemic clones of *E. faecalis* from orthopaedic implant infections produce stronger biofilms and have increased expression of the autolysin genes responsible for eDNA production than laboratory strains (134), suggesting there may be an association with the ability to produce eDNA and virulence.

1.5.2.2 ***Staphylococcus aureus***

Autolysis-mediated release of eDNA has also been demonstrated for staphylococcal biofilms (132, 135). The main peptidoglycan hydrolase in *S. aureus* is AtlA (136). AtlA can be proteolytically cleaved into two catalytically active enzymes, amidase (AM) and glucosaminidase (GL) (137). Deletion mutants of *atlA*, the AM domain and the GL domain result in decreased biofilm biomass and significantly less cell-associated eDNA. Both AM and GL play a unique role in biofilm formation through autolysis as Δ*atlA* strains can be partially complemented by AM and GL individually but not to wild-type levels (137). The majority of studies on eDNA-release in *S. aureus* have focussed on the whole autolysin AtlA.

AtlA is essential for fibronectin-binding-protein-mediated biofilm formation, which requires eDNA release (138). Along with its involvement in releasing eDNA, AtlA also has a role in excreting cytoplasmic proteins, whereby deletion of *atlA* results in a significant decrease in excreted proteins in the culture supernatant (139). As the cell lyses, its internal contents including cytoplasmic proteins are expelled into the milieu and this serves as a non-classical protein secretion mechanism. Another autolysin Aaa (140) functions as an emergency secondary autolysin, however its upregulation can not prevent many of the phenotypes associated with *atlA* deletion (141).

It is speculated that AtlA is regulated by the holin-like proteins CidAB and LrgAB in a positive and negative fashion, respectively (142). Evidence showing the impact of *cidAB* and *lrgAB* deletions on AtlA suggests the autolysin is translocated to the peptidoglycan layer by oligomerisation of CidAB, forming pores in the cytoplasmic membrane of *S. aureus* (135, 142, 143). Deletion of *lrgAB,* the anti-holin, results in increased extracellular peptidoglycan hydrolase activity (143). Biofilm biomass also increases in Δ*lrgAB* mutants (142), which is synonymous with cell death and eDNA release by AtlA (137). This regulatory system is minimally expressed in early exponential cultures, suggesting eDNA release at bacterial attachment and biofilm initiation may occur through another mechanism.

The *cid* operon is conserved in approximately 50% of bacterial species with sequenced genomes (144). This cell death pathway appears to be conserved in bacteria, plants and animals (145). Putative *cidAB* and *lrgAB* loci

were identified in *P. aeruginosa* (67) and are involved in cell death and timing of lysis.

1.5.2.3 ***Staphylococcus epidermidis***

The autolysin AtlE is responsible for eDNA production in *S. epidermidis* and is required for attachment and biofilm formation via eDNA release (121, 146-148). Qin *et al*. (121) demonstrated that eDNA released through AtlE-mediated cell lysis is required for initial attachment of bacterial cells to a substratum and early stage biofilm formation. Deletion of *atlE* dramatically reduces eDNA release and impairs biofilm formation. This process is thought to aid in attachment as the eDNA surrounds the cells and deletion of *atlE* or DNase I treatment prevents attachment (121). The addition of exogenous DNA in the mutant strain failed to stimulate biofilm formation, indicating that the DNA and possibly other constituents released from the cell during lysis are required to stimulate biofilm formation (121). A second autolysin in *S. epidermidis* has been discovered, Aae, but its role in eDNA release has not been explored (146).

Interestingly some research speculates that the autolysin itself is responsible for the initial attachment. AtlE is normally involved in turning over the cell wall by degrading old peptidoglycan (141). Since a transposon mutant of *atlE* was defective in attachment, further investigations have focused on the autolysin itself as the adhesin (149, 150). It has been identified to associate with the cell wall surface and activation of AtlE induces changes in cell surface hydrophobicity (150). It is unclear how or what domain of AtlE mediates primary attachment and stable surface binding. Since deletion of *altE* and DNase I treatment of wild-type strains results in identical phenotypes, and strong evidence shows eDNA mediating attachment and initiating biofilm formation in *S. epidermidis* (121), we can reason that eDNA and the autolysin itself are responsible for attachment in *S. epidermidis*.

1.5.2.4 ***Neisseria meningitidis***

Neisseria meningitidis also employs an autolysin-mediated mechanism for the release of eDNA, required for initial stages of biofilm attachment and development (151). The release of eDNA in this species relies upon the action of putative autolysins (MltA and MltB) and a cytoplasmic *N*-acetylmuramoyl-L-

alanine amidase (AmpD) responsible for cell wall recycling and involved in autolysis. Knock outs of the genes responsible, *mltA*, *mltB* and *ampD*, released significantly less eDNA than the wild-type strain (151). Inactivation of *ampD* in *N. meningitidis* reduces eDNA levels to half that produced by the wild-type strain, decreasing initial biofilm formation (151).

1.5.3 Bacteriophage- and prophage-mediated eDNA release

Another mechanism for the release of eDNA is through bacteriophage- or prophage-mediated lysis. About 20% of bacterial genomes are of viral origin (152-154), consisting of fully functional prophages, cryptic prophage and phage-like elements that have remained after incomplete excision of the phage or horizontal gene transfer events (155).

Prophages can be spontaneously induced in single cells within a population even in the absence of an external trigger (156), which may be the result of stochastic gene expression (157) or from a genuine SOS response (158). Prophage induction is often followed by lysis and was originally thought to be detrimental to the population. However, as these cells lyse, they produce and/or release public goods that aid in biofilm formation or virulence, which is evolutionarily advantageous. The release of public goods will be further discussed in section 1.9.

For bacteriophages or prophages to be released from the cell, the cell must lyse with the aid of phage-associated lytic proteins, mainly an endolysin and a holin (159-161). An endolysin is a peptidoglycan hydrolase that breaks down the peptidoglycan in the cell wall and must be transported to the periplasm by a holin (162). A holin is an integral membrane protein that forms an oligomer complex resembling a pore in the cytoplasmic membrane, allowing for the transport of an endolysin (163, 164).

1.5.3.1 **Prophage-mediated lysis in *Shewanella oneidensis***

eDNA release in *S. oneidensis* is mediated by three prophages (165), LambdaSo, MuSo1 and MuSo2 (166). Interestingly only LamdaSo and MuSo2 form infectious phage particles but all three are considered to be involved in the process (165). Deletion of all three prophages reduces eDNA production in submerged biofilms by 60%, almost equal to that produced by the wild-type

when treated with DNase I (165). Treatment of *S. oneidensis* biofilms with DNase I for a short time period reduces biofilm biomass by 50 – 60% regardless of the developmental stage (165). eDNA enhances the initial attachment of bacterial cells and is integral in biofilm formation in all developmental stages (165). Treatment of *S. oneidensis* biofilms with DNase I for a short time period reduces biofilm biomass by 50 – 60% regardless of the developmental stage (165). Addition of either herring sperm DNA or *S. oneidensis* chromosomal DNA does not restore biofilm formation in the triple prophage deletion mutant and it has been suggested that auxiliary factors may be necessary for eDNA to mediate cell-cell and cell-substratum interactions (165).

1.5.3.2 **Bacteriophage-mediated lysis in *Streptococcus pneumoniae***

Streptococcus pneumoniae phage SV1 and its associated lysin are responsible for releasing eDNA and enhancing biofilm formation (167). Deletion of the SV1 phage lysin leads to a decrease in cell lysis, eDNA release and biofilm formation (167). *S. pneumoniae* is able to maximise pneumococcal lysogenic phage release by coordinating the action of phage-encoded lysin and bacterial autolysin LytA (168). LytA-mediated pneumococcal lysis is essential for biofilm formation as its absence results in a diminished biofilm. Impairment of either the phage lysin or the bacterial lysin results in identical biofilm development to that formed by a non-lysogenic strain R36A (168). Addition of exogenous DNA to a lysogenic strain that is missing one lysin increases the biomass well above that produced by a lysogenic strain alone. Lysis through these lysins occurs at the early stages of development (168). Interestingly only 76% of *S. pneumoniae* clinical isolates have lysogenic phages present on the genome but eDNA is produced in all strains (169). This indicates that the phage lytic genes and not the phage itself may be responsible for eDNA production.

In *S. pneumoniae,* induction of natural competence triggers cell lysis and the release of eDNA. This is a coordinated event where the competence-stimulating peptide (CSP) induces expression of the autolysins LytA and LytC (168, 170). This results in the lysis of a sub-population of the cells in a biofilm and the remaining competent cells take up this eDNA. This has been proposed as a mechanism for genetic recombination in prokaryotes that resembles sexual

selection (170, 171), allowing for the spread of advantageous adaptions such as antibiotic resistance or virulence-associated factors (172-174).

1.6 eDNA release in *P. aeruginosa*

1.6.1 Quorum-sensing dependent eDNA release

Quorum sensing (QS) is a mechanism to control and regulate gene expression in a cell-density-dependent manner using signalling molecules (175). eDNA can be released from *P. aeruginosa* through quorum sensing-dependent mechanisms involving N-acyl-L-homo-serine lactones (AHL) and Pseudomonas quinolone signalling molecule (PQS) (92, 176). *P. aeruginosa* QS is mainly comprised of two systems, *lasRI* and *rhlRI,* which are responsible for the expression of a wide range of virulence factors (177, 178). A third system is based on 2-heptyl-3-hydroxy-4-quinolone, termed PQS (179) and believed to operate between the *las* and *rhl* systems (180).

PQS is known to trigger eDNA release in early phase planktonic growth through the induction of prophage, leading to cell lysis (92). PQS and AHL quorum sensing also promotes the synthesis of phenazines, which are modified to produce pyocyanin (181-183). Pyocyanin has been reported to be involved in eDNA release through its interaction with oxygen, creating reactive oxygen species (ROS) and consequently hydrogen peroxide (184). This can lead to changes in the redox balance, leading to cell death. Pyocyanin production peaks between 16-24 h of biofilm formation, however eDNA production by pyocyanin isn't enhanced until well after 24 h (184). Montanaro *et al*. (174) suggest that a pathway independent of QS could be responsible for basal levels of eDNA at the early stages of biofilm development in *P. aeruginosa*. eDNA production at the early stages of development is crucial for attachment and aggregation (79). However it is still unknown how eDNA release is regulated during early stages of biofilm development when quorum-sensing systems are not yet active.

1.6.2 Bacteriophage-mediated lysis in *P. aeruginosa*

In *P. aeruginosa*, the two component regulator BfmR is required for the transition to the maturation stage of biofilm development (185) and also controls

bacteriophage-mediated lysis and eDNA release via 'prevent-host-death' factor PhdA (186, 187). BfmR suppresses the transcription of *phdA*, resulting in impaired biofilm development and increased cell death (186). Transcriptome analysis of a *bfmR* mutant showed the pyocin gene cluster, which are cryptic bacteriophage, and filamentous Pf4 phage were significantly upregulated (186). Pf4 induction occurs in the mature biofilm, likely in response to ROS, and is associated with lysis within the biofilm structure, which leads to dispersal of the biofilm (176, 188). Along with lysis, large amounts of eDNA are released from the cells and integrated into the biofilm to maintain structural stability (186, 187). Interestingly the Pf4 phage is released from the cell by extrusion without lysis and has no associated endolysin. As eDNA release and cell death appears to occur at the same time as Pf4 infects neighbouring cells, it has been suggested that these two events are associated (176, 187, 189).

1.7 Prophage

Prophage are present in almost all sequenced pathogenic and non-pathogenic bacterial genomes and can constitute up to 20% of a bacteria's DNA (153). They contribute to survival against osmotic, oxidative and acid stresses, influence biofilm formation and increase resistance against some antibiotics (190) and as such prophage play an integral role in the growth and survival of bacteria in adverse conditions. Prophage induction usually occurs in the presence of an external or environmental trigger (156) but they can also be induced spontaneously, which may be the result of stochastic gene expression (157) or from activation of the SOS response (158).

1.7.1 Intrinsic induction of prophage

The SOS response is a global regulatory network in bacteria that is central to activating a response to DNA damage (191). The products of *lexA* (the repressor) and *recA* (the activator) genes control the SOS response to address the formation of single stranded DNA and DNA lesions (192). Induction of the SOS response is intrinsic to prophage induction (162, 193-195). Studies on SOS expression in single cells have shown that a small proportion of a clonal population will have the SOS response induced under normal conditions (196, 197). It has been suggested that prolonged SOS induction could lead to

the induction of prophages (196-198). In support of this, free phage particles appear in the supernatant of non-induced cultures of lysogenic bacteria (199). More recently, spontaneous induction of SOS in single cells of *Corynebacterium glutamicum* in standard culturing conditions was found to be sufficient enough to induce promoters of a prophage, including the associated lysin (196). For most bacteria, RecA is the main SOS response regulator (200, 201). In some bacteria lacking *recA*, there is still some induction of prophage, indicating that stochastic events may also take place (196). This is likely to play a minor role in phage release or production compared to SOS-induced pathways.

Even in a clonal population of cells, there will be cell-to-cell variation in gene expression. Spontaneous fluctuations in the levels of prophage repressors may be responsible for switching between lysogenic and lytic states (202). Stochastic gene expression is able to provide a selective advantage for a clonal population by increasing genotypic and phenotypic variation.

1.7.2 Extrinsic factors inducing prophage

Extrinsic factors can also induce prophages by damaging DNA (203). The most common agents that induce prophages through DNA damage include ROS, UV radiation and the effects of antibiotics such as mitomycin C (MMC) and fluoroquinolones (204-208). Interestingly, prophage induction in *S. pneumoniae* occurs through RecA but in an SOS-independent manner as it lacks an SOS-like system (209-211). Other environmental stresses including changes in pH, temperature and organic carbon, can also impact on phage induction. For example, upon exposure to low pH, phage production is induced in *Helicobacter pylori* whilst phage release decreases in *Lactococcus lactis* (212, 213).

1.7.3 Benefits of bacteriophage lysis on a population

Perhaps counter-intuitively, the production of prophages in bacteria can be seen as a survival strategy. To support this, deletion of nine cryptic phages in *E. coli* K-12 resulted in a strain that was more sensitive to antibiotics, displayed decreased biofilm formation and was unable to cope with osmotic stress (190). The SOS response is activated in cells experiencing DNA damage. By having only those cells with damaged DNA induce phage

production and cell lysis, it could be a strategy for maintaining the genetic integrity of the population whilst simultaneously releasing public goods. The release of public goods may benefit the surviving population by aiding in biofilm formation, which in turn may protect the surviving population from the original stress or future stressors (214). The release of public goods will be further discussed in section 1.9. Although this process is advantageous to the bacterial populations, its also presents an Achilles' heel where other bacteria and even the human host could exploit this pathway. At the same time it provides a great opportunity to develop novel therapies to target these highly conserved prophage elements.

1.8 The pyocin-associated endolysin Lys

Pyocins are bacteriocins produced by the *Pseudomonas* genus that are capable of killing the same or closely related species (215). They are cryptic bacteriophages, in that they produce a headless phage particle and are also referred to as tailocins (216). For pyocins to be released from the producing cell, the cell must lyse. This involves the holin *hol* (PA0614) and the endolysin *lys* (PA0629) (162). The holin proteins polymerise to form aqueous channels in the cytoplasmic membrane (163, 164), which allows for the nonspecific transport of the endolysin to the periplasm. The endolysin in the periplasm hydrolyses the peptidoglycan layer of the cell wall, which eventually results in lysis (162). Overexpression of both *hol* and *lys* in *E. coli* and *P. aeruginosa* results in lysis (162). Lys-mediated cell death may also be involved in the release of eDNA and will be the focus of this Thesis.

1.8.1 Regulation of pyocins

The regulatory system for pyocins resembles those of temperate bacteriophages, as R- and F-type pyocins share homology to tail genes of P2 and λ phage families, respectively, and the R-and F-pyocins share regulatory and lysis genes (Figure 1.4) (162). R- and F-type pyocin production is dependent on RecA and is induced by DNA damage caused by UV irradiation, genotoxic or oxidative stress including ROS from neutrophils, which activate the SOS response (217-221). In the absence of an inducer, synthesis is repressed to limit pyocin production to a few cells (156). The expression of the pyocin

genes are controlled by the SOS response regulator RecA and negatively regulated by PrtR (Figure 1.5). Under normal conditions, RecA is produced at min levels and the repressor PrtR inhibits the induction of the activator *prtN* (222, 223). In the presence of a mutagenic agent or endogenous stress (224), RecA production is upregulated and forms monomers, aiding in the auto-cleavage of PrtR, allowing *prtN* to be transcribed. PrtN then induces the transcription of the pyocin gene cluster including *lys* and *hol* (223). PrtR does not repress production of pyocins at the basal level but suppresses the enhanced production under stressful conditions, indicating that pyocin expression is not completely regulated by PrtR (223).

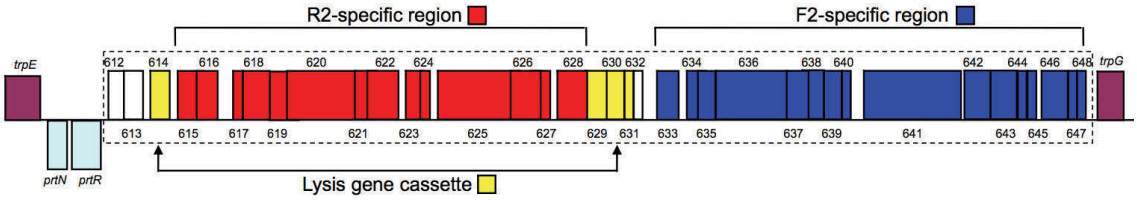


Figure 1.4: R- and F-pyocin gene cluster in *P. aeruginosa*.

Each box represents a gene between PA0609 and PA0649. The genes within the dotted line are co-transcribed together. The lysis cassette shown in yellow includes *hol* (PA0614), *lys* (PA0629) and two hypothetical proteins (PA0630 and PA0631). Adapted from (225).

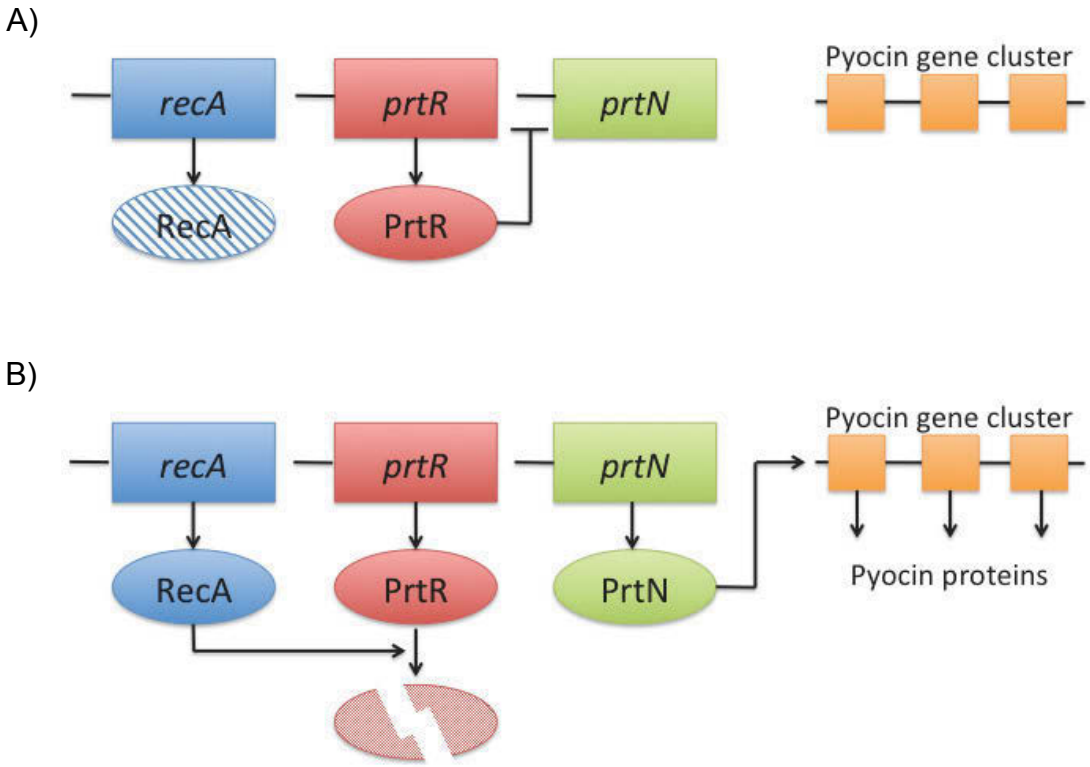


Figure 1.5: Regulation of the pyocin gene cluster.

A) Under normal conditions, PrtR inhibits the induction of the pyocin activator *prtN*. B) In the presence of a mutagenic agent, PrtR is cleaved with aid of RecA, allowing for *prtN* to be transcribed. This activates the expression of the pyocin gene cluster. Adapted from (222).

1.8.2 Induction of pyocins

Expression of the pyocin gene cluster can also be induced upon exposure to exogenous stressors including clinically relevant antibiotics. Transcriptome and DNA microarray analysis shows dramatic upregulation of the pyocin gene cluster in response to the fluoroquinolone antibiotic ciprofloxacin (193, 194). Ciprofloxacin causes DNA damage by preventing DNA from unwinding and duplicating (226). Brazas and Hancock (193) hypothesised that induction of the pyocin lysis cassette sensitises *P. aeruginosa* to genotoxic stress. This was later confirmed by Penterman *et al*. (195). They also proposed that induction of the pyocin lysis gene cassette is responsible for the lethality associated with ciprofloxacin. Whether the pyocin lysis gene cassette influences the sensitivity of *P. aeruginosa* to other clinically relevant antibiotics is unknown.

1.8.3 Transportation of Lys

For the pyocin endolysin Lys to cause lysis, it must be transported to the periplasm by a holin. There are currently three annotated holins in the *P. aeruginosa* genome, Hol, AlpB and CidAB (67, 162, 227).

Hol is likely to be the cognate holin for Lys, as it is encoded in the pyocin gene cluster along with *lys* (162). When Hol and Lys were first demonstrated to be responsible for cell lysis during release of pyocins, Lys alone without Hol was unable to cause lysis unless the inner membrane was permeabilised under the conditions examined (162).

AlpB is part of a recently discovered self-lysis pathway in *P. aeruginosa* related to bacteriophage lambda and can functionally substitute for bacteriophage lambda holin in *E. coli* (227). This pathway is dependent on DNA damage and only lyses a subset of the cells when activated. Lysis through this pathway is visually similar to bacteriophage-mediated lysis, with the formation of round cells that go on to lyse. eDNA is also released through this pathway in broth cultures (227). AlpB is regulated by the lexA-like regulator PA0906 and is upregulated in response to ciprofloxacin. Interestingly the endolysin responsible for hydrolysing the peptidoglycan has not been identified for this system.

CidAB is involved in cell death and the dispersal stage of submerged biofilm development in *P. aeruginosa* (67). Cell death through this pathway is also involved in the formation of a matrix cavity in the centre of microcolonies that may also be required for the release of surface-bound Psl, eDNA and Psl-degrading enzymes. Ma *et al*. (67) did not investigate whether cidAB-mediated cell death is responsible for eDNA production in these biofilms and no endolysin has been identified to be part of this cell death pathway.

Holins are considered to be non-specific transporters. There are no specific requirements for a particular holin to transport a particular endolysin, as it is unlikely they directly interact with each, making holins and endolysins interchangeable (228, 229). As the holins AlpB and CidAB have no known associated endolysins in *P. aeruginosa*, all three holins could possibly contribute to the transportation of Lys, providing redundancy.

1.9 Public goods associated with lysis and bacteriophage

For those systems that utilise cell lysis to release eDNA, other intracellular and membrane products are also released into the extracellular biofilm milieu simultaneously. These now extracellular products can be considered ‘public goods’ if they also benefit the surrounding bacterial community. ‘Public goods’ are products that are costly for the individual to produce but provide a benefit to the surrounding population (230). In the case of bacteria, the most costly form of public goods release would be through cell lysis. eDNA can be considered a public good that benefits communities of bacteria as it is likely produced by a subset of the population and aids in facilitating migration, biofilm formation and other interactions within the biofilm matrix.

Cell lysis may be an integral part of pathogenesis in bacterial biofilms for the release of virulence factors. *Salmonella typhimurium* requires cell lysis to release type III secretion systems and flagella to induce inflammation in the gut of the host as a way of eliminating the competing host microflora (231). In the case of *S. pneumoniae*, lysis is decreased in laboratory strains compared to clinical strains where pneumolysin release is more advantageous (214). These mechanisms of public goods release promote formation of cooperative sub-populations that lyse whilst preparing the ground for successful infection (231). Colicin-producing *E. coli* responds to DNA damage and starvation by producing colicin, which lyses the producing cell but also kills neighbouring competitors (232).

The production of bacteriophage-associated proteins like holins and lysins has mainly been implicated in lysis of the cell as a mechanism for the production of public goods. Interestingly in *Streptococcus mitis* the holin and lysin are mainly involved in the export of other phage-encoded proteins that are involved in binding to platelets during infection of heart tissue (233). The lysin is responsible for exporting the proteins PblA and PblB to the cell wall surface where these proteins aid in platelet binding and aggregation (234). Simultaneously the lysin becomes cell wall-associated through its interaction with choline in the cell wall and can directly interact with fibrinogen in the

surface of platelets having a greater effect on platelet binding than PblA and PblB (235). Homologues of PblA and PblB are also required for prophage release and adhesion to platelets in *E. faecalis* (236). From this example we can hypothesise that the lysin, holin or other bacteriophage-encoded genes that are involved in lysis and/or production of public goods may also have multi-functional roles themselves and aid in transportation or binding of other virulence factors.

The release of public goods through cell lysis is possibly a form of altruism whereby a subpopulation is sacrificed during the production of public goods in response to stress or environmental cues or merely occurs through stochastic gene expression. In either case, the lytic event benefits the remaining population by increasing its protection or virulence. As bacteriophage-mediated lysis releases various public goods, it provides an explanation as to why they have persisted within bacterial genomes.

1.10 Membrane vesicles

MVs are another integral component of bacterial biofilms and are ubiquitous in Gram-negative bacteria. They are bi-layered spherical structures, ranging from 50-300nm in diameter. In *P. aeruginosa,* they play a role in several biological functions such as cell-to-cell signalling, secretion of virulence determinants and insoluble molecules as well as stimulation of host immune responses (24, 101-105).

MVs from *P. aeruginosa* have been shown to carry DNA, phospholipase C, hemolysin, alkaline phosphatase, PQS, protease, β-lactamase, proelastase, endotoxin, lipopolysaccharide, autolysins and other cytosolic proteins, which can contribute to pathogenesis (24, 101-103, 237-239). The majority of extracellular proteins in *P. aeruginosa* planktonic cultures and biofilms are derived from MVs (240). DNA is a constituent of MVs and can be associated with the lumen or membrane surface of the MV (24, 106). MV surface bound DNA appears to be an electrostatic bridging component that is central to MV-bacterium-biofilm matrix interactions, stabilising the biofilm matrix (106).

Based on observations made via electron microscopy, MVs are formed through the blebbing (or ballooning) of the outer membrane, encapsulating outer membrane, inner membrane, periplasmic and even cytosolic contents as

they do so (101, 104). They have been observed to form in both planktonic and biofilm modes of growth in *P. aeruginosa* cultures (237). One proposed mechanism for their formation is that lipopolysaccharide gathers in localised regions on the outer membrane, forcing the membrane into a high-curvature structure, creating membrane blebs (237). In *P. aeruginosa* the majority of PQS is packaged into MVs and PQS has been proposed to mediate MV formation by directly interacting with lipopolysaccharide, resulting in localised membrane curvature (241, 242). Toyofuku *et al*. (243) recently demonstrated the formation of MVs by SOS-induced bacteriophage-related proteins under denitrifying conditions when PQS is not produced. The precise mechanism that leads to MV formation is yet to be elucidated.

Electron microscopy images show MVs on the surface of intact *P. aeruginosa* cells and it is unclear from the images whether the MVs are actively blebbing from the cell or if they are merely lying on the surface of the cell or an artefact of the imaging technique. Time-lapse microscopy of MV formation will determine whether blebbing is the main mechanism for their biogenesis. There are currently no known mechanisms for the transport of DNA into MVs by *P. aeruginosa*. This could also be applied to other bacterial species.

P. aeruginosa MVs have been shown to not only disperse within the biofilm matrix but also extend into the external environment surrounding the biofilm (238). This mechanism for distribution may help concentrate virulence factors and transport them to the host tissue without the bacterial cells or biofilm having to be in direct contact with the host tissue, thereby avoiding attack by the host's immune system (105, 239, 244, 245). This was demonstrated by the induction of innate and adaptive immune responses in mice in response to intragastric delivery of MVs (105). These MVs stimulated the immune response by delivering peptidoglycan to epithelial cells where it is recognised by the nucleotide-binding oligomerisation domain-containing protein 1 (NOD1), which is a cytoplasmic innate immune pattern recognition receptor in the host (105). This virulence mechanism allows the bacteria to initiate inflammatory processes in the host without direct interaction.

It has also been demonstrated that MVs have the capability to fuse and anneal with the outer membrane of bacterial cells, including the host's normal flora and other species, to release their contents into the receiving cells

periplasmic space (246). MVs containing peptidoglycan hydrolases can also lyse a fraction of the surrounding population (101, 239).

1.11 Explosive cell lysis

The production of eDNA has recently been examined in *P. aeruginosa* interstitial biofilms. eDNA production in these biofilms tends to be distributed in a punctate pattern (49). Time-lapse microscopy has shown the release of eDNA in sudden intense bursts (247). Occasionally a number of cells change from a rod-shaped cell to a flexible round cell (Figure 1.6). In many cases this now transformed cell undergoes an explosive lysis event that destroys the cell and expels the internal contents, including genomic DNA, into the biofilm milieu (Figure 1.6). This explosive event is always preceded by the formation of a flexible round cell. These lysis events have been determined to account for all the eDNA produced in actively expanding interstitial biofilms. The entire process of a rod-shaped cell rapidly transforming into a round-shaped cell and lysing in an explosive manner occurs in a matter of seconds. This novel process has been termed 'explosive cell lysis' (247).

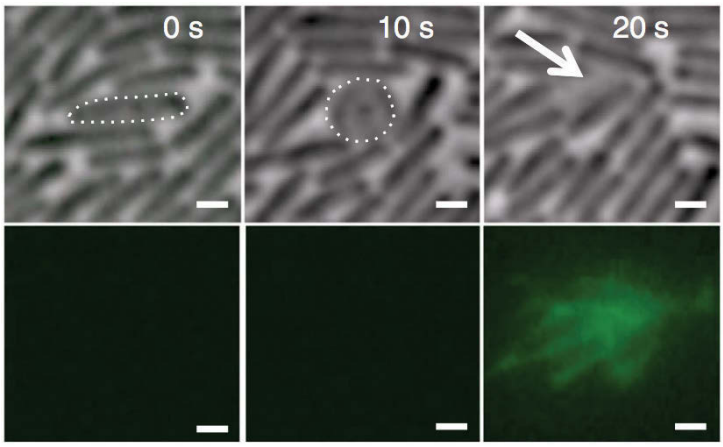


Figure 1.6: Explosive cell lysis in *P. aeruginosa* interstitial biofilms.
Interstitial biofilm of PAO1 cultured in the presence of eDNA stain TOTO-1. Time series of rod-to-round cell transition (dotted whit line, upper panels) and subsequent lysis releasing eDNA stained by TOTO-1 (green, lower panels). Time in s (top right), scale 1 µm.

It is well established that eDNA production in mature *P. aeruginosa* biofilms is regulated through the quorum sensing systems Las and Rhl (see section 1.5.2). Mutants of these systems were investigated to determine

whether they are involved in the formation of round cells, the process of explosive cell lysis and the production of eDNA in interstitial and saturated biofilms. These mutants did not display a defect in eDNA or spherical cell formation, indicating these mature eDNA-production systems do not play a role in explosive cell lysis at the early stages of biofilm formation (247). eDNA is released through this mechanism at the early stages of submerged biofilm development, before quorum-sensing systems would be in play.

Along with the release of DNA and other cytosolic contents, super-resolution imaging using OMX 3D-SIM revealed the presence of a large number of MVs at sites of explosive cell lysis (Figure 1.7A) (247). Interestingly time-lapse microscopy revealed that these MVs do not result from the blebbing of the outer membrane but form from fragments of shattered membrane of a cell undergoing explosive cell lysis, rapidly circularizing in the hydrophilic environment (Figure 1.7B). As these fragments circularize, they are able to capture compounds in the vicinity including cytoplasmic contents of the cell and eDNA (247). This mechanism of public goods release could explain how 50% of proteins in the biofilm matrix and 40% of MV-associated proteins are derived from the cytosol (240). This could also provide evidence for how cytosolic proteins without signal peptide sequences are found in the biofilm milieu.

One possible mechanism for explosive cell lysis could be the universal release and combined action of peptidoglycan hydrolases and/or autolysins like those known to be involved in eDNA production in other bacteria (see sections 1.5.2 and 1.5.3). These enzymes could be responsible for degradation of peptidoglycan, reducing the cells structural rigidity, causing the cell to ‘round up’ and lyse in a dramatic fashion. In support of this hypothesis, the process of explosive cell lysis is visually similar to lysis of *E. coli* cells by lambda bacteriophage (228, 248).

If this phenomenon were genetically encoded, only a portion of the population would express it. The mechanism for releasing public goods would also need to be an efficient process otherwise it would be counter intuitive. This behaviour would also need to be tightly regulated and may be dependent upon cell density and distinct characteristics of each population to prevent unnecessary death. Following bacterial attachment to a substratum, activation

of the major autolysins may rely on reaching a certain cell density. Once activated, the autolysins would induce lysis in a subset of the population.

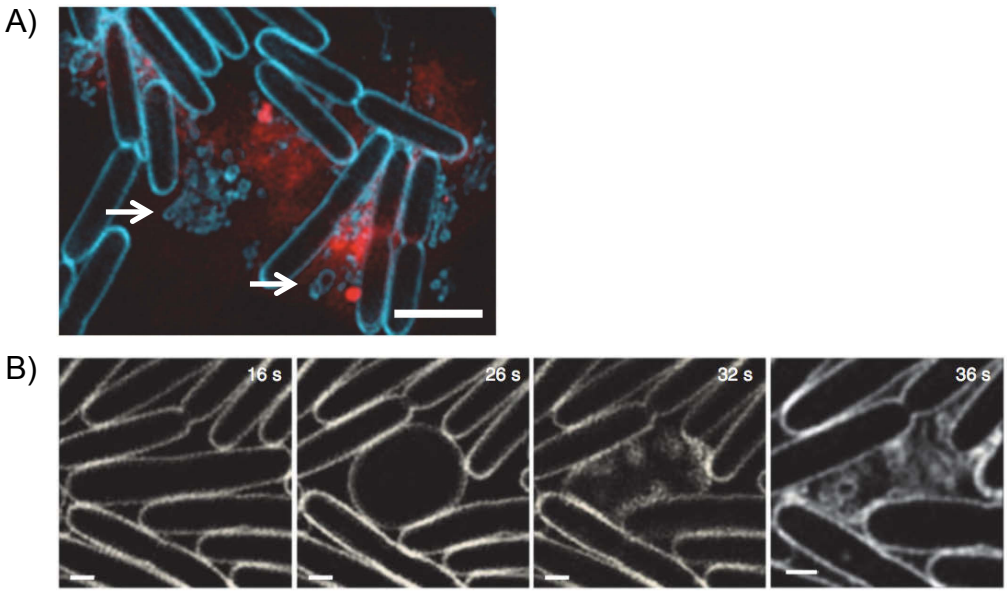


Figure 1.7: MVs in *P. aeruginosa* interstitial biofilms.

A) f3D-SIM of PAK interstitial biofilm in the presence of membrane stain FM1-43FX (blue) and eDNA stain Ethidium Homodimer-2 (red), scale 2 μm. Arrows indicate MVs stained with FM1-43FX. B) f3D-SIM time-series of live interstitial biofilms in the presence of FM1-43FX (white). Time in s (s), top right, scale bar, 0.5 μm. Adapted from (247).

Explosive cell lysis may be crucial for preparing the environment and substratum for establishing a biofilm and coping with external stressors. Bacteriophage and prophage can be induced through the SOS response and by genotoxic stressors. It is possible that explosive cell lysis also serves as a survival strategy in response to stress; cells that have compromised DNA could undergo explosive cell lysis to die in a way that is beneficial to the remaining population by releasing public goods such as eDNA that are essential for biofilm formation.

Explosive cell lysis could be a form of either fratricide or altruistic suicide. In the case a fratricide, two separate sub-populations would arise with one producing the ‘killing’ factor that targets the remaining population and they are protected from the killing factor by the production of an immunity protein. Altruistic suicide results from the death of a cell without a prompt in order to release public goods for the benefit of the community.

Explosive cell lysis could be a novel strategy for bacteria to release and distribute public goods and essential biofilm matrix components throughout the biofilm milieu for the benefit of the community by either fratricide or altruistic suicide (214, 231, 249). Explosive cell lysis could be an integral part of pathogenesis in a host organism. In the case of CF, the bacteria residing in the mucus in the lungs rarely directly interact with the lung epithelium (245). Explosive cell lysis and resulting MVs could be an infection mechanism responsible for distributing public goods and virulence factors throughout the biofilm and into the surrounding environment, eliminating the necessity for direct interaction with the substratum, competing bacteria or host tissue to cause cytotoxicity.

At the time this Thesis was initiated, it was determined that all instances of eDNA production in interstitial biofilms occurs through explosive cell lysis. As explosive cell lysis is visually similar to lysis of *E. coli* cells by lytic bacteriophage (248), it was hypothesized that an endolysin and holin would provide the mechanism for explosive cell lysis in *P. aeruginosa.* Bioinformatic analysis has determined that *P. aeruginosa* genome carries only one putative endolysin encoded by the *lys* gene (162). Interestingly, *lys* has also been reported to be involved in membrane vesicle formation (243) and pyocin related genes have been identified as major membrane vesicle-associated proteins (102, 240, 243, 250). As membrane vesicles form after explosive cell lysis (247), it is possible that Lys is responsible for mediating explosive cell lysis and eDNA release in *P. aeruginosa* biofilms. The role of Lys in explosive cell lysis will be explored in this Thesis.

1.12 Thesis scope and aims

P. aeruginosa is the fourth most commonly isolated nosocomial pathogen, often infecting immune-compromised individuals and implanted medical devices by forming biofilms. eDNA is a major component of the biofilm matrix, playing various roles in facilitating cell migration, adhesion and intercellular connectivity and is essential for biofilm development. In the absence of eDNA, biofilms fail to form, suggesting eDNA is produced during early stage biofilm development. However, the mechanism of its production at this developmental stage is unknown.

At the initiation of this Thesis, it was observed that during the early stages of development of submerged biofilms, as well as in actively expanding interstitial biofilms, that a small of cells spontaneously transform into round cells before rapidly lysing and releasing eDNA and cellular content into the extracellular milieu. This phenomenon is termed ‘explosive cell lysis’. This process accounts for all eDNA produced in these biofilms as well as a number of other ‘public goods’ including MVs that may benefit the biofilm community. As eDNA, MVs and extracellular proteins contribute to biofilm development and therefore virulence, explosive cell lysis may be a critical process for *P. aeruginosa*. Therefore, it is important to identify the mechanism mediating this process.

The aim of this Thesis is to identify and characterise the genes and cellular processes that lead to round cell formation, explosive cell lysis and eDNA release, and to better understand the role of eDNA in early stages of biofilm development. Chapter 3 of this Thesis identifies that the key enzyme required for the formation of round cells and eDNA release through explosive cell lysis is an endolysin related to cryptic prophage. Chapter 3 goes on to demonstrate explosive cell lysis as a cell death pathway under the control of SOS response regulator RecA. Chapter 4 explores the role of intrinsic and exogenous stress in explosive cell lysis and whether exposure to exogenous stressors, including clinically relevant antibiotics, induces the phenomenon. Chapter 5 investigates which holins mediate the translocation of the endolysin responsible for explosive cell lysis. Chapter 6 then examines the role of explosive cell lysis in submerged biofilm formation and determines that explosive cell lysis is necessary for the formation of young microcolonies in submerged biofilms and microcolony formation is spatially and temporally correlated to explosive cell lysis.

Chapter Two:

Materials and Methods

2 Materials and Methods

2.1 Media and Buffers

2.1.1 Na_2EDTA

A 0.5 M solution of ethylenediaminetetraacetic acid (EDTA) disodium salt dehydrate (Na_2EDTA, Sigma-Aldrich) was prepared by mixing 0.5 M of the solid in diH_2O. The pH was raised to 9 by the addition of NaOH to allow the solid to dissolve and then lowered to pH 8 with 10 M HCl. The solution was sterilised in an autoclave at 121 °C and 15 kPA for 20 min (unless otherwise stated, all autoclave settings are the same as those stated here) and stored at room temperature.

2.1.2 Tris Borate EDTA (TBE)

A 10× stock solution of TBE was prepared by dissolving a pre-made mixture of 0.89 M tris base, 0.89 M boric acid and 0.02 M 0.5 M EDTA (Medicargo AB, Sweden) in diH_2O. The solution was autoclaved and stored at room temperature. A 0.5× working solution was prepared by diluting the stock with diH_2O then stored at room temperature.

2.1.3 Phosphate Buffered Saline (PBS)

PBS was prepared by dissolving 1 PBS tablet (Medicargo AB, Sweden) per 100 mL diH_2O. The solution was sterilised by autoclaving and stored at room temperature.

2.1.4 Tris-Cl

A 1 M solution of Tris-Cl was made by dissolving 121.1 g/L of Tris base in diH_2O and adjusting the pH to 7.5 with 10 M HCl. The solution was autoclaved and stored at room temperature.

2.1.5 Tris-EDTA (TE) buffer

A 1 M solution of Tris-Cl (prepared in section 2.1.4) and a 0.5 M solution of EDTA (prepared in section 2.1.1) were diluted in 100 mL of diH_2O so that the

final concentrations of Tris-Cl and EDTA were 10 mM and 1 mM, respectively. The solution was adjusted to pH 8 with 10 M NaOH, the solution autoclaved and stored at room temperature.

2.1.6 Sodium chloride solution

A 6 M saturated solution of NaCl was prepared by mixing 350.64 g/L of NaCl (Sigma-Aldrich) with diH_2O. The solution was autoclaved and stored at room temperature.

2.1.7 Bacterial lysis solution

Bacterial lysis solution was prepared by mixing 1 M Tris-Cl (as prepared in section 2.1.4), 0.5 M Na_2EDTA (as prepared in section 2.1.1) and 6 M NaCl (as prepared in section 2.1.6) with diH_2O so that the final concentrations were 10 mM, 2 mM and 400 mM, respectively. The solution was autoclaved and stored at room temperature.

2.1.8 Proteinase K storage solution

Storage solution for Proteinase K was prepared by combining 1 M Tris-Cl at pH 7.5 (as prepared in section 2.1.4), 6 M NaCl (as prepared in section 2.1.6), 0.5 M Na_2EDTA (as prepared in section 2.1.1), 1 M $CaCl_2$ (Sigma-Aldrich) and 1 M Dithiothreitol (DTT) so that the final concentrations were 50 mM, 100 mM, 0.1 mM, 10 mM and 1 mM, respectively, with the addition of glycerol (50% v/v, Amresco LLC) and Triton X-100 (0.1 %, Sigma-Aldrich). Proteinase K stock was made up at a concentration of 10 mg/mL in the storage solution and stored at -20 °C.

2.1.9 Low salt Luria-Bertani (LB) Broth and Agar (LBA)

Low salt LB was composed of 10 g/L of tryptone (Oxoid), 5 g/mL of yeast extract (Oxoid) and 5 g/L of NaCl dissolved in diH_2O, autoclaved and then cooled before use and stored at room temperature. LBA was made with the addition of 1.5 % w/v bacteriological grade agar (Oxoid) which was autoclaved then cooled to 55 °C before the addition of heat liable additives such as antibiotics and poured into 92 mm Petri dishes (Sarstedt, Nümbrecht, Germany).

2.1.10 LBA with 5 % sucrose

LBA with 5 % sucrose in RO water (Sigma-Aldrich) was prepared for use in the *P. aeruginosa* conjugation to counter-select strains with vectors containing the *sacB* gene. This media was made by preparing a stock solution of 0.73 M sucrose, which was filter-sterilised using a 0.22 µm pore size filter. LBA was prepared as described in section 2.1.9 except NaCl was omitted and the volume adjusted to 80 % of the final volume. After the media had cooled to 55 °C, sucrose stock solution was added so that the final concentration of sucrose was 0.15 M (20 % of the final volume). The media was poured into 92 mm Petri dishes (Sarstedt, Nümbrecht, Germany).

2.1.11 Vogel-Bonner media (VBM) and agar (VBMA)

A 10× stock solution of Vogel-Bonner media was prepared by dissolving 2 g/L $MgSO_4.7H_2O$ (Sigma-Aldrich), 20 g/L citric acid (anhydrous) (Univar), 100 g/L K_2HPO_4 (Chem Supply) and 35 g/L $NaNH_5PO_4.4H_2O$ in RO water. The pH was adjusted to 7 using NaOH solution and autoclaved. To make VBMA, 1.5% bacteriological agar was prepared in 90% of the volume and autoclaved. After the agar had cooled to 55 °C, 10× VBM stock solution was added so that the final concentration of VBM was 1× (10 % of the final volume). The media was poured into 92 mm Petri dishes (Sarstedt, Nümbrecht, Germany).

2.1.12 Cation-Adjusted Mueller-Hinton Broth (CAMHB)

Twenty-one grams per litre of cation-adjusted Mueller-Hinton Broth powder (Oxoid) was dissolved in diH_2O and boiled for one min before autoclaving. The solution was cooled before use and stored at room temperature.

2.1.13 Gellan Gum-solidified nutrient media (TMGG)

Gellan gum-solidified nutrient media was prepared by addition of 4 g/L of tryptone (Oxoid), 2 g/L yeast extract (Oxoid), 2 g/L NaCl and 1 g/L $MgSO_4.7H_2O$ (Sigma-Aldrich) and dissolved in diH_2O. The solution was then heated and stirred on a magnetic stirrer and 8 g/L Gellan Gum (MP Biomedicals) was added slowly to ensure it completely dissolved. Upon boiling the media was

sterilised by autoclaving and stored at room temperature. This media will be referred to as twitching motility gellan gum (TMGG) (49).

2.1.14 Super optimal broth (SOB) media

SOB media was prepared by combining 20 g/L tryptone (Oxoid), 5 g/L yeast extract (Oxoid) and 0.5 g/L NaCl dissolved in diH_2O, with the pH adjusted to 7.5 by addition of KOH. The media was then sterilised by autoclaving and cooled before use. $MgSO_4$ and $MgCl_2$ in MQ water, which had been filter sterilised (0.22 µm pore size filter), were added at a final concentration of 10 mM and 4.6 mM, respectively to the media post autoclaving. The media was then stored at room temperature.

2.1.15 RF1 and RF2 buffer

RF1 was prepared by dissolving 0.1 M RbCl (Sigma-Aldrich), 0.06 M $MnCl_2.2H_2O$ (Sigma-Aldrich), 0.03 M KOAc (Sigma-Aldrich), 0.02 M $CaCl_2.2H_2O$ (Sigma-Aldrich) and 7 % w/v glycerol in MQ water, with the pH being adjusted to 5.8 with 2 M acetic acid. The solution was then filter-sterilised (0.22 µm pore size filter) and stored at 4 °C.

RF2 was prepared by dissolving 5.3 mM MOPS (Amresco LLC), 8.3 mM RbCl, 0.07 M $CaCl_2.2H_2O$, 7 % w/v glycerol in MQ water, then pH adjusted to 6.8 with 10 M NaOH. The solution was then filter-sterilised (0.22 µm pore size filter) and stored at 4 °C.

2.1.16 Deoxyribonuclease I (DNase I)

DNase I (D5025, Sigma) was dissolved in a storage buffer consisting of 10mM Tris-Cl pH 7.5, 10mM $CaCl_2$, 10mM $MgCl_2$, 50% glycerol to a stock concentration of 100,000 Kunitz units/mL. For experiments, DNase I was used at a final concentration of concentration of 100 Kunitz units/mL.

2.2 Bacterial strains and culture conditions

2.2.1 Bacterial strains

The bacterial strains used in this study are listed in Table 2.1.

Table 2.1: Strains used in this study

Strains	Relevant characteristics	Source
E. coli		
DH5α	*E. coli* strain for transformation (F^-, *lacZ*ΔM1, *recA1*)	TaKaRa
S17-1	Donor strain for conjugal transfer (*thi pro hdR recA chr::RP4-2*)	(251)
P. aeruginosa		
PAO1	Wild-type *P. aeruginosa* strain	(67)
PAO1Δ*alpB*	In frame deletion of *alpB*	This study
PAO1Δ*cidAB*	In frame deletion of *cidAB*	(67)
PAO1Δ*hol*	In frame deletion of *hol*	This study
PAO1Δ*cidAB*Δ*alpB*	In frame deletion of *cidAB* and *alpB*. Parent strain PAO1Δ*cidAB*	This study
PAO1Δ*cidAB*Δ*hol*	In frame deletion of cidAB and hol. Parent strain PAO1Δ*cidAB*	This study
PAO1Δ*hol*Δ*alpB*	In frame deletion of *hol* and *alpB*. Parent strain PAO1Δ*hol*	This study
PAO1Δ*cidAB*Δ*hol*Δ*alpB*	In frame deletion of *cidAB*, *hol* and *alpB*. Parent strain PAO1Δ*cidAB*Δhol	This study
PAO1Δ*lys*	In frame deletion of *lys*	(247)
PAO1Δ*prtN*	In frame deletion of *prtN*	(243)
PAO1Δ*pf4*	*pf4* prophage cassette inactivated by allelic displacement with gentamicin resistance (Gm^R)	(187)
PAO1Δ*recA*	In frame deletion of *recA*	(243)
PAK	Laboratory strain	D. Bradley, Memorial University of Newfoundlands, St John's, Canada
PA14	Laboratory strain	(252)
PA103	Laboratory strain	(253)
ATCC27853	Laboratory strain	Oxoid
CF57	Cystic fibrosis isolate	David Armstrong, Monash Medical Centre, Australia
CF219	Cystic fibrosis isolate	David Armstrong, Monash Medical Centre, Australia

CF227	Cystic fibrosis isolate	David Armstrong, Monash Medical Centre, Australia
CF497	Cystic fibrosis isolate	David Armstrong, Monash Medical Centre, Australia
CF581	Cystic fibrosis isolate	David Armstrong, Monash Medical Centre, Australia
CLIN66	Endotracheal aspirate	Peter Midolo, Monash Medical Centre, Australia
CLIN67	Chest fluid	Peter Midolo, Monash Medical Centre, Australia
PA14_07980::MAR2xT7	Conserved hypothetical protein	(254)
PA14_08000::MAR2xT7	Conserved hypothetical protein	(254)
PA14_08010::MAR2xT7	Putative baseplate assembly protein V	(254)
PA14_08030::MAR2xT7	Putative phage baseplate assembly protein	(254)
PA14_08040::MAR2xT7	Putative phage tail protein	(254)
PA14_08050::MAR2xT7	Putative tail fibre protein	(254)
PA14_08060::MAR2xT7	Putative tail fibre assembly protein	(254)
PA14_08070::MAR2xT7	Putative phage tail sheath protein	(254)
PA14_08090::MAR2xT7	Putative phage tail tube protein	(254)
PA14_08100::MAR2xT7	Conserved hypothetical protein	(254)
PA14_08120::MAR2xT7	Putative tail length determinator protein	(254)
PA14_08130::MAR2xT7	Putative tail formation protein	(254)
PA14_08180::MAR2xT7	Hypothetical protein	(254)
PA14_08210::MAR2xT7	Putative major tail protein V	(254)
PA14_08220::MAR2xT7	Hypothetical protein	(254)
PA14_08230::MAR2xT7	Hypothetical protein	(254)
PA14_08240::MAR2xT7	Putative tail length determination protein	(254)
PA14_08260::MAR2xT7	Putative minor tail protein L	(254)
PA14_08270::MAR2xT7	Conserved hypothetical protein	(254)
PA14_08280::MAR2xT7	Putative phage tail assembly protein	(254)
PA14_08300::MAR2xT7	Putative phage-related protein, tail component	(254)
PA14_08320::MAR2xT7	Conserved hypothetical protein	(254)

2.2.2 Storing and reviving bacteria

Overnight cultures of bacteria were mixed with 15 % v/v glycerol (Amresco LLC) and stored at -80 °C. Bacteria were revived from the frozen glycerol stock by streaking some of the frozen culture onto an LBA plate (supplemented with appropriate antibiotics) and incubating at the appropriate temperature overnight.

2.2.3 *E. coli* culture conditions

E. coli strains were cultured in LB. Cultures were incubated overnight at 37 °C, with broth cultures shaking at 250 rpm in an orbital shaker. Media was supplemented where appropriate with antibiotics at the following concentrations: 100 µg/mL ampicillin (Astral Scientific), 50 µg/mL kanamycin sulphate (Astral Scientific) and 10 µg/mL gentamicin sulphate (Sigma-Aldrich).

2.2.4 *P. aeruginosa* culture conditions

All *P. aeruginosa* strains were cultured in 2 mL of LB or CAMHB in 14 mL loosely capped polypropylene tubes (BD Falcon®) and cultured at 37 °C overnight in an orbital shaker at 250 rpm. For larger volumes (up to 10 mL) the strain was inoculated in a 50 mL tube (BD Falcon®) and the tube was sealed with Aeraseal™ (Excel Scientific Inc., Victorville, CA, USA) to allow for sufficient airflow, and cultured at 37 °C shaking at 250 rpm. Where appropriate, media was supplemented with antibiotics at the following concentrations: 100 µg/mL gentamicin sulphate (Sigma-Aldrich) and 250 µg/mL carbenicillin (Sigma-Aldrich).

2.2.5 Preparation of competent *E. coli* cells

E. coli competent cells were prepared as follows: *E. coli* was inoculated into 4 mL SOB media (as prepared in section 2.1.13) and grown overnight at 37 °C, shaking at 250 rpm. These overnight cultures were then added to 500mL fresh SOB media and grown at 37 °C, shaking at 250 rpm for 3 h. The cultures were then divided into 50 mL aliquots and incubated on ice for 30 min. The cells were the pelleted by centrifugation (3, 000 *g* for 10 min at 4 °C). Each pellet was resuspended in 8 mL RF1 buffer (as prepared in section 2.1.14) by

swirling, and all resuspended cells pooled and left on ice for 15 min. The pooled cells were then pelleted by centrifugation (3, 000 *g* for 10 min at 4°C) and the pellet resuspended in 20 mL RF2 buffer (as prepared in section 2.1.14). The resuspended cells were left on ice for 15 min and 500 µL aliquots transferred into 1.5 mL microfuge tubes. These aliquots were snap frozen in a dry ice/95 % ethanol bath and stored at -80 °C.

2.2.6 Preparation of electro-competent *P. aeruginosa* cells

P. aeruginosa electro-competent cells were prepared as follows: *P. aeruginosa* was inoculated into 2 mL CAMHB and grown overnight at 37 °C, shaking at 250 rpm. The cells were pelleted by centrifugation (3, 000 *g* for 10 min at 4 °C). The cell pellet was washed four times in 1 mL ice-cold 300 mM sucrose (Sigma-Aldrich) and finally resuspended in 200 µL ice-cold 300 mM sucrose. Five mircolitre of plasmid DNA was added to 80-100 µL of cells, incubated at room temperature for 5 min, and transferred to a 1 mm gap-width electroporation cuvette (BTX). After applying a pulse (settings: 25 µF; 200 Ω; 2.5 kV on a BTX 600 Electro Cell Manipulator; Harvard Apparatus Inc.), the cells were added to 1 mL CAMHB with 0.2 % glucose and incubated at 37 °C, shaking at 250 rpm for 1 h and then plated onto 1.5% LBA containing appropriate antibiotics.

2.3 Molecular Biology Techniques

2.3.1 Transformation of DNA into competent *E. coli* cells

DNA was shuttled into competent *E. coli* cells prepared in section 2.26. 50 µL aliquots of competent *E. coli* cells were thawed and incubated with plasmid DNA for 30 min on ice. After heat shock at 42 °C for 45 s, the cells were incubated on ice for 2 min. LB with 0.2 % glucose was added and the cells incubated at 37 °C for 1 h. The cells were serially diluted in LB and 100 µL aliquots spread onto 1.5% LBA containing the appropriate antibiotics.

2.3.2 Genomic DNA extraction

Five hundred microlitre of an overnight culture was pelleted by centrifugation (16, 200 *g* for 3 min at room temperature). One µL of 50 µg/mL

Proteinase K was added to 300 µL of bacterial lysis solution (as prepared in section 2.1.7) and used to resuspend the pelleted cells. This was incubated at 65 °C for 45 min with vortexing every 5 min. After incubation, the cell lysate was cooled to 37 °C and RNase was added (5 µg/µL) and incubated for 30 min. After incubation, 500 µL of 6 M NaCl (as prepared in section 2.1.6) was added to the cell lysate, which was then vortexed well. The cell lysate was then centrifuged (10, 000 *g* for 10 min at 4 °C). Two hundred and fifty µL of the supernatant was transferred into fresh microfuge tube containing 500 µL isopropanol (Sigma-Aldrich) with the contents of the tube mixed by inversion (30 times). The DNA was pelleted by centrifugation (10, 000 *g* for 10 min at 4 °C) and the pellet washed twice with 75 % ethanol (Sigma-Aldrich). The washed pellet was resuspended in TE buffer (as prepared in section 2.1.5).

2.3.3 Oligonucleotides

Oligonucleotides were purchased from Invitrogen (Life Technologies Cooperation) and were reconstituted with Baxter's water to a final concentration of 100 mM and stored at -20 °C. Oligonucleotides for Polymerase Chain Reaction were thawed and made to a concentration of 10 mM with Baxter's water and stored at -20 °C. Oligonucleotides used in this study are listed in Table 2.2.

2.3.4 Polymerase Chain Reaction (PCR)

Primers for flanking regions for *lys, alpB, cidAB* and *hol* were designed using MacVector (Oxford Molecular Group) (see Table 2.2) and amplified by PCR using 2.5 U of *Pfu Turbo* DNA Polymerase (Agilent Technologies), 1× *Pfu Turbo* buffer, 0.2 mM dNTPs (purchased as individual nucleotides from Promega from which a 5 mM mix was made and stored at -20 °C), dimethyl sulfoxide (DMSO) (1.5 % v/v, Sigma-Aldrich) and 0.2 µM of each primer (as prepared in section 2.3.3). PCR reactions were performed on an Eppendorf Mastercycler® (Eppendorf, Hamburg, Germany) using the following cycling conditions: initial 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s; annealing at the temperature indicated in Table 2.2 for 30 s; extension at 68 °C for 1 min/kb. A final extension at 68 °C for 4 min 30 s followed. The products were stored at 4 °C.

Table 2.2 Primers used in this study

Primers	Sequence 5' → 3'	Annealing Temperature
lys_F	AGTTCCTGATCGACTCGGTG	52 °C
lys_R	TGAGTCAGGATGGACATGGA	52 °C
alpB_F	TGACGCTATGGGACGATAAA	54 °C
alpB_R	GTACGTTCGTTCAATGCAGG	54 °C
cidAB_F	CTTTCTCCATCCCCGATTTC	54 °C
cidAB_R	TTTTGTCGTTATCGGATGCC	54 °C
hol_F	TTCTTGTAAAGGTGCGTCCC	54 °C
hol_R	GCATGGTTGACTCCTTCGAT	54 °C

2.3.5 Agarose gel electrophoresis

Nucleic acids were visualised by agarose gel electrophoresis. Agarose gels (0.7 % or 1 % w/v) were made with biotechnology grade agarose (Amresco LLC) and melted in 0.5× TBE (as prepared in section 2.1.2). After the gel had cooled to 50 °C, 1 U/mL of Gel Red™ Nucleic Acid Gel Stain (Gel Red, Biotium) was added. Gels were submerged in 0.5× TBE and samples were loaded with bromophenol blue loading dye (2.5 g/L bromophenol blue (Sigma-Aldrich), 400 g/L sucrose (Sigma-Aldrich), in diH_2O). Gels were visualised using Kodak EDAS 290 Electrophoresis Documentation and Analysis System with a DC290 Camera (Eastman Kodak Company).

2.3.6 Isolation of DNA from agarose gels

DNA fragments were isolated by running DNA on a 1 % agarose gel (as described in section 2.4.5). Fragments were then visualised with a UV light source and excised with a scalpel. DNA was extracted from the gel using a HiYield™ Gel/PCR DNA mini kit (Real Biotech Corporation) as per the manufacturer's instructions. DNA was eluted in TE buffer and stored at 4 °C.

2.3.7 Isolation of plasmid DNA

Plasmids were isolated from overnight cultures using QIAprep® Spin Miniprep Kit (Qiagen) following the manufacturer's instructions. Plasmid DNA was eluted in TE buffer and stored at 4 °C.

2.3.8 Restriction endonuclease digestion

Restriction enzymes were purchased from New England Biolabs (Australia) and stored at -20 °C. All reactions were incubated at 37 °C for 2 h. Each reaction consisted of 2 µL of 10× buffer, 1 µL of enzyme, 2 µL of 1 mg/mL Bovine serum albumin (BSA) and 1 µg of DNA and the volume was made up to 20 µL using Baxter's water. The digest was run on an agarose gel (see section 2.3.5) to test for complete digestion before the enzyme was heat inactivated.

2.3.9 Ligation

Ligations were performed using a T4 DNA ligase kit (New England Biolabs) using 10-20 ng of vector DNA and 20-40 ng of insert DNA in a total volume of 20 µL. To this volume, 2 µL of 10× ligase buffer, and 1 µL (2 U) of ligase enzyme were added. The reaction was incubated at room temperature for 1 h or at 4 °C overnight.

2.3.10 Construction of plasmids

The plasmids used in this study are listed in Table 2.3. Complementing plasmids were constructed by cloning the wild-type gene synthesised by GeneArt Gene Synthesis (Thermo Scientific, Carlsbad, CA, USA) into the multiple cloning site of pJN105 using *Spe*I and *Sac*I restriction enzymes from New England Biolabs (Australia). IbpA-YFP fusion gene obtained pAc1.8 (255) was shuttled into the multiple cloning site of pUCP24 for use in *P. aeruginosa*.

Table 2.3 Plasmids used in this study

Plasmids	Relevant characteristics	Source
pJN105	Broad host range arabinose inducible gene expression vector (Gm^R)	(256)
pJN105*alpB*	pJN105 with wild-type *alpB* (Gm^R)	This study
pJN105*cidAB*	pJN105 with wild-type *cidAB* (Gm^R)	This study
pJN105*hol*	pJN105 with wild-type *hol* (Gm^R)	(247)
pJN105*lys*	pJN105 with wild-type *lys* (Gm^R)	(247)
pJN105*lys**	E51V substitution in putative active site of Lys	(247)
pRIC380	Suicide vector (Amp^R)	(257)
pPS856	*FRT* cassette vector (Gm^R)	(258)
pFLP2	Site-specific excision vector (Gm^R)	(258)
pUCP24	Cloning vector (Gm^R)	(259)

pAc1.8	IbpA-YFP fusion on pAc plasmid derived from pUC18-YFP	(255)
pMLAC-G	*lac* promoter region fused to *eGFP* in pMEXGFP	(247)
pMHOL-G	*hol* promoter region fused to *eGFP* in pMEXGFP	(247)
pALH3	1kb upstream and 1kb downstream flanking regions of *hol.* A *Hind*III site in between the flanking regions and a *Spe*I site on the 5' end and 3' end of this 2 kb fragment, synthesised in pMK-RQ; Km^R	This study
pALH5	pALH3 containing *FRT-*Gm^R*-FRT* from pPS856 cloned into *Hind*III site; Gm^R, Km^R	This study
pALH7	3.1 kb *Spe*I insert from pALH5 cloned into pRIC380; Gm^R, Ap^R	This study
pALH9	1kb upstream and 1kb downstream flanking regions of *alpB.* A *Hind*III site in between the flanking regions and a *Spe*I site on the 5' end and 3' end of this 2 kb fragment, synthesised in pMK-RQ; Km^R	This study
pALH10	pALH9 containing *FRT-*Gm^R*-FRT* from pPS856 cloned into *Hind*III site; Gm^R, Km^R	This study
pALH11	3.1 kb *Spe*I insert from pALH10 cloned into pRIC380; Gm^R, Ap^R	This study

2.3.11 Construction of allelic exchange mutants

In-frame deletions of *hol* and *alpB* were constructed in *P. aeruginosa* strain PAO1 by allelic exchange using the Flp-FRT recombination system for site specific excision of chromosomal sequences as previously described (258) to construct the strains listed in Table 2.1. Briefly, 1 kb sections that flanked the *hol* and *alpB* regions were synthesised by GeneArt Gene Synthesis (Thermo Scientific, Carlsbad, CA, USA), creating pALH3 and pALH9, respectively (Figure 2.1A). This also included 100 bp of the 5` and 3` ends of the target gene with a *Hind*III site introduced in the middle. The *FRT-*Gm^R cassette from pPS856 (243) was sub-cloned into the internal *Hind*III site of pALH3 and pALH9 resulting in pALH5 and pALH10 containing the flanking regions of *hol* and *alpB*

separated by the *FRT-Gm*^{R} cassette (Figure 2.1B). The resultant clones were then digested with *Spe*I and cloned into the suicide vector pRIC380 resulting in pALH7 and pALH11 (Figure 2.1B). The pRIC380 vector contains the genes *sacB*/*sacR*, which promote sensitivity to sucrose and *oriT* which enables conjugal transfer. The resultant clones were transformed into the *E. coli* donor strain S17-1 in in preparation for conjugal transfer into *P. aeruginosa* PAO1 strains The *E. coli* donor and *P. aeruginosa* recipient strains were grown in LB with the appropriate antibiotics for 6-8 h at 37 °C, shaking at 250 rpm and spread plated onto 1.5% LBA with *E. coli* and *P. aeruginosa* incubated in humidified containers overnight at 37 °C and 42 °C, respectively. Bacterial lawns of the *E. coli* donor and *P. aeruginosa* recipient were scraped up with an inoculation loop and mixed for 10 min on 1.5% LBA before 24 h incubation at 37 °C in a humidified container. The mating mixture of *E. coli* and *P. aeruginosa* were scraped up with an inoculation loop and resuspended in LB before being serially diluted and spread plated onto VBMA (refer to section 2.1.11) containing the appropriate antibiotics for selection of antibiotic resistant *P. aeruginosa* colonies. VBMA plates were incubated at 37 °C for 24-72 h.

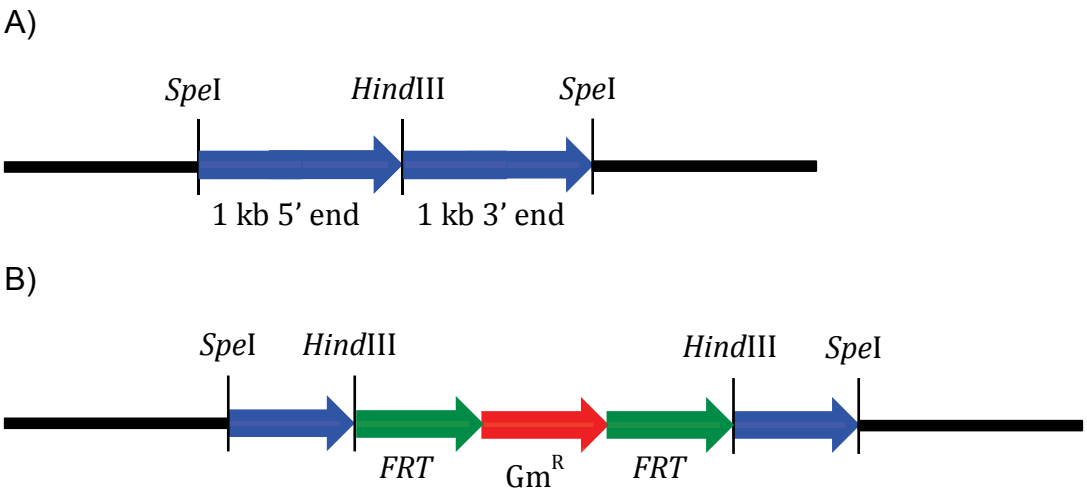


Figure 2.1: Maps of vectors used in creating deletion mutants.

A) Vector map for synthesised vectors pALH3 and pALH9. The blue genes indicate the 1 kb upstream and downstream sections of the respective genes with the remaining section replaced by a *Hind*III site. B) Vector map for vectors pALH5, pALH7, pALH10 and pALH11. The blue genes indicate the 1 kb upstream and downstream sections of the respective genes. The green genes indicate *FRT* sequences and the red gene indicates a gentamicin resistance gene (Gm^R).

The transconjugants were plated onto 1.5% LBA without sodium chloride and supplemented with 5% sucrose and containing gentamicin to select for colonies in which the plasmid had excised while leaving the homologously recombined *hol*::Gm^R or *alpB*::Gm^R alleles in the chromosome. The Gm^R gene was then excised using the pFLP2 plasmid that expresses the Flp recombinase as described previously (258) creating *P. aeruginosa* strains with the *hol* or *alpB* regions deleted and replaced with an FRT sequence. Allelic exchange mutants were confirmed by PCR (refer to section 2.3.4) of isolated chromosomal DNA. All the plasmids used in this Thesis are listed in Table 2.3 and the primers used are in Table 2.2.

2.4 Interstitial biofilm assays

2.4.1 TMGG interstitial biofilm setup

Microscopic twitching motility was assayed on gellan gum-solidified nutrient media (TMGG) (as prepared in section 2.1.12) coated microscope slides as follows: TMGG was melted in a microwave and poured into 50 mL tubes (BD Falcon®) which had been pre-heated to 65 °C. To prevent setting, TMGG was kept at 65 °C, and used on the same day it was melted. Microscope slides (Hurst Scientific Pty. Ltd.) were flame-sterilised in 95 % ethanol and placed in a 150 mm petri dish (Greiner Bio-One) on a level 65 °C heating platform. Five millilitre aliquots of gellan TMGG were poured and spread in an even layer across the surface of four sterilised microscope slides. Fluorescent stains and 0.2 % L-arabinose were added to the molten media immediately prior to pouring where indicated. Fluorescent stains used in this study include the nucleic acid stain TOTO-1 iodide (1 μM; Life Technologies) or the eDNA and dead cell stain ethidium homodimer-2 (EthHD-2; 1 μM; Biotium) The petri dish containing the TMGG-coated microscope slides was then transferred to a level platform at room temperature and the media allowed to fully set before being stored at 4 °C for no more than a week. Prior to use in interstitial biofilm assays, each set of TMGG-coated microscope slides was dried in a biosafety cabinet and media removed from the edges (~5 mm) of each microscope slide to allow the slide to sit completely level on the microscope stage. A small inoculum of the strain of interest was taken from a fresh overnight LBA plate

culture and applied to the TMGG-coated slide. A sterilised 22 x 22 mm or 22 x 32 mm coverslip (0.13-0.16 mm thick) (Menzel Glaser, Germany) was carefully placed onto the solidified media and the slide incubated at 37 °C in a humidified chamber for 4-6 h. The slides were imaged on a Nikon Ti inverted research microscope with a 100x 1.45 numerical aperture (NA) PlanApo objective, using NIS Elements acquisition software (Nikon Instruments, Tokyo, Japan), solid state illumination (Lumencor, Beaverton, OR, USA), and Cascade 1Kx1K EMCCD camera (Photometrics) and fitted with an environmental chamber (ClearState Solutions, Mt Waverley, VIC, Australia).

2.4.2 TMGG interstitial biofilm with saturated filter disc

A filter disc diffusion assay was used to examine eDNA release in *P. aeruginosa* interstitial biofilms in response to chemical gradients. The chemicals examined were mitomycin C (MMC; Sigma-Aldrich), ciprofloxacin (Cip; Sigma-Aldrich), gentamicin sulphate (Gm; Sigma-Aldrich) and colistin (CL; Sigma-Aldrich). TMGG-coated microscope slides were prepared for inoculation as in section 2.5.1. A filter disc (6 mm, GE Healthcare) was saturated with 30 µL of the chemical of interest and incubated aseptically at room temperature within a 92 mm petri dish for 20 min. The saturated disc was moved to a dry area of the plate and left to dry for 10 min. The soaked disc was then applied to a TMGG-coated microscope slide (prepared in section 2.4.1) and incubated at room temperature for 1 h. A small inoculum of the strain of interest from a fresh overnight LBA plate culture was inoculated on the solidified media at 5 mm from the edge of the filter disc. A sterile 22 x 22 mm coverslip (0.13-0.16 mm thick) (Menzel Glaser, Germany) was carefully placed onto the solidified media at the edge of the filter disc, and the slide incubated at 37 °C for 4-6 h in a humidified chamber. The slides were imaged on a Nikon Ti inverted research microscope and analysed as described in section 2.4.1.

2.4.3 Interstitial biofilm analysis

2.4.3.1 Number of eDNA release sites per mm^2

For quantitative analysis of eDNA release sites in interstitial biofilms, series of overlapping images spanning the outermost leading edge through to the main colony were obtained (Nikon Ti; 100x objective) and stitched using the

NIS Elements acquisition software (Nikon Instruments, Tokyo, Japan). The number of eDNA release sites across the biofilm monolayer was enumerated manually and the area of the images covered by the biofilm calculated by auto-thresholding using FIJI (260). This was performed in biological triplicate with 10 images from each replicate.

2.4.3.2 **Area of biofilm covered by eDNA**

A series of overlapping images spanning the outermost leading edge through to the main colony were obtained (Nikon Ti; × 100 objective) and stitched using the NIS Elements acquisition software (Nikon Instruments, Tokyo, Japan). The area covered by eDNA and the area of the biofilm comprised of cells was identified by auto-thresholding using FIJI (260). This was performed in biological triplicate with 10 images from each replicate.

2.4.3.3 **BacFormatics analysis**

Interstitial biofilms for BacFormatics analysis were imaged on Olympus IX71 inverted research microscope with × 100 1.4 NA UPlanFLN objective, FViewII monochromatic camera and AnalySIS Research acquisition software (Olympus Australia, Notting Hill, VIC, Australia) fitted with an environmental chamber (Solent Scientific, Segensworth, UK). Quantitative analysis of cell morphotypes in interstitial biofilms was performed using an in-house program, BacFormatics v0.7 (source code available at https://github.com/ithreeMIF/BacFormatics) that was developed in MATLAB (The MathWorks Inc., Natick, MA, USA). Analysis was carried out as previously described (247). This was performed in biological duplicates with 25 images from each replicate.

2.4.3.4 **Number of dark spots and IbpA-YFP spots**

Random fields of view of interstitial biofilms were imaged on Nikon Ti inverted research microscope with a 100x 1.45 NA PlanApo objective, using NIS Elements acquisition software (Nikon Instruments, Tokyo, Japan). For 200 cells, the number of dark spots present in each cell and the number of IbpA-YFP fluorescent spots in each cell. The number of dark spots that co-localised with IbpA-YFP fluorescence was also counted. This was performed in biological duplicates.

2.4.3.5 **Frequency of explosive cell lysis events**

Phase-contrast time-lapse microscopy was used to determine the frequency of explosive cell lysis events. Interstitial biofilms were setup according to section 2.4.1 and the leading edge of the biofilm was imaged on Nikon Ti inverted research microscope with a 100x 1.45 numerical aperture (NA) PlanApo objective, using NIS Elements acquisition software (Nikon Instruments, Tokyo, Japan). One frame was taken every second for 20 min before moving to a new area of the biofilm for a total of 100 min. Time-lapse images were viewed in FIJI (260) and the number of explosive cell lysis events, consisting of a rod-shaped cell transforming into a round cell and lysing, over 100 min were recorded. This was performed in biological replicates.

2.5 Sessile biofilm assays

2.5.1 Microcolony formation in submerged biofilms

1 mL of an overnight broth culture was transferred to a sterile microfuge tube and pelleted by centrifugation (13, 000 rpm for 1 min). The pelleted cells were washed three times with fresh CAMHB. The washed cells were diluted to an equivalent of 1/100 in 2 mL CAMHB in a 14 mL loosely capped polypropylene tube (BD Falcon®) and cultured at 37 °C for 2 h shaking at 250 rpm. The cultures were diluted to an equivalent of 1/100 in CAMHB and 300 µL transferred to an µ-Slide 8 well ibiTreat microscopy chamber (ibidi, GmbH, Germany) and incubated statically at 37 °C for the indicated time. DNase I (D5025, Sigma) was used at 100 Kunitz units/mL (refer to section 2.1.15 for DNase I preparation). *P. aeruginosa* PAO1 chromosomal DNA prepared using the methods in section 2.3.2 was added to a final concentration of 1 µg/mL. This was performed in biological triplicate with 10 images from each replicate.

2.5.1.1 **Microcolony formation over time**

To observe eDNA release and microcolony formation during the development of the submerged biofilms, the biofilm culture media included either eDNA stain TOTO-1 iodide (1 µM; Life Technologies, Grand Island, NY, USA) or the eDNA and dead cell stain EthHD-2 (1 µM; Biotium) and time-lapse imaging commenced immediately with phase contrast and wide-field fluorescent

microscopy (Nikon Ti inverted research microscope, × 100 objective). Fifty eDNA release sites for each strain were followed over 0-7 h. The *x* and *y* coordinates of eDNA release sites and microcolonies present (aggregates greater 100 μm^2) were recorded using FIJI (260). The area covered by eDNA was identified by auto-thresholding using FIJI (260). Microcolony size was determined by identifying "Particles" using auto-thresholding in FIJI (260). Integrated density was used to measure eDNA fluorescence over time in FIJI (260). This was performed in biological triplicate following six locations in each replicate.

2.5.1.2 **Microcolony formation at 8 h**

To visualise biofilm formation after 8 h static culture, wells were washed with fresh media. CAMHB containing eDNA and dead cell stain EthHD-2 was added to the wells and biofilms at the substratum were imaged with phase contrast and wide-field fluorescence microscopy (Olympus IX71, × 40 objective). To analyse the frequency of microcolonies in submerged biofilms, random images of the substrate surface were obtained and "Particles" (microcolonies) greater than 100 mm^2 identified by auto-thresholding using FIJI (260). This was performed in biological triplicate with 10 images from each replicate.

2.5.2 Microtitre plate static biofilm assay

Biofilm biomass was examined using a microtitre plate assay with crystal violet staining (261). Overnight cultures of *P. aeruginosa* were diluted to an equivalent of 1/100 in CAMHB and 100 µL added to the wells of a 96-well microtitre plate (Microtest-96, BD). The plates were covered with Aeraseal™ (Excel Scientific Inc.) and incubated statically at 37 °C for 24 h. Plates were then washed three times with PBS and stained with crystal violet (0.2 % w/v) for 1 h, incubated at room temperature on an orbital shaker. The crystal violet was then removed and the wells washed three times with PBS. The crystal violet stain was extracted with 30 % (v/v) acetic acid and the plates incubated at room temperature for 40 min on an orbital shaker. The optical density (OD) of the extracted stain was measured at OD_{595} for each well using a plate reader (Biotek) and the value was used as relative biofilm biomass. Each sample and

control was assayed in five individual wells within each of the biological triplicates.

2.6 Cell viability assays

2.6.1 Colony Forming Units (CFU)

Aliquots of broth cultures were serially diluted in PBS in triplicate and plated in 20 µL spots on 1.6 % high salt-LBA plates. Once dried, the plates were incubated at 37 °C for 16-24 h. The number of CFU/mL was then calculated from the technical triplicates. This was performed in biological triplicates.

2.6.2 Minimum Inhibitory Concentration (MIC)

The MIC for antibiotics was determined using the broth micro-dilution protocol from (262) for ciprofloxacin (CIP; Sigma-Aldrich), gentamicin sulphate (GM; Sigma-Aldrich) and colistin (CL; Sigma-Aldrich). Overnight broth cultures of were diluted to an equivalent of 1/100 in fresh CAMHB. Two-fold dilutions of 100 µL of antibiotics were setup in triplicate in a 96-well microtitre plate (Microtest-96, BD). Five microlitre of the diluted broth cultures was used to inoculate the wells of each antibiotic concentration. The plates were covered with Aeraseal™ (Excel Scientific Inc.) and incubated statically at 37 °C for 24 h. The lowest antibiotic concentration with no visible growth was reported as the MIC. The crystal violet staining method described in section 2.5.2 was used to determine biofilm biomass. Each sample and control was assayed in five individual wells within each of the biological triplicates.

2.6.3 Minimum Bactericidal Concentration (MBC)

The MBC for antibiotics was determined using the broth micro-dilution protocol from (262) for ciprofloxacin (Cip; Sigma-Aldrich), gentamicin sulphate (Gm; Sigma-Aldrich) and colistin (CL; Sigma-Aldrich). Following on from the methods in section 2.6.2 in determining the MIC, 100 µL aliquots were taken from wells with no visible growth. These aliquots were serially diluted in sterile PBS and plated in 20 µL spots on 1.6 % high salt-LBA plates. Once dried, the

plates were incubated at 37 °C for 24 h. The lowest antibiotic concentration with no growth was stated as the MBC. This was performed in biological triplicates.

2.6.4 Time kill curves

One millilitre of an overnight broth culture was pelleted by centrifugation (13, 000 rpm for 1 min) in a sterile microfuge tube. The pellet was washed three times with fresh CAMHB and finally resuspended in 1 mL fresh CAMHB. The washed cells were diluted to an equivalent of 1/100 in 2 mL CAMHB in a 14 mL loosely capped polypropylene tubes (BD Falcon®) and cultured at 37 °C for 2 h shaking 250 rpm. Cells were diluted to an equivalent of 1/4 in fresh CAMHB and the following antibiotics were added at bactericidal concentrations; ciprofloxacin (CIP; Sigma-Aldrich), gentamicin sulphate (GM; Sigma-Aldrich) and colistin (CL; Sigma-Aldrich). The culture was gently mixed and incubated statically at 37 °C. At each time point 100 µL aliquots were used to perform CFU counts (see section 2.6.1) in biological triplicate.

This was also performed in a µ-Slide 8 well ibiTreat microscopy chamber (ibidi, GmbH, Germany) to observe cell morphology and viability with Live/Dead staining. Syto 9 (Life Technologies) and EthHD-2 (Biotium) were added to the cultures along with the antibiotics and transferred to the chamber slide. Time-lapse imaging commenced immediately with phase contrast and wide-field fluorescent microscopy (Nikon Ti inverted research microscope, × 100 objective). The number of live and dead cells present, along with rod-shaped and round cells was identified using FIJI (260).

2.7 Phage plaque assays

Phage production was quantified by performing a phage plaque assay using the top-layer agar method (187). Interstitial biofilms were setup following the methods in section 2.4.1 and incubated overnight at 37 °C. The coverslips were removed and the biofilm washed off the coverslip with sterile PBS into a 50 mL tube (BD Falcon®). One millilitre of the resuspended biofilm was transferred to a microfuge tube and centrifuged at 16, 000 *g* for 5 min. The supernatant was filtered through a 0.2 µm pore-size syringe filter (Merck Millipore, Vic, AUS) and serially diluted in PBS. Sterile PBS was included as a 'no phage' control. Top agar lawns were prepared with PAO1 and PAO1Δ*pf4*

overnight broth cultures by mixing 500 µL of the culture in 4.5 mL molten 0.8% LBA prior to pouring on 1.5% LBA. Twenty microlitre aliquots were spotted onto the 0.8% LBA top agar and allowed to dry before incubation at 37 °C for 16-18 h. The number of plaques present as seen by a zone of clearing was counted and the number of plaque forming units per mL (PFU/mL) was calculated.

2.8 Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 (La Jolla, CA, USA). Unpaired *t*-test with Welch's correction was used for comparisons as indicated in figure legends under graphs.

Chapter Three:

Identification of Genes Involved in Explosive Cell Lysis

3 Identification of Genes Involved in Explosive Cell Lysis in Interstitial Biofilms

3.1 Publication information and other author contributions

This data in this Chapter contributed to the following publication:

L. Turnbull*, M. Toyofuku*, A. L. Hynen, M. Kurosawa, G. Pessi, N. K. Petty, S. R. Osvath, G. Cárcamo-Oyarce, E. S. Gloag, R. Shimoni, U. Omasits, S. Ito, X. Yap, L. G. Monahan, R. Cavaliere, C. H. Ahrens, I. G. Charles, N. Nomura, L. Eberl & C. B. Whitchurch, Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nature Communications* **7**, Article number: 11220 (2016); doi:10.1038/ncomms11220.

* Denotes equal contribution.

The results presented in this Chapter were all generated and analysed by Amelia L. Hynen with the following exceptions:

- Construction of the PAO1Δ*lys* and PAO1Δ*recA* strains: Masanori Toyofuku, University of Tsukuba, Japan
- Construction of the pJN105/*lys*, pJN105/*lys**, pMLAC-G, pMHOL-G plasmids: Masanori Toyofuku, University of Tsukuba, Japan
- Computer vision BacFormatics analysis for cell morphology: Raz Shimoni, University of Technology Sydney, Australia
- Bioinformatics identifying *lys* as a putative endolysin: Nicola K. Petty, University of Technology Sydney, Australia.

3.2 Introduction

Bacteria are often found growing as biofilms rather than as free-living planktonic cells. Biofilms are comprised of a sessile aggregate of cells, encased in a matrix of extracellular polymeric substances produced by the cells. This Thesis focuses on two forms of biofilms, submerged and interstitial. Submerged biofilms form on abiotic surfaces immersed in liquid culture media whilst interstitial biofilms develop at the interface between a solidified nutrient media and an abiotic surface in a thin film of fluid (30, 44). The most commonly seen components of these biofilm matrices include eDNA, lipids, proteins, exopolysaccharides and MVs (24, 54). eDNA is an essential biofilm matrix component in many bacterial species (79, 85, 120, 121, 132, 151, 165, 167, 168, 263). In *P. aeruginosa*, eDNA is essential for the development of submerged biofilm microcolonies (79) and facilitates cell-cell and cell-matrix interactions that stabilise the multicellular communities throughout submerged biofilm development (70, 84, 86, 92, 95). In interstitial biofilms, eDNA facilitates twitching-mediated motility biofilm expansion by aiding in efficient traffic flow and organisation of cells (49). It is the most abundant polymer of the biofilm matrix and its production levels vary between strains, including clinical isolates (63, 81).

The mechanisms for eDNA production in mature biofilms are well understood (refer to section 1.6.). The mechanism for eDNA release in interstitial biofilms is now beginning to be understood. Gloag *et al.* (49) observed the formation of punctate foci of eDNA in interstitial biofilms, that is suggestive of discrete, single cells releasing eDNA through lysis. At the time this Thesis was initiated, it had been determined that this punctate release of eDNA in interstitial and submerged biofilms occurs through explosive cell lysis (Turnbull and Whitchurch, personal communication). The mechanism of explosive cell lysis and the role of explosive cell lysis-mediated eDNA release during submerged biofilm formation were unknown.

The process of explosive cell lysis involves a subset of the bacterial population undergoing a transition from a rod-shaped cell to a round morphotype (refer to Figure 1.6) (247). In many cases this now transformed cell undergoes an explosive lysis event that destroys the cell and expels the internal

contents, including its genomic DNA, into the biofilm milieu. Simultaneously, as the cell explodes, MVs form from shattered membrane fragments circularising in the hydrophilic environment. The entire process from morphology transition to explosion occurs in a matter of seconds. This process has been observed during the early stages of submerged and interstitial biofilm development and accounts for all eDNA being produced in the early stages of development in interstitial biofilms (247).

Two common forms of lysis-mediated eDNA production in bacteria are through autolysin- and bacteriophage-mediated systems. Autolysis is carried out by peptidoglycan hydrolases encoded in the bacteria's genome (128, 264). Bacteriophage-mediated lysis occurs through the action of bacteriophage-related enzymes, namely an endolysin and a holin (168). For both cases, disruption of the peptidoglycan layer in the cell wall leads to structural instability, after which osmotic forces can cause abrupt disruption of the cell leading to lysis (265). Lysis usually occurs in a subset of the population and releases the internal contents of the cell including eDNA.

As explosive cell lysis is visually similar to lysis of *E. coli* cells by lytic bacteriophage (248), it was hypothesized that an endolysin and holin would provide the mechanism for explosive cell lysis in *P. aeruginosa.* Bioinformatic analysis has determined that the *P. aeruginosa* genome carries only one putative endolysin encoded by the *lys* gene (PA0629) (162).

This endolysin Lys is part of the R- and F-pyocin gene cluster. Pyocins are bacteriocins produced by *Pseudomonas* genus that are capable of killing the same or closely related species (215). They are cryptic bacteriophages, in that they produce a headless phage particle (162). For pyocins to be released from the cell, the cell must lyse, involving the action of the Lys endolysin and the Hol holin (162). The holin proteins polymerise in the inner membrane to form a pore (163, 164). These pores allow for the non-specific transport of the endolysin to the periplasm where the endolysin can degrade the peptidoglycan layer, which leads to lysis. Overexpression of both *lys* and *hol* in *E. coli* and *P. aeruginosa* results in lysis (162).

This Thesis Chapter details the investigation into the involvement of pyocins and more specifically Lys in eDNA release through explosive cell lysis.

3.3 Results

3.3.1 Explosive cell lysis in *P. aeruginosa* interstitial biofilms

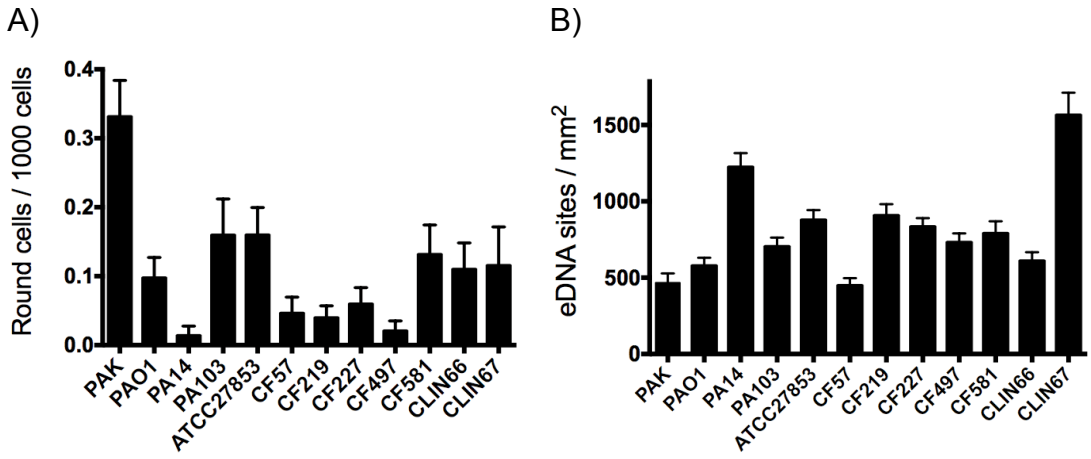
We investigated the occurrence of explosive cell lysis in various strains of *P. aeruginosa* to determine whether this is a conserved phenomenon in both clinical isolates and laboratory strains. The production of eDNA was examined in interstitial biofilms. Interstitial biofilms allow for the formation of a monolayer of cells where the cell morphology and distribution of eDNA can be clearly observed without interference from other cells (49, 247).

To identify sites of eDNA release, interstitial biofilms were cultured in the presence of cell-impermeant nucleic acid stain Ethidium Homodimer-2 (EthHD-2) (refer to section 2.4.1). This cell-impermeant stain allows for the visualisation of DNA in the extracellular milieu of the biofilms. As eDNA is released from the cell, the EthHD-2 in the media stains the eDNA and appears as punctate foci. Interstitial biofilms of a selection of laboratory strains and clinical isolates (Table 3.1) cultured in the presence of EthHD-2 were analysed for the frequency of round cells and eDNA release sites throughout the actively expanding biofilm. Both round cell morphotypes and eDNA release sites can be used as indicators of explosive cell lysis events. Computer vision (BacFormatics) was used to analyse and identify cell morphotypes in the interstitial biofilms and categorise them as round or rod shaped (refer to section 2.4.3.3) (247). The number of eDNA release sites across the biofilm were enumerated and normalised to the area covered by the biofilm (refer to sections 2.4.3.1 and 2.4.3.2).

Table 3.1: Laboratory and clinical strains examined for presence of explosive cell lysis

Strain	Source
PAO1	Laboratory strain ATCC 15692 (American Type Culture Collection)
PAK	Laboratory strain (D. Bradley, Memorial University of Newfoundlands, St John's, Canada)
PA14	Laboratory strain (252)
PA103	Laboratory strain (253)
ATCC27853	Laboratory strain (Oxoid)
CF57	Cystic fibrosis isolate
CF219	Cystic fibrosis isolate
CF227	Cystic fibrosis isolate
CF497	Cystic fibrosis isolate
CF581	Cystic fibrosis isolate
CLIN66	Endotracheal aspirate
CLIN67	Chest fluid

Round cells were present in all *P. aeruginosa* strains examined in varying frequencies from 1 per 3, 000 to 1 per 100, 000 rod-shaped cells (Figure 3.1 A). The variation in frequency of round cells present at any point in time could be explained by shorter or longer survival times of round cells as reported by Turnbull *et al.* (247). eDNA throughout the interstitial biofilms was observed as bright punctate foci stained by EthHD-2 (Figure 3.1C). This was identical to the punctate pattern observed in PAK interstitial biofilms reported by Gloag *et al*. (49). Turnbull *et al*. (247) showed that these are evidence of explosive cell lysis i.e. that all eDNA released in interstitial biofilms is released through explosive cell lysis. Each strain varied in the number of eDNA release sites across the interstitial biofilm confirming this phenomenon occurs in different *P. aeruginosa* strains (Figure 3.1B). The number of eDNA release sites was calculated per mm^2 of biofilm as computer vision analysis was unable to identify all cells across large sections of the biofilm. There appears to be no difference in the frequency of explosive cell lysis events and the strains type as the ranges of eDNA release sites were similar between laboratory and clinical strains. Comparing the Figures 3.1A and 3.1B, differences in number of round cells and number of eDNA release sites could be explained by the stability and survival time of the round cells. Turnbull *et al*. (247) demonstrated that round cells could survive for different lengths of time and therefore only a proportion of the round cells undergoing explosive cell lysis will be visible at any one point in time. Explosive cell lysis appears to be a conserved process across laboratory and clinical strains of *P. aeruginosa* allowing us to explore this phenomenon in different strains when necessary.



C)

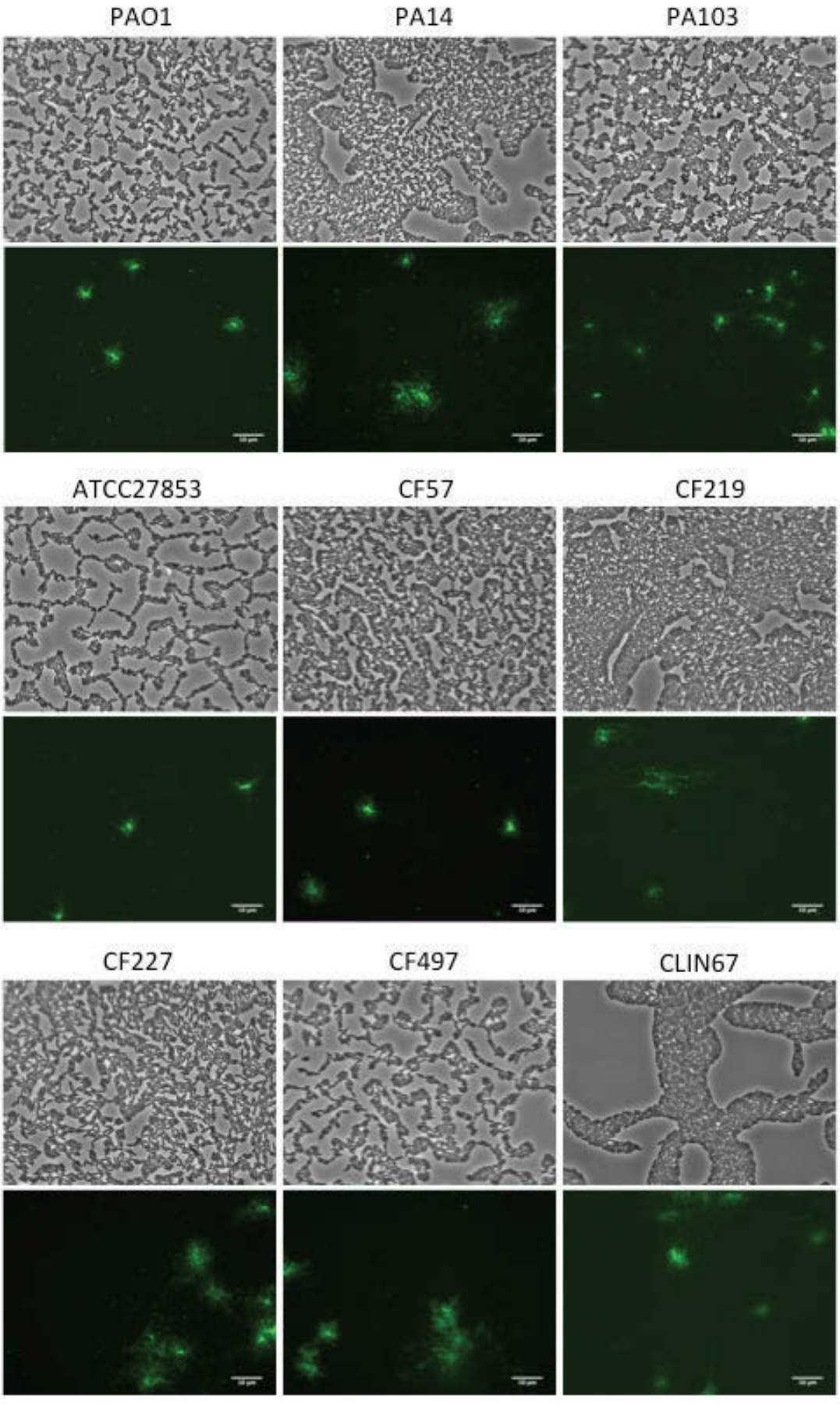


Figure 3.1: Explosive cell lysis is conserved across *P. aeruginosa* strains.

A) Proportions of cells with round cell morphotype in interstitial biofilm monolayers of *P. aeruginosa* strains. Computer vision (BacFormatics) was used to identify approximately 30,000 cells in each experiment and characterise these as having either rod or round morphotypes; *n* = 3, mean ± s.e.m. B) eDNA release sites in interstitial biofilms of *P. aeruginosa* strains. The number of eDNA release sites were normalised to area covered by the biofilm; *n* = 3, mean ± s.e.m. C) Phase-contrast (top) and EthHD-2 stained eDNA (bottom) of interstitial biofilms of laboratory strains (PAO1, PA14, PA103 and ATCC27853), four CF clinical isolates (CF57, CF219, CF227 and CF497) and a non-CF clinical isolate (CLIN67), scale bar 10 µm. All strains are capable of producing eDNA throughout actively expanding interstitial biofilms through explosive cell lysis and this occurs at varying degrees as demonstrated by different proportions of round cells and the number of eDNA release sites across the biofilm.

3.3.2 Expression of the pyocin lysis cassette

To determine if the pyocin gene cluster is involved in the explosive cell lysis process, we first examined the correlation between pyocin lysis gene cassette expression and eDNA release events using green fluorescent protein (GFP) transcriptional plasmids pMLAC-G and pMHOL-G. These plasmids contain either *lac* or *hol* promoter regions fused to *eGFP* in a pMEXGFP vector (243, 247). pMLAC-G was used as a control for constitutive expression of the GFP transcriptional fusions under the *lac* promoter. The plasmid pMHOL-G was used to identify expression of the lysis genes of the pyocin gene cluster, with GFP expression under the control of *hol*, the first lysis gene in the pyocin cluster (refer to Figure 1.4). These plasmids were transformed into PAO1 and the resulting strains cultured as interstitial biofilms in the presence of cell-impermeant nucleic acid stain EthHD-2 to stain for eDNA as an indicator of explosive cell lysis events.

The control expression vector pMLAC-G displayed homogenous GFP expression levels in all cells in PAO1 throughout the interstitial biofilms, demonstrating the constitutive expression of *lac* in all cells (Figure 3.2).

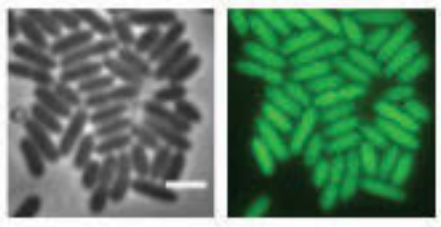


Figure 3.2: pMLAC-G is expressed in all cells of PAO1 interstitial biofilms.

Phase-contrast (left), GFP fluorescence of pMLAC-G (green, right), scale bar 2 µm.

GFP expression in cells containing the transcriptional fusion plasmid pMHOL-G indicates gene expression of the pyocin lysis genes. In PAO1 pMHOL-G, rod-shaped cells expressing high levels of GFP were observed (Figure 3.3). These high GFP expressing cells were observed to transform into a round cell and explode, releasing eDNA as seen by EthHD-2 staining (Figure 3.3). We followed 74 explosive cell lysis events and found that all of the exploding cells had high levels of GFP expression compared to neighbouring rod-shaped cells, indicating that expression of the pyocin lysis gene cassette is upregulated in these cells that undergo explosive cell lysis. Only those cells expressing high levels of GFP underwent explosive cell lysis and only those cells exploding were responsible for eDNA production. This GFP reporter can be used in further investigations as an indicator to identify cells that will undergo explosive cell lysis.

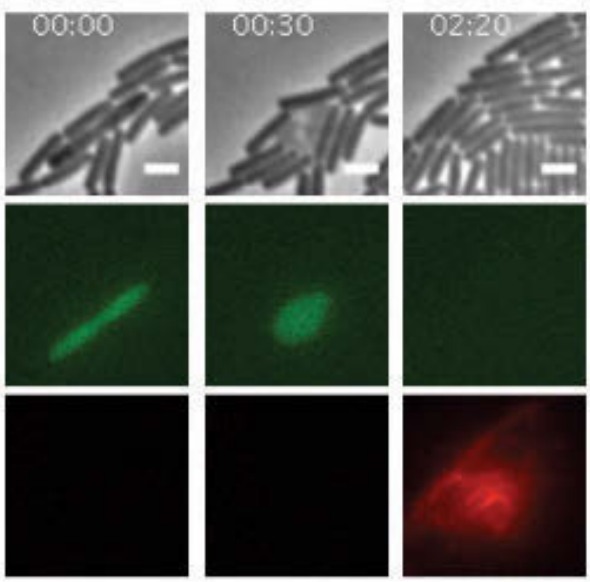


Figure 3.3: PAO1 pMHOL-G expressing high levels of GFP undergo explosive cell lysis.

PAO1 pMHOL-G interstitial biofilms imaged with phase-contrast (upper panels), GFP fluorescence (green, middle panels) and Ethidium homodimer-2 stained eDNA (red, lower panels), scale bar 2 µm. Time is indicated in upper left corner in min. Cells that express high levels of GFP undergo explosive cell lysis and produce eDNA.

3.3.3 Involvement of R- and F-pyocins in explosive cell lysis

These observations of cells containing the pMHOL-G plasmid expressing high levels of GFP prior to undergoing explosive cell lysis indicate that the pyocin lysis gene cassette was upregulated in exploding cells. The main role of the lysis genes in the pyocin cluster is to breakdown the peptidoglycan in order to lyse the cell and allow for the release of pyocins. Therefore we examined whether the pyocins themselves were the cause or instigator of explosive cell lysis. R- and F-pyocins are located adjacent to the lysis genes and are co-transcribed (223, 225). Although it is widely believed that pyocin producing strains are insensitive to their own pyocins, under certain conditions they can become susceptible (247).

To explore if the pyocins themselves are required for explosive cell lysis, we examined eDNA release in interstitial biofilms of structural mutants of R- and F-pyocins. Twenty-two strains from the PA14 MAR2xT7 transposon insertion mutant library (254) (see Table 3.2) were screened for defects in eDNA production in interstitial biofilms. Interstitial biofilms of these strains were cultured in the presence of cell-impermeant nucleic acid specific stain TOTO-1 to identify explosive cell lysis events. This cell-impermeant stain allows for the visualisation of DNA in the extracellular milieu of the biofilms. The number of eDNA sites were enumerated and normalised to the area covered by the actively expanding biofilm. The strains were categorised based on the number of eDNA release sites as ‘wild-type’, ‘increased’ or ‘decreased’ in comparison to PA14 parent strain (see Figure 4.1C for PA14 eDNA release in interstitial biofilm). If a strain was statistically significantly different from the PA14 wild-type using a student *t*-test, it was categorised as ‘increased’ or ‘decreased’ accordingly.

All the R- and F-pyocin structural mutants examined were capable of producing wild-type levels of eDNA (Table 3.2). This indicates that single structural components of the R- and F-pyocins are not essential for explosive cell lysis. Although these pyocins may be released simultaneously as the cell lyses, these results indicate that they are not required for explosive cell lysis.

Table 3.2: Mutants of structural components of R- and F-pyocins screened for eDNA release.

Strain	Gene Description	PAO1 Ortholog	eDNA release
PA14_07980::MAR2xT7	Conserved hypothetical protein	PA0613	Wild-type
PA14_08000::MAR2xT7	Conserved hypothetical protein	PA0615	Wild-type
PA14_08010::MAR2xT7	Putative baseplate assembly protein V	PA0616	Wild-type
PA14_08030::MAR2xT7	Putative phage baseplate assembly protein	PA0618	Wild-type
PA14_08040::MAR2xT7	Putative phage tail protein	PA0619	Wild-type
PA14_08050::MAR2xT7	Putative tail fibre protein	PA0620	Wild-type
PA14_08060::MAR2xT7	Putative tail fibre assembly protein	PA0621	Wild-type
PA14_08070::MAR2xT7	Putative phage tail sheath protein	PA0622	Wild-type
PA14_08090::MAR2xT7	Putative phage tail tube protein	PA0623	Wild-type
PA14_08100::MAR2xT7	Conserved hypothetical protein	PA0624	Wild-type
PA14_08120::MAR2xT7	Putative tail length determinator protein	PA0625	Wild-type
PA14_08130::MAR2xT7	Putative tail formation protein	PA0626	Wild-type
PA14_08180::MAR2xT7	Hypothetical protein	PA0630	Wild-type
PA14_08210::MAR2xT7	Putative major tail protein V	PA0633	Wild-type
PA14_08220::MAR2xT7	Hypothetical protein	PA0634	Wild-type
PA14_08230::MAR2xT7	Hypothetical protein	PA0635	Wild-type
PA14_08240::MAR2xT7	Putative tail length determination protein	PA0636	Wild-type
PA14_08260::MAR2xT7	Putative minor tail protein L	PA0638	Wild-type
PA14_08270::MAR2xT7	Conserved hypothetical protein	PA0639	Wild-type
PA14_08280::MAR2xT7	Putative phage tail assembly protein	PA0640	Wild-type
PA14_08300::MAR2xT7	Putative phage-related protein, tail component	PA0641	Wild-type
PA14_08320::MAR2xT7	Conserved hypothetical protein	PA0647	Wild-type

* No defects were identified in the mutants tested indicating that the production of functional pyocins is not required for eDNA release through explosive cell lysis.

These results together with the observations that show expression of the pyocin lysis genes prior to explosive cell lysis suggest that the lysis genes alone may be responsible for explosive cell lysis.

3.3.4 The endolysin Lys mediates explosive cell lysis

As the pyocin lysis genes are upregulated prior to explosive cell lysis events, we investigated the role of the putative endolysin Lys as the enzyme responsible for this process. The other genes in the lysis gene cassette are the holin Hol and hypothetical proteins PA0630 and PA0631 (refer to Figure 1.4).

As Hol is a transporter, and PA0630 and PA0631 have undefined functions, we initially concentrated on Lys to determine its role in explosive cell lysis.

A deletion mutant of *lys* in PAO1 (247) was examined for its ability to undergo explosive cell lysis. Interstitial biofilms of PAO1 and PAO1Δ*lys* were cultured in the presence of cell impermeant nucleic acid specific stain TOTO-1 to identify eDNA release through explosive cell lysis (refer to section 2.4.1). The number of eDNA release sites were enumerated and normalised to the area covered by the actively migrating biofilm (refer to sections 2.4.3.1 and 2.4.3.2).

In PAO1, eDNA can be seen in punctate patterns throughout the actively expanding biofilm (Figure 3.4 A). The *lys* deletion mutant showed a complete defect in eDNA production across the actively expanding biofilm (Figure 3.4). To confirm the requirement of *lys* for eDNA production in interstitial biofilms, the deletion was complemented with the wild-type gene *in trans* (pJN105*lys*). eDNA production in PAO1Δ*lys* pJN105*lys* was restored to wild-type levels (Figure 3.4). We then examined whether merely the presence of the enzyme or the activity of the enzyme is essential for eDNA production by complementing PAO1Δ*lys* with pJN105*lys**, a plasmid containing *lys* with an E51V substitution in the putative active site of the enzyme. Complementation with the catalytic mutant did not restore eDNA production in PAO1Δ*lys*, indicating the enzymatic action of *lys* is essential for eDNA production in actively expanding biofilms (Figure 3.4).

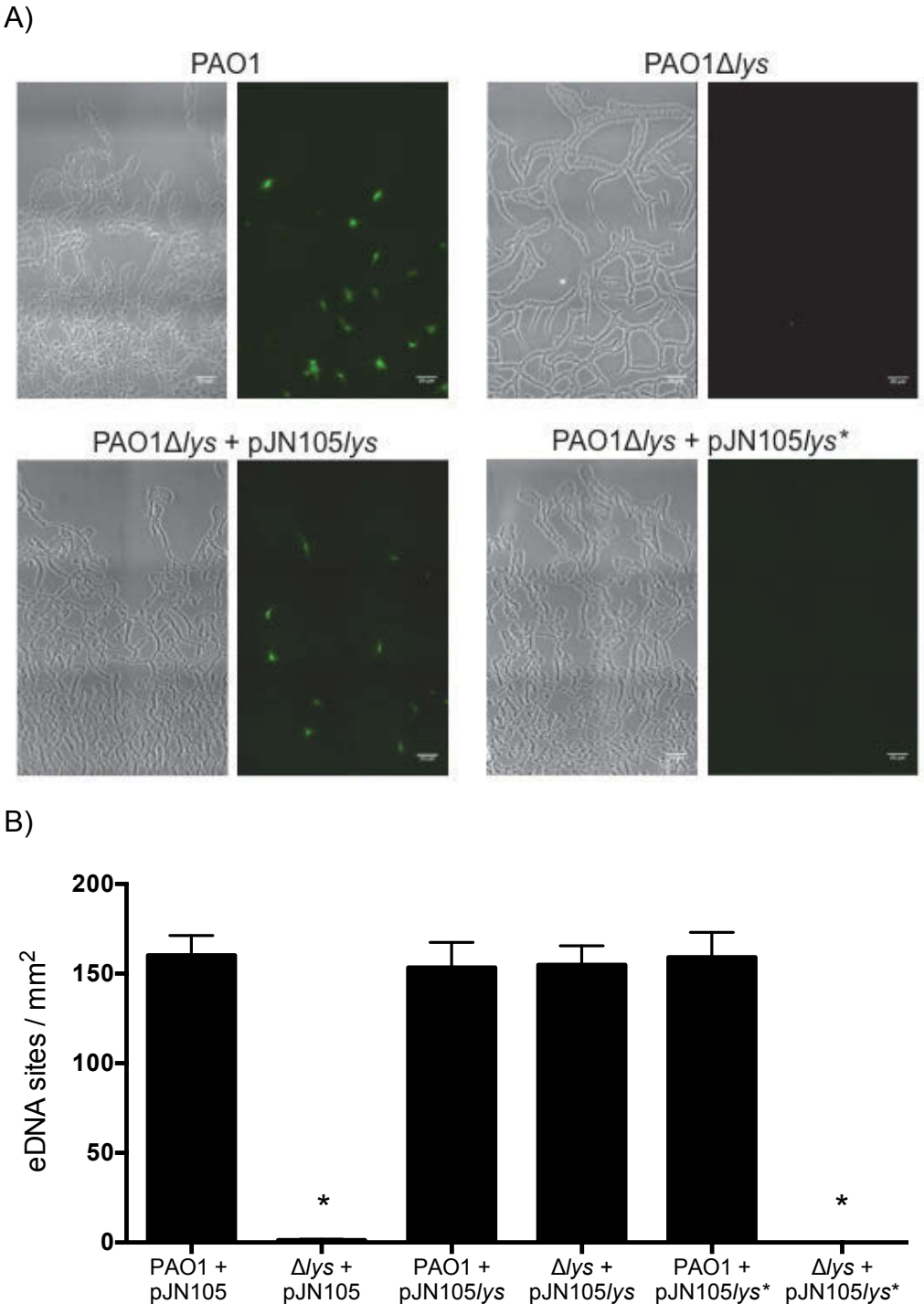


Figure 3.4: The pyocin endolysin Lys is required for eDNA production through explosive cell lysis

A) Phase-contrast (left) and TOTO-1 stained eDNA (green, right) of interstitial biofilms of PAO1 and PAO1Δ*lys* containing pJN105 (vector control), pJN105*lys* (complementation plasmid) or pJN105*lys** (catalytic mutant), scale bar 20 µm. Lys is required for eDNA production in interstitial biofilms and complementation *in trans* with *lys* restores eDNA production. Complementation with *lys**, carrying a point mutation in the putative active site of the enzyme, did not restore eDNA release in PAO1Δ*lys*. B) eDNA release sites in interstitial biofilms. The

number of eDNA release sites were normalised to the area covered by the biofilm; *n* = 30, mean ± s.e.m. * *P* < 0.0001, unpaired *t*-test with Welch's correction compared to PAO1 pJN105.

As demonstrated in this Chapter, the pyocin lysis genes are upregulated prior to explosive cell lysis events (see section 3.3.2). pMHOL-G was transformed into PAO1Δ*lys* to determine if the remaining pyocin lysis genes were being expressed, without lysing the cell. Interstitial biofilms of PAO1Δ*lys* containing pMLAC-G or pMHOL-G were cultured in the presence of cell-impermeant nucleic acid stain EthHD-2 to identify eDNA release (see section 2.4.1.

The control expression vector pMLAC-G displayed homogenous GFP expression levels in all cells in PAO1Δ*lys* throughout the interstitial biofilms, demonstrating the constitutive expression of *lac* in all cells (Figure 3.5).

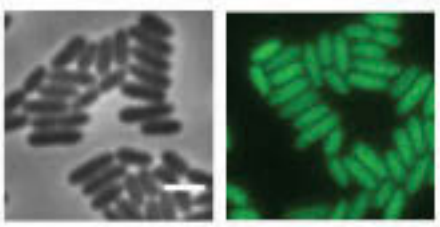


Figure 3.5: pMLAC-G is expressed in all cells of *P. aeruginosa* PAO1Δ*lys* interstitial biofilms
Phase-contrast (left), GFP fluorescence of pMLAC-G (green, right), scale bar 2 μm.

Although PAO1Δ*lys* pMHOL-G did not produce any eDNA, a sub-population of rod cells expressing high levels of GFP was observed (Figure 3.6). These GFP expressing cells failed to undergo explosive cell lysis when tracked for up to 15 min. This demonstrates that although the remaining pyocin lysis genes are expressed, the endolysin is solely responsible for the morphological change and lysis.

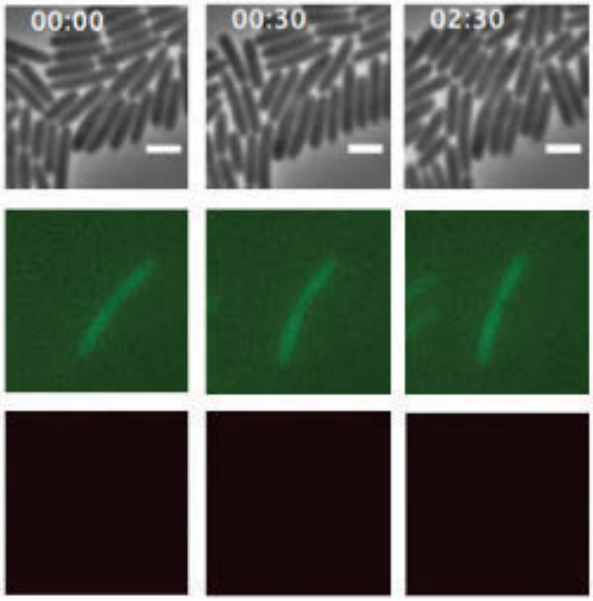


Figure 3.6: PAO1Δ*lys* pMHOL-G expressing high levels of GFP fail to undergo explosive cell lysis.

Phase-contrast (upper panels), GFP fluorescence (green, middle panels) and Ethidium homodimer-2 stained eDNA (red, lower panels), scale bar 2 μm. Time is indicated in upper left corner in min. Cells expressing high levels of GFP do not lyse and no eDNA is produced in these biofilms.

3.3.5 Regulation of explosive cell lysis

Genes in the pyocin cluster are regulated by RecA, PrtR and PrtN (223). Under normal conditions, PrtR represses activation of the activator PrtN, preventing transcription of the pyocin gene cluster. Under periods of stress initiated by a mutagenic agent, RecA is upregulated. RecA aids in the auto-cleavage of PrtR, which then allows for the transcription of *prtN*. Once synthesised, PrtN then activates the transcription of the remaining pyocin genes. However, under normal conditions, the expression of *recA* is leaky and allows for the production of pyocins at minute levels (222, 223).

As RecA is a regulator of the pyocin gene cluster, we examined if RecA is also a regulator of Lys-mediated explosive cell lysis. Interstitial biofilms of PAO1Δ*recA* were cultured in the presence of cell impermeant nucleic acid specific stain TOTO-1 to identify explosive cell lysis-mediated eDNA release (refer to section 2.4.1). The number of eDNA release sites were enumerated

and normalised to the area covered by the actively expanding biofilm (refer to sections 2.4.3.1 and 2.4.3.2).

PAO1Δ*recA* showed a defect in eDNA production in interstitial biofilms (Figure 3.7). This suggests RecA is a regulator of explosive cell lysis and mediates the expression of the endolysin responsible for this process.

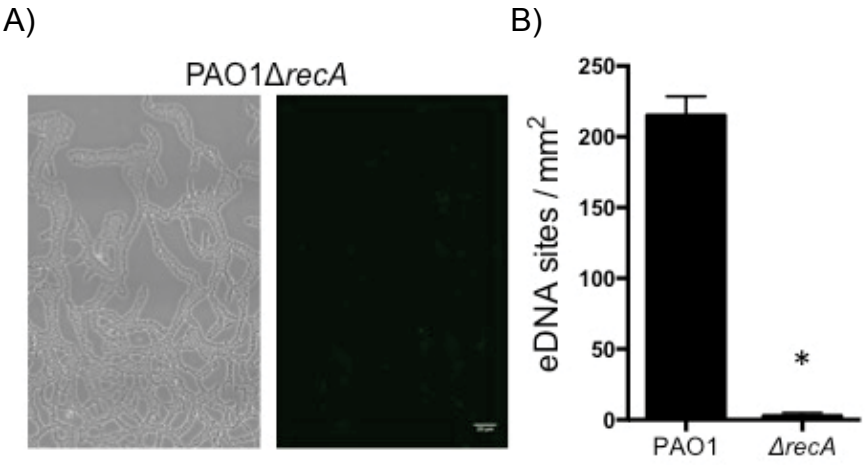


Figure 3.7: Explosive cell lysis is regulated by *recA*.

A) Phase-contrast (left) and TOTO-1 stained eDNA (green, right) of PAO1Δ*recA* interstitial biofilm, scale bar 20 μm. B) eDNA release sites normalised to area covered by the biofilm; *n* = 30, mean ± s.e.m. **P* < 0.0001, unpaired *t*-test with Welch's correction. The deletion of *recA* abrogated eDNA release.

To confirm RecA is the regulator for all the pyocin lysis genes, pMHOL-G was shuttled into PAO1Δ*recA* and interstitial biofilms of PAO1Δ*recA* containing pMLAC-G or pMHOL-G were cultured in the presence of cell-impermeant nucleic acid stain EthHD-2 to identify eDNA release.

The control expression vector pMLAC-G showed homogenous GFP expression throughout interstitial biofilms of PAO1Δ*recA* (Figure 3.8). With the pMHOL-G transcriptional fusion, PAO1Δ*recA* did not express any detectable GFP and did not produce any eDNA (Figure 3.9). This indicates that eDNA production and pyocin lysis gene expression is tightly regulated by *recA*. Although RecA is the activator for the transcription of the pyocin gene cluster, it is also an essential SOS response regulator (221). A RecA-mediated SOS response to an internal stressor (224) may be responsible for inducing explosive cell lysis.

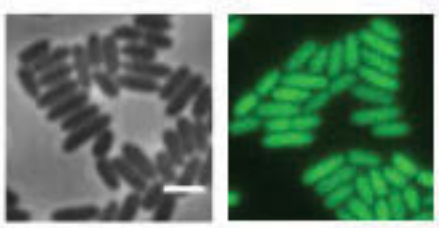


Figure 3.8: pMLAC-G is expressed in all cells of PAO1Δ*recA* interstitial biofilms.

Phase-contrast (left), GFP fluorescence of pMLAC-G (green, right), scale bar 2 μm.

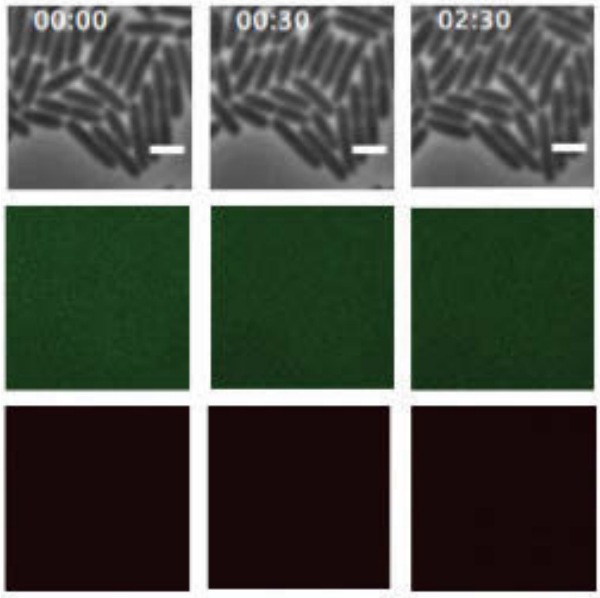


Figure 3.9: PAO1Δ*recA* pMHOL-G does not express GFP.

Phase-contrast (upper panels), GFP fluorescence (green, middle panels) and Ethidium homodimer-2 stained eDNA (red, lower panels), scale bar 2 μm. Time is indicated in upper left corner in min. PAO1Δ*recA* does not express any GFP, undergo explosive cell lysis nor produce eDNA.

3.4 Discussion

Explosive cell lysis is a novel mechanism for the production of eDNA in biofilms of *P. aeruginosa*. In this Chapter we have demonstrated that this process is mediated by the pyocin endolysin Lys and is under the control of RecA, accounting for all the eDNA produced in the early stages of interstitial biofilm development.

3.4.1 Conservation of lys

All clinical and laboratory *P. aeruginosa* strains examined were capable of undergoing explosive cell lysis, suggesting that this is a conserved phenomenon. *Lys* is highly conserved in all complete genomes available in the

public genome databases (EMBL/GenBank/DDBJ) (247) even though pyocins are not produced in all *P. aeruginosa* strains (266). For *P. aeruginosa* to maintain *lys* on the genome in the absence of pyocins, *lys* must provide a survival advantage.

All *P. aeruginosa* strains examined produced eDNA including both PAO1 and PA14 used in the study. However, there were differences in numbers of round cells and eDNA release sites which suggests that there may be differences in how explosive cell lysis events are regulated in different strains.

Explosive cell lysis could be considered a form of programmed cell death. Programmed cell death is a regulated process that mediates a cell death pathway, which confers an advantage to the organism and this typically occurs in multicellular organisms (249, 264, 267). In the case of explosive cell lysis, the organism can be considered to be the multicellular biofilm and the advantage it confers is the production of public goods including eDNA. This process may confer a fitness advantage where the ability to undergo explosive cell lysis aids in the survival of the community, possibly through biofilm formation. To explore the potential advantage of eDNA release through explosive cell lysis, the role of this in biofilm formation will be examined in Chapter 6 of this Thesis.

It is yet to be determined whether explosive cell lysis is a programmed cell death pathway initiated by fratricide or altruistic suicide (268). In the case of fratricidal killing, the biofilm community would differentiate into separate populations where one produces killing factors that target the remaining population to initiate lysis and are themselves protected from the killing factors by immunity proteins (126). The pyocins are a possible candidate for a fratricidal process but we have shown that pyocin structural mutants are not defective in explosive cell lysis. For altruistic suicide, a cell would die for the benefit of the community on its own accord in order to release public goods (269). This is the most likely case for explosive cell lysis. Neighbouring cells are unaffected by explosive cell lysis and the morphological changes and GFP expression of the pyocin lysis gene cassette seen in this Chapter are cell-specific. Explosive cell lysis may be induced by internal stressors (224) or may be a death strategy for old cells, at a point where it is no longer possible for that cell to live (270).

Although these programmed cell death pathways are commonly seen in eukaryotic development, it is becoming widely recognised that prokaryotic organisms use similar pathways as well (127, 145, 249, 267, 268). It seems illogical that a single-celled organism would undergo cell death or even maintain genes that confer this pathway. However as most bacteria grow in a community like a biofilm, this programmed cell death confers a greater advantage beyond that single cell.

Programmed cell death pathways like explosive cell lysis could also facilitate release of public goods like MVs (247), virulence factors (271) or other essential biofilm matrix components necessary for colonisation of hosts or abiotic surfaces (227). Explosive cell lysis may also be a survival strategy for the remaining population; lysis of a subset of the population could prevent dissemination of phage or promote biofilm formation, enhancing survival of the population as a whole (272).

3.4.2 Regulation of explosive cell lysis

The SOS response regulator RecA regulates the production of pyocins and the associated lysis genes (162, 221, 273). As explosive cell lysis is cell-specific and occurs in a small proportion of the population, the SOS response inducing Lys production may arise from an internal stressor or source. It is therefore possible that explosive cell lysis may be upregulated under stressful conditions (162, 193-195, 224, 225). Addition of exogenous stressors may induce explosive cell lysis and increase the proportion of cells undergoing Lys-mediated lysis. This will be explored in Chapter 4 of this Thesis.

Explosive cell lysis, like other forms of eDNA release, does not necessarily rely upon phage or phage-like particles to lyse the cell (120, 121, 132). This Chapter demonstrates that structural elements of the pyocins are not necessary for explosive cell lysis. Interestingly, Toyofuku *et al*. (243) did not observe the presence of pyocin particles in transmission electron microscopy analysis of membrane vesicles produced through the action of Lys, even though R-type pyocin proteins are often identified in membrane vesicle fractions (102, 240, 274). The production of R- and F-pyocins appears more frequently in clinical situations than environmental (275). As we cannot rule out that the release of pyocins coincides with explosive cell lysis, it's possible that the higher

frequency of eDNA release sites per mm^2 of biofilm in clinical strains is correlated with pyocin release. Future work should explore the correlation between pyocin release and explosive cell lysis to determine whether pyocins and eDNA are released through this same cell death pathway and if the lysis gene cassette is independently regulated from the pyocin structural genes.

3.4.3 The process of explosive cell lysis

The use of pMHOL-G transcriptional fusion allowed us to track cells over time that could eventually undergo explosive cell lysis. GFP expression in the presence of this transcriptional fusion was visible in rod-shaped cells for a period of time before any cell shape change was observed. This suggests that enzymatic action of Lys may have to reach a threshold before the structural integrity of peptidoglycan is disrupted. Following this, the cell can no longer maintain its shape and collapses into a round cell. The turgor pressure of the cell or differences in osmotic pressure may be responsible for the eventual explosion and release of eDNA (265).

Most endolysins require a holin to transport it to the periplasm where it degrades the peptidoglycan (159-162). Hol is likely to be the cognate holin for Lys as it is encoded in the R- and F-pyocin gene cluster along with *lys*. In addition to Hol, two other putative holins AlpB (227) and CidAB (67) have been identified in *P. aeruginosa*. As these holins have been implicated in cell death pathways in *P. aeruginosa* biofilms, they may also contribute to the transportation of Lys. Chapter 5 of this Thesis investigates the involvement of these holins in explosive cell lysis.

eDNA is essential for the formation of *P. aeruginosa* submerged biofilms, where removal of eDNA by the DNA-degrading enzyme DNase I prevents biofilms from forming (79) (refer to section 1.3.4). At the initiation of this Thesis, Turnbull and Whitchurch (personal communication) had observed the punctate release of eDNA through explosive cell lysis events at the early stages of submerged biofilm development. The role of explosive cell lysis-mediated eDNA release during submerged biofilm formation will be investigated in Chapter 6 of this Thesis.

Chapter Four:
Induction of Explosive Cell Lysis:
Intrinsic and Exogenous Stress

4 Induction of Explosive Cell Lysis: Intrinsic and Exogenous Stress

4.1 Publication information and other author contributions

The data in section 4.3.2.1 and the data on ciprofloxacin contributed to the following publication:

L. Turnbull*, M. Toyofuku*, A. L. Hynen, M. Kurosawa, G. Pessi, N. K. Petty, S. R. Osvath, G. Cárcamo-Oyarce, E. S. Gloag, R. Shimoni, U. Omasits, S. Ito, X. Yap, L. G. Monahan, R. Cavaliere, C. H. Ahrens, I. G. Charles, N. Nomura, L. Eberl & C. B. Whitchurch, Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nature Communications* **7**, Article number: 11220 (2016); doi:10.1038/ncomms11220.

* Denotes equal contribution.

The data presented in this Chapter was generated and analysed by Amelia L. Hynen with the following exceptions:

- Construction of the PAO1Δ*lys* strain: Masanori Toyofuku, University of Tsukuba, Japan.
- Construction of the pJN105*lys*, pMLAC-G, pMHOL-G plasmids: Masanori Toyofuku, University of Tsukuba, Japan.
- IbpA-YFP fusion protein (255) was obtained from Ariel Lindner, Institut National de la Santé et de la Recherche Médicale, France.
- PAO1Δ*pf4* (187) was obtained from Scott Rice, Nanyang Technological University, Singapore.

4.2 Introduction

Explosive cell lysis is a novel phenomenon for the release of eDNA in early stage biofilm development of *P. aeruginosa* (247). It involves single cells in a population transforming from a rod-shaped cell to a round morphotype and lysing in an explosive manner in a matter of seconds. This process is responsible for eDNA release in the early stages of interstitial and submerged biofilm development. Chapter 3 of this Thesis demonstrated that explosive cell lysis is mediated by the endolysin Lys, which is encoded within the cryptic bacteriophage R- and F-pyocin gene cluster. Lys is responsible for lysing the cell by hydrolysing the peptidoglycan layer in the cell wall, leading to a morphological change and lysis (247).

Bacteriophage-mediated lysis can be initiated from spontaneous prophage induction (156), which can be stimulated from intrinsic or extrinsic factors. Intrinsic factors include the activation of SOS response (158) and stochastic gene expression (157). Extrinsic induction occurs upon exposure to DNA damaging agents (203). The most common agents that induce prophage through DNA damage include ROS, UV radiation and the effects of antibiotics such as mitomycin C (MMC) and fluoroquinolones (204-208). These agents also activate the SOS response (217-221).

Expression of the pyocin gene cluster including *lys* is regulated by the SOS response (217-221). Expression of the cluster is dependent on the main SOS response regulator RecA and can be induced by DNA damage caused by UV irradiation, genotoxic or oxidative stress including ROS from neutrophils (217-221). Under non-inducing conditions, pyocins are produced by a small proportion of cells in the population (156). In Chapter 3 of this Thesis, we demonstrated that SOS is involved in explosive cell lysis.

As we see only single cells undergoing explosive cell lysis under normal conditions, an internal stressor may be responsible for stimulating the SOS response, leading to expression of *lys* and explosive cell lysis. We have observed the formation of dark spots in cells that undergo explosive cell lysis. These dark spots may be inclusion bodies. Inclusion bodies can form from natural transcription and translation errors and comprise of damaged, partially- or mis-folded proteins (276). Formation of inclusion bodies increases under

stressful conditions (255) and can also be sites of viral replication and early viral assembly (277). In this Chapter, we investigated whether these dark spots are putative inclusion bodies that could form an internal stressor.

In addition to the pyocin gene cluster, the filamentous phage Pf4 has been reported to be associated with cell death and eDNA release in mature PAO1 and PA14 *P. aeruginosa* biofilms (176, 186, 187, 278). This phage is extruded through the cell wall without lysing the cell and has no known associated endolysin. Pf4 phage has an unusual property that unlike other phage it can infect cells of the clonal population from which it was produced (176). Pf4 phage is released from the cell by extrusion however infection by Pf4 phage has been associated with lysis (176). Infection and lysis of bacterial cells by lytic bacteriophage is visually similar to explosive cell lysis (228, 248). It is possible that explosive cell lysis under inducing or non-inducing conditions could be in part due to Pf4 phage infection and lysis. Explosive cell lysis could also provide a mechanism for the release of Pf4 phage in addition to extrusion through the cell wall. Interestingly, a screen of Pf-like sequences (including PfU, Pf1, Pf4 and Pf5) in 241 laboratory and clinical strains of *P. aeruginosa* showed that Pf4 phage elements are only present in 22% of the strains examined and 19% of strains had no Pf-like sequences (279). In Chapter 3 of this Thesis we demonstrated that explosive cell lysis appears to be a conserved process in various laboratory and clinical strains of *P. aeruginosa*. Therefore Pf4 phage is unlikely to contribute to explosive cell lysis.

Pyocins and their associated genes are known to be upregulated during exposure to the fluoroquinolone antibiotic ciprofloxacin (193, 194). Fluoroquinolones act on bacteria by preventing DNA from unwinding and duplicating (226). A study by Cirz *et al.* (194) examined the global transcriptional response to clinically relevant doses of the fluoroquinolone ciprofloxacin and observed the strong upregulation of pyocin gene cluster with the endolysin *lys* upregulated 100-fold upon exposure to ciprofloxacin (194). Brazas and Hancock (193) hypothesised that induction of the pyocin lysis cassette sensitises *P. aeruginosa* to genotoxic stress. They proposed that induction of the lysis gene cassette is responsible for the lethality associated with ciprofloxacin exposure (193). It is therefore possible that explosive cell lysis is the mechanism of cell death in response to ciprofloxacin treatment.

As we demonstrated in Chapter 3 of this Thesis explosive cell lysis is mediated by an endolysin that is part of a cryptic bacteriophage cluster (R- and F-pyocins) and is regulated by the SOS response, the addition of exogenous stressors may induce explosive cell lysis and increase eDNA release in biofilms. In this Chapter we explored the induction of explosive cell lysis by exposing biofilms to exogenous stressors, including different classes of antibiotics, to determine what agents induce explosive cell lysis and whether it is through activation of the SOS response.

The aim of this Chapter is to explore sources of intrinsic and exogenous stress for their involvement in inducing explosive cell lysis.

4.3 Results

4.3.1 Internal stressors

As explosive cell lysis also occurs under non-inducing conditions, the explosive cell lysis pathway may be activated in single cells by an internal stressor. Time-lapse microscopy of cells undergoing explosive cell lysis shows that some cells have dark spots or dark polar-regions and under MMC stress, these dark spots become more prominent (Figure 4.1). These interstitial biofilms were cultured in the presence of filter discs saturated with RO water or MMC (500 µg/mL) and imaged after 4-6 h incubation (refer to section 2.4.2). We hypothesised that these dark spots may be inclusion bodies and may correlate with explosive cell lysis. Similar dark spots and dark polar-regions have been documented in aging cells of *E. coli* (255). These spots in *E. coli* are thought to be inclusion bodies, aggregates of mis-folded or partially folded proteins in the cell, indicating an old or stressed cell (255, 280). Inclusion bodies similar to these are also present in and the cause of age-related diseases in humans including Alzheimer's disease, Parkinson's disease and Huntington's disease (281-283). In the case of humans, aggregation of mis-folded proteins can reach toxic levels in the cell, inducing mutations and may lead to the incidence of neurodegenerative disease (283). These inclusion bodies in bacteria and humans may form an internal stress, activating the SOS response in single cells. In the case of *P. aeruginosa*, this may lead to explosive cell lysis. This could account for the single cell-specific nature of explosive cell lysis under

non-inducing conditions. Inclusion bodies can be formed from aggregation of mis-folded proteins due to natural transcription and translation errors (276) and can also form from viral replication and appear to be the site of early viral assembly (277). The formation of inclusion bodies is also induced under stressful conditions (276). We therefore investigated whether these dark spots were inclusion bodies and if they formed from the aggregation of phage particles or the aggregation of mis-folded proteins.

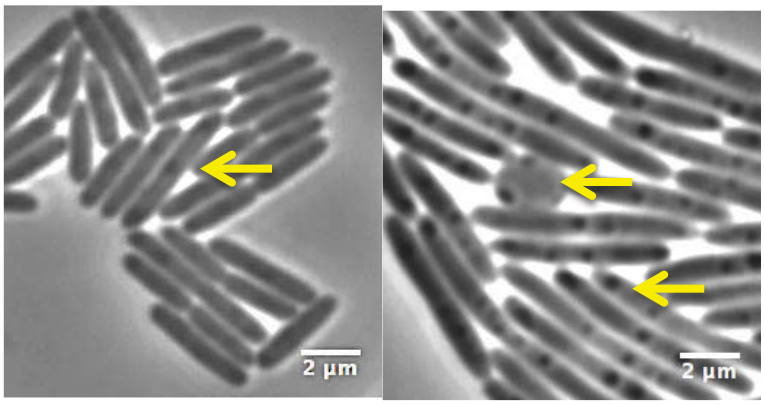


Figure 4.1: Dark spots in cells exposed to water and MMC.

Dark spots and polar regions can be seen in PAO1 cells under non-inducing (water, left) and inducing (MMC, right) conditions. Yellow arrows indicate dark spots and polar regions visible in rod-shaped and round cells, scale 2 µm. Dark spot formation is induced by MMC.

4.3.1.1 **Involvement of pyocin particles in formation of dark spots**

The pyocin lysis gene cluster is upregulated prior to explosive cell lysis and the cell producing the pyocins must lyse for the particles to be released. We explored whether the dark spots observed are formed by pyocins, forming aggregates in the cell as part of the assembly process and for their release from the cell. Partially folded elements of the Enterobacteria phage P22 are responsible for the aggregation of tailspike proteins and the formation of inclusion bodies (284, 285). We therefore examined the correlation between the presence of the pyocin structural components and the formation of these dark spots in eleven structural mutants of R- and F-pyocins from the PA14 MAR2xT7 transposon insertion mutant library (Table 4.1) (254). Interstitial biofilms of these pyocin structural mutants were cultured in the presence of filter discs saturated with water or MMC (500 µg/mL) and imaged after 4-6 h incubation (refer to section 2.4.2).

The presence or absence of dark spots was recorded for each strain (Table 4.1). In all mutants examined, there was no visible defect in the formation of these dark spots, suggesting that the formation of these dark spots is not dependent on synthesis of pyocin structural elements in inducing and non-inducing conditions (Table 4.1).

Table 4.1: Mutants of structural components of R- and F-pyocins screened for internal dark spots.

Strain	Gene Description	PAO1 Ortholog	Visible dark spots (water/MMC)
PA14_08040::MAR2xT7	Putative phage tail protein	PA0619	+/+
PA14_08050::MAR2xT7	Putative tail fibre protein	PA0620	+/+
PA14_08300::MAR2xT7	Putative phage-related protein, tail component	PA0641	+/+
PA14_08090::MAR2xT7	Putative phage tail tube protein	PA0623	+/+
PA14_08210::MAR2xT7	Putative major tail protein V	PA0633	+/+
PA14_08260::MAR2xT7	Putative minor tail protein L	PA0638	+/+
PA14_08010::MAR2xT7	Putative baseplate assembly protein V	PA0616	+/+
PA14_08000::MAR2xT7	Conserved hypothetical protein	PA0615	+/+
PA14_08030::MAR2xT7	Putative phage baseplate assembly protein	PA0618	+/+
PA14_08060::MAR2xT7	Putative tail fibre assembly protein	PA0621	+/+
PA14_08070::MAR2xT7	Putative phage tail sheath protein	PA0622	+/+

* + = dark spots visible, - = dark spots absent. All strains were capable of forming dark spots when exposed to water or 500 µg/mL MMC.

4.3.1.2 **Involvement of Pf4 phage in explosive cell lysis and dark spot formation**

P. aeruginosa Pf4 phage has been proposed to be involved in eDNA release in mature biofilms (186) although the mechanism by which this occurs is unclear. Prophages can be spontaneously induced in single cells within a population even in the absence of an external trigger (156). The addition of exogenous stressors also induces the production of Pf4, leading to cell lysis (278). As the formation of inclusion bodies is associated with the replication and assembly of viral particles (277, 284, 285), Pf4 phage particles could be involved in their formation. Pf4 phage particles may form aggregates prior to their release from the cell, resulting in the formation of these dark spots. We therefore investigated if, under the conditions tested, production of Pf4 phage

causes an internal stressor in the form of dark spots and is associated with explosive cell lysis.

Interstitial biofilms of PAO1 and the Pf4 phage cassette deletion mutant PAO1Δ*pf4* (187) were cultured in the presence of filter discs saturated with water or 500 µg/mL MMC and biofilms were imaged after 4-6 h (refer to section 2.4.2). The number of dark spots was counted for 200 cells from random fields of view (refer to section 2.4.3.4).

There was no significant difference in the formation of dark spots in the presence and absence of the Pf4 phage cassette under non-inducing and inducing conditions (Figure 4.2). This suggests that production of Pf4 phage in interstitial biofilms is not responsible for the formation of dark spots.

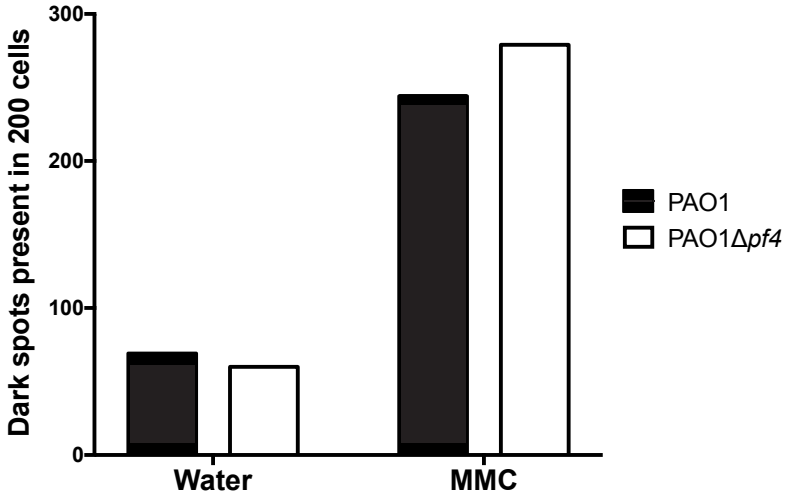


Figure 4.2: Pf4 phage production does not contribute to the formation of dark spots in PAO1.

Total number of dark spots observed in 200 cells of PAO1 (black) and PAO1Δ*pf4* (white) exposed to water and 500 µg/mL MMC.

Although Pf4 phage appears to not be involved in the formation of dark spots, it may still contribute to explosive cell lysis. The Pf4 phage has no associated endolysin and is extruded through the cell wall (176). Once released from the cell it is able to infect neighbouring cells and result in lysis (176). Infection and lysis of bacterial cells by lytic bacteriophage is visually similar to explosive cell lysis (228, 248). It is possible that explosive cell lysis under inducing or non-inducing conditions could be in part due to Pf4 phage infection and lysis. Explosive cell lysis could also provide a mechanism for the release of Pf4 phage in addition to extrusion through the cell wall.

To examine if Pf4 phage contributes to explosive cell lysis-mediated eDNA release in interstitial biofilms, interstitial biofilms of PAO1 and PAO1Δ*pf4* were cultured for 4-6 h in the presence of cell-impermeant nucleic acid specific stain EthHD-2 to identify eDNA release (refer to section 2.4.2). The number of eDNA release sites per mm^2 of biofilm was calculated (refer to section 2.4.3.1).

There was no significant difference in the number of eDNA release sites across the biofilms of PAO1 and PAO1Δ*pf4* (Figure 4.3A and B). This indicates the production of Pf4 phage does not contribute to explosive cell lysis-mediated eDNA release in interstitial biofilms.

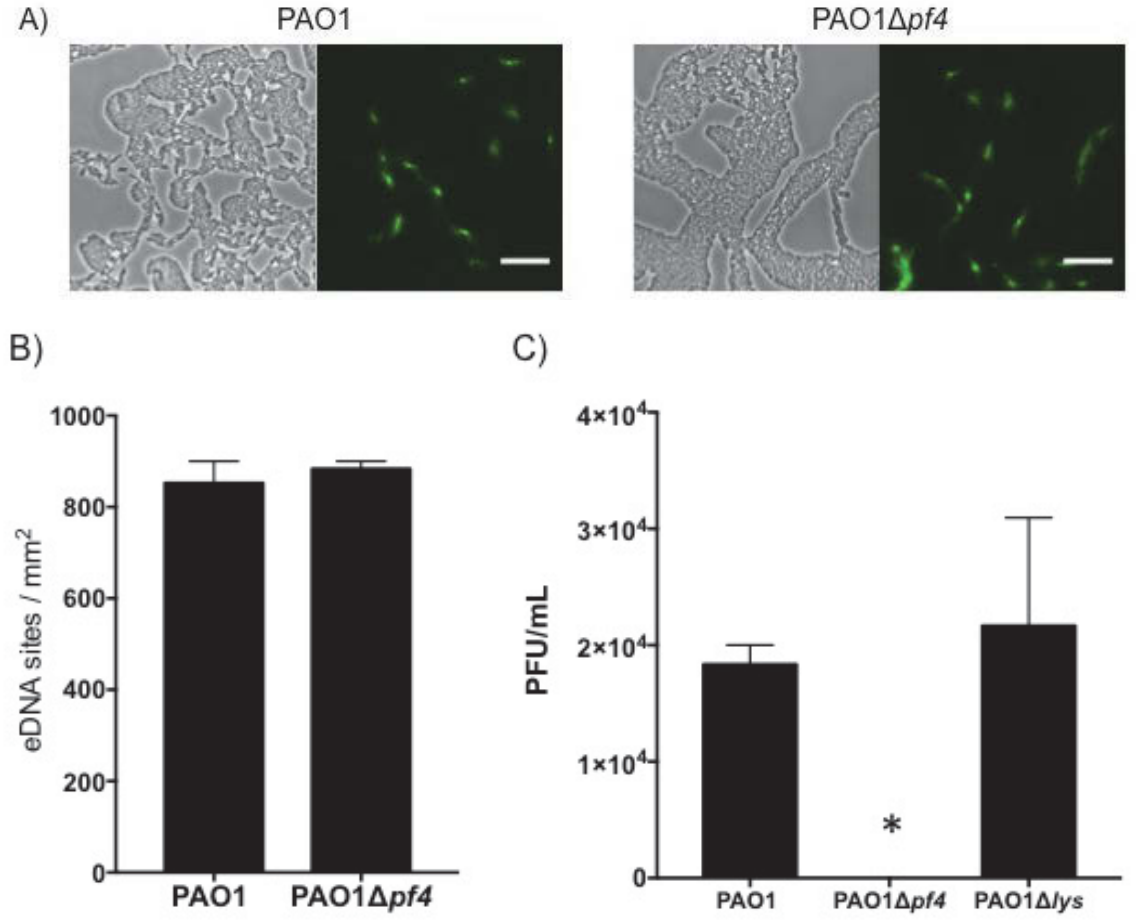


Figure 4.3: Pf4 phage is not required for explosive cell lysis-mediated eDNA release.

A) Interstitial biofilms of PAO1 and PAO1Δ*pf4* with phase-contrast (left) and EthHD-2 stained eDNA stained (green, right), scale bar 20 μm. B) eDNA release sites per mm^2 interstitial biofilm; n = 30, mean ± s.e.m. P = 0.5458, unpaired t-test with Welch's correction. C) Plaque-forming units per mL (PFU/mL) detected from interstitial biofilms of PAO1, PAO1Δ*pf4* and PAO1Δ*lys*; n = 3, mean ± s.e.m. * P = 0.0082, unpaired t-test with Welch's correction compared to PAO1.

Pf4 phage production occurs in mature flow biofilms (176, 187) and has been detected as early as 48 h (186). However Pf4 production has not been examined in interstitial biofilms. To confirm Pf4 phage is also released in interstitial biofilms and to determine if Lys-mediated explosive cell lysis is necessary for its release, Pf4 phage production was quantified by performing a phage plaque assay using the top-layer agar method (refer to section 2.7). Interstitial biofilms of PAO1, PAO1Δ*pf4* and PAO1Δ*lys* were cultured overnight. These biofilms were collected, resuspended in PBS and the filtered supernatant was spotted onto lawns of PAO1Δ*pf4* to detect presence of Pf4 phage as described by Rice *et al.* (187).

Supernatants of PAO1 and PAO1Δ*lys* produced phage plaques on a PAO1Δ*pf4* lawn, indicating phage release in interstitial biofilms of these strains (Figure 4.3C). No plaques were formed from the supernatant of PAO1Δ*pf4*, indicating any phage detected in PAO1 and PAO1Δ*lys* supernatants are likely due to Pf4 phage. This is the first description of Pf4 phage being detected in interstitial biofilms. As there was no significant difference in the number of plaques formed by PAO1 and PAO1Δ*lys*, we can infer that Lys is not necessary for the release of Pf4 phage and that Pf4 release is independent of explosive cell lysis under the conditions tested.

4.3.1.3 **Involvement of mis-folded proteins in formation of dark spots**

The small heat shock protein inclusion body protein A (IbpA) chaperones damaged or partially folded proteins to form inclusion bodies (286, 287). An IbpA-YFP fusion protein was utilised by Lindner *et al.* (255) to identify co-localisation of IbpA and inclusion bodies in *E. coli* formed under stress-inducing and non-inducing conditions. We sub-cloned the fusion protein gene into the *Pseudomonas* vector pUCP24 for use in *P. aeruginosa* (refer to section 2.3.10). The resultant plasmid, pUCP24::IbpA-YFP, was transformed into PAO1 and was used to determine if the dark spots and dark polar regions seen in PAO1 under inducing and non-inducing conditions are inclusion bodies of protein origin.

Interstitial biofilms of PAO1 pUCP24::IbpA-YFP were cultured in the presence of filter discs saturated with water or 500 µg/mL MMC and biofilms

were imaged after 4-6 h. The number of dark spots and IbpA-YFP spots were counted for 200 cells from random fields of view (refer to section 2.4.3.4).

Under non-inducing conditions, in 200 cells, 49 dark spots were visible, there were 39 points of IbpA-YFP expression and 12 of the dark spots co-localised with IbpA-YFP expression (Figure 4.4). Only 24% of the dark spots co-localised with IbpA-YFP expression, suggesting only a small proportion of the visible dark spots are formed from protein aggregation.

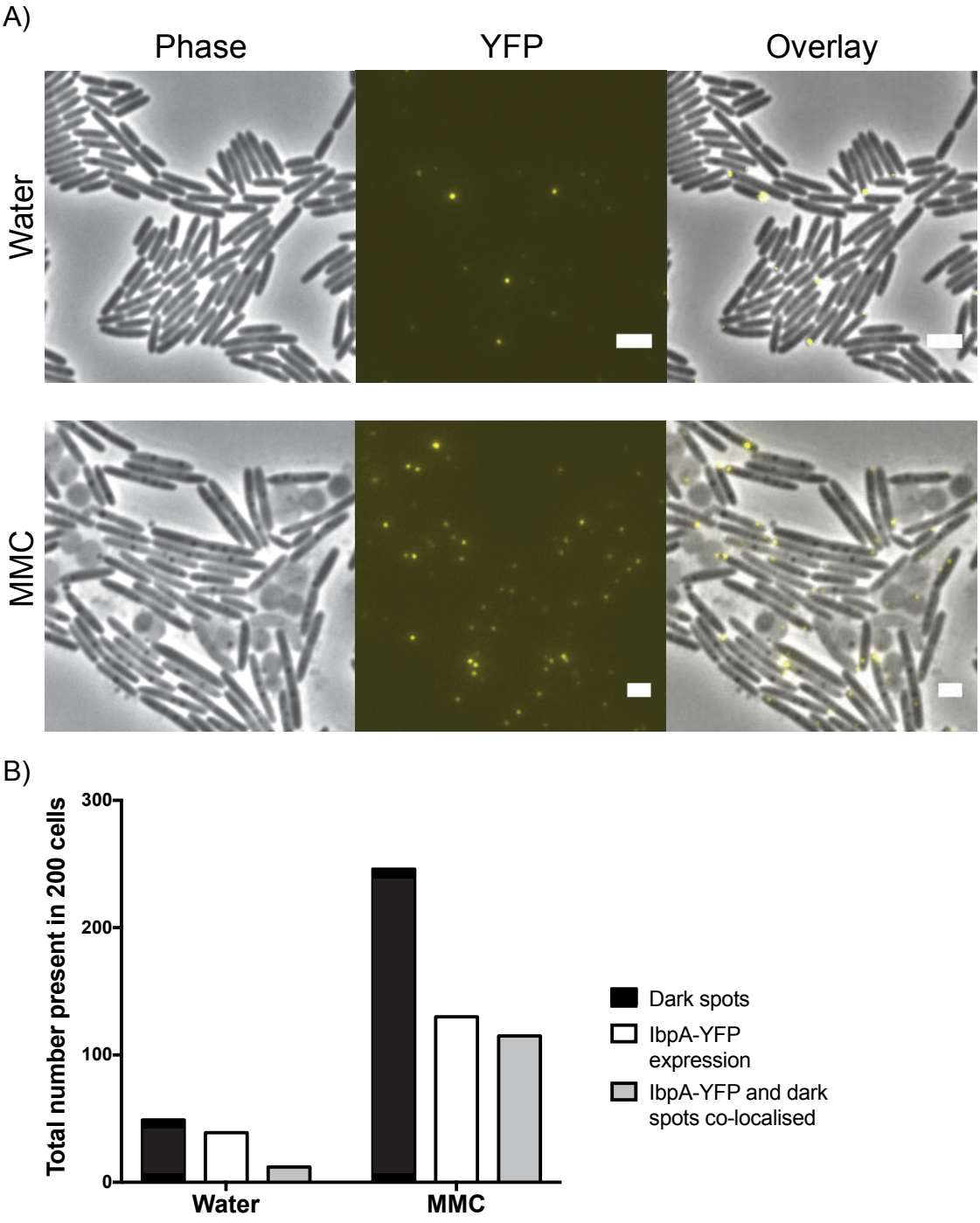


Figure 4.4: IbpA-YFP chaperone co-localises with some dark spots in PAO1.

A) Phase contrast (left), YFP (yellow, middle) and overlay of phase-contrast and YFP (right) of interstitial biofilms of PAO1 pUCP24::IbpA-YFP cultured in the presence of water (top panels) and 500 µg/mL MMC (lower panels), scale 2 µm. B) Total number of dark spots, IbpA-YFP expression and co-localised spots in 200 cells exposed to water and 500 µg/mL MMC.

In 200 cells exposed to MMC, 246 dark spots were visible, there were 130 points of IbpA-YFP expression and 115 of the dark spots co-localised with IbpA-YFP expression (Figure 4.4). The average number of dark spots per cell increased from 0.245 to 1.23 spots per cell under inducing conditions. Interestingly, the majority of dark spots in the presence of MMC co-localised with IbpA-YFP expression. This suggests genotoxic stress increases the production of partially folded or mis-folded proteins and the formation of inclusion bodies. As not all the dark spots visible in induced and non-induced conditions co-localised with IbpA-YFP expression, we cannot conclude that these inclusion bodies are always due to mis-folded proteins.

Together this data suggests the visible dark spots may be formed by aggregation of partially folded proteins, typically seen in the formation of inclusion bodies in aging and stressed cells (255) and are unlikely to be aggregates of pyocin and Pf4 phage particles. The formation of these inclusion bodies may be an indicator of cells experiencing stress and may also be a stressor themselves. Further investigation is required to determine if the formation of inclusion bodies directly correlates to the explosive cell lysis pathway.

4.3.2 Effect of exogenous stressors on explosive cell lysis

Expression of the pyocin gene cluster is dependent on the main SOS response regulator RecA and can be induced by DNA damage caused by UV irradiation, genotoxic or oxidative stress including ROS from neutrophils (217-221). In this section, we explore the effect of various exogenous stressors as inducers of explosive cell lysis.

4.3.2.1 **Genotoxic stress**

We first examined MMC as a genotoxic stressor to induce explosive cell lysis as it causes DNA damage, which activates the SOS response and is known to induce the pyocin gene cluster (162, 204, 273). Interstitial biofilms of

PAO1 were grown in the presence of cell-impermeant nucleic acid specific stain TOTO-1 to identify eDNA release and filter discs saturated with either water or 500 µg/mL MMC (refer to section 2.4.2). As the interstitial biofilms grow and expand, the bacteria are exposed to increasing concentrations of MMC. After 4-6 h of growth in the presence of water or MMC, the saturated discs were removed and the biofilm imaged as per section 2.4.3.2.

As the cells encounter higher concentrations of MMC, the number of explosive cell lysis events dramatically increases, compared to biofilms exposed to water (Figure 4.5). This can be seen by an increase in the number of round cells present and an increase in the area of the biofilm covered by eDNA, as seen by TOTO-1 staining.

As it is difficult to determine the number of eDNA release sites in the MMC exposed biofilms, eDNA production levels were quantified by calculating the area of the biofilm (area covered by cells) covered by eDNA as a percentage (refer to section 2.4.3.2). The percentage of PAO1 biofilm covered by eDNA increased from 5% to 70% upon exposure to MMC (Figure 4.5B). Together this demonstrates that explosive cell lysis can be induced by DNA damage caused by MMC.

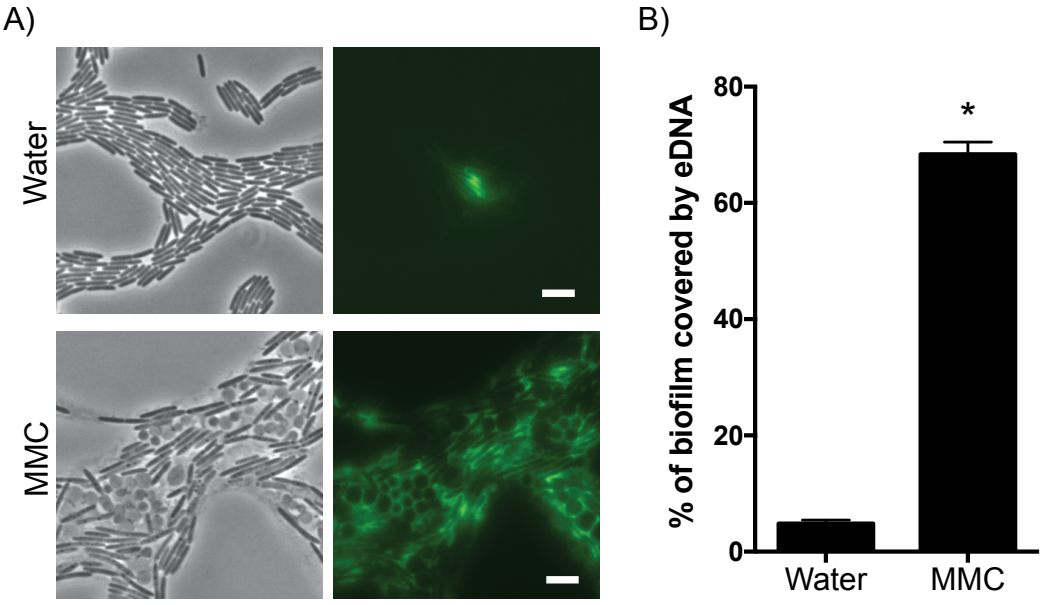


Figure 4.5: Mitomycin C induces round cell formation and explosive cell lysis

Phase-contrast (left panels) and TOTO-1 stained eDNA (right panels, green) of interstitial biofilms of PAO1 exposed to water and 500 µg/mL of MMC, scale 5 µm. The number of round cells and explosive cell lysis events increases upon exposure to MMC. (C) Percentage of the

biofilm covered by eDNA increases upon MMC exposure. *n* = 30, mean ± s.e.m. * *P* < 0.0001, unpaired *t*-test with Welch's correction.

In Chapter 3 of this Thesis, the expression of the pyocin lysis gene cassette was observed by high GFP expression of the GFP reporter pMHOL-GFP in cells that undergo explosive cell lysis (refer to section 3.3.2). In this plasmid, the promoter region of the first gene in the pyocin lysis cassette is fused to GFP (247). Previous reports show the pyocin gene cluster is upregulated under genotoxic stress (162, 273). Here we used pMHOL-GFP to confirm that expression of the pyocin lysis gene cassette increases in cells undergoing explosive cell lysis in response to MMC stress (500 µg/mL). pMLAC-GFP was used as a control for constitutive expression of the GFP transcriptional fusions under the *lac* promoter. Interstitial biofilms of PAO1 containing either pMLAC-G or pMHOL-G were cultured in the presence of water or MMC.

In Figure 4.6A, all cells with pMLAC-GFP expressed high levels of GFP in the presence and absence of MMC, demonstrating constitutive expression of *lac* in all cells under both conditions. Round cells produced under MMC stress also expressed pMLAC-GFP. Expression of pMHOL-GFP was limited to single cells under non-inducing conditions (Figure 4.6B). When exposed to MMC, the majority of cells containing pMHOL-GFP expressed high levels of GFP. Round cells containing pMHOL-GFP in the presence and absence of MMC also expressed high levels of GFP. This demonstrates that cells undergoing explosive cell lysis in response to genotoxic stress have increased expression levels of the pyocin lysis gene cassette.

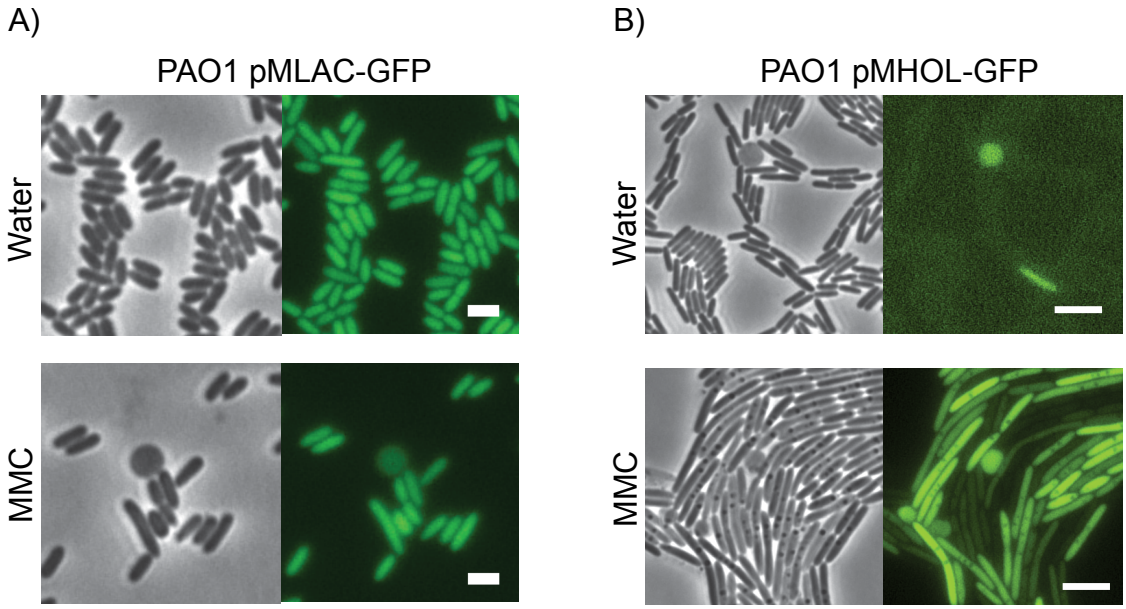
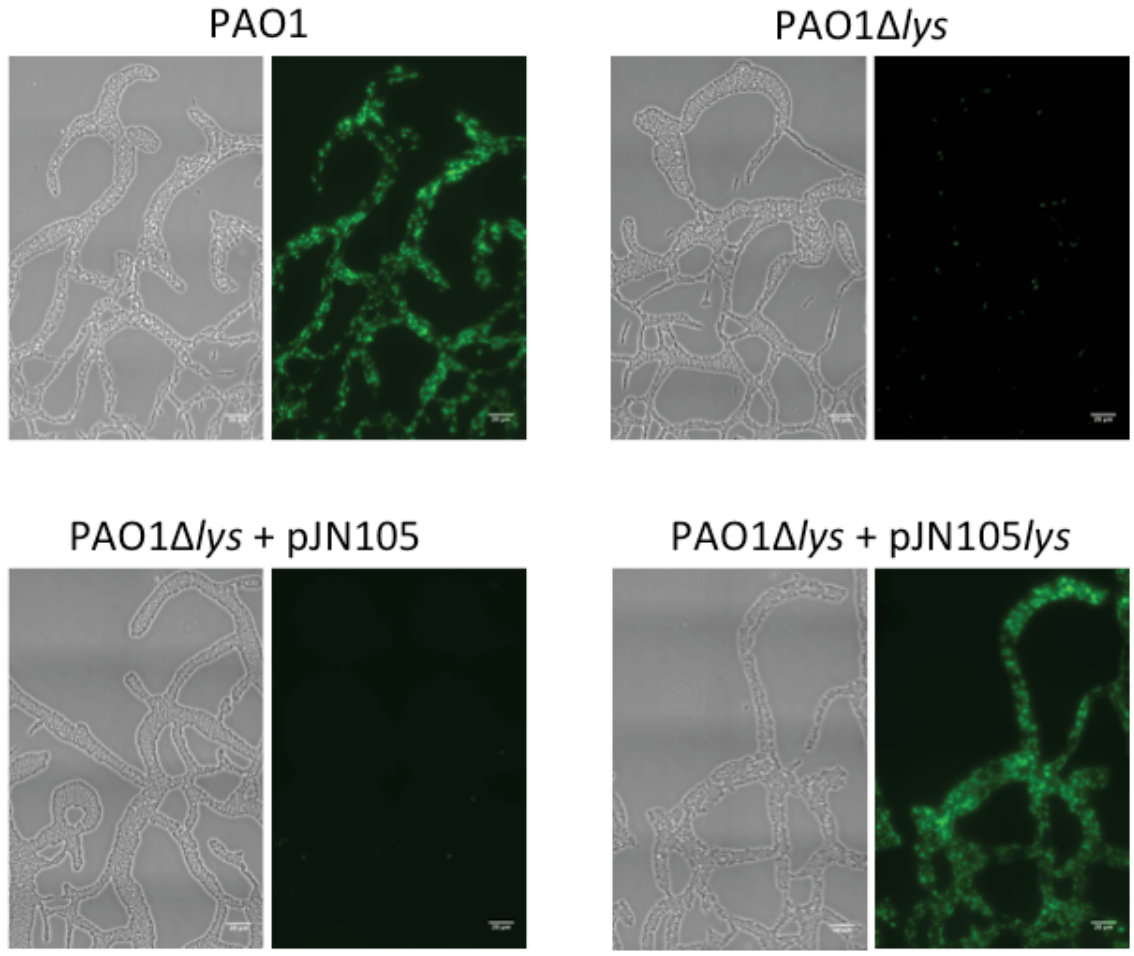


Figure 4.6: Expression of the pyocin lysis gene cassette increases under genotoxic stress.

A) Phase contrast (left) and GFP fluorescence of pMLAC-GFP (green, right) of PAO1 pMLAC-GFP interstitial biofilms cultured in the presence of water (top panels) and 500 µg/mL of mitomycin C (bottom panels), scale 2 µm. B) Phase contrast (left) and GFP fluorescence of pMHOL-GFP (green, right) of PAO1 pMLAC-GFP interstitial biofilms cultured in the presence of water (top panels) and 500 µg/mL of mitomycin C (bottom panels), scale 5 µm.

To confirm that explosive lysis is mediated by Lys in response to genotoxic stress, we cultured PAO1Δ*lys* interstitial biofilms in the presence of TOTO-1 and water or MMC (500 µg/mL) (refer to section 2.4.3.2). As the cells approached increasing concentrations of MMC, PAO1Δ*lys* did not undergo explosive cell lysis and no eDNA was released (Figure 4.7). Providing the wild-type *lys* gene *in trans* complemented genotoxic stress-induced explosive cell lysis, restoring eDNA production to wild-type levels (Figure 4.7B).

A)



B)

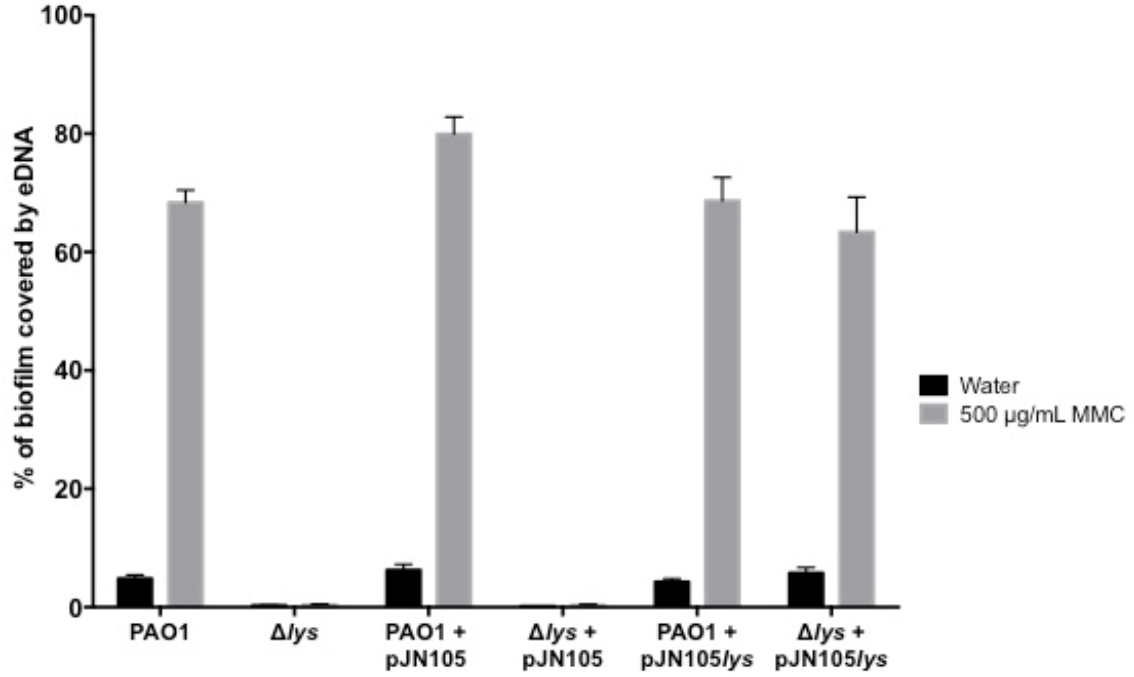


Figure 4.7: *Lys* is required for stress-induced explosive cell lysis

A) Phase-contrast (left panels) and TOTO-1 stained eDNA (green, right) interstitial biofilms exposed to 500 µg/mL of MMC, scale 20 µm. B) Percentage of interstitial biofilms of PAO1 and PAO1Δ*lys* containing pJN105 (vector control), pJN105/*lys* (complementation plasmid) covered by eDNA when exposed to water (black) or 500 µg/mL MMC (grey); *n* = 30, mean ± s.e.m.

These results indicate that explosive cell lysis can be induced through genotoxic stress and is mediated by Lys under inducing and non-inducing conditions.

4.3.2.2 **Antibiotic stress**

Other DNA damaging compounds including fluoroquinolone antibiotics are also known to induce pyocin production and upregulate *lys* (194, 195, 217-221, 225). It has been proposed that the pyocin lysis gene cassette is involved in ciprofloxacin-mediated cell death (193). It is possible that *P. aeruginosa* cells die through the explosive cell lysis cell death pathway when exposed to ciprofloxacin. It is unknown if the pyocin lysis gene cassette also influences the sensitivity of *P. aeruginosa* to other clinically relevant antibiotics. No investigations have been conducted examining the cell morphology of live cells exposed to bactericidal antibiotics. This section explores if Lys is involved in antibiotic-induced cell death and if explosive cell lysis is strictly inducible through DNA damage.

As PAO1Δ*lys* does not undergo explosive cell lysis, we used this as a control against PAO1 to examine the effect of explosive cell lysis on the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) for classes of antibiotics commonly used against *P. aeruginosa*; fluoroquinolones (ciprofloxacin), aminoglycosides (gentamicin) and polymyxins (colistin). Fluoroquinolones act on bacteria by preventing DNA from unwinding and duplicating (226), aminoglycosides inhibit protein synthesis (288) and polymyxins disrupt the structure of the bacterial membrane by interacting with phospholipids (289).

MIC and MBC of ciprofloxacin, gentamicin and colistin were performed on PAO1 and PAO1Δ*lys* to determine if explosive cell lysis has an effect on antibiotic sensitivity and susceptibility (refer to sections 2.6.2 and 2.6.3). The MIC and MBC for PAO1 and PAO1Δ*lys* were identical for all three antibiotics

tested (Table 4.2). This demonstrates that susceptibility to different antibiotic classes is not affected by the ability to undergo explosive cell lysis.

Table 4.2: Minimal inhibitory concentration and minimal bactericidal concentration are not effected by explosive cell lysis.

Minimal Inhibitory Concentration

Strain	Ciprofloxacin	Gentamicin	Colistin
PAO1	0.1 µg/mL	4 µg/mL	4 µg/mL
PAO1Δ*lys*	0.1 µg/mL	4 µg/mL	4 µg/mL

Minimal Bactericidal Concentration

Strain	Ciprofloxacin	Gentamicin	Colistin
PAO1	0.2 µg/mL	8 µg/mL	8 µg/mL
PAO1Δ*lys*	0.2 µg/mL	8 µg/mL	8 µg/mL

4.3.2.2.1 Time kill curves in planktonic cultures

As there was no difference in MIC and MBC for PAO1 and PAO1Δ*lys* for different antibiotic classes, we investigated whether cell death was occurring through the same pathway in both strains. Initially we examined the cell morphology of PAO1 and PAO1Δ*lys* planktonic cultures to determine if explosive cell lysis occurs under these conditions.

Log-phase cultures of PAO1 and PAO1Δ*lys* were exposed to bactericidal concentrations of ciprofloxacin, gentamicin or colistin for 6 h and examined microscopically (refer to section 2.6.4). These cultures were stained with Live/Dead stains Syto 9 and EthHD-2 to indicate cell viability and the cell populations of 6 locations were tracked over the 6 h for the number of live and dead cells. The number of round cells was also recorded as an indicator of cells undergoing explosive cell lysis. Cell viability was also verified with aliquots of the cultures plated out for the number of colony forming units per mL (CFU/mL) (refer to section 2.6.1).

Under normal conditions (i.e. in absence of antibiotic) PAO1 and PAO1Δ*lys* are rod-shaped cells and the whole population is stained by Syto 9 (Figure 4.8). No cells were stained with the dead cell stain EthHD-2. Over 6 h, the number of CFU/mL increases gradually.

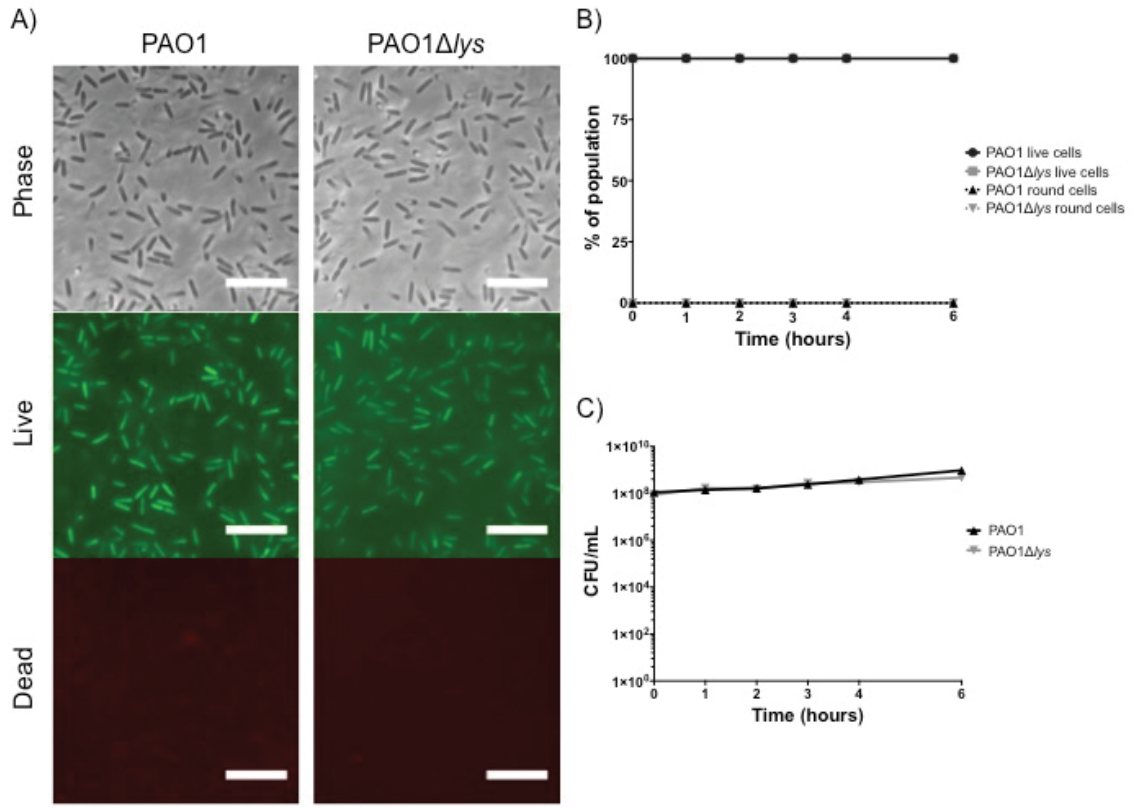


Figure 4.8: Cell morphology and viability over 6 h.

A) Planktonic cultures of PAO1 and PAO1Δ*lys* at 6 h, showing phase contrast (top panels), live cells stained with Syto 9 (green, middle panels) and dead cells stained with EthHD-2 (red, bottom panels), scale 10 μm. B) Planktonic cultures were examined for cell viability and morphology. Percentage of the population that are live cells and percentage of the population that are round. *n* = 18, mean ± s.e.m. C) Colony forming units per mL (CFU/mL) was used to determine cell viability of PAO1 and PAO1Δ*lys*. *n* = 3, mean ± s.e.m.

4.3.2.2.1.1 Ciprofloxacin

Over the course of 6 h, the majority of PAO1 cells exposed to bactericidal concentrations of ciprofloxacin (0.2 μg/mL) are stained with the live stain Syto 9, with the occasional dead cell present as seen by EthHD-2 staining (Figure 4.9). Coinciding with this, the proportion of round cells present increased over time and the round cells were stained with Syto 9. (Figure 4.9A). The number of live PAO1 cells identified by Syto 9 staining remains relatively constant over the time course and the proportion of round cells increases (Figure 4.9B). Conversely, the number of viable cells as determined by CFU/mL shows a significant decrease over time (Figure 4.9C). From this data, we can infer that explosive cell lysis is involved in ciprofloxacin-mediated cell death.

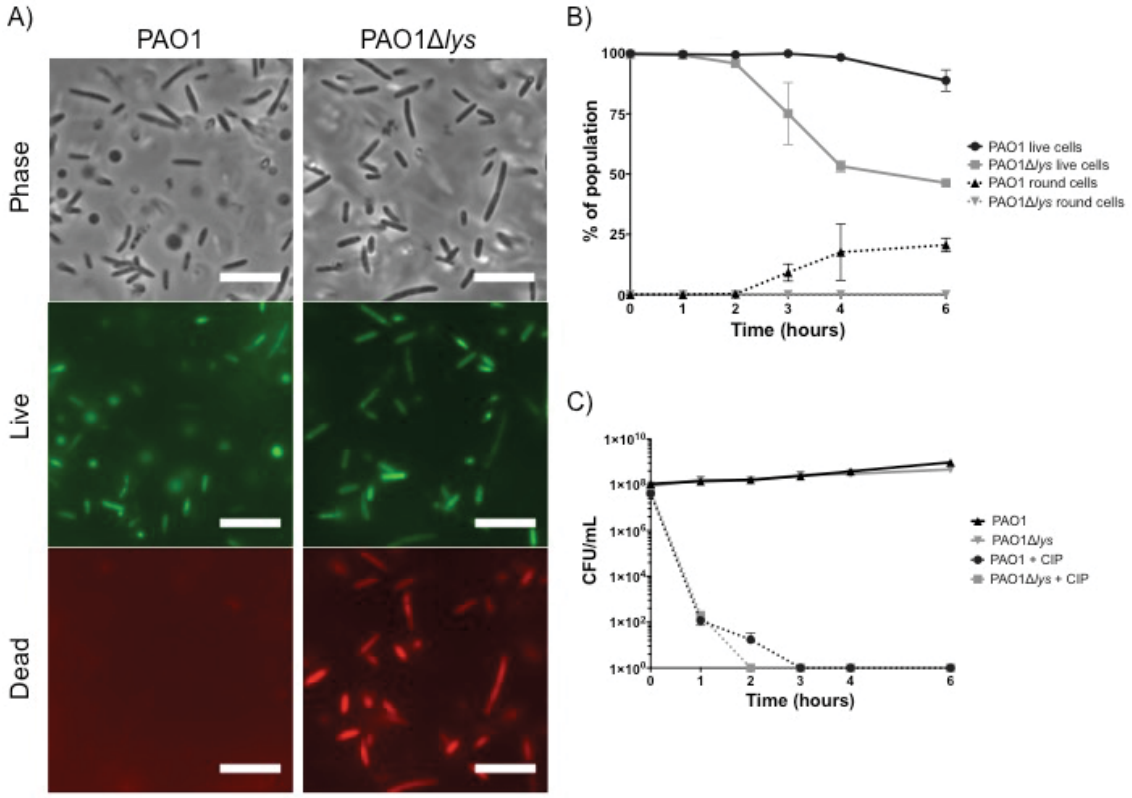


Figure 4.9: Bactericidal concentrations of ciprofloxacin induces explosive cell lysis.

A) Planktonic cultures of PAO1 and PAO1Δ*lys* exposed to 0.2 µg/mL ciprofloxacin at 6 h, showing phase contrast (top panels), Syto 9 staining live cells (green, middle panels) and EthHD-2 staining dead cells (bottom panels, red), scale 10 µm. B) Planktonic cultures exposed to 0.2 µg/mL ciprofloxacin were examined for cell viability and morphology. Percentage of the population that are live cells and percentage of the population that are round. *n* = 18, mean ± s.e.m. C) Colony forming units per mL (CFU/mL) was used to determine cell viability of PAO1 and PAO1Δ*lys* exposed to 0.2 µg/mL ciprofloxacin (CIP). *n* = 3, mean ± s.e.m.

To confirm this, we also examined cell death in PAO1Δ*lys.* For PAO1Δ*lys* the proportion of cells stained with the dead cell stain EthHD-2 increased over time with no round cells visible at any time point (Figure 4.9). The number of viable cells, as determined by CFU/mL, also decreased at a similar rate to PAO1. This indicates there is no change in susceptibility to ciprofloxacin in the absence of the explosive cell lysis pathway. It appears that in the absence of the explosive cell lysis pathway, *P. aeruginosa* undergoes a visually different form of cell death whereby the bacteria remain as a rod-shape.

4.3.2.2.1.2 Gentamicin

Exposure to bactericidal concentrations of gentamicin (8 µg/mL) caused cells of PAO1 and PAO1Δ*lys* to die at a similar rate and in a visually similar manner (Figure 4.10). The number of cells stained with dead cell stain EthHD-2 gradually increased over 6 h in PAO1 and PAO1Δ*lys*. These dead cells were rod-shaped and no round cells were visible across the time course (Figure 4.10B). Cell viability as determined by CFU/mL decreased at a similar rate for both strains indicating explosive cell lysis has no effect on susceptibility to gentamicin for up to 6 h (Figure 4.10C). Gentamicin appears to have a slow effect on killing. Gentamicin-mediated cell death in PAO1 and PAO1Δ*lys* appears to be independent of explosive cell lysis.

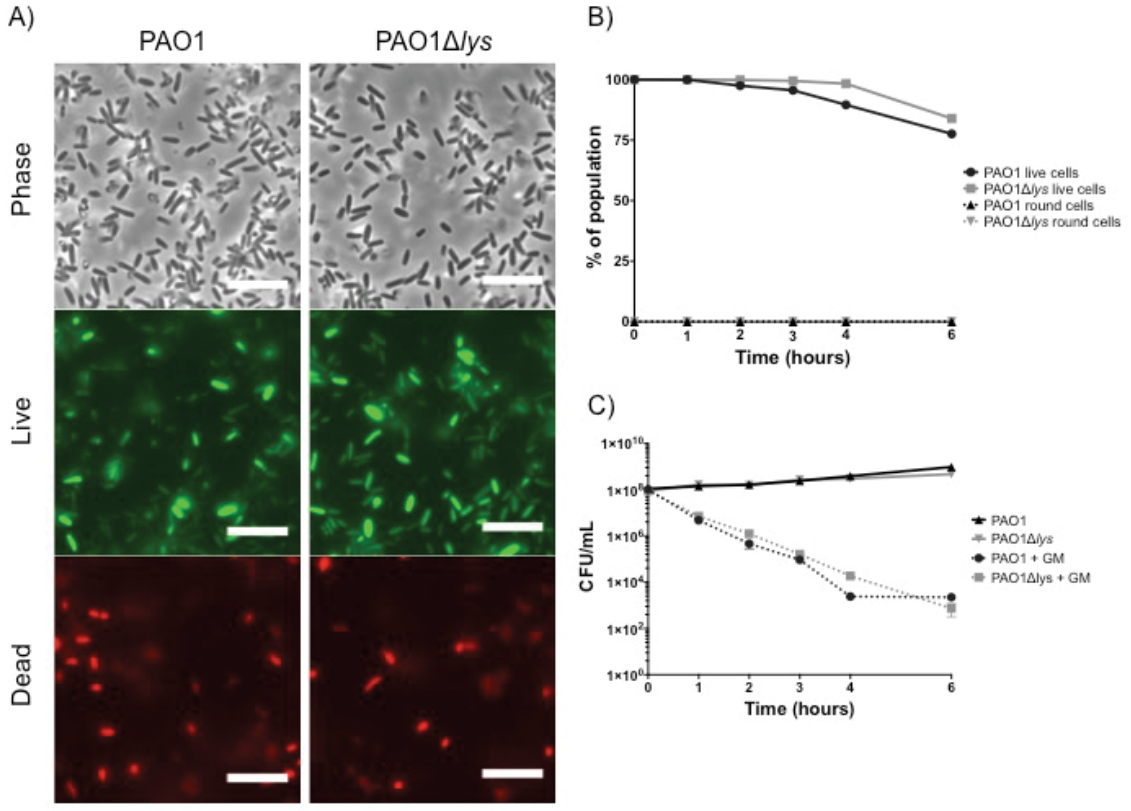


Figure 4.10: Explosive cell lysis plays no role in gentamicin-mediated cell death.
A) Planktonic cultures of PAO1 and PAO1Δ*lys* exposed to 8 µg/mL gentamicin at 6 h, showing phase contrast (top panels), Syto 9 staining live cells (green, middle panels) and EthHD-2 staining dead cells (bottom panels, red), scale 10 µm. B) Planktonic cultures exposed to 8 µg/mL gentamicin were examined for cell viability and morphology. Percentage of the population that are live cells and percentage of the population that are round. *n* = 18, mean ± s.e.m. C) Colony forming units per mL (CFU/mL) was used to determine cell viability of PAO1 and PAO1Δ*lys* exposed to 8 µg/mL gentamicin (GM). *n* = 3, mean ± s.e.m.

4.3.2.2.1.3 Colistin

Bactericidal concentrations of colistin (8 µg/mL) had a dramatic effect on PAO1 and PAO1Δ*lys.* Live/Dead staining of the two strains showed the number of dead cells increased exponentially until all cells were stained with EthHD-2 by 4 h (Figure 4.11A and B). The cells appear to die as rod-shaped cells and then go on to lyse, leaving behind cellular debris (Figure 4.11A), similar to electron microscopy images of the effect of polymyxin B on *E. coli* and *Salmonella typhimurium* (290, 291). After one h of exposure to bactericidal concentrations of colistin, no cells were viable for both strains as seen by CFU/mL (Figure 4.11C). Explosive cell lysis has no effect on the susceptibility to colistin and colistin-induced cell death.

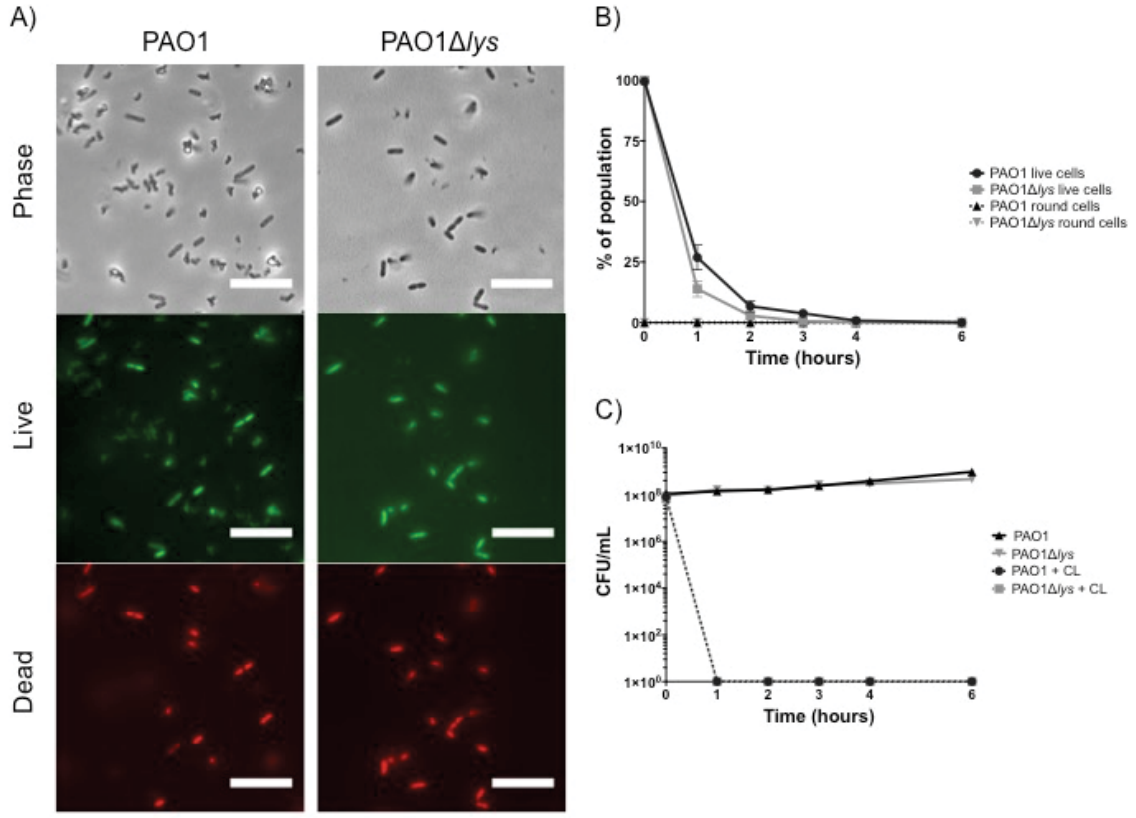


Figure 4.11: Explosive cell lysis plays no role in colistin-mediated cell death.

A) Planktonic cultures of PAO1 and PAO1Δ*lys* exposed to 8 µg/mL colistin at 6 h, showing phase contrast (top panels), Syto 9 staining live cells (green, middle panels) and EthHD-2 staining dead cells (bottom panels, red), scale 10 µm. B) Planktonic cultures exposed to 8 µg/mL colistin were examined for cell viability and morphology. Percentage of the population that are live cells and percentage of the population that are round. *n* = 18, mean ± s.e.m. C) Colony forming units per mL (CFU/mL) was used to determine cell viability of PAO1 and PAO1Δ*lys* exposed to 8 µg/mL colistin (CL). *n* = 3, mean ± s.e.m.

4.3.2.2.2 Interstitial biofilms

We also examined the effect of these antibiotics in interstitial biofilms to verify that explosive cell lysis is only induced by the genotoxic stressors MMC and ciprofloxacin and determine if eDNA was being released as a consequence of antibiotic exposure.

Interstitial biofilms of PAO1 and PAO1Δ*lys* were cultured in the presence of EthHD-2 to identify eDNA release and dead cells in the biofilms. Filter discs saturated with water, 100 µg/mL ciprofloxacin, 100 µg/mL gentamicin or 100 µg/mL colistin were also placed on these biofilms to establish a concentration gradient (refer to section 2.4.2). After 4-16 h, the biofilms were imaged and eDNA release and cell viability was examined.

Exposing interstitial biofilms of PAO1 and PAO1Δ*lys* to bactericidal concentrations of ciprofloxacin induces similar responses to MMC exposure (refer to section 4.3.2.1) (Figure 4.12). After 4 h of growth, the majority of the cells in PAO1 become round and under go explosive cell lysis, releasing eDNA into the biofilm milieu as indicated by EthHD-2 staining. In PAO1Δ*lys*, no cells under go explosive cell lysis and there is no eDNA released. Using the dead cell stain, EthHD-2, we can see that a few cells in the PAO1Δ*lys* have taken up the stain. No rod-shaped dead cells are visible in PAO1, indicating that all cell death in interstitial biofilms when exposed to bactericidal concentrations of ciprofloxacin is through explosive cell lysis in the presence of Lys.

No round cells or eDNA was observed in interstitial biofilms of PAO1 and PAO1Δ*lys* exposed to bactericidal concentrations of gentamicin and colistin, indicating these antibiotics do not induce explosive cell lysis in interstitial biofilms. At 4-6 h when MMC and ciprofloxacin cause the majority of the cell population to explode, gentamicin and colistin had no effect on eDNA release or cell viability. To see any effect of these antibiotics, the interstitial biofilms had to be grown for 16 h. Gentamicin and colistin may be slower to diffuse across the media and slower to penetrate the biofilm (112, 292). After 16 h, all the cells exposed to colistin had taken up EthHD-2 and maintained their rod-shaped structure. Cells exposed to gentamicin also maintained their rod-shape but only a small proportion of cells were stained with EthHD-2. Along with this, no eDNA release was observed upon exposure to gentamicin and colistin, demonstrating

gentamicin- and colistin-induced cell death is independent of explosive cell lysis.

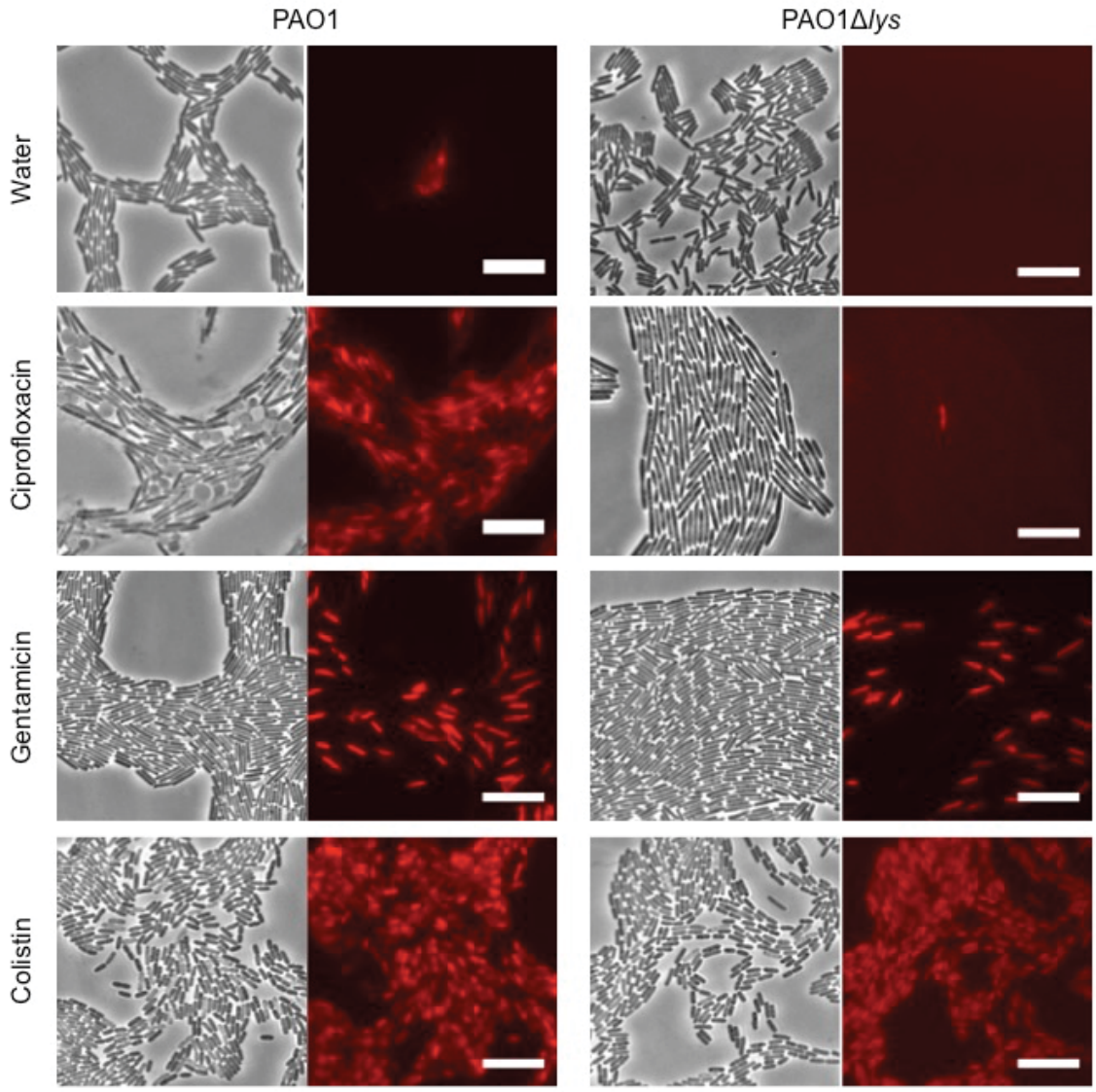


Figure 4.12: Antibiotic-induced cell death in interstitial biofilms.

Interstitial biofilms of PAO1 and PAO1Δ*lys* cultured in the presence of water, 100 µg/mL ciprofloxacin, 100 µg/mL gentamicin or 100 µg/mL colistin. Phase contrast (left) and EthHD-2 stained dead cells and eDNA (red, right), scale 10 µm.

4.3.2.2.3 Submerged biofilm biomass

Sub-minimal inhibitory concentrations (sub-MIC) of antibiotics are known to induce biofilm formation in many bacterial species (293-299). Sub-MIC levels of ciprofloxacin are known to induce biofilm formation in *P. aeruginosa* (293). We investigated if explosive cell lysis is involved in sub-MIC induced biofilm formation for ciprofloxacin, gentamicin and colistin.

PAO1 and PAO1Δ*lys* static biofilms were cultured in the presence of sub-MIC to MBC concentrations of ciprofloxacin, gentamicin and colistin in a 96-well polystyrene microtitre plate (refer to section 2.5.2). After 8 h of biofilm formation, the wells were washed with PBS and stained with crystal violet to determine biofilm biomass (261). The optical density (OD) at 595 nm of the extracted crystal violet was used as relative biofilm biomass.

Figure 4.13 shows the biofilm biomass of PAO1 and PAO1Δ*lys* exposed to various concentrations of ciprofloxacin, gentamicin and colistin. Biofilm biomass of PAO1Δ*lys* at 8 h in the absence of antibiotics was reduced compared to PAO1. For all antibiotic treatments, PAO1Δ*lys* biomass was less than that of PAO1 at lower concentrations. This difference in biomass may be attributed to defects in explosive cell lysis, which may be important in biofilm formation. At concentrations just below the MIC for the respective antibiotics, there was no difference in biofilm biomass between PAO1 and PAO1Δ*lys*. This suggests that sub-MIC induced biofilm formation, in response to antibiotic stress, is independent of explosive cell lysis.

4.3.2.2.3.1 Ciprofloxacin

Sub-MIC concentrations of ciprofloxacin induced biofilm formation in PAO1 (Figure 4.13A). Biomass steadily increased with increasing concentrations of ciprofloxacin, peaking at 0.01 µg/mL, resulting in double the biomass of untreated biofilms. This confirms what has been previously reported for ciprofloxacin-induced biofilm formation (293). Interestingly, biomass remained relatively constant in PAO1Δ*lys* at 0.0001 and 0.005 µg/mL ciprofloxacin. At 0.01 µg/mL biomass increased to similar biomass as PAO1 at the same concentration. As PAO1Δ*lys* is unable to undergo explosive cell lysis and produce eDNA in the presence and absence of ciprofloxacin, these results suggest that explosive cell lysis does not contribute to enhancement of biofilm formation at concentrations just below the MIC.

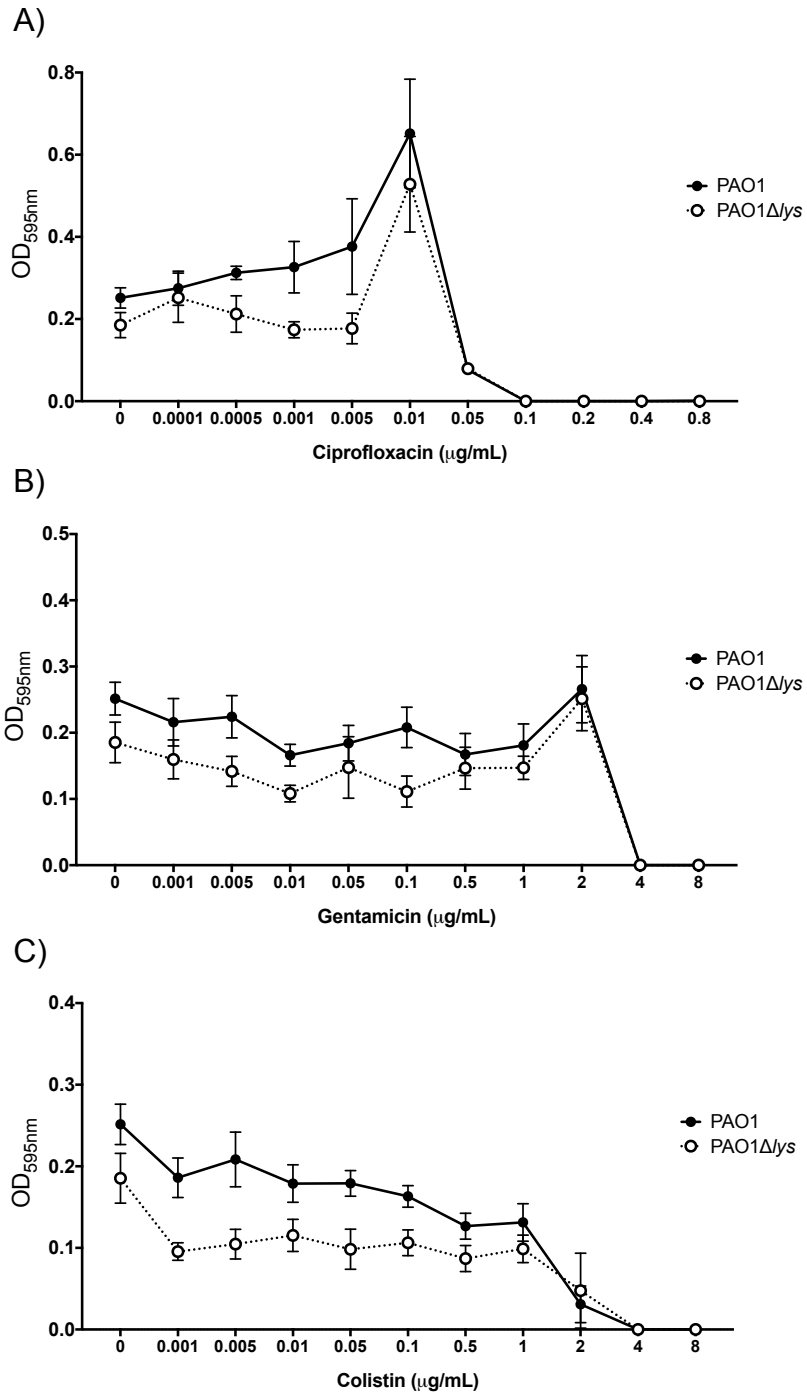


Figure 4.13: Biofilm biomass in response to antibiotic exposure.

Biomass of PAO1 and PAO1Δ*lys* biofilms cultured in the presence of various concentrations of A) ciprofloxacin, B) gentamicin and C) colistin, grown in 96-well polystyrene microtitre plates for 8 h. Biomass was stained with crystal violet and relative biomass presented as optical density (OD) at 595nm. *n* = 3, mean ± s.e.m.

4.3.2.2.3.2 Gentamicin

Gentamicin caused a decrease in biomass in PAO1 at concentrations between 0.001 and 1 μg/mL (Figure 4.13B). At 2 μg/mL, just below the MIC,

biomass increased back to untreated levels. A similar trend was observed for PAO1Δ*lys*, where biomass was always less than PAO1 at lower concentrations and biomass was almost the same as PAO1 at 2 µg/mL. Although we can not conclude gentamicin induced biofilm formation at 8 h, a longer incubation period may corroborate data presented by Linares *et al.* (293).

4.3.2.2.3.3 Colistin

Sub-MIC concentrations of colistin did not induce biofilm formation in PAO1 and PAO1Δ*lys* (Figure 4.13C). Biomass of PAO1 steadily decreased with increasing concentrations of colistin. PAO1Δ*lys* biomass decreased at 0.001 µg/mL and remained relatively constant and decreased further at 2 µg/mL, at the same concentration PAO1 biomass dropped.

4.4 Discussion

This Chapter explored the induction of explosive cell lysis through intrinsic and exogenous stressors. Intrinsic stressors include the presence of inclusion bodies that form from the aggregation of mis-folded or partially folded proteins. Exogenous stressors that cause DNA damage induce Lys-mediated explosive cell lysis and eDNA release. Stressors that target other aspects of the bacterial cell do not impact upon explosive cell lysis and deletion of *lys* does not influence the sensitivity of *P. aeruginosa* to antibiotic challenge.

As we only see single cells undergoing explosive cell lysis under non-inducing conditions, an internal stressor may be responsible for the expression of the pyocin lysis gene cassette. The formation of inclusion bodies can also serve as an intracellular stressor (283) therefore we investigated if dark spots observed in cells undergoing explosive cell lysis were inclusion bodies formed from the aggregation of pyocin particles, Pf4 phage or mis-folded proteins. We determined the production of pyocin structural elements and Pf4 phage had no effect on the formation of these putative inclusion bodies under normal and stressful conditions. Under genotoxic stress, the majority of these putative inclusion bodies are of protein origin, as seen by co-localisation with the fluorescently tagged small heat shock protein IbpA. It is yet to be determined what the inclusion bodies are under normal conditions as only a small proportion of the dark spots co-localised with IbpA-YFP and deletion of pyocin

structural elements and Pf4 phage had no effect on their formation. As stress can induce explosive cell lysis, it is possible that these dark spots form in response to an internal stress or alternatively act as an internal stressor themselves, inducing single cells to undergo the explosive cell lysis pathway. As the pyocin lysis gene cassette transcription is under the control of RecA, it is highly likely a cell undergoing explosive cell lysis is experiencing stress.

We also investigated the involvement of Pf4 phage in explosive cell lysis. Pf4 phage is involved in cell death in mature biofilm development and dispersal of PAO1 and PA14 (176, 186, 187). Pf4-mediated lysis is considered an important mechanism for eDNA production in mature biofilms of *P. aeruginosa* (186, 187). We determined that there was no defect in explosive cell lysis-mediated eDNA release in young biofilms in the absence of Pf4. Lys-mediated lysis is also not responsible for releasing the phage in early stage interstitial biofilm development. Further investigations will need to be performed on mature stage biofilm development of submerged biofilms to determine if Pf4 infection causes cell death through explosive cell lysis in later stages of development. Addition of purified Pf4 phage to mature biofilms of PAO1 and PAO1Δ*lys* will show if lysis Pf4-mediated lysis is associated with explosive cell lysis.

At the initiation of this Thesis, it was known that the pyocin gene cluster is upregulated upon exposure to genotoxic stress through activation of the SOS response (162, 193, 194, 273). Here we demonstrated that in the presence of the DNA damaging agents MMC and ciprofloxacin, the pyocin lysis gene cassette is upregulated and Lys-mediated explosive cell lysis is responsible for cell death. Bacterial cells of PAO1 and PAO1Δ*lys* appear longer when exposed to genotoxic stressors MMC and ciprofloxacin. These stressors activate the RecA-mediated stress response and this response can lead to a block in cell division in bacteria, resulting in longer cells (300). The induction of Lys-mediated explosive cell lysis appears to be specific to DNA damage, possibly through the creation of reactive oxygen species (ROS). Many antibiotics including ciprofloxacin have been demonstrated to increase reactive oxygen species in bacterial cells upon exposure, leading to cell death (301-303). It is therefore possible that other ROS generating compounds such as hydrogen peroxide could also induce explosive cell lysis. This will need to be explored in

the future as ROS are commonly produced by neutrophils and macrophages in an infection setting (304).

Other forms of stress, like protein synthesis inhibition by gentamicin and perturbation of the cell wall by colistin, appear to have no effect on explosive cell lysis or eDNA release. High-throughput screening of cell morphology and transcriptome analysis of the SOS response upon exposure to different antibiotics could illuminate which other compounds induce explosive cell lysis.

For the antibiotics examined, it appears that sub-MIC-induced biofilm formation is independent of explosive cell lysis at concentrations just below the MIC where the cells would be experiencing the most stress. At lower concentrations, a difference in biomass between PAO1 and PAO1Δ*lys* was observed for all antibiotics. This may be due to the potential involvement of explosive cell lysis in biofilm formation rather than stress inducing explosive cell lysis and influencing biofilm formation.

Antibiotic-induced biofilm formation occurs in many bacterial species and although there appears to be no single mechanism responsible, a global response to cell stress seems to play a role in many bacteria (305). The mechanism may involve SOS response as more antimicrobial agents have been found to induce SOS while triggering stress-inducible biofilm formation (306). In light of the data presented in this Chapter, the SOS response may be activated in reaction to antibiotic stress at lower concentrations where explosive cell lysis had an impact on biofilm biomass. At higher concentrations, just under the MIC, other mechanisms are likely to be responsible for inducing biofilm formation.

Brazas and Hancock (193) hypothesised that induction of this lysis cassette sensitises *P. aeruginosa* to genotoxic stress. They proposed that induction of the phage lytic system in the pyocin gene cluster is responsible for the lethality with ciprofloxacin induction. They examined the impact of pyocin tail fibre proteins and determined that they impacted upon ciprofloxacin susceptibility and did not examine the effect of the lytic genes. As demonstrated in Chapter 3, these tail fibre proteins play no role in explosive cell lysis (247) and are unlikely to be responsible for ciprofloxacin-induced cell death. Two other genes that are part of the pyocin lysis gene cassette, PA0630 and PA0631 have unknown functions and may be involved in this process.

A paradox exists whereby SOS-induced genes that combat damage also induce pyocin synthesis and co-expression of lysis proteins (232). The induction of pyocins during periods of stress and DNA damage may be a selective advantage. The cells that undergo explosive cell lysis experience stress, triggered by internal or external stressors. The cells reach a threshold where they are no longer able to repair DNA damage. The damaged DNA would activate the SOS response and consequently the pyocin gene cluster. This ultimately leads to explosive cell lysis and destruction of the cell. This could help maintain the genetic integrity of the clonal population and at the same time provide public goods to aid in the survival of the remaining community. This was demonstrated by biofilm biomass increasing at low concentration of ciprofloxacin.

Treatment of bacterial biofilms or infections with DNA damaging compounds could cause an upregulation of encoded prophages, leading to increased lysis, and release of public goods and virulence factors. The use of MMC and fluoroquinolones to treat enterohemorrhagic *E. coli* (EHEC) infections was found to cause the induction of Shiga toxin-producing phage (Stx-phage) and promote the production of Shiga toxin encoded by the phage (307-311). The natural immune response can also cause induction of the phage through ROS produced by neutrophils (307). Epidemiological studies have identified an association between these antibiotic treatments and an increased severity of EHEC infections through Stx-phage induction (311-313). Explosive cell lysis in *P. aeruginosa* infections could be induced and cause the release of virulence-associated public goods in response to treatment with DNA damaging agents and the natural response of the host's immune system. For future treatment developments, we will need to understand how treatments may impact on phage induction and potentially exacerbate the infection.

Chapter Five:
Contribution of Holins in Explosive Cell Lysis

5 Contribution of Holins in Explosive Cell Lysis

5.1 Publication information and other author contributions

The data presented in this Chapter was generated and analysed by Amelia L. Hynen with the following exceptions:

- PAO1Δ*cidAB* strain was obtained from Daniel Wozniak, The Ohio State University, United States of America.
- Construction of pJN105*alpB* and pJN105*cidAB*: Amelia L. Hynen and James Lazenby, University of Technology Sydney, Australia.

5.2 Introduction

A novel mechanism for the release of eDNA termed explosive cell lysis was recently reported for interstitial and submerged biofilms. Turnbull *et al.* (247) demonstrated eDNA production in real time *in situ* during the early stages of submerged and interstitial biofilm development, where eDNA is released from single cells in a population that under go explosive cell lysis (247). In Chapter 3 of this Thesis we showed that this process is mediated by the endolysin Lys encoded within the R- and F-pyocin gene cluster (247).

For an endolysin to reach the periplasm where it can break down the peptidoglycan in the cell wall, a holin must transport it through the inner membrane (159-162). The holin forms an oligomer complex resembling a pore in the cytoplasmic membrane, allowing for the non-specific transport of the endolysin (163, 164). Without a holin to transport it, the endolysin remains harmless in the cytosol (162). There are no specific requirements for a particular holin to transport a particular endolysin as it is unlikely that they directly interact with each other, making holins and endolysins interchangeable (228, 229). In the *P. aeruginosa* genome there are three annotated holins that could be responsible for the transport of Lys; *hol*, *alpB* and *cidAB* (67, 162, 227).

Hol is likely to be the cognate holin for Lys as it is encoded in the pyocin gene cluster along with *lys*. When Hol and Lys were first demonstrated to be responsible for cell lysis during release of pyocins, Lys alone without Hol was unable to cause lysis unless the inner membrane was permeabilised under the conditions examined (162). Hol has previously been associated with membrane vesicle production in broth cultures under normal and denitrifying conditions (243) and more recently Lys-mediated explosive cell lysis was reported to be involved in membrane vesicle production under these conditions (247). It is therefore likely that Hol contributes to the transport of Lys during explosive cell lysis under these conditions. Two other holins, *alpB* and *cidAB*, have been identified in the *P. aeruginosa* genome and are also implicated in cell death pathways that lead to lysis and eDNA release (67, 227). It is not known if these holins are involved in explosive cell lysis. Since an endolysin for either of these

pathways has not been identified, it is possible that Lys may also be associated with these lysis pathways.

AlpB is part of a recently discovered self-lysis pathway in *P. aeruginosa* related to bacteriophage lambda and can functionally substitute for bacteriophage lambda holin in *Escherichia coli* (227). This pathway is dependent on DNA damage and only lyses a subset of the cells in the population when activated. Lysis in this pathway is visually similar to explosive cell lysis, where cells appear to lose structural integrity and form round cells before they lyse. However the lysis event occurs over longer time frames of up to 460 min. Lysis through this pathway is also associated with an increase in eDNA production in broth culture supernatant (227).

The *cid* operon is conserved in around 50% of bacterial species that have sequenced genomes (144) and programmed cell death through this operon appears to be a conserved pathway in bacteria, plants and animals (145). *CidAB* is well described in *S. aureus* to be involved in eDNA production through cell lysis (132, 142). In *P. aeruginosa* it is involved in the dispersal stage of submerged biofilms (67). A tentative link has been made between eDNA production and CidAB-mediated cell death in *P. aeruginosa* but CidAB-mediated eDNA release was not examined (67). The genes *cidA* and *cidB* are translationally coupled and are likely to be functionally related. The CidA protein is the holin and the CidB protein is a homologous multi-spanning membrane protein of unknown function (135, 143).

As discussed in section 1.8.1, RecA, PrtR and PrtN regulate the pyocin gene cluster. Under normal conditions, PrtR represses activation of the activator PrtN, preventing transcription of the pyocin gene cluster. Under periods of stress initiated by a mutagenic agent, RecA is upregulated. RecA aids in the auto-cleavage of PrtR, which then allows for the transcription of *prtN*. Once synthesised, PrtN then activates the transcription of the remaining pyocin genes. It is therefore possible that *prtN* is involved in mediating explosive cell lysis.

This aim of this Chapter is to determine the involvement of the holins Hol, AlpB and CidAB, and the pyocin gene cluster regulator PrtN in explosive cell lysis.

5.3 Results

5.3.1 Involvement of holins in translocating Lys

We investigated the involvement of Hol in Lys-mediated explosive cell lysis, as it is likely to be the cognate holin for Lys. Explosive cell lysis results in the formation of punctate foci of eDNA in actively expanding interstitial biofilm monolayers and can be used as a marker of explosive cell lysis events (247). We cultured interstitial biofilms of an unmarked deletion of *hol* (PAO1Δ*hol*) (see section 2.3.11) in the presence of eDNA stain EthHD-2 and quantified the number of eDNA release sites across the actively expanding biofilm (see sections 2.4.1 and 2.4.3.1). The number of eDNA release sites were compared to the wild-type parent PAO1 and to the explosive cell lysis defective strain PAO1Δ*lys* (Figure 5.1). Deletion of *hol* reduced the number of eDNA release sites across the interstitial biofilm compared to PAO1 (Figure 5.1A and B). The reduction in eDNA release in PAO1Δ*hol* could be restored back to wild-type levels when wild-type *hol* was provided *in trans* (Figure 5.1C). The reduction in eDNA release in the Δ*hol* strain was statistically significant but eDNA release was not as abrogated as PAO1Δ*lys*, indicating that the Hol holin alone is not essential for explosive cell lysis, but contributes to the process.

Two other holins, *alpB* and *cidAB*, have been identified in the *P. aeruginosa* genome and have been implicated in cell death pathways (67, 227). These holins were examined for their involvement in explosive cell lysis. We studied eDNA release in strains with unmarked deletions of *alpB* or *cidAB* (refer to section 2.3.11). Interstitial biofilms of PAO1Δ*alpB* and PAO1Δ*cidAB* were cultured in the presence of eDNA stain EthHD-2 as a marker of explosive cell lysis events (see sections 2.4.1 and 2.4.3.1). The resulting eDNA production levels were compared to PAO1 and PAO1Δ*lys* (Figure 5.2). Interestingly PAO1Δ*alpB* and PAO1Δ*cidAB* also showed decreases in eDNA release, similar to PAO1Δ*hol,* where the reduction was statistically significant but not as severely abrogated as PAO1Δ*lys* (Figure 5.2A and B). eDNA release was increased above wild-type levels in PAO1Δ*alpB* (Figure 5.2C) and eDNA release was restored back to wild-type levels in PAO1Δ*cidAB* (Figure 5.2D) when the respective wild-type genes were provided *in trans*. As explosive cell

lysis-mediated eDNA release was reduced but not abrogated in these two holin deletions, these two holins, along with Hol, may be involved in translocating Lys in a coordinated manner.

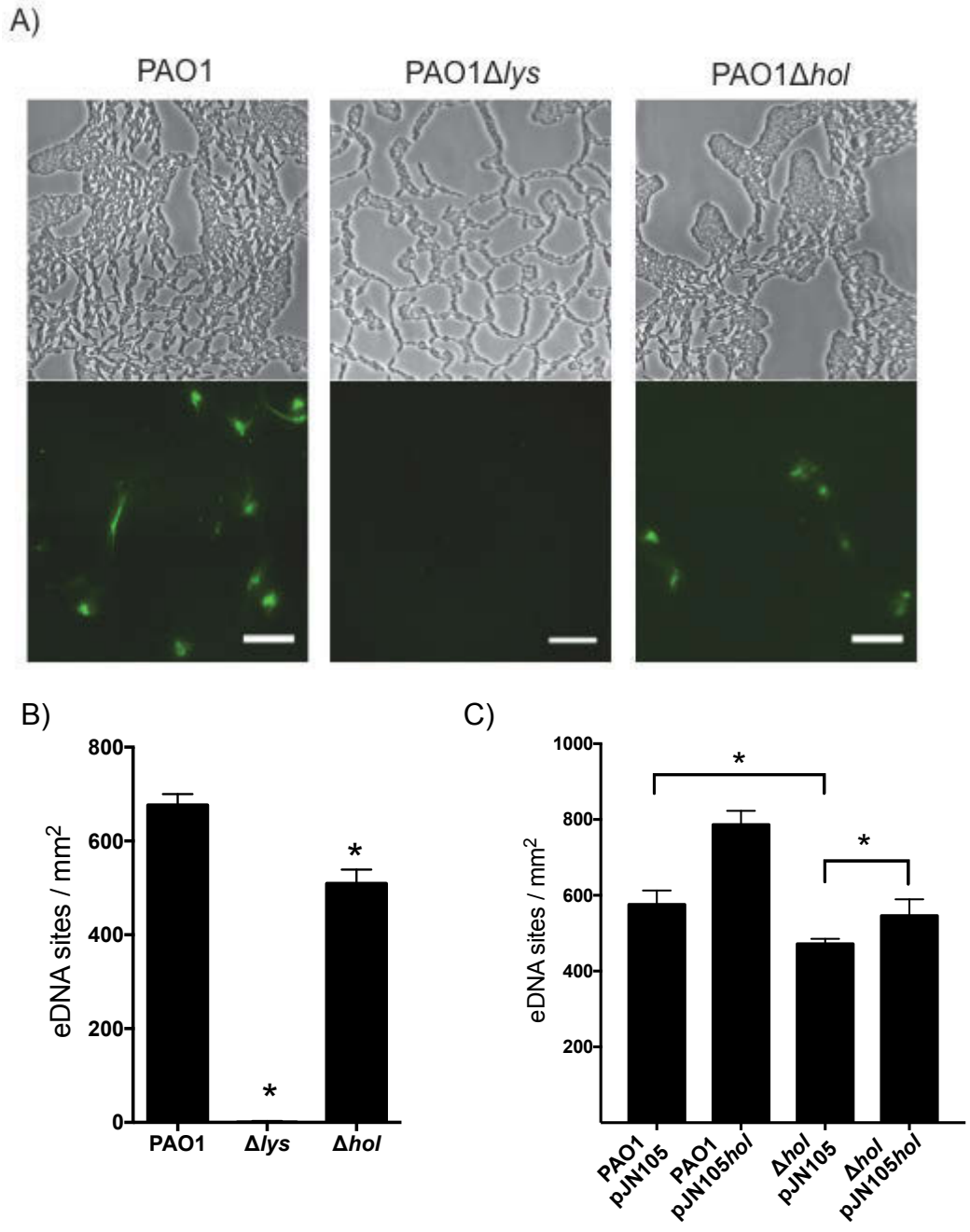


Figure 5.1: The cognate holin of Lys is not essential for explosive cell lysis in interstitial biofilms.

A) Phase-contrast (left) and Ethidium homodimer-2 stained eDNA (green, right) of interstitial biofilms of PAO1, PAO1Δ*lys* and PAO1Δ*hol*; scale bar, 20 μm. (B) Deletion of *hol* the cognate holin of Lys is not sufficient to abrogate explosive cell lysis-mediated eDNA release in interstitial biofilms; n = 30; mean ± s.e.m. * P < 0.0001, unpaired t-test with Welch's correction.

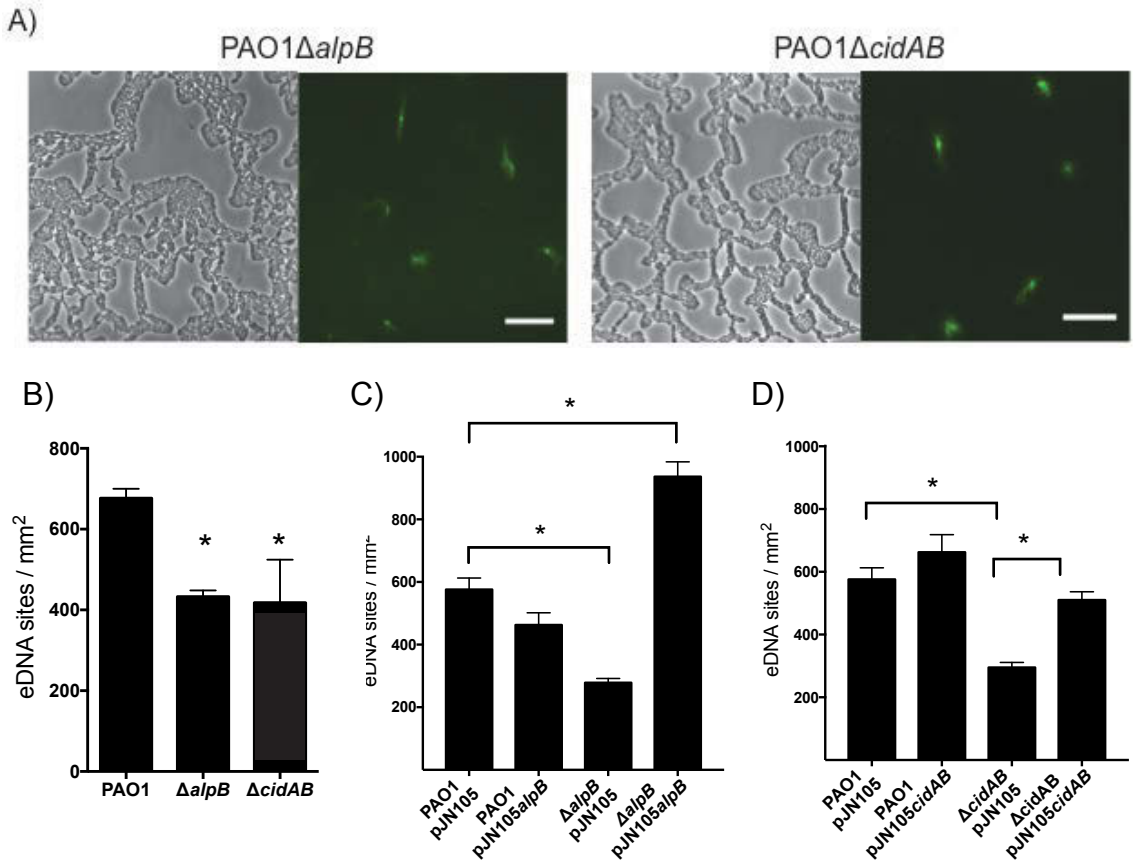


Figure 5.2: Holins AlpB and CidAB contribute to explosive cell lysis.

A) Phase-contrast (left) and Ethidium homodimer-2 stained eDNA (green, right) of interstitial biofilms of PAO1Δ*alpB* and PAO1Δ*cidAB*; scale bar, 20 µm. B) eDNA release is reduced with the deletion of *alpB* and *cidAB*; *n* = 30; mean ± s.e.m, * *P* < 0.0001, unpaired *t*-test with Welch's correction, compared to PAO1. C) A*lpB* contributes to explosive cell lysis-mediated eDNA release; *n* = 30; mean ± s.e.m.. * *P* < 0.0001, unpaired *t*-test with Welch's correction. D) *CidAB* is also involved in eDNA production in interstitial biofilms; *n* = 30; mean ± s.e.m., * *P* < 0.0001, unpaired *t*-test with Welch's correction.

As single deletions of these three holins resulted only in a relatively small reduction in eDNA release through explosive cell lysis and they all appear to be involved to a certain extent, it suggests there may be redundancy in the holin function. To explore this further, we created double and triple unmarked deletions of the three holins to determine which combinations of these holins are necessary for explosive cell lysis. The following unmarked deletion strains were created and interstitial biofilms of these strains were examined for eDNA production through explosive cell lysis; PAO1Δ*cidAB*Δ*alpB*, PAO1Δ*alpB*Δ*hol*, PAO1Δ*cidAB*Δ*hol* and PAO1Δ*cidAB*Δ*hol*Δ*alpB* (refer to sections 2.3.11, 2.4.1

and 2.4.3.1). All of the double deletion strains had a greater reduction in eDNA release sites compared to the parental PAO1 and the single holin deletions (Figure 5.3). eDNA release was reduced by half in PAO1Δ*cidAB*Δ*alpB* and PAO1Δ*cidAB*Δ*hol,* and by 87% in PAO1Δ*hol*Δ*alpB* (Figure 5.3B). This may be due to Hol and AlpB having greater roles in the transport of Lys than CidAB. The triple deletion showed an almost complete defect in eDNA production similar to PAO1Δ*lys*. This indicates that all three holins contribute to eDNA production through explosive cell lysis in *P. aeruginosa* interstitial biofilms. To our knowledge, this is the first evidence of the involvement of multiple holins in a single cell death pathway.

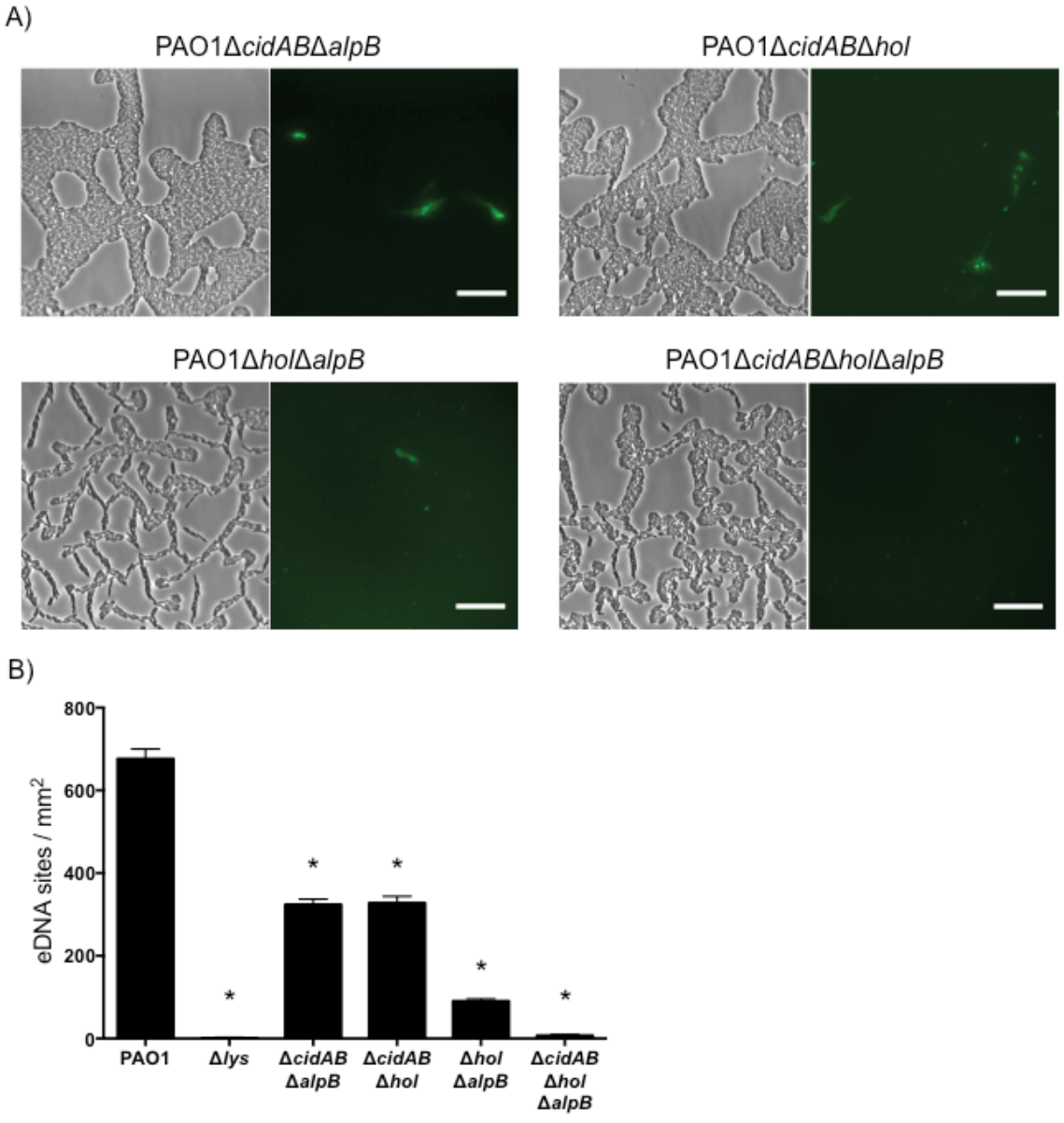


Figure 5.3: Three holins contribute to explosive cell lysis.

A) Phase-contrast (left) and Ethidium homodimer-2 stained eDNA (green, right) of interstitial biofilms of PAO1Δ*cidAB*Δ*alpB*, PAO1Δ*cidAB*Δ*hol*, PAO1Δ*hol*Δ*alpB* and PAO1Δ*cidAB*Δ*hol*Δ*alpB*; scale bar, 20 μm. (B) Deletion of all three holin is required to abrogate explosive cell lysis; $n = 30$; mean ± s.e.m. * $P < 0.0001$, unpaired *t*-test with Welch's correction compared to PAO1.

eDNA release was abrogated in the triple holin deletion mutant, suggesting that there are no other holins involved. To further examine this, we complemented the triple holin deletion with *lys* under the control of an arabinose-inducible promoter and induced its expression. If there are other unknown holins involved in the process, we would expect that eDNA production would increase under inducing conditions (0.02% arabinose) in the triple holin deletion. In PAO1 with *lys in trans*, eDNA release was increased under inducing conditions (Figure 5.4), demonstrating that arabinose induced the expression of *lys* and resulted in an increase of explosive cell lysis. Interestingly no eDNA was produced in the triple deletion with *lys in trans* under inducing and non-inducing conditions (Figure 5.4). This confirms that all three holins are essential for the translocation of Lys for explosive cell lysis and there are no other holins involved in the process.

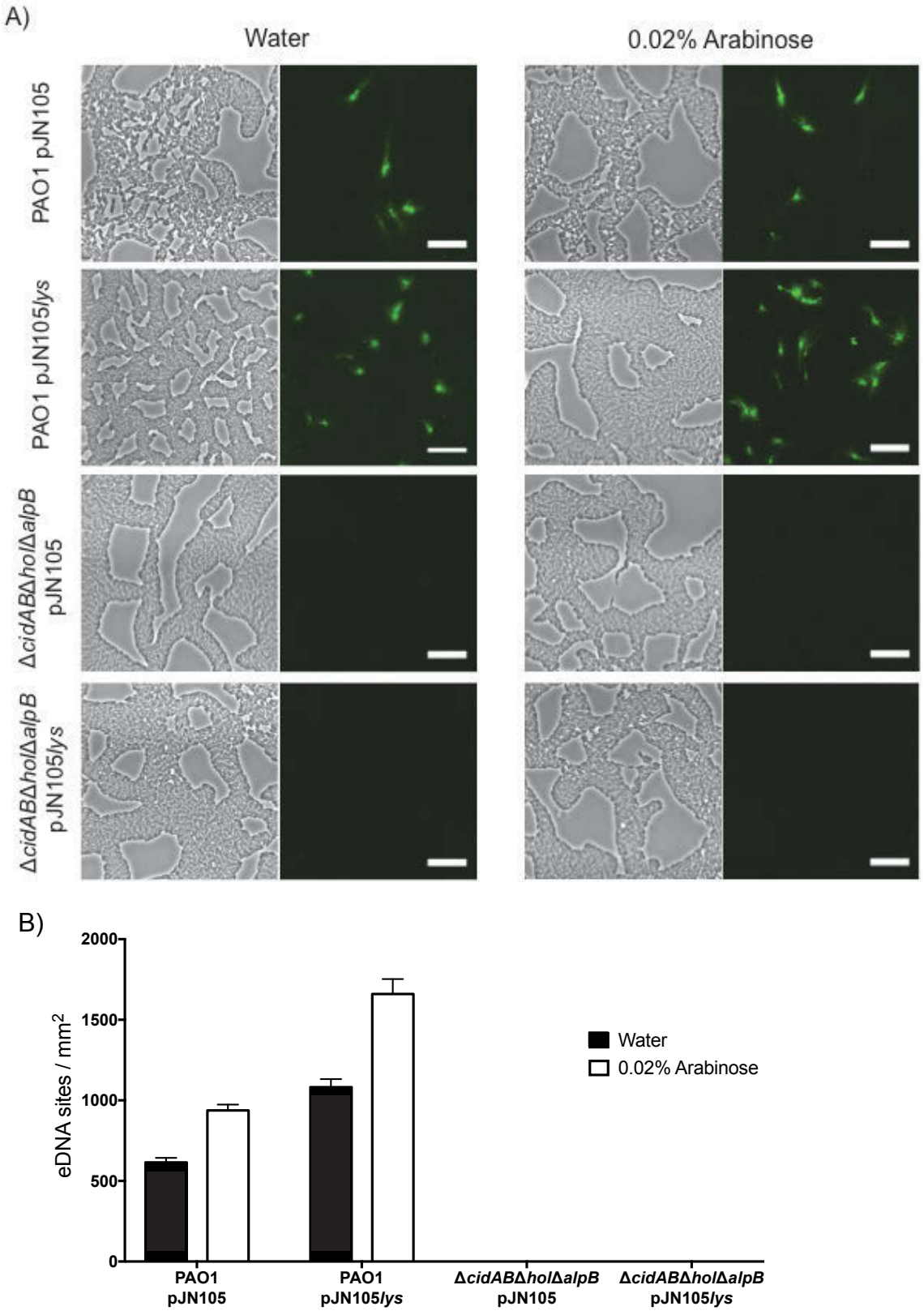


Figure 5.4: Induction of *lys* in triple holin deletion.

A) Phase-contrast (left) and Ethidium homodimer-2 stained eDNA (green, right) of interstitial biofilms of PAO1 and PAO1Δ*cidAB*Δ*hol*Δ*alpB* containing either pJN105 (vector control) or pJN105*lys* cultured in the presence or absence of 0.02% arabinose; scale bar 20 µm. (B) Three

holins are required for explosive cell lysis-mediated eDNA release under inducing and non-inducing conditions; *n* = 30; mean ± s.e.m.

As holins are non-specific transporters, it's possible that they are all able to transport Lys and therefore provide functional compensation and redundancy. To investigate whether they are able to functionally compensate for the loss of each other, we complemented single holin deletions with a different holin i.e. PAO1Δ*alpB* with *cidAB in trans,* PAO1Δ*cidAB* with *hol in trans* etc. Interstitial biofilms of these various complementation combinations were cultured in the presence of EthHD-2 and the number of eDNA release sites for each strain was quantitated (see sections 2.4.1 and 2.4.3.1). eDNA release in PAO1Δ*alpB* could be complemented with *cidAB* and *hol* provided *in trans*, restoring eDNA levels back to wild-type (Figure 5.5A). Complementation of PAO1Δ*cidAB* with *alpB in trans* could restore eDNA release back to PAO1 levels but the presence of *hol in trans* did not restore eDNA release (Figure 5.5B). eDNA production in PAO1Δ*hol* was restored by the addition of *cidAB* and not *alpB in trans* (Figure 5.5C). Altogether this shows that although these holins are functionally similar, they may not necessarily compensate for each other, indicating that each of them may have unique contributions to the process of explosive cell lysis.

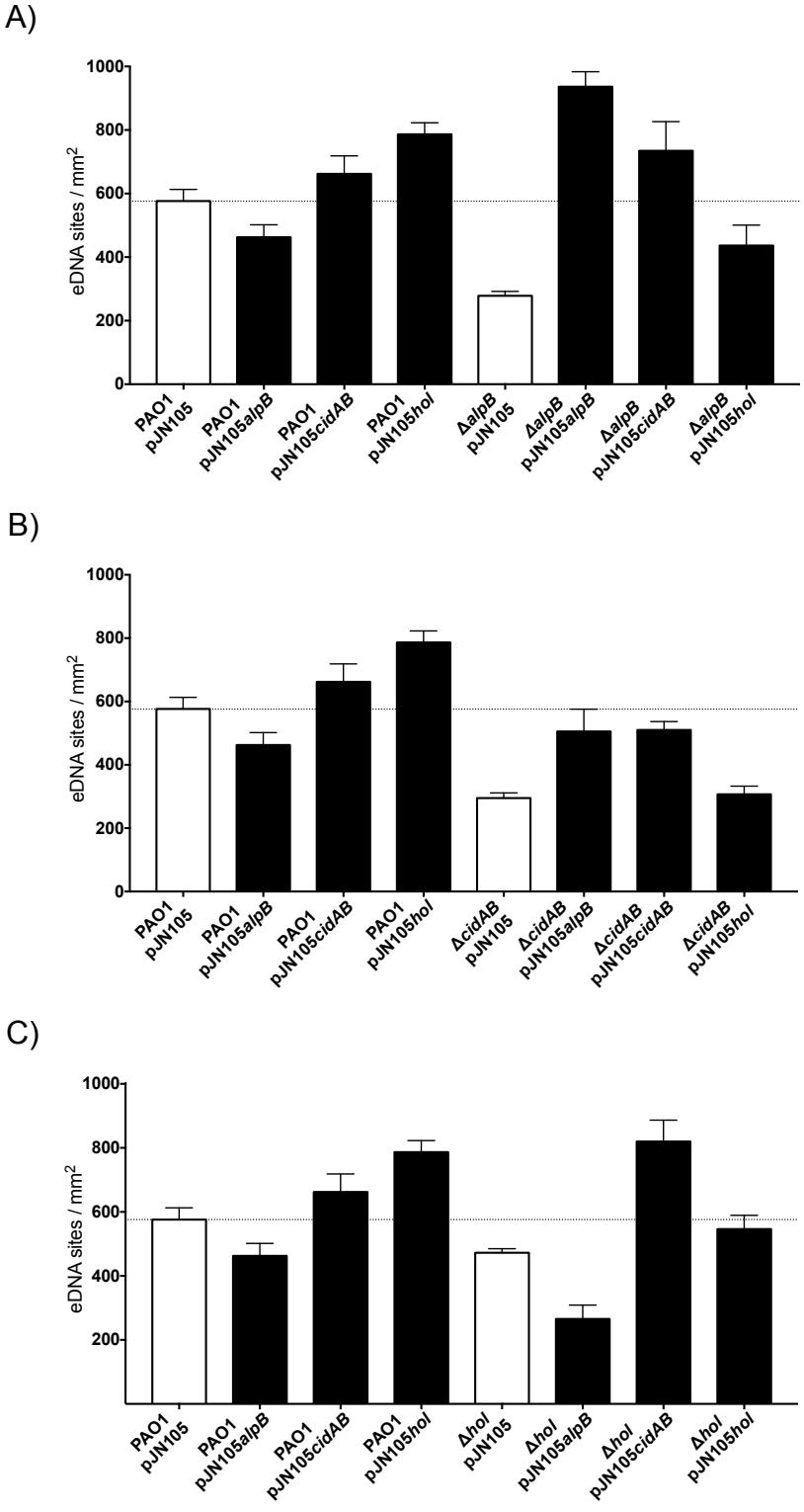


Figure 5.5: Each holin plays a unique role in explosive cell lysis-mediated eDNA release.

Restoring eDNA release in (A) Δ*alpB*, (B) Δ*cidAB* and (C) Δ*hol* with another holin *in trans* depends on the complementing holin. Dotted line represents PAO1 eDNA release levels; *n* = 30; mean ± s.e.m.

5.3.2 Holins involved in induction of explosive cell lysis

In Chapter 4 of this Thesis we demonstrated that explosive cell lysis is induced under genotoxic stress with the addition of MMC (247). To determine which of these holins are also involved in stress-induced explosive cell lysis, interstitial biofilms of the single and triple holin deletions were grown in the presence of eDNA stain EthHD-2 to identify eDNA release and filter discs saturated with either water or 500 µg/mL MMC (refer to section 2.4.2). As the biofilms of PAO1, PAO1Δ*alpB*, PAO1Δ*cidAB* and PAO1Δ*hol* approached increasing concentrations of MMC, explosive cell lysis was induced and large amounts of eDNA was released in these strains as seen by EthHD-2 staining (Figure 5.6). Interestingly, PAO1Δ*cidAB*Δ*hol*Δ*alpB* produced no eDNA, identical to PAO1Δ*lys*. This demonstrates that the loss of one holin is not sufficient to reduce stress-induced explosive cell lysis, indicating functional redundancy under stress-induced conditions.

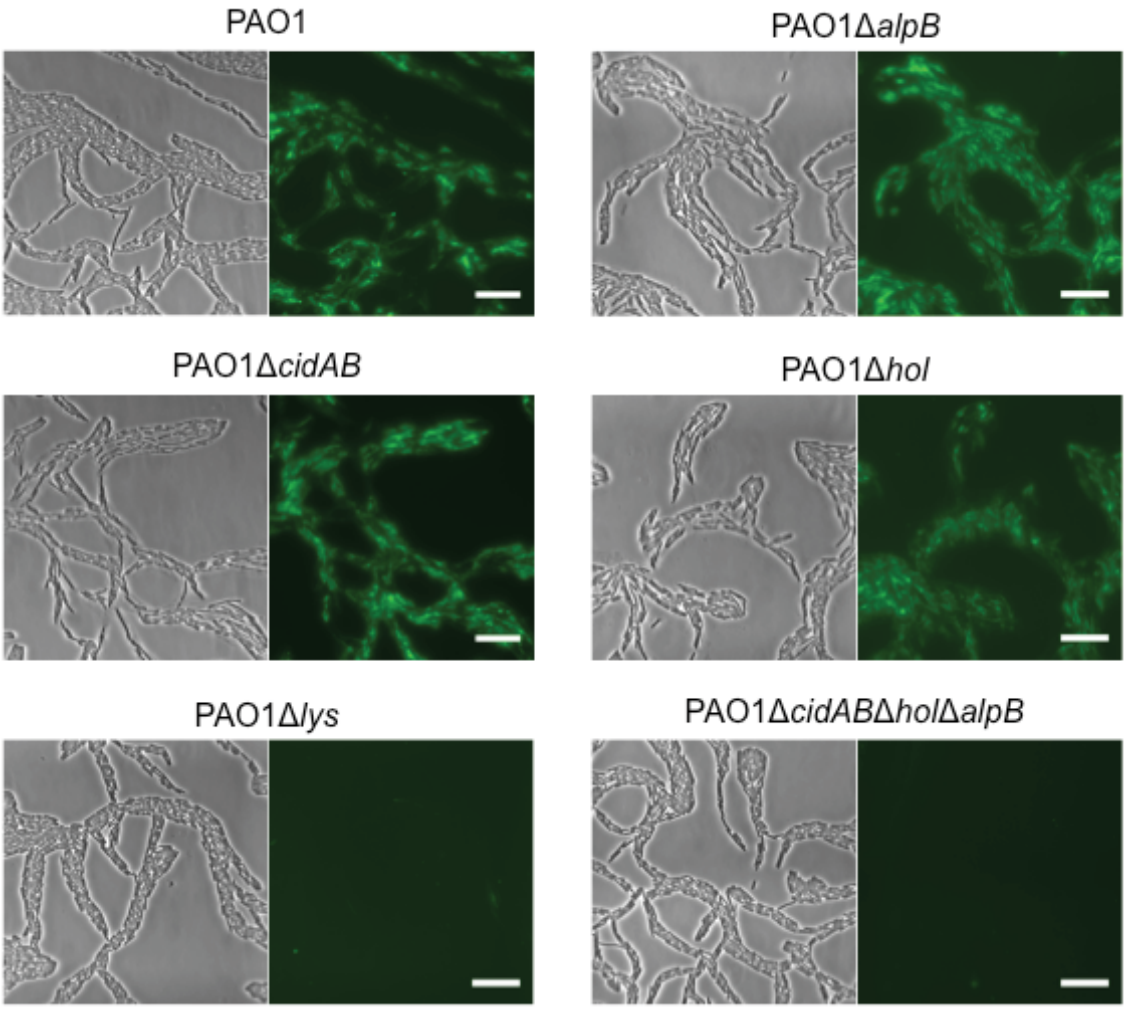


Figure 5.6: Holins are involved in stress induced explosive cell lysis.
Phase-contrast (left) and Ethidium homodimer-2 stained eDNA (green, right) of interstitial biofilms of PAO1, PAO1Δ*alpB*, PAO1Δ*cidAB*, PAO1Δ*hol*, PAO1Δ*lys* and PAO1Δ*cidAB*Δ*hol*Δ*alpB* in the presence of filter discs saturated in MMC; scale bar, 20 μm.

5.3.3 Frequency of explosive cell lysis

We also analysed the frequency of round cells in a population at a point in time to determine the frequency of cells undergoing explosive cell lysis. Randomly selected fields of view of interstitial biofilms of PAO1, the single, double and triple holin deletions were imaged with phase-contrast microscopy and computer vision analyses were utilised to identify cell morphotypes and categorise them as rod or round cells (refer to section 2.4.3.3) (247). In PAO1, approximately 1 in every 2,500 cells undergoes explosive cell lysis (Figure 5.7). Deletion of any single holin dramatically decreases the number of round cells in the population, down to 1 in every 12,000, 22,000 and 12,000 cells in PAO1Δ*alpB*, PAO1Δ*cidAB* and PAO1Δ*hol*, respectively (Figure 5.7). Double deletions resulted in further reduction of round cells, with no round cells found in PAO1Δ*hol*Δ*alpB* and the triple deletion, identical to PAO1Δ*lys*. The number of round cells present in the population at any point in time is dependent on the frequency of explosive events and the survival time of the round cells. As the frequency of round cells did not decrease proportionally to the decrease in eDNA release events in the deletion mutants, the survival time of the round cells may have changed.

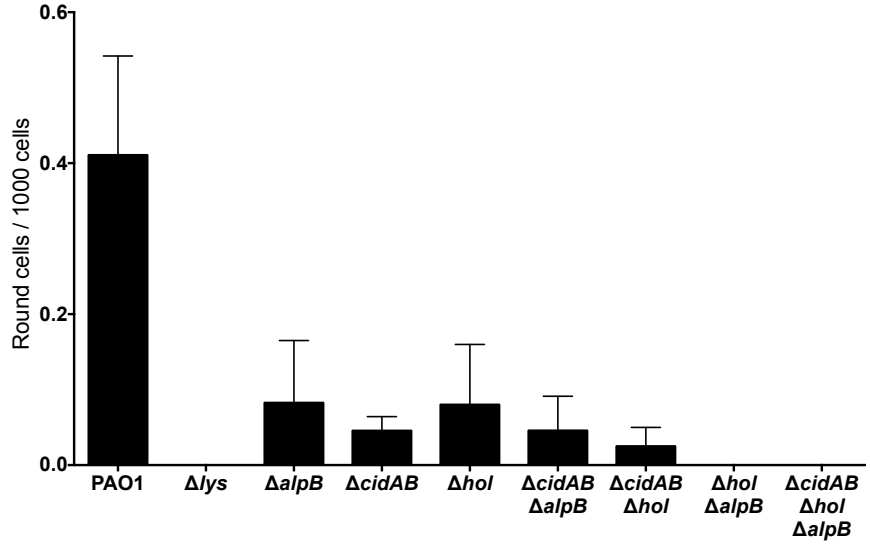


Figure 5.7: Frequency of round cells in interstitial biofilms.

Proportions of round cells in interstitial biofilm monolayers of PAO1, PAO1Δ*lys,* PAO1Δ*alpB*, PAO1Δ*cidAB*, PAO1Δ*hol*, PAO1Δ*cidAB*Δ*alpB*, PAO1Δ*cidAB*Δ*hol*, PAO1Δ*hol*Δ*alpB* and PAO1Δ*cidAB*Δ*hol*Δ*alpB.* Computer vision was used to identify cells in 60 random images of each strain and classified as having either rod or round morphotype (247). The total number of cells for each strain was PAO1 47092*,* PAO1Δ*alpB* 82814, PAO1Δ*cidAB* 137584, PAO1Δ*hol* 67830, PAO1Δ*cidAB*Δ*alpB* 89368, PAO1Δ*cidAB*Δ*hol* 87846, PAO1Δ*hol*Δ*alpB* 111344 and PAO1Δ*cidAB*Δ*hol*Δ*alpB* 2022; *n* = 30; mean ± s.e.m.

Phase-contrast time-lapse microscopy was also performed to determine the frequency of explosive cell lysis events. The leading edge portion of interstitial biofilms of PAO1, the single and double holin deletions were tracked for 100 min and the number of explosive cell lysis events for each strain was recorded. Deletion of each holin individually reduced the number of explosive events in 100 min compared to PAO1 (Figure 5.8). Interestingly PAO1Δ*cidAB* had fewer explosive events than PAO1Δ*alpB* and PAO1Δ*hol.* Furthermore, PAO1Δ*cidAB*Δ*hol* had similar number of events as PAO1Δ*cidAB*, suggesting *cidAB* is more important than *hol* in mediating the initial explosive cell lysis events occurring in the biofilm. PAO1Δ*cidAB*Δ*alpB* and PAO1Δ*hol*Δ*alpB* had the least lysis events suggesting *alpB* in coordination with *cidAB* and *hol* is responsible for the majority of lysis events occurring in the biofilm.

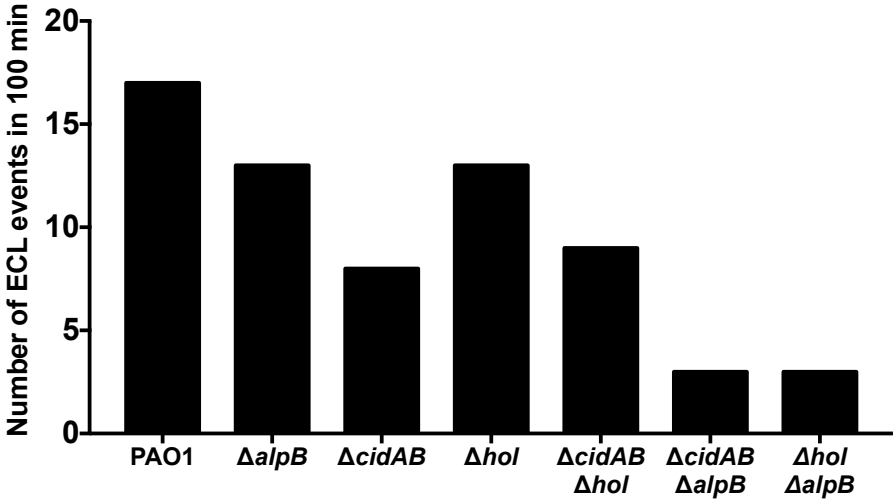


Figure 5.8: Holins affect round cell and lysis frequency.

Number of explosive cell lysis (ECL) events in 100 min (min) decreases with deletion of holins. Interstitial biofilms were cultured for 4 h before imaging commenced.

5.3.4 Involvement of PrtN

The *lys* and *hol* genes are both encoded as part of the pyocin gene cluster and PrtN has been shown to regulate expression of this cluster (223). McFarland *et al*. (227) used a *prtN* deletion mutant to demonstrate that the pyocin gene cluster was not involved in the novel self-lysis pathway controlled by the *alp* operon, under the assumption that *prtN* is required for pyocin-associated cell death (223). They reported no defect of the AlpB-mediated cell death pathway in PAO1Δ*prtN* and therefore concluded the pyocin gene cluster is not involved in this pathway. However the results in this Chapter show that Lys is likely to be the mediator of AlpB-mediated lysis. In addition to this, McFarland *et al*. (227) show that a significant number of cells lyse in the absence of the *alp* operon under DNA damaging conditions in a similar manner to cells undergoing explosive cell lysis in DNA damaging conditions. This may be due to the induction of explosive cell lysis as well. We therefore explored the involvement of *prtN* by testing the assumption that deletion of *prtN* will abrogate explosive cell lysis.

We examined explosive cell lysis-mediated eDNA release in PAO1Δ*prtN* interstitial biofilms to determine whether PrtN is involved in explosive cell lysis (refer to section 2.4.1 and 2.4.3.1). Interestingly, PAO1Δ*prtN* showed a 17% reduction in eDNA release in interstitial biofilms with PAO1Δ*prtN* showing 179 eDNA release sites per mm^2 of interstitial biofilm compared to 215 for PAO1. However this reduction is not sufficient to confirm that it is required for pyocin-associated cell death through explosive cell lysis under these conditions (Figure 5.9). This indicates that PrtN may not be a regulator of *lys* and explosive cell lysis. The use of *prtN* as an indicator of pyocin gene cluster expression may not be accurate for *lys* expression. Together this suggests *lys* may have a different regulatory pathway than other genes in the pyocin cluster. It is yet to be determined whether *lys* is regulated directly by RecA or a different intermediate other than PrtN.

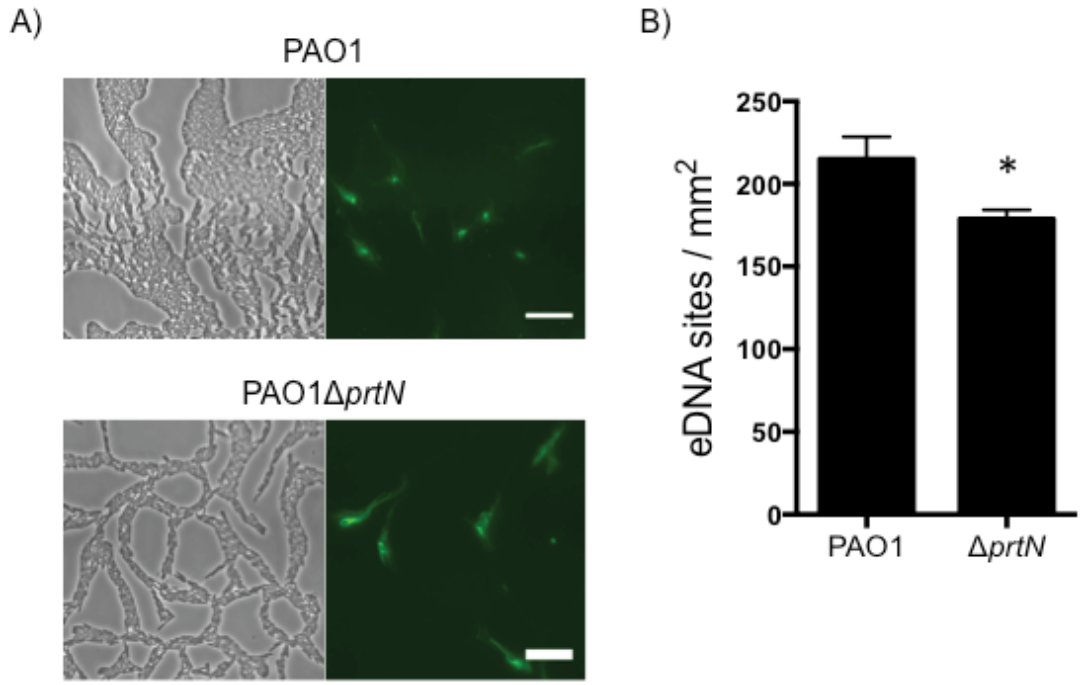


Figure 5.9: Pyocin gene cluster regulator PrtN not required for explosive cell lysis.

A) Phase-contrast (left) and Ethidium homodimer-2 stained eDNA (green, right) of interstitial biofilms of PAO1 and PAO1Δ*prtN*; scale bar, 20 μm. B) PrtN is not an important regulator of explosive cell lysis; n = 30; mean ± s.e.m. * P = 0.0168, unpaired t-test with Welch's correction.

5.4 Discussion

In this Chapter, we have shown that explosive cell lysis is mediated by the action of three independent holins, Hol, AlpB and CidAB. Deletion of each holin individually was not sufficient to abrogate explosive cell lysis-mediated eDNA release in interstitial biofilms. eDNA production was only abrogated when all three holins were deleted. These three holins appear to work in coordination to release eDNA in interstitial biofilms under inducing and non-inducing conditions. It is most likely that these holins contribute to explosive cell lysis by transporting the endolysin to the periplasm.

For most bacteriophage lytic systems a minimum of a holin and an endolysin are required (159-161). In the majority of cases an endolysin is unable to cause cell lysis without the aid of a holin to transport it to the cytosol (162). The studies that identified the holins AlpB and CidAB in *P. aeruginosa* did not identify an endolysin responsible for the respective cell death pathways (67, 227). The findings of this Chapter suggest that both AlpB and CidAB can

transport Lys and therefore Lys may also be involved in the AlpB- and CidAB-mediated cell death pathways. It is yet to be determined whether these three holins share a regulatory pathway, when each holin is expressed and how each holin is responsible for transporting Lys. To our knowledge, this is the first investigation into the involvement of multiple holins in a single cell death pathway. We found that although these holins are functionally similar, it is possible that they each play a unique role in explosive cell lysis, as not all holins could compensate the loss of another holin. It is also possible that each holin pathway is involved in explosive cell lysis in a sequential manner, which may encourage continual eDNA release during biofilm formation. Future examination of the temporal expression of these holins will further determine when each holin is involved in the explosive cell lysis process. In addition, quantifying protein expression levels with Western blotting of the holins in each single holin deletion mutant background could help further understand how expression of the holins change when one holin is removed. In addition, the use of promoter reporters of each holin could be used to follow gene expression.

We also further investigated the involvement of the pyocin cluster regulator PrtN. Deletion of *prtN* did not show a sufficient defect in eDNA production for it to be conclusively confirmed to be a regulator of explosive cell lysis, indicating *lys* may have a different regulatory pathway than other genes in the pyocin gene cluster. This is significant as McFarland *et al*. (227) used a *prtN* deletion to demonstrate that the pyocin-associated cell death pathway was independent of the AlpB-mediated cell death. Furthermore the involvement of Lys in the AlpB-mediated cell death pathway cannot be ruled out either. As explosive cell lysis and AlpB-mediated cell death are visually similar, these two pathways may both use the endolysin Lys as the mediator. As no endolysin has been identified in the involvement of AlpB-mediated cell death, examining cell death in PAO1Δ*lys* under the conditions tested by McFarland *et al*. (227) will determine if Lys is also responsible for this cell death pathway. Performing qRT-PCR may further elucidate how the endolysin and holins are regulated. It is possible that *hol* is regulated by PrtN and the other holins are likely to have their own regulators as described by Ma *et al.* (67) and McFarland *et al.* (227). RecA or another intermediate regulator may also be responsible for activating the production of each holin as well, which could be identified through qRT-PCR.

Taken together, our data demonstrates that holins from three previously described cell death pathways all contribute to the transportation of Lys, leading to explosive cell lysis. The current assumption is that bacteriophage lytic systems are mutually exclusive even though they utilise functionally similar proteins. We have shown there can be overlap of independent pathways where each holin has a unique contribution to one cell death pathway.

Chapter Six:

Explosive Cell Lysis Mediates Microcolony Formation

6 Explosive Cell Lysis Mediates Microcolony Formation

6.1 Publication information and other author contributions

The data in sections 6.3.3 and 6.3.4 of this Chapter contributed to the following publication:

L. Turnbull*, M. Toyofuku*, A. L. Hynen, M. Kurosawa, G. Pessi, N. K. Petty, S. R. Osvath, G. Cárcamo-Oyarce, E. S. Gloag, R. Shimoni, U. Omasits, S. Ito, X. Yap, L. G. Monahan, R. Cavaliere, C. H. Ahrens, I. G. Charles, N. Nomura, L. Eberl & C. B. Whitchurch, Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nature Communications* **7**, Article number: 11220 (2016); doi:10.1038/ncomms11220.

* Denotes equal contribution.

The results presented in this Chapter were all generated and analysed by Amelia L. Hynen with the following exceptions:

- Construction of the PAO1Δ*lys* strain and pJN105*lys* plasmid: Masanori Toyofuku, University of Tsukuba, Japan.

6.2 Introduction

eDNA is an essential biofilm matrix component of *P. aeruginosa* biofilms, crucial to the development of submerged biofilms (79), facilitating cell-cell and cell-matrix interactions that stabilise the multicellular communities (70, 84, 86, 92, 95). eDNA release in mature stages of biofilm development is regulated through QS-dependent mechanisms involving AHL and PQS (92, 176) and is also mediated by the filamentous Pf4 phage (186). eDNA release through these systems is the result of lysis of a sub-population of cells (92, 171, 314). However, these QS-dependent systems and filamentous phage are only in play during the mature stages of biofilm development. It is evident that eDNA is produced at the early stages of biofilm development, before QS systems are in play, as demonstrated by the necessity of eDNA in the formation of biofilms (79). The mechanisms of eDNA production during the early stages of biofilm formation and its role in microcolony formation is not well understood.

In Chapter 3 we demonstrated that eDNA production in interstitial biofilms is mediated by the Lys endolysin encoded within the R- and F-pyocin gene cluster. As eDNA is required to form submerged biofilms (79) and explosive cell lysis has been observed in planktonic culture and during the early stages of biofilm development (Turnbull and Whitchurch, personal communication), we hypothesised that Lys-mediated explosive cell lysis may play a role in the initial stages of biofilm formation. The aim of this Chapter is to examine the role of explosive cell lysis mediated-eDNA release in microcolony formation during the early stages of biofilm development.

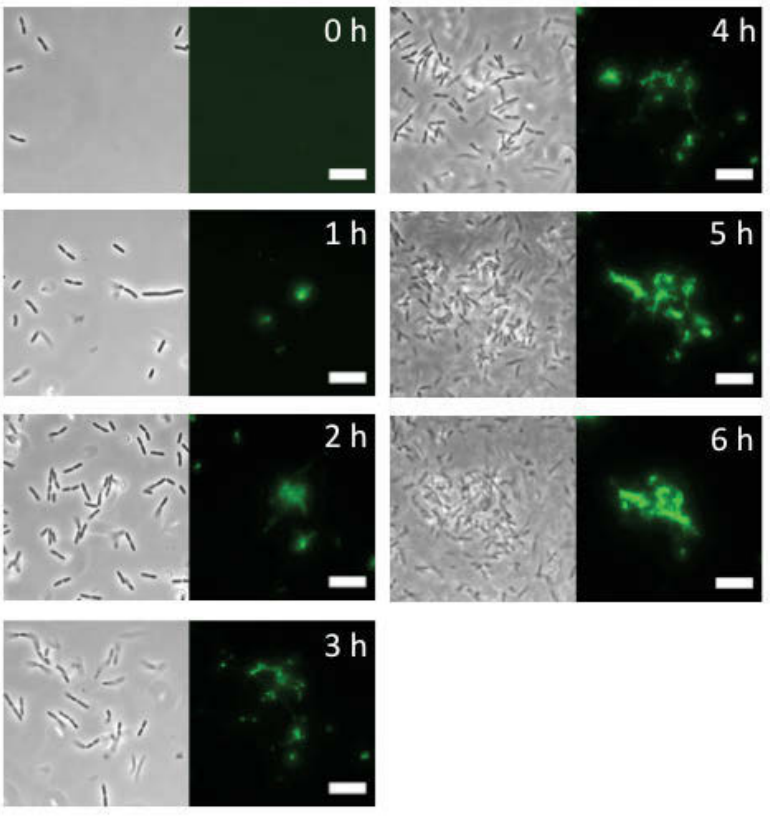
6.3 Results

6.3.1 eDNA release is necessary for microcolony formation

To determine if eDNA release is correlated with the formation of microcolonies, we performed time-lapse microscopy of submerged biofilm formation in PAO1 with the nucleic acid stain TOTO-1 to stain eDNA and recorded the times that eDNA was released and microcolonies first appeared (see sections 2.5.1 and 2.5.1.1).

We observed that explosive cell lysis occurs at the substratum, releasing eDNA, which can be seen by staining with TOTO-1 (Figure 6.1A). eDNA is first released at 1-1.5 h after inoculation, well before any microcolonies are formed. Bacterial aggregates begin to appear at 3-4 h, a couple of h after the initial explosive cell lysis events (Figure 6.1A and B). These bacterial aggregates then form microcolonies > 100 μm^2 by 6 h. We followed 50 of these eDNA release sites over 6 h and recorded their *x* and *y* coordinates as well as the coordinates of microcolonies that were present at 6 h (Figure 6.1C) (refer to section 2.5.1.1). We observed the formation of 32 microcolonies over 6 h. All of these microcolonies had formed at sites of previous eDNA release, indicating the deposition of eDNA initiates the formation of microcolonies.

A)



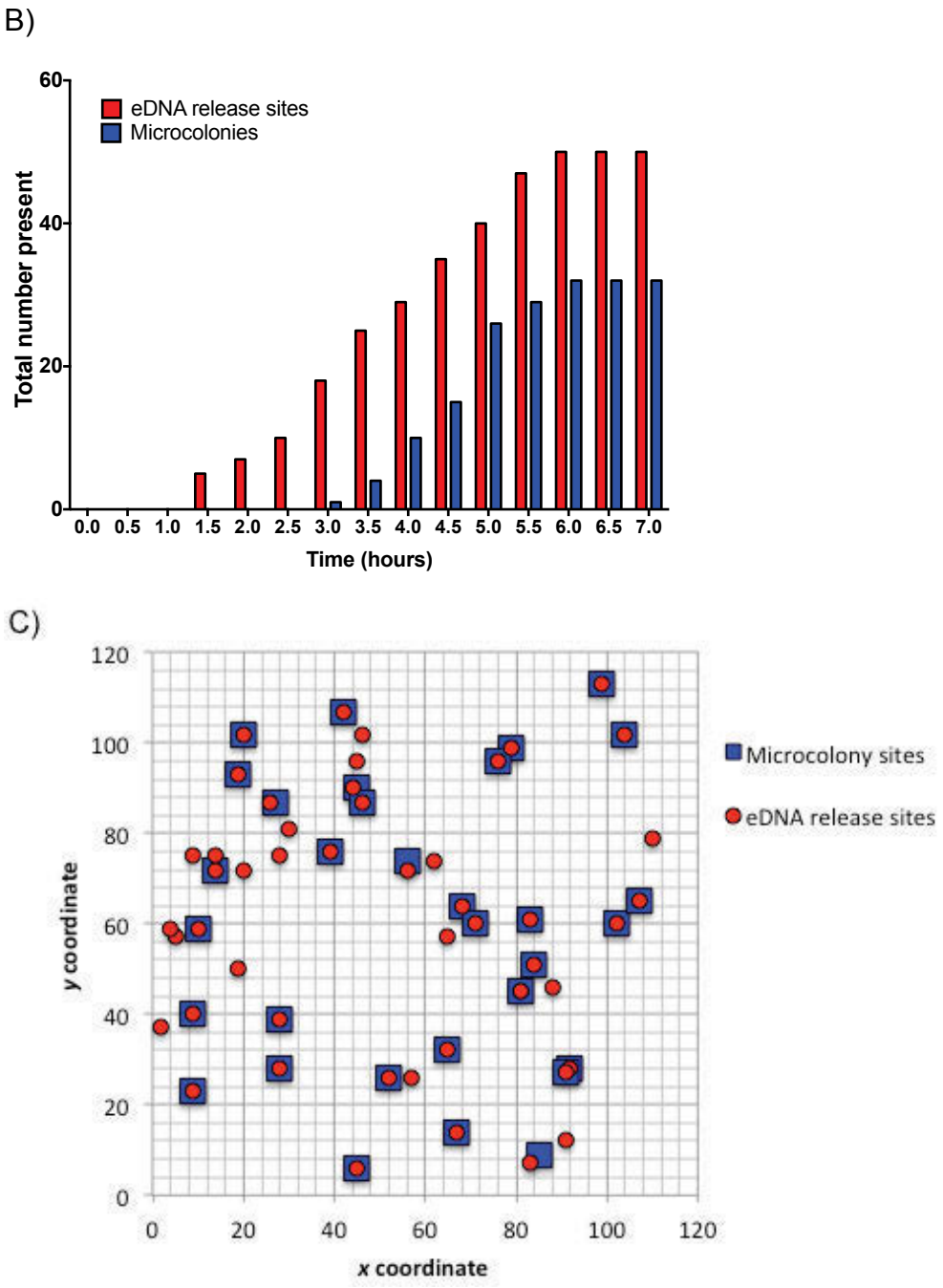


Figure 6.1: Explosive cell lysis initiates microcolony formation in PAO1.

A) Microcolony formation over time in PAO1. eDNA stained with TOTO-1 (green), time indicated in h (h), scale bar, 10 µm. B) Total number of eDNA release sites and microcolonies formed over time in PAO1. C) Coordinates of eDNA release sites and microcolonies. 50 eDNA release events were followed over 6 h and their x and y coordinates were recorded (•, red). The coordinates of microcolonies at 6 h were recorded (■, blue), with a cut off size of > 30 µm^2. If an eDNA release site overlays with a microcolony, they were considered to co-localise.

6.3.2 Continual eDNA release

The formation of microcolonies at eDNA release sites may be dependent on continual production of eDNA through multiple explosive cell lysis events at the same location to create sufficient amounts of eDNA to build microcolonies and stabilise their structures. This is supported by the appearance of multiple eDNA spots and the increase in fluorescence intensity throughout microcolony formation in Figure 6.1A. To examine this further, eDNA fluorescence was measured by integrated density (the product of area and mean grey value) and the size of the microcolonies identified in 6.3.1 were measured (see section 2.5.1.1).

Over time, the fluorescence of eDNA increases steadily in PAO1 as measured by integrated density (Figure 6.2). Similarly, microcolony size also increases over time, suggesting that the release of more eDNA is correlated with increase in microcolony size. Together this demonstrates that eDNA is produced before the formation of microcolonies and suggests that microcolony formation may be temporally and spatially correlated to explosive cell lysis-mediated eDNA release.

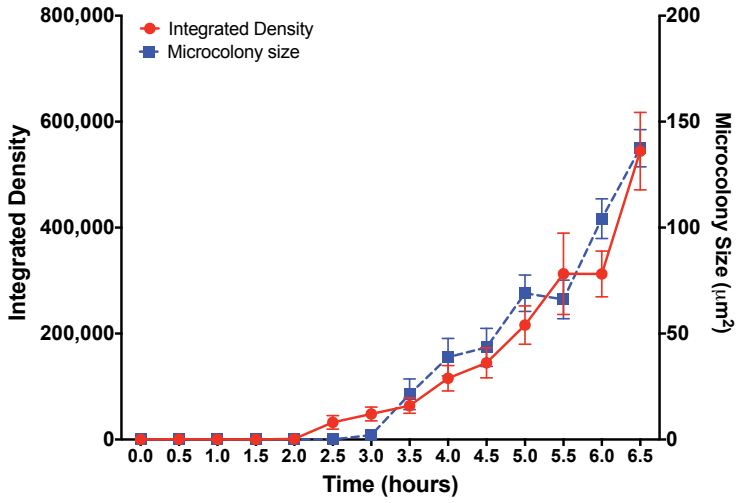


Figure 6.2: Sequential release of eDNA during microcolony formation.
eDNA fluorescence as measured by integrated density and microcolony size (μm^2) over time in the development of PAO1 submerged biofilms. *n* = 32, mean ± s.e.m.

6.3.3 Explosive cell lysis is required for microcolony formation

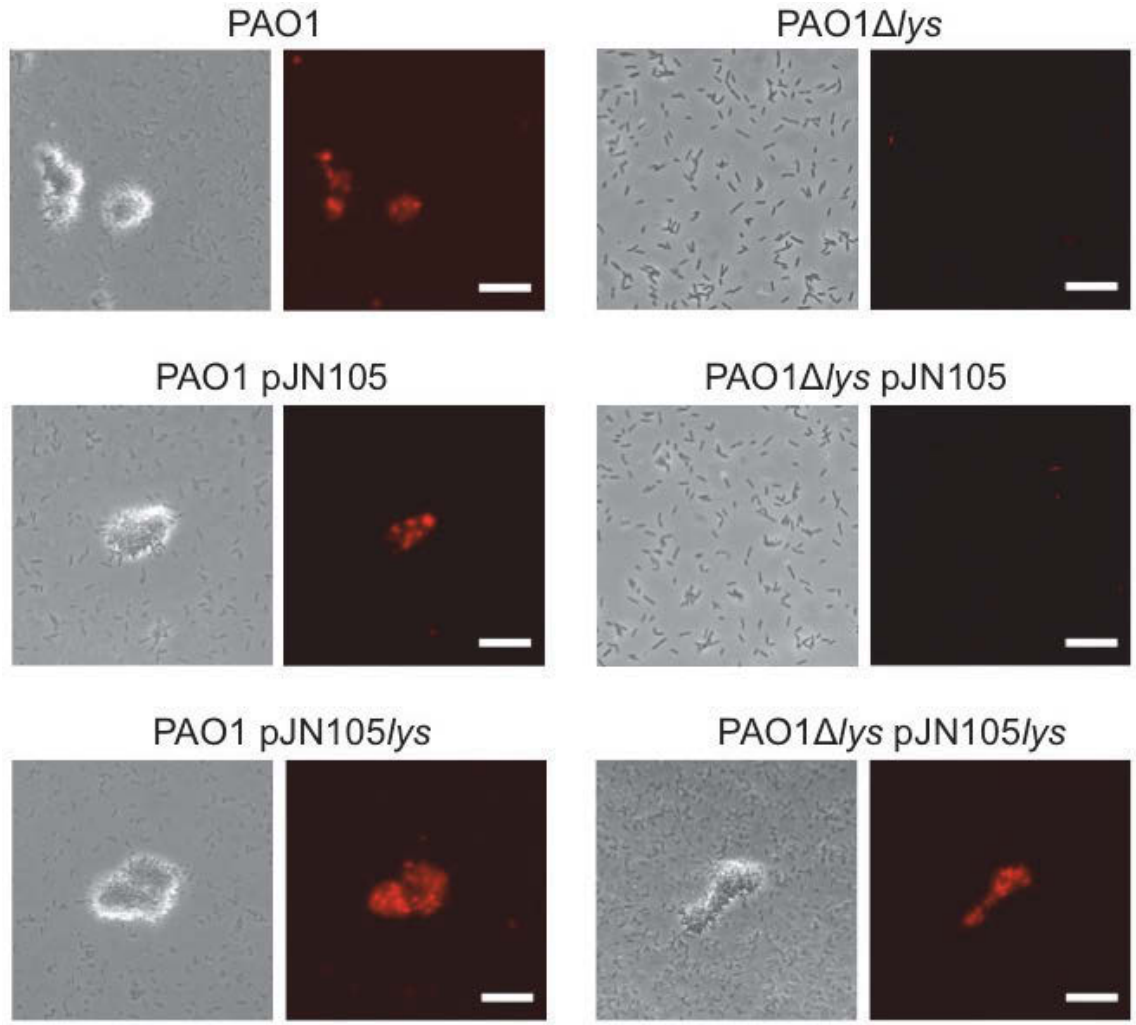
As microcolony formation is dependent on eDNA release, we investigated whether this was via Lys-mediated explosive cell lysis. Submerged

biofilms of PAO1 and PAO1Δ*lys* were cultured statically for 8 h and the resulting microcolony structures attached to the substratum were stained with the eDNA stain EthHD-2 to identify areas of eDNA (refer to section 2.5.1). An 8 h incubation period was chosen to examine the early stages of biofilm formation as eDNA release is crucial in these early stages and eDNA is likely to come from explosive cell lysis than other sources such as quorum-sensing mechanisms. From random fields of view, microcolonies were identified as multi-cellular structures ≥ 100 μm^2, the number of microcolonies present was counted and the number of microcolonies per mm^2 was calculated (refer to section 2.5.1.2).

After 8 h, PAO1 formed numerous microcolonies across the substratum, with eDNA throughout the structures (Figure 6.3A). PAO1Δ*lys* was severely defective compared to the wild-type, producing no microcolonies or eDNA at the substratum (Figure 6.3 A and B). Microcolony formation and eDNA production could be restored by complementing PAO1Δ*lys* with *lys* provided *in trans,* demonstrating *lys* is required for eDNA production and microcolony formation in submerged biofilms of *P. aeruginosa*.

As previously discovered by Whitchurch *et al.* (79), DNase I has the ability to prevent biofilm formation at the early stages of biofilm development. We examined whether the addition of DNase I prevented microcolony formation under these conditions. Submerged biofilms of PAO1 and PAO1Δ*lys* were cultured in the presence of DNase I (100 Kunitz units/mL) and after 8 h the attached microcolony structures were stained with eDNA stain EthHD-2 to identify areas of eDNA (see sections 2.5.1 and 2.5.1.2). As shown in Figure 6.4, PAO1 cultured in the presence of DNase I prevented the formation of microcolonies, resulting in the same phenotype as PAO1Δ*lys*. PAO1Δlys formed no microcolonies in the presence and absence of DNase I. Since PAO1Δ*lys* is just as defective as the addition of DNase I in PAO1, it suggests that all eDNA in the initial stages of biofilm development is released via Lys. Together this confirms that eDNA is required for microcolony formation under these conditions and that Lys-mediated explosive cell lysis is responsible for all eDNA released in submerged biofilm development.

A)



B)

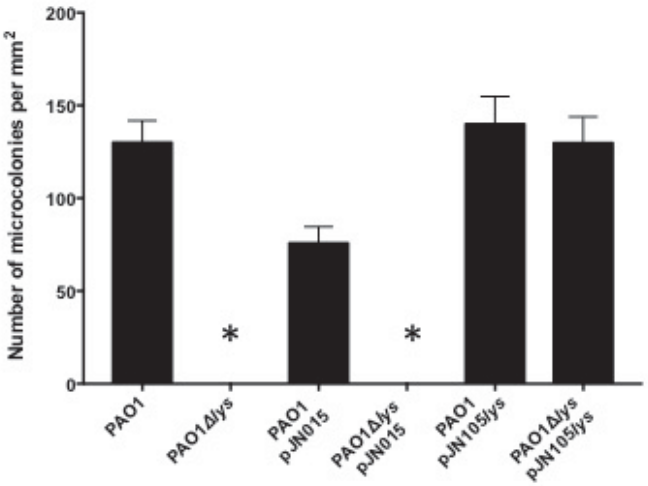


Figure 6.3: Lys-mediated explosive cell lysis is essential for microcolony formation up to 8 h.

A) Microcolony structures in 8 h submerged biofilms of PAO1 and PAO1Δ*lys*, alone or containing vector control (pJN105) or complementation plasmid (pJN105*lys*). Representative

phase contrast (left) and eDNA (EthHD-2, right, red) images, scale bar 10 µm. B) Microcolonies per mm^2 in 8 h submerged biofilms of PAO1 and PAO1Δ*lys. n* = 30, mean ± s.e.m. * *P* < 0.0001, unpaired *t*-test with Welch's correction compared to PAO1.

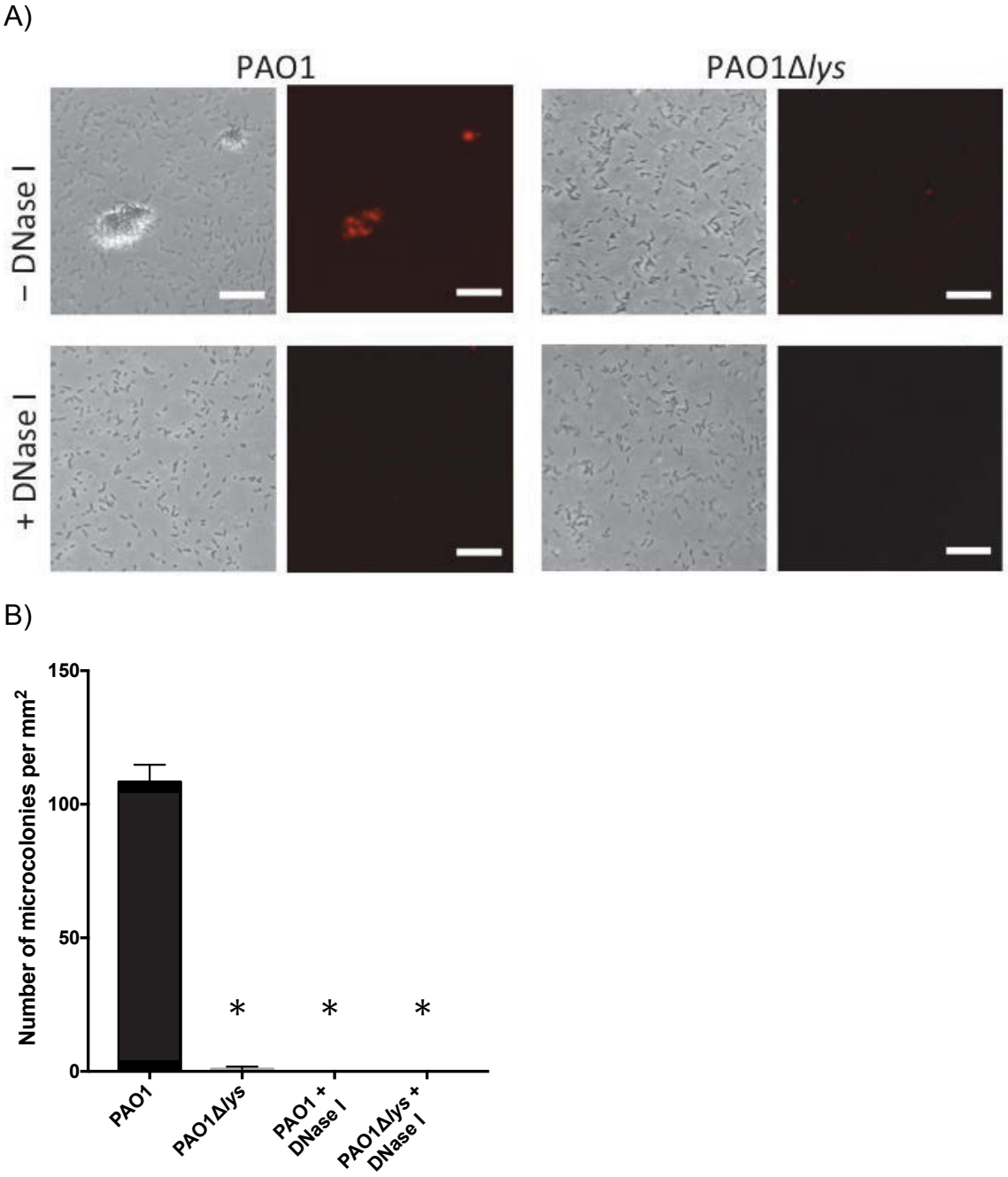


Figure 6.4: eDNA is required for the formation of microcolonies.

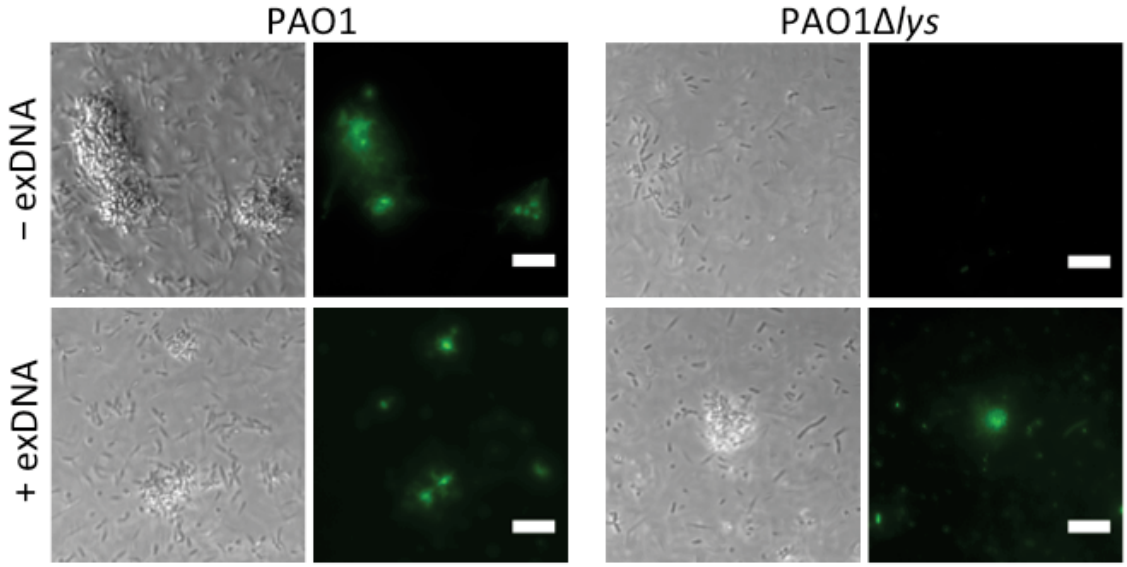
A) Microcolony formation in 8 h submerged biofilms of PAO1 and PAO1Δ*lys* cultured in the presence or absence of DNase I (100 Kunitz units/mL). Representative images of phase contrast (left) and eDNA (EthHD-2, red, right). B) Microcolonies per mm^2 in 8 h submerged biofilms of PAO1 and PAO1Δ*lys* in the presence or absence of DNase I; *n* = 30, mean ± s.e.m. * *P* < 0.0001, unpaired *t*-test with Welch's correction, compared to PAO1.

6.3.4 Effect of exogenous DNA on microcolony formation

Other studies on the requirement of eDNA for submerged biofilm formation in different bacterial species have also investigated whether the addition of exogenous DNA can enhance or restore biofilm formation in the wild-type and defective strains, respectively (167, 263). We examined whether exogenously added DNA alone is sufficient to restore microcolony formation in PAO1Δ*lys* by culturing the bacteria in the presence of purified *P. aeruginosa* PAO1 genomic DNA for 8 h (final concentration 1 µg/mL) (see section 2.3.2).

As shown in Figure 6.5, addition of exogenous DNA was able to partially rescue biofilm formation in PAO1Δ*lys* but it was not sufficient to restore microcolony formation in PAO1Δ*lys* back to wild-type levels. Interestingly, exogenous DNA significantly inhibited microcolony formation in PAO1 (Figure 6.5B). This suggests that eDNA needs to be produced in concentrated foci at the substratum as previously observed in section 6.3.1 and/or other matrix components that are released through explosive cell lysis are also required to initiate microcolony formation.

A)



B)

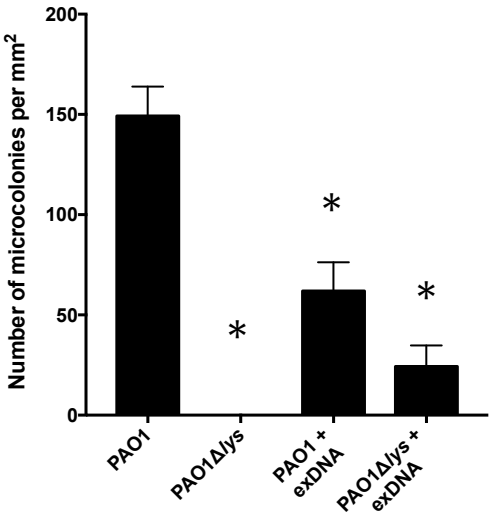


Figure 6.5: Exogenously added DNA is not sufficient to restore microcolony formation in PAO1Δ*lys*.

A) Microcolonies in 8 h submerged biofilms of PAO1 and PAO1Δ*lys* cultured in the presence or absence of exogenously added DNA (exDNA) at a final concentration of 1 µg/mL. Representative phase contrast (right) and eDNA (EthHD-2, green, right) images; scale bar 10 µm. B) Microcolonies per mm^2 in 8 h submerged biofilms of PAO1 and PAO1Δ*lys* in the presence or absence of exDNA; n = 30, mean ± s.e.m. * P < 0.0001, unpaired t-test with Welch's correction, compared to PAO1.

6.3.5 Holins involved in microcolony formation

As Lys-mediated explosive cell lysis is required for the formation of microcolonies and we showed in Chapter 5 that the three holins Hol, AlpB and CidAB could each contribute to the translocation of Lys in interstitial biofilms, we examined the importance of these three holins in the formation of microcolonies in submerged biofilms. Submerged biofilms of PAO1, PAO1Δ*lys*, PAO1Δ*alpB*, PAO1Δ*cidAB,* PAO1Δ*hol* and PAO1Δ*alpB*Δ*cidAB*Δ*hol* were cultured for 8 h after which the attached microcolony structures were stained with EthHD-2 to identify the presence of eDNA (refer to sections 2.5.1 and 2.5.1.2).

Microcolony structures were formed by PAO1 with eDNA present throughout the microcolony (Figure 6.6A). Significantly fewer microcolony structures were formed by the single holin deletions, with eDNA in the centre of these structures (Figure 6.6A and B). These microcolonies were also significantly smaller than those formed by PAO1 (Figure 6.6C) possibly to due fewer explosive events occurring. Microcolony formation could also be

complemented in the single deletion mutants by providing the wild-type gene *in trans* (Figure 6.7). The triple deletion was as defective in forming microcolonies and producing eDNA at the substratum as the explosive cell lysis defective PAO1Δ*lys* strain (Figure 6.6A and B).

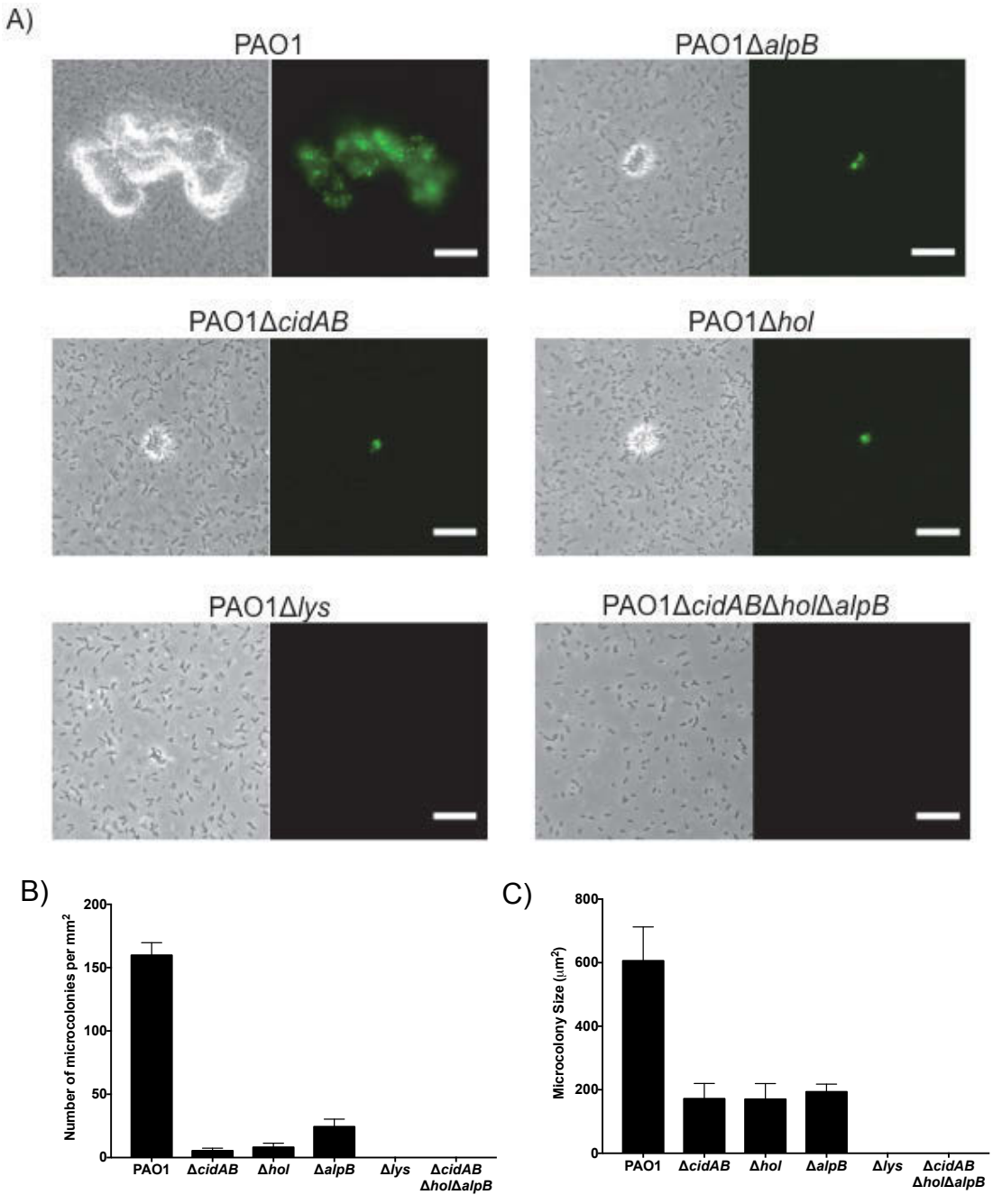


Figure 6.6: Holins affect microcolony formation and size.

A) Representative phase contrast (top) and eDNA (EthHD-2, bottom) of microcolonies formed at 8 h in PAO1, PAO1Δ*lys* and PAO1Δ*cidAB*Δ*hol*Δ*alpB*; scale bar, 10 μm. B) Number of microcolonies per mm^2 in 8 h submerged biofilms; *n* = 30; mean ± s.e.m. C) Microcolony size in 8 h submerged biofilms; *n* = 30; mean ± s.e.m.

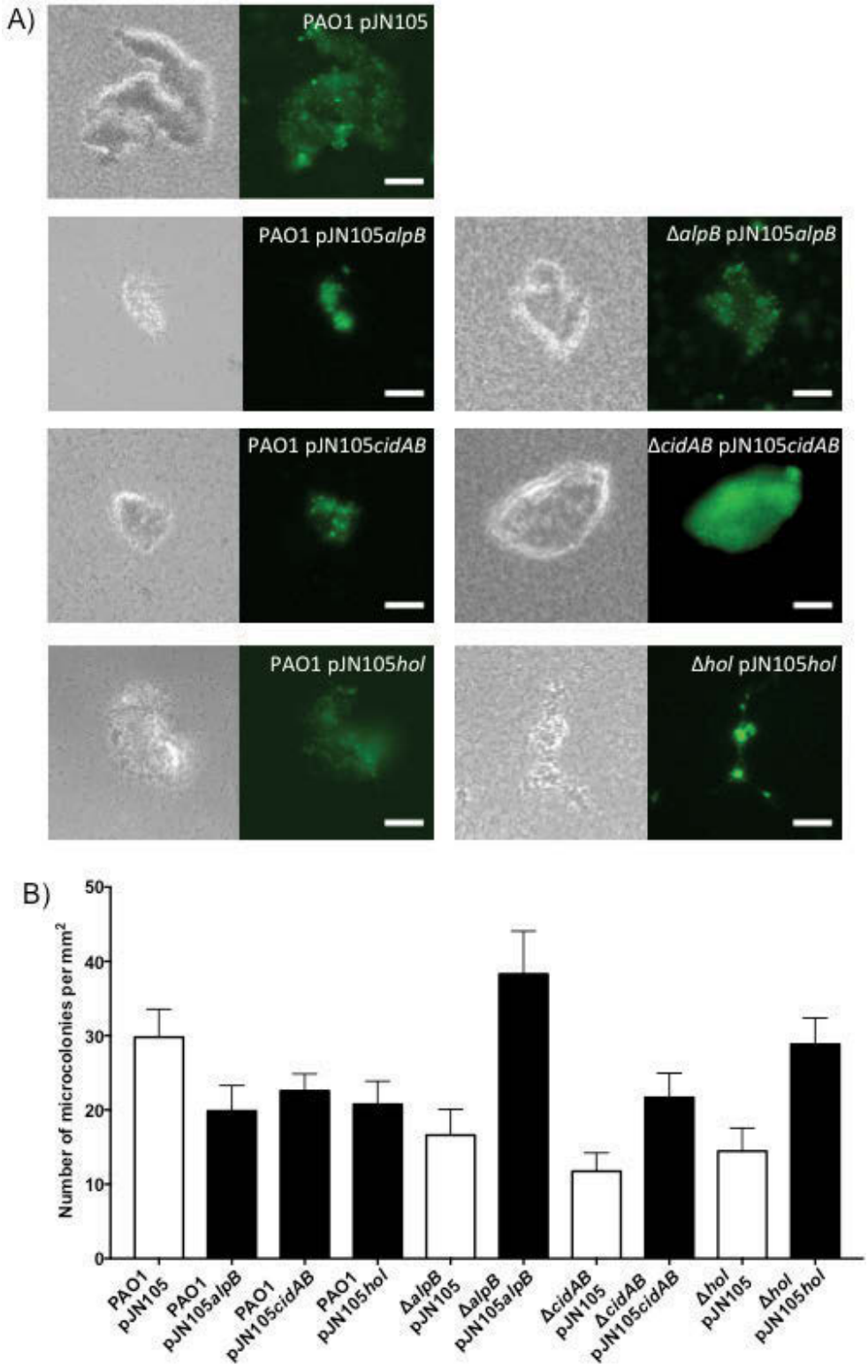


Figure 6.7: Complementation restores microcolony formation in single holin deletions.

A) Phase-contrast (left) and eDNA (EthHD-2, green, right) of microcolonies formed at 8 h by PAO1, PAO1Δ*alpB*, PAO1Δ*cidAB* and PAO1Δ*hol* with pJN105 (vector control) or complementing plasmids pJN105*alpB,* pJN105*cidAB* and pJN105*hol,* scale bar 20 µm. B) Number of microcolonies formed per mm^2; *n* = 30; mean ± s.e.m.

Microcolony formation over time was also examined in the single holin deletions to determine if these holins were important in the timing of eDNA release and microcolony formation in submerged biofilms. PAO1Δ*alpB*, PAO1Δ*cidAB* and PAO1Δ*hol* were cultured in the presence of eDNA stain EthHD-2 and 50 eDNA release sites were followed over 6 h for each strain. The *x* and *y* coordinates of the eDNA release sites and microcolonies at 6 h were recorded. The total number of eDNA release sites and microcolonies present at each time point was also recorded (refer to section 2.5.1.1).

Less eDNA was produced by each holin deletion, resulting in small microcolonies (Figures 6.8 – 6.11). For all the single holin deletions, fewer eDNA release sites gave rise to microcolonies than PAO1 (PAO1Δ*alpB* 21 out of 50; PAO1Δ*cidAB* 19 out of 50; PAO1Δ*hol* 22 out of 50) (Figures 6.8 – 6.10) (refer to section 6.3.1 for PAO1). Interestingly there was no significant difference in the timing of eDNA release, as eDNA first appeared at similar times between the strains and PAO1 (Figure 6.11 A, C and E) however these eDNA release sites were smaller, consisting of less eDNA than PAO1 (Figure 6.11 B, D and F). Microcolonies appeared at a similar time frame as that observed for PAO1 however fewer microcolonies were formed by the holin deletions and they were smaller (Figure 6.11 B, D and F).

We hypothesised that microcolony formation may be dependent upon sequential explosive events utilising all three holins for continual eDNA release as more eDNA is released over time in PAO1 (refer to section 6.3.1). This may strengthen the attachment of bacterial aggregates, allowing them to form larger aggregates. To confirm this, the fluorescence of eDNA over time as determined by integrated density was measured for PAO1Δ*alpB,* PAO1Δ*cidAB* and PAO1Δ*hol* (refer to section 2.5.1.1). Figure 6.11 B, D and F shows the integrated density of eDNA fluorescence increases in a step-wise manner over time in all strains. Interestingly, eDNA fluorescence starts to decrease at 6 – 6.5 h in the single holin deletions whilst it continually increases in PAO1. This may be the result of the eDNA being dislodged from the substratum as it is only produced in small amounts by the holin deletions.

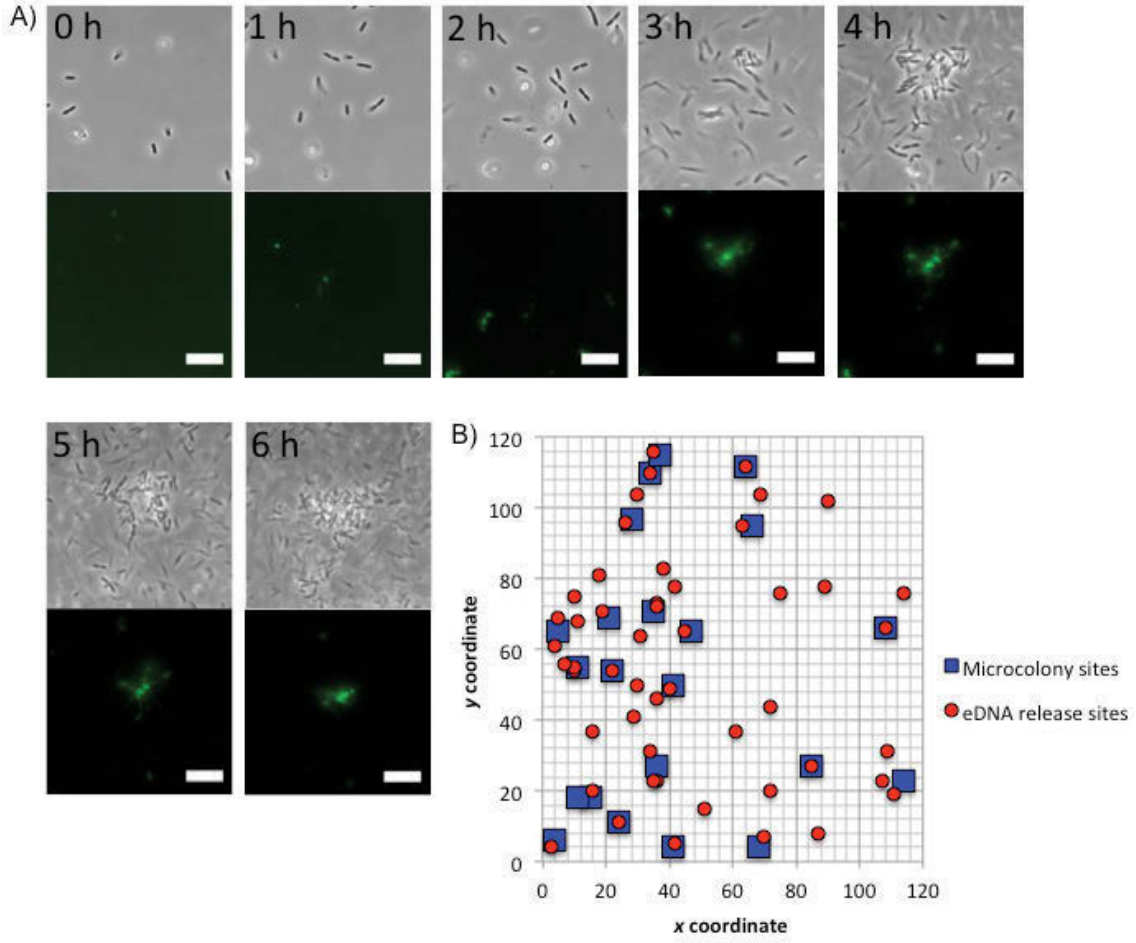


Figure 6.8: Microcolony formation over time in PAO1Δ*alpB*.

A) Microcolony formation over time in PAO1. eDNA stained with Ethidium homodimer-2 (red), time indicated in h (h), scale bar, 10 µm. B) Coordinates of eDNA release sites and microcolonies. 50 eDNA release events were followed over 6 h and their x and y coordinates were recorded (•, red). The coordinates of microcolonies at 6 h were recorded (■, blue), with a cut off size of > 30 μm^2. If an eDNA release site overlays with a microcolony, they were considered to co-localise.

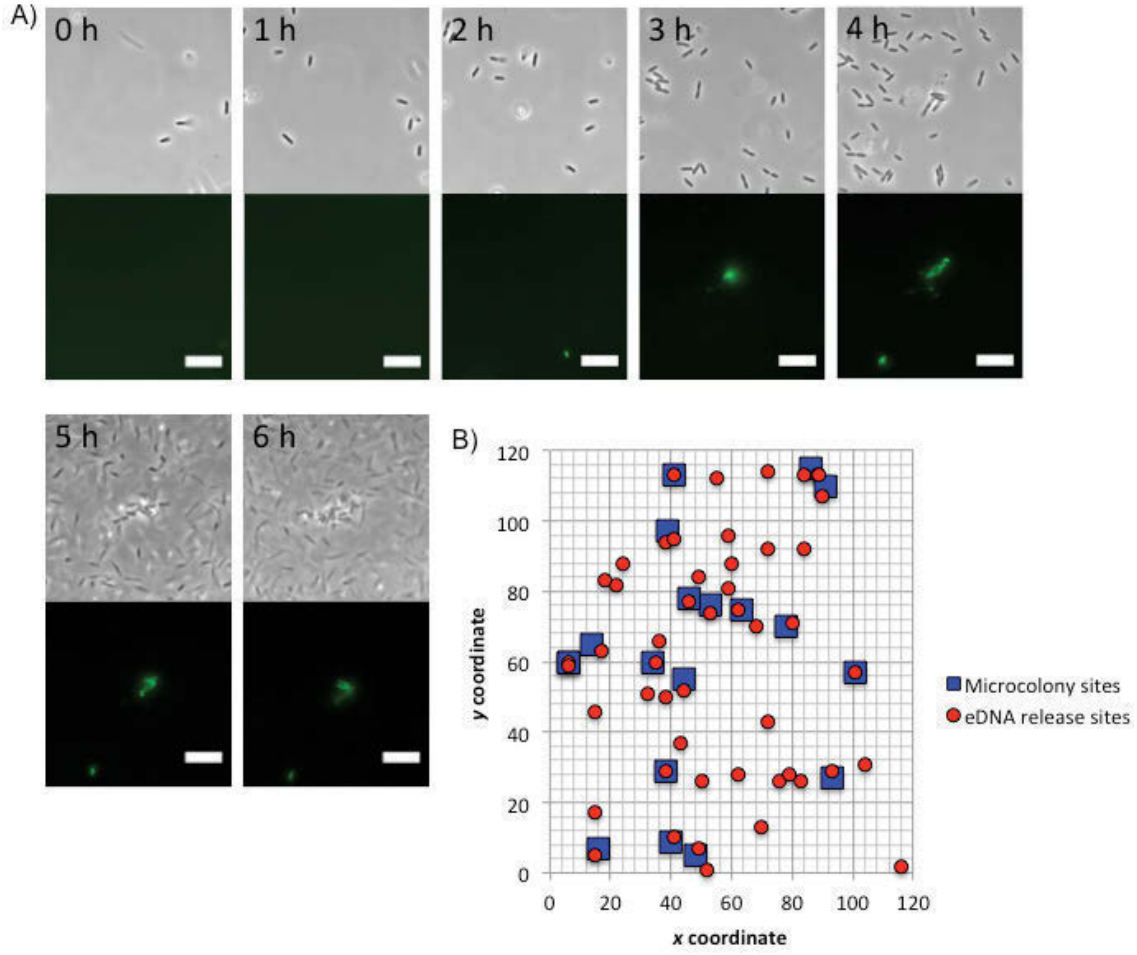


Figure 6.9: Microcolony formation over time in PAO1Δ*cidAB*.

A) Microcolony formation over time in PAO1. eDNA stained with Ethidium homodimer-2 (red), time indicated in h (h), scale bar, 10 µm. B) Coordinates of eDNA release sites and microcolonies. 50 eDNA release events were followed over 6 h and their x and y coordinates were recorded (•, red). The coordinates of microcolonies at 6 h were recorded (■, blue), with a cut off size of > 30 µm^2. If an eDNA release site overlays with a microcolony, they were considered to co-localise.

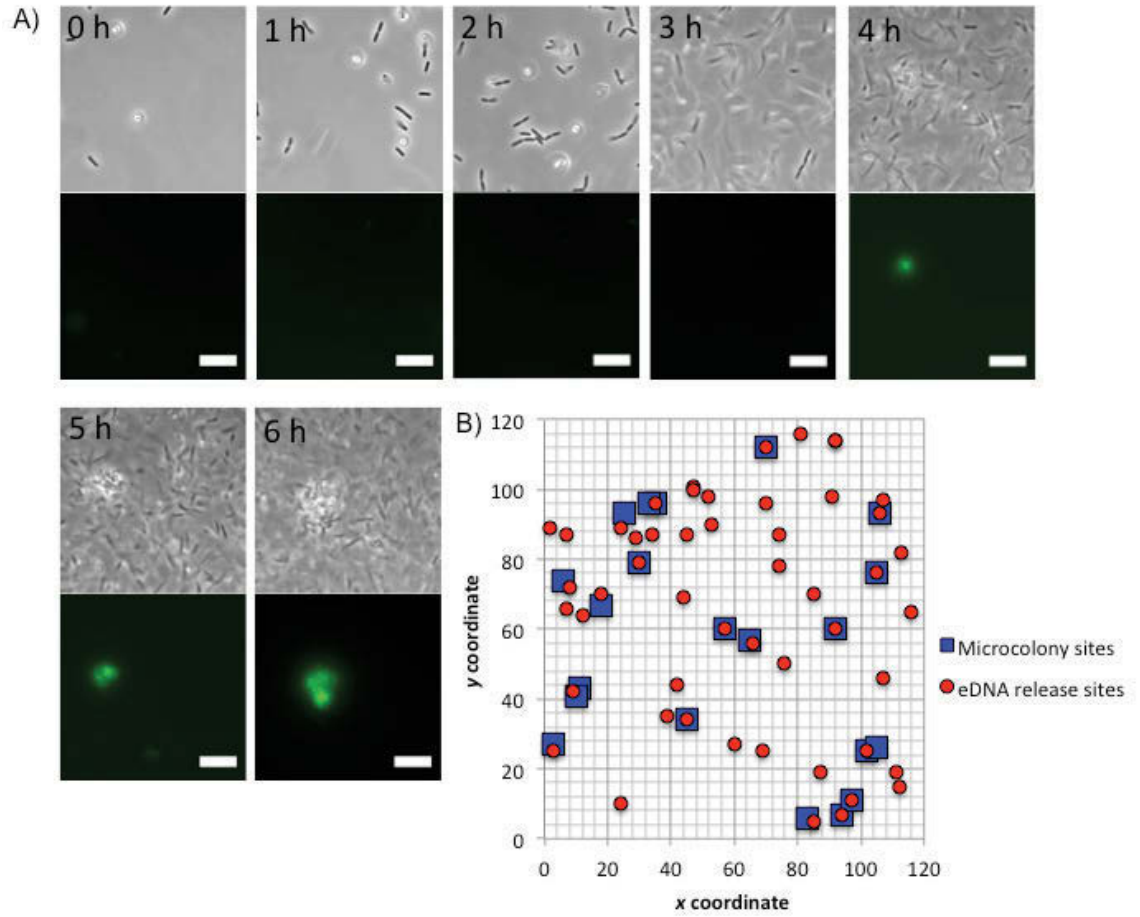


Figure 6.10: Microcolony formation over time in PAO1Δ*hol*.

A) Microcolony formation over time in PAO1. eDNA stained with Ethidium homodimer-2 (red), time indicated in h (h), scale bar, 10 µm. B) Coordinates of eDNA release sites and microcolonies. 50 eDNA release events were followed over 6 h and their x and y coordinates were recorded (•, red). The coordinates of microcolonies at 6 h were recorded (■, blue), with a cut off size of > 30 μm^2. If an eDNA release site overlays with a microcolony, they were considered to co-localise.

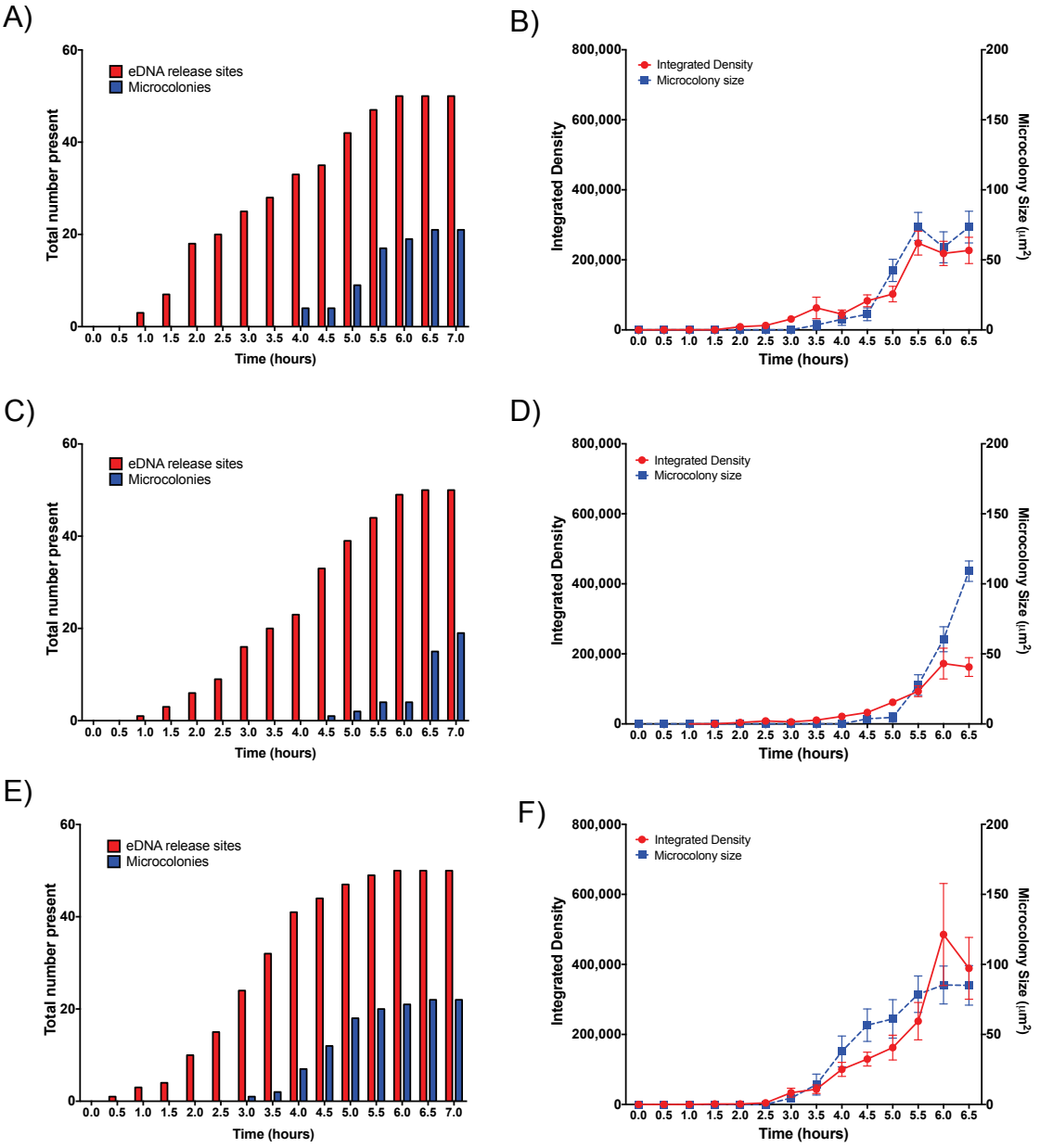


Figure 6.11: Time of eDNA release and microcolony formation.

Total number of eDNA release sites and microcolonies present in A) PAO1Δ*alpB*, C) PAO1Δ*cidAB* and D) PAO1Δ*hol*. 50 eDNA release sites for each strain were followed over the period of 7 h. eDNA fluorescence as measured by integrated density and microcolony size (μm^2) over time in the development of B) PAO1Δ*alpB* (*n* = 21), D) PAO1Δ*cidAB* (*n* = 19) and F) PAO1Δ*hol* (*n* = 22) submerged biofilms; mean ± s.e.m.

6.4 Discussion

In this Chapter, we have shown that explosive cell lysis is required for the formation of microcolonies in the early stages of submerged biofilm development and sequential explosive events are required for the formation of larger microcolonies.

Previous studies on the formation of submerged biofilms of *P. aeruginosa* have focused on how eDNA is produced and its role in established biofilms. We show in this Chapter that eDNA is produced within the first hours of biofilm initiation and produced through explosive cell lysis. Explosive cell lysis events are integral in the formation of bacterial aggregates and these aggregates go on to form microcolonies. When explosive cell lysis is disrupted through the deletion of the endolysin or holins, microcolony formation is severely affected.

Future investigations will examine mature biofilm formation in PAO1Δ*lys*. It is possible that mature PAO1Δ*lys* biofilms do not have the same morphology as PAO1 as eDNA is used in creating the architecture of mature microcolonies as mentioned in section 1.4. As shown in Figure 4.13, PAO1Δ*lys* is capable of forming biofilms with less biomass and these may have a different architecture than PAO1 and possibly produce flat biofilms.

It is also possible that each holin pathway is involved in explosive cell lysis in a sequential manner, which encourages continual eDNA at one site during microcolony formation. Future examination of the temporal expression of these holins will further determine when each holin is involved in the explosive cell lysis process and in the building of microcolonies.

The addition of exogenous DNA throughout microcolony development can have opposing effects in different bacteria depending on the predominant role of the eDNA and the interactions eDNA has with other components in the biofilm matrix. In *Streptococcus pneumoniae* exogenous DNA alone increases biofilm biomass in a dose-dependent manner (167), suggesting that eDNA is the main biofilm building component. In some cases eDNA needs to interact with other matrix components to restore biofilm formation, like N-acetyl glucosamine along with exogenous eDNA is required to restore biofilm biomass in a deficient strain of *Listeria monocytogenes* (263).

eDNA is crucial for the formation of microcolonies from the initiation stages (79), however the addition of exogenous DNA was not sufficient to restore microcolony formation under the conditions tested in this Thesis Chapter. This may be due to one of two reasons, (i) eDNA needs to be produced in concentrated foci for the bacteria to attach to and/or (ii) other components or public goods released through explosive cell lysis are also required for the formation of microcolonies. As shown in this Chapter, the release of small amounts of eDNA in discrete foci by the single holin deletions is sufficient for the formation of small microcolonies. Simultaneously, eDNA interacts with many public goods produced within a biofilm which may also be required for the formation of microcolonies. One such example is the DNA-binding protein IHF as it is important in stabilising the biofilm structures of *P. aeruginosa, H. influenzae* and *E. coli* (315-318). Interestingly IHF is a cytosolic protein that moonlights as an extracellular protein although it has no known signal peptide sequence to direct it to the general secretory pathway (102, 103, 315). It is plausible that IHF is released through explosive cell lysis. Quantification of IHF in the extracellular environment of PAO1 and PAO1Δ*lys* will determine if IHF is released through explosive cell lysis. Future investigations into early stage microcolony development could also examine protein-eDNA interactions through the addition of proteases.

The involvement of three holins in transporting Lys may allow for the continual release of eDNA throughout microcolony formation. This appears to be a vital factor in determining whether a particular eDNA release site will give rise to a microcolony. The requirement of continual eDNA release for microcolony formation may be analogous to a 'rich-get-richer' model that Zhao *et al.* (69) proposed for the exopolysaccharide Psl, whereby Psl is laid down by single cells and as cells cluster around the Psl, they produce more creating a positive-feedback loop. The results presented here show that deletion of any one holin limits sequential explosive cell lysis events and therefore significantly affects the number and size of the microcolonies formed. Future work will investigate the temporal expression of each holin to explore if each holin plays a different role in each stage of biofilm development. Creation of fluorescent protein reporters for each holin will show when each holin is expressed throughout biofilm development. Furthermore, future investigations will examine

how these holins impact on mature biofilm development and whether they produce the three-dimensional mushroom structure typical of submerged *P. aeruginosa* biofilms.

Chapter Seven:

General Discussion

7 General Discussion

P. aeruginosa is an opportunistic pathogen that causes acute and chronic infections in a range of immune-compromised patients (3-7). It is also a common cause of infections associated with implanted medical devices, making it responsible for half of health care-associated infections (12, 13). Formation of biofilms by *P. aeruginosa* is often associated with the chronic nature of infections caused by this pathogen (4, 5) and one of the main constituents of these biofilms is eDNA (63, 81). The production of eDNA is essential for the formation of biofilms in hydrated or submerged environments, facilitating cell-cell and cell-matrix interactions, stabilising the multicellular aggregates during submerged biofilm development (70, 79, 84, 86, 92, 95). eDNA also facilitates active expansion of interstitial biofilms where it is produced in punctate pattern throughout the biofilm, ensuring efficient traffic flow from the colony to the leading edge (49). The release of eDNA in mature biofilms has been well documented where QS molecules AHL and PQS, along with the filamentous Pf4 phage mediate this process (92, 176, 186) but the mechanism of eDNA release in early stage biofilm development before QS systems and Pf4 phage are active is largely unknown. At the beginning of this body of work, Turnbull *et al.* observed a novel phenomenon they termed explosive cell lysis. This process involves a single cell in a population rapidly changing morphology from rod-shaped to round-shaped and then lysing in an explosive manner. This lysis results in the expulsion of the internal contents of the cell, including DNA. MV formation was observed as a result of shattered membrane fragments annealing, capturing the cellular contents in the vicinity. The mechanisms underlying explosive cell lysis were unknown at the initiation of this Thesis. As explosive cell lysis is a novel biological process that has only recently been discovered, a detailed understanding of this fascinating new aspect of pathogen biology is warranted as it may lead to the development of novel approaches to combat *P. aeruginosa* infections.

The focus of this Thesis has been on elucidating the genes and processes involved in explosive cell lysis and the role of eDNA in early stage development of *P. aeruginosa* submerged biofilms. This included an investigation that identified the pyocin-associated endolysin Lys as a mediator

and the SOS response regulator RecA as a regulator of explosive cell lysis. The work presented in this Thesis also investigated the effect internal and exogenous stressors had on inducing explosive cell lysis, determining only agents that cause DNA damage induce explosive events and eDNA release. Three holins that are part of separate cell death pathways were all identified to contribute to the transportation of Lys to the periplasm, leading to explosive cell lysis and eDNA release. Finally, it was determined that explosive cell lysis is necessary for microcolony formation in submerged biofilm development, with sequential eDNA release through explosive events controlling microcolony formation spatially and temporally.

Overall, the results of this Thesis revealed the genes and processes necessary for explosive cell lysis, a novel mechanism for the release of eDNA in *P. aeruginosa* biofilms. This Thesis also extends our knowledge of the role of eDNA in biofilm formation. The key conclusions and outcomes of this work, as well as future avenues of investigation are discussed below.

7.1 Explosive cell lysis as a novel phenomenon

Chapter 3 of this Thesis identified the pyocin-associated endolysin Lys as the enzyme responsible for mediating explosive cell lysis as determined by abrogation of explosive cell lysis and eDNA release in interstitial and submerged biofilms when *lys* was deleted. Investigations into the regulation of explosive cell lysis determined the process is regulated by the SOS response regulator RecA, a known regulator of the pyocin gene cluster (221). In Chapter 5 of this Thesis, the regulation of Lys-mediated explosive cell lysis was further explored by examining the involvement of PrtN as a regulator as it is a known regulator of the pyocin gene cluster and is under the control of RecA (223). In Chapter 5, deletion of *prtN* was found to have no effect on explosive cell lysis, indicating *lys* may have a different regulatory pathway than other genes in the pyocin cluster. This finding is significant as a *prtN* deletion was used by McFarland *et al.* (227) to demonstrate that the pyocin gene cluster wasn't involved in AlpB-mediated cell death, whereas our findings indicate that this is not an accurate indicator of *lys* expression and may therefore not be a valid assumption. Further work is required to determine the regulation pathway of *lys* as it may be regulated directly by RecA or may be regulated by a different

intermediate than PrtN. As the transcription start sites have been mapped, we can follow gene expression by RNAseq. Chromatin immunoprecipitation (ChIP) (319) techniques could also be used to identify where on the genome RecA binds, revealing which sequences may be involved in regulating *lys*. In addition, examining the role of the SOS response repressor LexA will aid in understanding the regulation of explosive cell lysis. It is unlikely that deletion of *lexA* will have a significant affect on explosive cell lysis as the transcriptional expression of *lys* was similar in PAO1 and PAO1Δ*lexA* when both strains were exposed to bactericidal concentrations of ciprofloxacin (194). Since all cells are capable of producing Lys yet only a small proportion of cells undergo explosive cell lysis, regulation of *lys* may be key in determining which cells undergo lysis. Understanding the exact regulatory pathway controlling explosive cell lysis will aid in the development of potential antimicrobial treatments targeting this process, which appears to be a conserved pathway across *P. aeruginosa* isolates.

Explosive cell lysis may also be a conserved phenomenon across different bacterial species as a mechanism for the release of eDNA, public goods and the biogenesis of MVs. Future investigations will examine the ability of other clinically relevant bacterial species to undergo explosive cell lysis and determine whether previously described bacteriophage- and autolysin-mediated cell death pathways are visually similar to explosive cell lysis. Phase-contrast and fluorescence microscopy, in addition to OMX 3D-SIM super resolution microscopy examining the production of eDNA and MVs in early stage biofilm development of organisms such as the 'ESKAPE' pathogens *Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumanni,* and *Enterobacter sp.* may reveal that explosive cell lysis is integral in the pathogenesis of notorious drug resistant bacteria, providing us with a new avenue for the development of antimicrobial therapies for these prevalent bacteria.

Along with the release of eDNA, other public goods that are integral in biofilm formation or virulence and infection of a host may also be released during Lys-mediated explosive cell lysis. Analysing the secretome of PAO1 and PAO1Δ*lys* will provide evidence and insight into the various public goods that are produced through this process. The IHF protein that plays an integral role in

stabilising biofilms produced within CF lungs (320) and in *P. aeruginosa, H. influenzae* and *E. coli* biofilms by interacting with eDNA (315-318), may also be released through explosive cell lysis as it has no known signal peptide sequences that would direct it to the general secretory pathway (102, 103, 315). To identify if production of IHF and its association with eDNA occurs during explosive cell lysis, immunofluorescence microscopy performed with OMX 3D-SIM super-resolution with IHF anti-sera and eDNA stains will determine the presence and location of IHF at sites of explosive cell lysis. Similarly, quantification of IHF production in *lys* deletion mutants will determine if IHF is released through explosive cell lysis. Treatment solutions have been hypothesised for targeting IHF as a novel therapy for CF lung infections (320) and recombinant human DNase is currently being used to disrupt *P. aeruginosa* lung infections by hydrolysing eDNA (98, 321) and is most effective in patients with mild to moderate lung disease (322). If IHF and eDNA were both produced through explosive cell lysis, an antimicrobial that targets explosive cell lysis could potentially aid in significantly improving mucociliary clearance and substantially improve the outcomes for patients with more severe disease progression that are not viable candidates for current treatments.

Along with being responsible for production of various public goods, the endolysin itself could also be directly involved in pathogenesis. The endolysin responsible for transporting the platelet-binding proteins PblA and PblB to the cell wall of *S. mitis* becomes cell wall-associated during the process and is then able to directly interact with fibrinogen in the surface of platelets, having a greater effect on platelet binding than PblA and PblB, which is important in the pathogenesis of infective endocarditis (235). The endolysin Lys and the holins involved in explosive cell lysis in *P. aeruginosa* may also have multi-functional roles that may contribute to virulence.

Once the cell has exploded, shattered fragments of the cell wall circularise to form MVs and capture the surrounding contents (247). As MVs have been implicated in cell-to-cell signalling, secretion of virulence factors and stimulation of host immune responses (105, 239, 244, 245, 323), explosive cell lysis and the resulting MVs could be an effective and integral step in pathogenesis of *P. aeruginosa*. They could be responsible for distributing concentrated packages of public goods and virulence factors throughout the

biofilm and into the surrounding environment, eliminating the necessity for direct interaction with the substratum, competing bacteria or host tissue to cause cytotoxicity (238, 246). This is supported by the work of Bauman and Kuehn (245), where they demonstrated that MVs associate with and are internalised by lung epithelial cells, contributing to the inflammatory response during infection. Similarly, MVs delivered to gastric epithelium of mice induces the innate and adaptive immune response as they deliver peptidoglycan, which initiates inflammatory processes after interaction with NOD1 (105).

7.2 Stress and explosive cell lysis

Chapter 4 of this Thesis investigated the induction of explosive cell lysis by intrinsic and exogenous stressors. As we established that explosive cell lysis is regulated by the SOS response regulator RecA in Chapter 3, Chapter 4 focused on induction of explosive cell lysis through exogenous stressors since expression of RecA and the pyocin gene cluster is upregulated upon exposure to agents that cause DNA damage (162, 221, 273). Although ciprofloxacin is known to induce the SOS response and *lys* expression, no investigations have directly examined if the pyocin lysis gene cassette impacts upon sensitivity to ciprofloxacin and other clinically relevant antibiotics, potentially impacting the efficacy of antibiotic treatment. We determined that only agents that cause DNA damage, including MMC and ciprofloxacin, induce explosive cell lysis in the biofilm population and other antibiotics, such as gentamicin and colistin, which target other aspects of the cell, have no effect on explosive cell lysis. Interestingly, we found that deletion of *lys* had no effect on the MIC and MBC of the three antibiotics tested and cell death occurred at the same rate in PAO1 and PAO1Δ*lys*. As we found there was no increase in susceptibility to ciprofloxacin in the absence of *lys* as Brazas and Hancock (193) predicted, other components of the pyocin lysis cluster might be responsible for ciprofloxacin sensitivity. There are currently two genes in the pyocin lysis gene cassette, PA0630 and PA0631, which have no known function and it is possible that they are involved in ciprofloxacin-mediated cell death as they are upregulated upon ciprofloxacin exposure, potentially contributing to ciprofloxacin sensitivity (193). Generating in-frame deletions of each of these

genes may identify their potential role in ciprofloxacin-induced cell death and possibly explosive cell lysis.

Exposing *P. aeruginosa* biofilms to DNA damaging agents induces explosive cell lysis and the release of large amounts of eDNA and DNA damaging compounds like ROS produced by neutrophils and macrophages could also induce explosive cell lysis during infection of a host. The induction of explosive cell lysis during the initiation of an infection could enhance the survival of the bacterial population and be an effective defence strategy, whereby attack from the host's immune system induces explosive cell lysis and the release of eDNA, leading to the formation of biofilms with larger biomass. Along with the immune response, drug treatments that target DNA replication or induce DNA damage may also stimulate explosive cell lysis and result in exacerbated infections. In support of this, epidemiological studies have found that treatment of EHEC infections with MMC and fluoroquinolones in cancer patients caused the induction of Stx-phages and production of the Shiga toxin (307-311), which increased the severity of these infections (311-313). To gain further understanding of the importance of the explosive cell lysis pathway in an infection setting and the impact the immune response has on explosive cell lysis, numerous types of infection models will need to be performed including but not limited to tissue-culture infection models to examine the interactions of *P. aeruginosa* with neutrophils and macrophages, invertebrate and vertebrate models and CF lung infection, covering the innate and adaptive immune responses. Another infection model that should be explored is catheter-associated urinary tract infections as eDNA has been found to be the major constituent of these biofilms in a murine model (77) and eDNA facilitates interstitial biofilm expansion (49), which is believed to be associated with infection of implanted medical devices. Treating these infection models with fluoroquinolones will also determine if ciprofloxacin-induced biofilm formation also occurs *in vivo*.

We could exploit this cell death pathway in targeted drug therapy through one of two approaches, either (i) inducing lysis through DNA interfering compounds or (ii) inhibiting explosive cell lysis in biofilms. Induction of explosive cell lysis as a treatment could potentially result in a massive release of virulence factors, exacerbating already established infections. Inhibiting explosive cell

lysis would be more of a preventative or prophylactic treatment. It would block explosive cell lysis from occurring, preventing the production of public goods that are essential for the formation of biofilms. Future research could be directed towards developing antimicrobial treatments that target behaviours that are costly to the individual but are essential for the population to efficiently invade and infect (324).

As only single cells undergo explosive cell lysis in non-inducing conditions, we hypothesised that an internal stressor may be responsible for triggering explosive events. We had observed the formation of dark spots in the cells that undergo explosive cell lysis. It was proposed that these dark spots were inclusion bodies, which form in stressed or aging cells. We initially investigated if these dark spots were the sites of viral particle assembly by examining the formation of dark spots in Pf4 phage and pyocin structural deletion mutants as they may form aggregates in the cell prior to their release. We found no correlation between dark spot formation and the presence of Pf4 phage and pyocin structural elements under inducing and non-inducing conditions, suggesting they are not involved in the process. Since inclusion bodies are also known to form from the aggregation of partially or mis-folded proteins, a fluorescent fusion protein of the small shock protein IbpA, known to co-localise with inclusion bodies of protein origin, was utilised to identify whether these dark spots were inclusion bodies formed from protein aggregation. The IbpA-YFP fusion protein co-localised with a small proportion of the dark spots visualised in non-inducing conditions and was associated with the majority of dark spots formed under genotoxic stress. Due to this variation in association, it is inconclusive if these dark spots are identical between cells and under different conditions. Fluorescent protein fusions with other small heat shock proteins like DnaK and ClpB that are involved in reversing protein aggregation (276) in combination with IbpA-YFP will conclusively determine whether all these dark spots are inclusion bodies formed from protein aggregation. In addition to the protein fusions, time-lapse microscopy observing the formation of the inclusion bodies could also aid in determining their identity and their effect on explosive cell lysis.

7.3 Transportation of Lys

In Chapter 5 of this Thesis, we investigated the contribution of three holins in mediating explosive cell lysis and determined that the three holins Hol, AlpB and CidAB all contribute to explosive cell lysis. To our knowledge, this is the first evidence of the involvement of multiple holins involved in one cell death pathway and shows that multiple cell death pathways can intersect with functionally identical proteins. It would be interesting to examine the temporal expression of the holins to elucidate when each holin is involved in transporting Lys, whether it is simultaneously or sequentially. Construction of fluorescent protein reporters of the holin promoters like that of pMHOL-GFP or genomic transcriptional *eGFP* fusion (247), will show the expression of each holin in real time during explosive events.

Each holin appears to have a unique contribution to explosive cell lysis as complementation of a single holin deletion with the wild type gene of another holin was not always sufficient to restore eDNA release. As each holin is involved in transporting Lys, their regulatory systems may be interlinked. AlpB and CidAB do not have associated endolysins so Lys could also be responsible for death through these holin-mediated cell death pathways as well. Examination of a *lys* deletion under the conditions which the AlpB- and CidAB-mediated cell death pathways are active will elucidate whether Lys is responsible for cell death in these systems.

Explosive cell lysis mediated by these holins may also effect the ability to form biofilms in a host as part of an infection as McFarland *et al*. (227) demonstrated that disruption of the *alp* operon reduced colonisation of the murine lung. Therefore disruption of explosive cell lysis may also reduce or even prevent colonisation of host tissue.

7.4 Submerged biofilm development

Chapter 5 also explored the role of explosive cell lysis-mediated eDNA release in submerged microcolony development and determined that explosive cell lysis controls the spatial and temporal formation of microcolonies with sequential lysis through the three holins involved in explosive events required for the formation of large microcolonies.

eDNA released through explosive cell lysis appears to influence microcolony formation in a similar manner to that described for the exopolysaccharide Psl, whereby cells preferentially associate with the matrix component deposited on the substratum, resulting in the aggregation of cells at these deposition sites and increased production of the matrix component in a 'rich-get-richer' model (69). To further demonstrate that this model can also be applied to eDNA release, the combination of fluorescent staining of eDNA and cell tracking algorithms could trace the exploration of cells across a surface, mapping the interactions of eDNA and tracking how bacteria movement is influenced by eDNA that leads to microcolony formation. Simultaneously staining for the Psl and Pel exopolysaccharides with Psl-staining *Hippeastrum hybrid* lectin from amaryllis (HHA) (67) and Pel-specific *Wisteria floribunda* lectin stain (71) will illuminate whether these exopolysaccharides are produced at the same time as eDNA through explosive cell lysis or after eDNA has been deposited and microcolonies have formed.

Explosive cell lysis may also be involved in eDNA production and cell death in the mature stages of submerged biofilm development and possibly dispersal. As we are unable to grow mature biofilms that are defective in explosive cell lysis, we could utilise an arabinose-inducible pBAD-based complementation plasmid in PAO1Δ*lys*, which would allow for the repression of *lys* expression with the addition of glucose. Alternatively, creation of a fluorescently tagged Lys protein will show when Lys is produced at high levels immediately prior to explosive events, identifying if explosive cell lysis also contributes to eDNA production in mature biofilms and cell death during dispersal.

7.5 Overall conclusions

Explosive cell lysis is a newly discovered strategy for the production and distribution of essential biofilm matrix components throughout the biofilm. This study, to our knowledge, is the first direct observation of processes by which cellular contents are liberated into the extracellular matrix of bacterial biofilms.

Explosive cell lysis accounts for all the eDNA produced in actively expanding biofilms and during the early stages of saturated biofilm development

(247). The process is dependent upon the pyocin-associated endolysin Lys and multiple holins that are themselves part of different previously reported cell death pathways of *P. aeruginosa* (67, 162, 227). An internal or exogenous stressor or DNA damage to individual cells causes the induction of *recA* expression. RecA then induces the production of Lys, either directly or through and unknown intermediary, as it is not under the same regulation as other pyocin associated genes. The endolysin is transported to the periplasm via holins Hol, AlpB and CidAB. It is yet to be determined if each holin plays a specific role for the transport of Lys, how they are regulated to transport Lys or if they are temporally regulated. In the periplasm, Lys hydrolyses the peptidoglycan, leading to structural instability. Once a threshold is reached, where the cell can no longer maintain its shape, the cell shape collapses into a round cell. These round cells survive on average 5-10 s before lysing rapidly in an explosive manner. The internal contents of the cell are efficiently released and expelled into the biofilm milieu, including genomic DNA, proteins and MVs. This eDNA is crucial for the formation of microcolony structures in saturated biofilm development, where spatial and temporal development of the microcolonies is dependent upon its release.

Overall the work presented in this Thesis adds to our understanding of the complex nature of eDNA production in early stage biofilm development and we suggest explosive cell lysis and the resulting public goods released are likely to significantly contribute to the success of *P. aeruginosa* in an infection setting. While further work is required to fully understand the regulation and impact of explosive cell lysis, the work in this Thesis provides a foundation for further investigation, which may contribute to the development of targeted treatments for biofilm-associated infections caused by *P. aeruginosa* and possibly other pathogens.

Chapter Eight:

References

8 References:

1. **Rahme LG, Ausubel FM, Cao H, Drenkard E, Goumerov BC, Lau GW, Mahajan-Miklos S, Plotnikova J, Tan MW, Tsongalis J, Walendziewicz CL, Tompkins RG.** 2000. Plants and animals share functionally common bacterial virulence factors. Proceedings of the National Academy of Sciences **97:**8815-8821.
2. **Kazmierczak BI, Mostov K, Engel JN.** 2001. Interaction of bacterial pathogens with polarized epithelium. Annual review of microbiology **55:**407-435.
3. **Lyczak JB, Cannon CL, Pier GB.** 2002. Lung infections associated with cystic fibrosis. Clinical Microbiology Reviews **15:**194-222.
4. **Costerton JW, Stewart PS, Greenberg EP.** 1999. Bacterial Biofilms: a common cause of persistent infections. Science **284:**1318-1322.
5. **Lyczak JB, Cannon CL, Pier GB.** 2000. Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. Microbes and Infection **2:**1051-1060.
6. **Chatzinikolaou I, Abi-Said D, Bodey GP, Rolston KVI, Tarrand JJ, Samonis G.** 2000. Recent experience with *Pseudomonas aeruginosa* bacteremia in patients with cancer: Retrospective analysis of 245 episodes. Archives of Internal Medicine **160:**501-509.
7. **Lipsky BA, Tabak YP, Johannes RS, Vo L, Hyde L, Weigelt JA.** 2010. Skin and soft tissue infections in hospitalised patients with diabetes: culture isolates and risk factors associated with mortality, length of stay and cost. Diabetologia **53:**914-923.
8. **Bleves S, Viarre V, Salacha R, Michel GP, Filloux A, Voulhoux R.** 2010. Protein secretion systems in *Pseudomonas aeruginosa*: A wealth of pathogenic weapons. International Journal of Medical Microbiology **300:**534-543.
9. **Planquette B, Timsit JF, Misset BY, Schwebel C, Azoulay E, Adrie C, Vesin A, Jamali S, Zahar JR, Allaouchiche B, Souweine B, Darmon M, Dumenil AS, Goldgran-Toledano D, Mourvillier BH, Bedos JP, Group OS.** 2013. *Pseudomonas aeruginosa* ventilator-associated pneumonia. predictive factors of treatment failure. American Journal of Respiratory and Critical Care Medicine **188:**69-76.
10. **Flores-Mireles AL, Walker JN, Caparon MG, Hultgren SJ.** 2015. Unrinary tract infections: epidemiology, mechanisms of infection and treatment options. Nature Reviews Microbiology **13:**269-284.
11. **Germiller JA, El-Kashlan HK, Shah UK.** 2005. Chronic *Pseudomonas aeruginosa* infections in cochlear implants. Otology and Neurotology **26:**196-2001.
12. **Guggenbichler JP, Assadian O, CBoeswald M, Kramer A.** 2011. Incidence and clinical implication of nosocomial infections associated with implantable biomaterials - catheters, ventilator-associated pneumonia, unrinary tract infections. GMS Krankenhausehygiene Interdisziplinär **6:**1-19.
13. **Richards MJ, Edwards JR, Culver DH, Gaynes RP.** 1999. Nosocomial infections in medical intensive care units in the United States. Critical Care Medicine **27:**887-892.

14. **Nordmann P, Naas T, Fortineau N, Poirel L.** 2007. Superbugs in the coming new decade; multidrug resistance and prospects for treatment of *Staphylococcus aureus, Enterococcus* spp. and *Pseudomonas aeruginosa* in 2010. Current opinion in microbiology **10:**436-440.
15. **Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature **406:**959-964.
16. **Van Delden C, Iglewski BH.** 1998. Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. Emerging Infectious Diseases **4:**551-560.
17. **Livermore DM.** 2002. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: Our worst nightmare? Antimircobial Resistance **34:**634-640.
18. **Monahan LG, Turnbull L, Osvath SR, Birch D, Charles IG, Whitchurch CB.** 2014. Rapid conversion of *Pseudomonas aeruginosa* to sperhical cell morphotype facilitates tolerance to carbapenems and penicillins but increases susceptibility to antimicrobial peptides. Antimicrobial Agents and Chemotherapy **58:**1956-1962.
19. **Donlan RM, Costerton JW.** 2002. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Reviews **15:**167-193.
20. **Anthony M, Rose B, Pegler MB, Elkins M, Service H, Thamotharampillai K, Watson J, Robinson M, Bye P, Merlino J, Harbour C.** 2002. Genetic analysis of *Pseudomonas aeruginosa* isolates from the sputa of Australian adult cystic fibrosis patients. Journal of Clinical Microbiology **40:**2772-2778.
21. **Fluge G, Ojeniyi B, Høiby D, A., Ciofu O, Hunstad E, Haanes OC, Storrøsten OT.** 2001. Typing of Psuedomonas aeruginosa strains in Norwegian cystic fibrosis patients. Clinical Microbiology and Infection **7:**238-243.
22. **Hogardt M, Heesemann J.** 2013. Microevolution of *Pseudomonas aeruginosa* to a chronic pathogen of the cystic fibrosis lung. Current topics in microbiology and immunology **358:**91-118.
23. **Simoes M.** 2011. Antimicrobial strategies effective against infectious bacterial biofilms. Current Medicinal Chemistry **18:**2129-2145.
24. **Schooling SR, Beveridge TJ.** 2006. Membrane vesicles: an overlooked component of the matrices of biofilms. Journal of bacteriology **188:**5945-5957.
25. **Decho AW.** 2000. Microbial biofilms in intertidal systems: an overview. Continental Shelf Research **20:**1257-1273.
26. **Hall-Stoodley L, Costerton JW, Stoodley P.** 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nature Reviews Microbiology **2:**95-108.
27. **Flemming HC.** 1993. Biofilms and environmental protection. Water Science and Technology **27:**1-10.

28. **Haussler S, Fuqua C.** 2013. Biofilms 2012: new discoveries and significant wrinkles in a dynamic field. Journal of bacteriology **195:**2947-2958.
29. **Gule NG, Begum NM, Klumperman B.** 2016. Advances in biofouling mitigation: A review. Critical Reviews in Environmental Science and Technology **46:**535-555.
30. **Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ.** 1987. Bacterial biofilms in nature and disease. Annual review of microbiology **41:**435-464.
31. **Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM.** 1995. Microbial biofilms. Annual review of microbiology **49:**711-745.
32. **Donlan RM.** 2001. Biofilm formation: a clincally relevant microbiological process. Clinical Infectious Diseases **33:**1387-1392.
33. **Donlan RM.** 2001. Biofilms and device-associated infections. Emerging Infectious Diseases **7:**277-281.
34. **Parsek MR, Singh PK.** 2003. Bacterial biofilms: an emerging link to disease pathogenesis. Annual review of microbiology **57:**677-701.
35. **Vergidis P, Patel R.** 2012. Novel approaches to the diagnosis, prevention, and treatment medical device-associated infections. Infectious Disease Clinics of North America **26:**173-186.
36. **Lappin-Scott H, Burton S, Stoodley P.** 2014. Revealing a world of biofilms--the pioneering research of Bill Costerton. Nature Reviews Microbiology **12:**781-787.
37. **Moreau-Marquis S, Bomberger JM, Anderson GG, Swiatecka-Urban A, Ye S, O'Toole GA, Stanton BA.** 2008. The DeltaF508-CFTR mutation results in increased biofilm formation by *Pseudomonas aeruginosa* by increasing iron availability. American Journal of Physiology - Lung Cellular and Molecular Physiology **295:**L25-37.
38. **Stoodley P, Sauer K, Davies DG, Costerton JW.** 2002. Biofilms as complex differentiated communities. Annual review of microbiology **56:**187-209.
39. **Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG.** 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. Journal of bacteriology **184:**1140-1154.
40. **Klausen M, Aaes-Jørgensen A, Molin S, Tolker-Nielsen T.** 2003. Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. Molecular microbiology **50:**61-68.
41. **Branda SS, Vik S, Friedman L, Kolter R.** 2005. Biofilms: the matrix revisited. Trends in microbiology **13:**20-26.
42. **Vanysacker L, Declerck P, Bilad MR, Vankelecom IFJ.** 2014. Biofouling on microfiltration membranes in MBRs: Role of membrane type and microbial community. Journal of Membrane Science **453:**394-401.
43. **Flemming HC.** 2011. Microbial biofouling: unsolved problems, insufficient approaches, and possible solutions, vol. 5. Springer Berlin Heidelberg.

44. **Semmler ABT, Whitchurch CB, Mattick JS.** 1999. Re-examination of twitching motility in *Pseudomonas aeruginosa*. Microbiology **145:**2863-2873.
45. **Mattick JS.** 2002. Type IV pili and twitching motility. Annual review of microbiology **56:**289-314.
46. **Merz AJ, So M, Sheetz MP.** 2000. Pilus retraction powers bacterial twitching motility. Nature **407:**98-102.
47. **Whitchurch CB.** 2006. *Pseudomonas*, vol. 4. Springer.
48. **Turnbull L, Whitchurch CB.** 2014. Motility assay: twitching motility. Methods in Molecular Biology **1149:**73-86.
49. **Gloag ES, Turnbull L, Huang A, Vallotton P, Wang H, Nolan LM, Mililli L, Hunt C, Lu J, Osvath SR, Monahan LG, Cavaliere R, Charles IG, Wand MP, Gee ML, Prabhakar R, Whitchurch CB.** 2013. Self-organization of bacterial biofilms is facilitated by extracellular DNA. Proceedings of the National Academy of Sciences **110:**11541-11546.
50. **Stickler DJ.** 2008. Bacterial biofilms in patients with indwelling urinary catheters. Nature Clinical Practice Urology **5:**598-608.
51. **Veerachamy S, Yarlagadda T, Manivasagam G, Yarlagadda PK.** 2014. Bacterial adherence and biofilm formation on medical implants: a review. Prceedings of the Institution of Mechanical Engineers **228:**1083-1099.
52. **Kearns DB.** 2010. A field guide to bacterial swarming motility. Nature Reviews Microbiology **8:**634-644.
53. **Flemming HC, Neu TR, Wozniak DJ.** 2007. The EPS matrix: the "house of biofilm cells". Journal of bacteriology **189:**7945-7947.
54. **Flemming HC, Wingender J.** 2010. The biofilm matrix. Nature Reviews Microbiology **8:**623-633.
55. **Martin DW, Schurr MJ, Mudd M, Govan J, Holloway B, Deretic V.** 1993. Mechanism of conversion to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. Proceedings of the National Academy of Sciences **90:**8377-8381.
56. **Pier G.** 1998. *Pseudomonas aeruginosa*: a key problem in cystic fibrosis. ASM News **6:**339-347.
57. **Govan JRW, Deretic V.** 1996. Microbial pathogenesis in Cystic Fibrosis: muciod *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiological Reviews **60:**539-574.
58. **Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S, Givskov M, Parsek MR.** 2001. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. Journal of bacteriology **183:**5395-5401.
59. **Wozniak DJ, Wyckoff TJO, Starkey M, Keyser R, Azadi P, O'Toole GA, Parsek MR.** 2003. Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. Proceedings of the National Academy of Sciences **100:**7907-7912.
60. **Nivens DE, Ohman DE, Williams J, Franklin MJ.** 2001. Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. Journal of bacteriology **183:**1047-1057.
61. **Stapper AP, Narasimhan G, Ohman DE, Barakat J, Hentzer M, Molin S, Kharazmi A, Hoiby N, Mathee K.** 2004. Alginate production affects

Pseudomonas aeruginosa biofilm development and architecture, but is not essential for biofilm formation. Journal of Medical Microbiology **53:**679-690.

62. **Ma L, Jackson KD, Landry RM, Parsek MR, Wozniak DJ.** 2006. Analysis of *Pseudomonas aeruginosa* conditional psl variants reveals roles for the psl polysaccharide in adhesion and maintaining biofilm structure postattachment. Journal of bacteriology **188:**8213-8221.
63. **Matsukawa M, Greenberg EP.** 2004. Putative exopolysaccharide synthesis genes influence *Pseudomonas aeruginosa* biofilm development. Journal of bacteriology **186:**4449-4456.
64. **Jackson KD, Starkey M, Kremer S, Parsek MR, Wozniak DJ.** 2004. Identification of psl, a locus encoding a potential exopolysaccharide that is essential for *Pseudomonas aeruginosa* PAO1 biofilm formation. Journal of bacteriology **186:**4466-4475.
65. **Friedman L, Kolter R.** 2004. Two genetic loci produce distinct carbohydrate-rich structural components of the *Pseudomonas aeruginosa* biofilm matrix. Journal of bacteriology **186:**4457-4465.
66. **Overhage J, Schemionek M, Webb JS, Rehm BH.** 2005. Expression of the psl operon in *Pseudomonas aeruginosa* PAO1 biofilms: PslA performs an essential function in biofilm formation. Applied and environmental microbiology **71:**4407-4413.
67. **Ma L, Conover M, Lu H, Parsek MR, Bayles K, Wozniak DJ.** 2009. Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. PLoS pathogens **5:**e1000354.
68. **Wang S, Parsek MR, Wozniak DJ, Ma LZ.** 2013. A spider web strategy of type IV pili-mediated migration to build a fibre-like Psl polysaccharide matrix in *Pseudomonas aeruginosa* biofilms. Environmental microbiology **15:**2238-2253.
69. **Zhao K, Tseng BS, Beckerman B, Jin F, Gibiansky ML, Harrison JJ, Luijten E, Parsek MR, Wong GC.** 2013. Psl trails guide exploration and microcolony formation in *Pseudomonas aeruginosa* biofilms. Nature **497:**388-391.
70. **Wang S, Liu X, Liu H, Zhang L, Guo Y, Yu S, Wozniak DJ, Ma LZ.** 2015. The exopolysaccharide Psl-eDNA interaction enables the formation of a biofilm skeleton in *Pseudomonas aeruginosa*. Environmental microbiology reports **7:**330-340.
71. **Jennings LK, Storek KM, Ledvina HE, Coulon C, Marmont LS, Sadovskaya I, Secor PR, Tseng BS, Scian M, Filloux A, Wozniak DJ, Howell PL, Parsek MR.** 2015. Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the *Pseudomonas aeruginosa* biofilm matrix. Proceedings of the National Academy of Sciences **112:**11353-11358.
72. **Friedman L, Kolter R.** 2003. Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. Molecular microbiology **51:**675-690.
73. **Colvin KM, Gordon VD, Murakami K, Borlee BR, Wozniak DJ, Wong GC, Parsek MR.** 2011. The pel polysaccharide can serve a structural and protective role in the biofilm matrix of *Pseudomonas aeruginosa*. PLoS pathogens **7:**e1001264.

74. **Yang L, Hu Y, Liu Y, Zhang J, Ulstrup J, Molin S.** 2011. Distinct roles of extracellular polymeric substances in *Pseudomonas aeruginosa* biofilm development. Environmental microbiology **13:**1705-1717.
75. **Colvin KM, Irie Y, Tart CS, Urbano R, Whitney JC, Ryder C, Howell PL, Wozniak DJ, Parsek MR.** 2012. The Pel and Psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix. Environmental microbiology **14:**1913-1928.
76. **Vasseur P, Vallet-Gely I, Soscia C, Genin S, Filloux A.** 2005. The pel genes of the *Pseudomonas aeruginosa* PAK strain are involved at early and late stages of biofilm formation. Microbiology **151:**985-997.
77. **Cole SJ, Records AR, Orr MW, Linden SB, Lee VT.** 2014. Catheter-associated urinary tract infection by *Pseudomonas aeruginosa* is mediated by exopolysaccharide-independent biofilms. Infection and immunity **82:**2048-2058.
78. **Okshevsky M, Meyer RL.** 2013. The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms. Critical reviews in microbiology **41:**341-352.
79. **Whitchurch CB, Tolker-Nielsen T, Ragas P, Mattick JS.** 2002. Extracellular DNA required for bacterial biofilm formation. Science **295:**1487.
80. **Ma L, Wang S, Wang D, Parsek MR, Wozniak DJ.** 2012. The roles of biofilm matrix polysaccharide Psl in mucoid *Pseudomonas aeruginosa* biofilms. FEMS immunology and medical microbiology **65:**377-380.
81. **Nemoto K, Hirota K, Murakami K, Taniguti K, Murata H, Viducic D, Miyake Y.** 2003. Effect of varidase (Streptodornase) on biofilm formed by *Pseudomonas aeruginosa*. Chemotherapy **49:**121-125.
82. **Mulcahy H, Charron-Mazenod L, Lewenza S.** 2010. *Pseudomonas aeruginosa* produces an extracellular deoxyribonuclease that is required for utilization of DNA as a nutrient source. Environmental microbiology **12:**1621-1629.
83. **Das T, Krom BP, van der Mei HC, Busscher HJ, Sharma PK.** 2011. DNA-mediated bacterial aggregation is dictated by acid–base interactions. Soft Matter **7:**2927.
84. **Das T, Sharma PK, Busscher HJ, van der Mei HC, Krom BP.** 2010. Role of extracellular DNA in initial bacterial adhesion and surface aggregation. Applied and environmental microbiology **76:**3405-3408.
85. **Das T, Sharma PK, Krom BP, van der Mei HC, Busscher HJ.** 2011. Role of eDNA on the adhesion forces between *Streptococcus mutans* and substratum surfaces: influence of ionic strength and substratum hydrophobicity. Langmuir : the ACS journal of surfaces and colloids **27:**10113-10118.
86. **Das T, Sehar S, Manefield M.** 2013. The roles of extracellular DNA in the structural integrity of extracellular polymeric substance and bacterial biofilm development. Environmental microbiology reports **5:**778-786.
87. **Berne C, Kysela DT, Brun YV.** 2010. A bacterial extracellular DNA inhibits settling of motile progeny cells within a biofilm. Molecular microbiology**:**no-no.
88. **Mulcahy H, Charron-Mazenod L, Lewenza S.** 2008. Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. PLoS pathogens **4:**e1000213.

89. **Das T, Sehar S, Koop L, Wong YK, Ahmed S, Siddiqui KS, Manefield M.** 2014. Influence of calcium in extracellular DNA mediated bacterial aggregation and biofilm formation. PloS one **9:**e91935.
90. **Chen CZ, Cooper SL.** 2002. Interactions between dendrimer biocides and bacterial membranes. Biomaterials **23:**3359-3368.
91. **de Kerchove AJ, Elimelech M.** 2008. Calcium and magnesium cations enhance the adhesion of motile and nonmotile *Pseudomonas aeruginosa* on alginate films. Langmuir : the ACS journal of surfaces and colloids **24:**3392-3399.
92. **Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, Molin S, Givskov M, Tolker-Nielsen T.** 2006. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Molecular microbiology **59:**1114-1128.
93. **Klausen M, Heydorn A, Ragas P, Lambertsen L, Aaes-Jørgensen A, Molin S, Tolker-Nielsen T.** 2003. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. Molecular microbiology **48:**1511-1524.
94. **van Schaik EJ, Giltner CL, Audette GF, Keizer DW, Bautista DL, Slupsky CM, Sykes BD, Irvin RT.** 2005. DNA binding: a novel function of *Pseudomonas aeruginosa* type IV pili. Journal of bacteriology **187:**1455-1464.
95. **Barken KB, Pamp SJ, Yang L, Gjermansen M, Bertrand JJ, Klausen M, Givskov M, Whitchurch CB, Engel JN, Tolker-Nielsen T.** 2008. Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. Environmental microbiology **10:**2331-2343.
96. **Smalyukh, Il, Butler J, Shrout JD, Parsek MR, Wong GC.** 2008. Elasticity-mediated nematiclike bacterial organization in model extracellular DNA matrix. Physical Review E: Statistical, Nonlinear, Biological, and Soft Matter Physics **78:**030701.
97. **Walker TS, Tomlin KL, Worthen GS, Poch KR, Lieber JG, Saavedra MT, Fessler MB, Malcolm KC, Vasil ML, Nick JA.** 2005. Enhanced *Pseudomonas aeruginosa* biofilm development mediated by human neutrophils. Infection and immunity **73:**3693-3701.
98. **Shak S, Capon DJ, Hellmiss R, Marsters SA, Baker CL.** 1990. Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. Proceedings of the National Academy of Sciences **87:**9188-9192.
99. **Ranasinha C, Assoufi B, Shak S, Christiansen D, Fuchs H, Empey D, Geddes D, Hodson M.** 1993. Efficacy and safety of short-term administration of aerosolised recombinant human DNase I in adults with stable stage cystic fibrosis. The Lancet **342:**199-202.
100. **Lethem MI, James SL, Marriott C, Burke JF.** 1990. The origin of DNA associated with mucus glycoproteins in cystic fibrosis sputum. European Respiratory Journal **3:**19-23.
101. **Ellis TN, Kuehn MJ.** 2010. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. Microbiology and Molecular Biology Reviews **74:**81-94.
102. **Choi DS, Kim DK, Choi SJ, Lee J, Choi JP, Rho S, Park SH, Kim YK, Hwang D, Gho YS.** 2011. Proteomic analysis of outer membrane

vesicles derived from *Pseudomonas aeruginosa*. Proteomics **11:**3424-3429.
103. **Kadurugamuwa JL, Beveridge TJ.** 1995. Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. Journal of bacteriology **177:**3998-4008.
104. **McBroom AJ, Kuehn MJ.** 2007. Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. Molecular microbiology **63:**545-558.
105. **Kaparakis M, Turnbull L, Carneiro L, Firth S, Coleman HA, Parkington HC, Le Bourhis L, Karrar A, Viala J, Mak J, Hutton ML, Davies JK, Crack PJ, Hertzog PJ, Philpott DJ, Girardin SE, Whitchurch CB, Ferrero RL.** 2010. Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells. Cellular microbiology **12:**372-385.
106. **Schooling SR, Hubley A, Beveridge TJ.** 2009. Interactions of DNA with biofilm-derived membrane vesicles. Journal of bacteriology **191:**4097-4102.
107. **Smirnov VV, Kiprianova A.** 1990. Bacteria of the *Pseudmonas* genus. Naukova Dumka, Kiev, USSR.
108. **Wilson R, Sykes DA, Watson D, Rutman A, Taylor GW, Cole PJ.** 1988. Measurement of *Pseudomonas aeruginosa* phenazine pigments in sputum and assessment of their contribution to sputum Sol toxicity for respiratory epithelium. Infection and immunity **56:**2515-2517.
109. **Ramos I, Dietrich LE, Price-Whelan A, Newman DK.** 2010. Phenazines affect biofilm formation by *Pseudomonas aeruginosa* in similar ways at various scales. Research in Microbiology **161:**187-191.
110. **Das T, Kutty SK, Kumar N, Manefield M.** 2013. Pyocyanin facilitates extracellular DNA binding to *Pseudomonas aeruginosa* influencing cell surface properties and aggregation. PloS one **8:**e58299.
111. **Borriello G, Werner E, Roe F, Kim AM, Ehrlich GD, Stewart PS.** 2004. Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. Antimicrobial Agents and Chemotherapy **48:**2659-2664.
112. **Mah TFC, O'Toole GA.** 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in microbiology **9:**34-39.
113. **Fux CA, Costerton JW, Stewart PS, Stoodley P.** 2005. Survival strategies of infectious biofilms. Trends in microbiology **13:**34-40.
114. **Chiang WC, Nilsson M, Jensen PO, Hoiby N, Nielsen TE, Givskov M, Tolker-Nielsen T.** 2013. Extracellular DNA shields against aminoglycosides in *Pseudomonas aeruginosa* biofilms. Antimicrobial Agents and Chemotherapy **57:**2352-2361.
115. **Lewenza S.** 2013. Extracellular DNA-induced antimicrobial peptide resistance mechanisms in *Pseudomonas aeruginosa*. Frontiers in microbiology **4:**21.
116. **Kaplan JB, LoVetri K, Cardona ST, Madhyastha S, Sadovskaya I, Jabbouri S, Izano EA.** 2012. Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in staphylococci. Journal of Antibiotics **65:**73-77.

117. **Hu W, Li L, Sharma S, Wang J, McHardy I, Lux R, Yang Z, He X, Gimzewski JK, Li Y, Shi W.** 2012. DNA builds and strengthens the extracellular matrix in *Myxococcus xanthus* biofilms by interacting with exopolysaccharides. PloS one **7:**e51905.
118. **Nakamura Y, Itoh T, Matsuda H, Gojobori T.** 2004. Biased biological functions of horizontally transferred genes in prokaryotic genomes. Nature Genetics **36:**760-766.
119. **Thomas CM, Nielsen KM.** 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nature Reviews Microbiology **3:**711-721.
120. **Thomas VC, Thurlow LR, Boyle D, Hancock LE.** 2008. Regulation of autolysis-dependent extracellular DNA release by *Enterococcus faecalis* extracellular proteases influences biofilm development. Journal of bacteriology **190:**5690-5698.
121. **Qin Z, Ou Y, Yang L, Zhu Y, Tolker-Nielsen T, Molin S, Qu D.** 2007. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology **153:**2083-2092.
122. **Kreth J, Vu H, Zhang Y, Herzberg MC.** 2009. Characterization of hydrogen peroxide-induced DNA release by *Streptococcus sanguinis* and *Streptococcus gordonii*. Journal of bacteriology **191:**6281-6291.
123. **Hamilton HL, Dominguez NM, Schwartz KJ, Hackett KT, Dillard JP.** 2005. *Neisseria gonorrhoeae* secretes chromosomal DNA via a novel type IV secretion system. Molecular microbiology **55:**1704-1721.
124. **Zweig M, Schork S, Koerdt A, Siewering K, Sternberg C, Thormann K, Albers SV, Molin S, van der Does C.** 2014. Secreted single-stranded DNA is involved in the initial phase of biofilm formation by *Neisseria gonorrhoeae*. Environmental microbiology **16:**1040-1052.
125. **Steichen CT, Cho C, Shao JQ, Apicella MA.** 2011. The *Neisseria gonorrhoeae* biofilm matrix contains DNA, and an endogenous nuclease controls its incorporation. Infection and immunity **79:**1504-1511.
126. **Claverys JP, Havarstein LS.** 2007. Cannibalism and fratricide: mechanisms and raisons d'etre. Nature Reviews Microbiology **5:**219-229.
127. **Hochman A.** 1997. Programmed cell death in prokaryotes. Critical reviews in microbiology **23:**207-214.
128. **Shockman GD, Daneo-Moore L, Kariyama R, Massidda O.** 1996. Bacterial walls, peptidoglycan hydrolases, autolysins, and autolysis. Microbial Drug Resistance **2:**95-98.
129. **Dmitriev B, Toukach F, Ehlers S.** 2005. Towards a comprehensive view of the bacterial cell wall. Trends in microbiology **13:**569-574.
130. **Kitano K, Tuomanen EI, Tomasz A.** 1986. Transglycosylase an endopeptidase participate in the degredation of murein during autolysis of *Escherichia coli*. Journal of bacteriology **167:**759-765.
131. **Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, Hayes E, Heuser J, Dodson KW, Caparon MG, Hultgren SJ.** 2009. Contribution of autolysin and Sortase a during *Enterococcus faecalis* DNA-dependent biofilm development. Infection and immunity **77:**3626-3638.
132. **Rice KC, Mann EE, Endres JL, Weiss EC, Cassat JE, Smeltzer MS, Bayles KW.** 2007. The cidA murein hydrolase regulator contributes to

DNA release and biofilm development in *Staphylococcus aureus*. Proceedings of the National Academy of Sciences **104:**8113-8118.

133. **Thomas VC, Hiromasa Y, Harms N, Thurlow L, Tomich J, Hancock LE.** 2009. A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of *Enterococcus faecalis*. Molecular microbiology **72:**1022-1036.
134. **Arciola CR, Baldassarri L, Campoccia D, Creti R, Pirini V, Huebner J, Montanaro L.** 2008. Strong biofilm production, antibiotic multi-resistance and high gelE expression in epidemic clones of *Enterococcus faecalis* from orthopaedic implant infections. Biomaterials **29:**580-586.
135. **Rice KC, Firek BA, Nelson JB, Yang SJ, Patton TG, Bayles KW.** 2003. The *Staphylococcus aureus* cidAB Operon: Evaluation of Its Role in Regulation of Murein Hydrolase Activity and Penicillin Tolerance. Journal of bacteriology **185:**2635-2643.
136. **Oshida T, Sugai M, Komatsuzawa H, Hong YM, Suginaka H, Tomasz A.** 1995. A *Staphylococcus aureus* autolysin that has an N-acetylmuramoyl-L-alanine amidase domain and an endo-f8-N-acetylglucosaminidase domain: Cloning, sequence analysis, and characterization. Proceedings of the National Academy of Sciences **92:**285-289.
137. **Bose JL, Lehman MK, Fey PD, Bayles KW.** 2012. Contribution of the *Staphylococcus aureus* Atl AM and GL murein hydrolase activities in cell division, autolysis, and biofilm formation. PloS one **7:**e42244.
138. **Houston P, Rowe SE, Pozzi C, Waters EM, O'Gara JP.** 2010. Essential role for the major autolysin in the fibronectin-binding protein-mediated *Staphylococcus aureus* biofilm phenotype. Infection and immunity **79:**1153-1165.
139. **Pasztor L, Ziebandt AK, Nega M, Schlag M, Haase S, Franz-Wachtel M, Madlung J, Nordheim A, Heinrichs DE, Gotz F.** 2010. Staphylococcal major autolysin (Atl) is involved in excretion of cytoplasmic proteins. Journal of Biological Chemistry **285:**36794-36803.
140. **Heilmann C, Hartleib J, Hussain MS, Peters G.** 2005. The multifunctional *Staphylococcus aureus* autolysin aaa mediates adherence to immobilized fibrinogen and fibronectin. Infection and immunity **73:**4793-4802.
141. **Biswas R, Voggu L, Simon UK, Hentschel P, Thumm G, Gotz F.** 2006. Activity of the major staphylococcal autolysin Atl. FEMS Microbiology Letters **259:**260-268.
142. **Ranjit DK, Endres JL, Bayles KW.** 2011. *Staphylococcus aureus* CidA and LrgA proteins exhibit holin-like properties. Journal of bacteriology **193:**2468-2476.
143. **Groicher KH, Firek BA, Fujimoto DF, Bayles KW.** 2000. The *Staphylococcus aureus* lrgAB operon modulates murein hydrolase activity and penicillin tolerance. Journal of bacteriology **182:**1794-1801.
144. **Bayles KW.** 2007. The biological role of death and lysis in biofilm development. Nature Reviews Microbiology **5:**721-726.
145. **Wang J, Bayles KW.** 2013. Programmed cell death in plants: lessons from bacteria? Trends in Plant Science **18:**133-139.
146. **Heilmann C, Thumm G, Chhatwal GS, Hartleib J, Uekotter A, Peters G.** 2003. Identification and characterization of a novel autolysin (Aae)

with adhesive properties from *Staphylococcus epidermidis*. Microbiology **149:**2769-2778.
147. **Heilmann C, Hussain M, Peters G, Götz F.** 1997. Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystrene surface. Molecular microbiology **24:**1013-1024.
148. **Christner M, Heinze C, Busch M, Franke G, Hentschke M, Bayard Duhring S, Buttner H, Kotasinska M, Wischnewski V, Kroll G, Buck F, Molin S, Otto M, Rohde H.** 2012. sarA negatively regulates *Staphylococcus epidermidis* biofilm formation by modulating expression of 1 MDa extracellular matrix binding protein and autolysis-dependent release of eDNA. Molecular microbiology **86:**394-410.
149. **Zoll S, Patzold B, Schlag M, Gotz F, Kalbacher H, Stehle T.** 2010. Structural basis of cell wall cleavage by a staphylococcal autolysin. PLoS pathogens **6:**e1000807.
150. **Otto M.** 2014. Physical stress and bacterial colonization. FEMS microbiology reviews **38:**1250-1270.
151. **Lappann M, Claus H, van Alen T, Harmsen M, Elias J, Molin S, Vogel U.** 2010. A dual role of extracellular DNA during biofilm formation of *Neisseria meningitidis*. Molecular microbiology **75:**1355-1371.
152. **Hatfull GF, Hendrix RW.** 2011. Bacteriophages and their genomes. Current Opinion in Virology **1:**298-303.
153. **Casjens S.** 2003. Prophages and bacterial genomics: what have we learned so far? Molecular microbiology **49:**277-300.
154. **Canchaya C, Fournous G, Brüssow H.** 2004. The impact of prophages on bacterial chromosomes. Molecular microbiology **53:**9-18.
155. **Dobrindt U, Hochhut B, Hentschel U, Hacker J.** 2004. Genomic islands in pathogenic and environmental microorganisms. Nature Reviews Microbiology **2:**414-424.
156. **Duport C, Baysse C, Michel-Briand Y.** 1995. Molecular characterisation of pyocin S3, a novel S-type pyocin from *Pseudomonas aeruginosa*. Journal of Biological Chemistry **270:**8920-8927.
157. **Elowitz MB, Levine AJ, Sigga ED, Swain PS.** 2002. Stochastic gene expression in a single cell. Science **297:**1183-1186.
158. **Little JW.** 1990. Chance phenotypic variation. Trends in Biochemical Sciences **15:**138.
159. **Xu M, Struck DK, Deaton J, Wang IN, Young R.** 2004. A signal-arrest-release sequence mediates export and control of the phage P1 endolysin. Proceedings of the National Academy of Sciences **101:**6415-6420.
160. **Young R.** 2002. Bacteriophage holins: deadly diversity. Journal of Molecular Microbiology and Biotechnology **4:**21-36.
161. **Young RF, White RL.** 2008. Lysis of the host by bacteriophage, vol. 3. Academic Press, Boston, USA.
162. **Nakayama K, Takashima K, Ishihara H, Shinomiya T, Kageyama M, Kanaya S, Ohnishi M, Murata T, Mori H, Hayashi T.** 2000. The R-type pyocin of *Pseudomonas aeruginosa* is related to P2 phage, and the F-type is related to lambda phage. Molecular microbiology **38:**213-231.
163. **Zagotta MT, Wilson DB.** 1990. Oligomerization of the bacteriophage lambda S protein in the inner membrane of *Escherichia coli*. Journal of bacteriology **172:**912-921.

164. **Young R.** 1992. Bacteriophage lysis: mechanism and regulation. Microbiological Reviews **56:**430-481.
165. **Gödeke J, Paul K, Lassak J, Thormann KM.** 2011. Phage-induced lysis enhances biofilm formation in *Shewanella oneidensis* MR-1. ISME Journal **5:**613-626.
166. **Heidelberg JF, Paulsen IT, Nelson KE, Gaidos EJ, Nelson WC, Read TD, Eisen JA, Seshadri R, Ward N, Methe B, Clayton RA, Meyer T, Tsapin A, Scott J, Beanan M, Brinkac L, Daugherty S, DeBoy RT, Dodson RJ, Durkin AS, Haft DH, Kolonay JF, Madupu R, Peterson JD, Umayam LA, White O, Wolf AM, Vamathevan J, Weidman J, Impraim M, Lee K, Berry K, Lee C, Mueller J, Khouri H, Gill J, Utterback TR, McDonald LA, Feldblyum TV, Smith HO, Venter JC, Nealson KH, Fraser CM.** 2002. Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. Nature biotechnology **20:**1118-1123.
167. **Carrolo M, Frias MJ, Pinto FR, Melo-Cristino J, Ramirez M.** 2010. Prophage spontaneous activation promotes DNA release enhancing biofilm formation in *Streptococcus pneumoniae*. PloS one **5:**e15678.
168. **Moscoso M, Claverys JP.** 2004. Release of DNA into the medium by competent *Streptococcus pneumoniae*: kinetics, mechanism and stability of the liberated DNA. Molecular microbiology **54:**783-794.
169. **Severina E, Ramirez M, Tomasz A.** 1999. Prophage carriers as a molecular epidemiological marker in *Streptococcus pneumoniae*. Journal of Clinical Microbiology **37:**3308-3315.
170. **Steinmoen H, Knutsen E, Havarstein LS.** 2002. Induction of natural competence in *Streptococcus pneumoniae* triggers lysis and DNA release from a subfraction of the cell population. Proceedings of the National Academy of Sciences **99:**7681-7686.
171. **Spoering AL, Gilmore MS.** 2006. Quorum sensing and DNA release in bacterial biofilms. Current opinion in microbiology **9:**133-137.
172. **Sykes R.** 2010. The 2009 Garrod lecture: the evolution of antimicrobial resistance: a Darwinian perspective. Journal of Antimicrobial Chemotherapy **65:**1842-1852.
173. **Dillard JP, Seifert HS.** 2001. A variable genetic island specific for *Neisseria gonorrhoeae* is involved in providing DNA for natural trasnformation and is found more often in disseminated infection isolates. Molecular microbiology **41:**263-277.
174. **Montanaro L, Poggi A, Visai L, Ravaioli S, Campoccia D, Speziale P, Arciola CR.** 2011. Extracellular DNA in biofilms. International Journal of Artificial Organs **34:**824-831.
175. **Whiteley M, Lee KM, Greenberg EP.** 1999. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences **96:**13904-13909.
176. **Webb JS, Thompson LS, James S, Charlton T, Tolker-Nielsen T, Koch B, Givskov M, Kjelleberg S.** 2003. Cell death in *Pseudomonas aeruginosa* biofilm development. Journal of bacteriology **185:**4585-4592.
177. **Passador L, Cook JM, Gambello MJ, Rust L, Iglewski BH.** 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science **260:**1127-1130.

178. **Latifi A, Winson MK, Foglino M, Bycroft BW, Stewart GSAB, Lazdunski A, Williams P.** 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. Molecular microbiology **17:**333-343.
179. **Pesci EC, Milbank JBJ, Pearson JP, McKnight S, Kende AS, Greenberg EP, Iglewski BH.** 1999. Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences **96:**11229-11234.
180. **McKnight S, Iglewski BH, Pesci EC.** 2000. The *Pseudomonas* quinolone signal regulates *rhl* quorum sensing in *Pseudomonas aeruginosa*. Journal of bacteriology **182:**2702-2708.
181. **Venkataraman A, Rosenbaum M, Arends JBA, Halitschke R, Angenent LT.** 2010. Quorum sensing regulates electric current generation of *Pseudomonas aeruginosa* PA14 in bioelectrochemical systems. Electrochemistry Communications **12:**459-462.
182. **Price-Whelan A, Dietrich LE, Newman DK.** 2006. Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. Nature Chemical Biology **2:**71-78.
183. **Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G, Thomashow LS.** 2001. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. Journal of bacteriology **183:**6454-6465.
184. **Das T, Manefield M.** 2012. Pyocyanin promotes extracellular DNA release in *Pseudomonas aeruginosa*. PloS one **7:**e46718.
185. **Petrova OE, Sauer K.** 2009. A novel signaling network essential for regulating *Pseudomonas aeruginosa* biofilm development. PLoS pathogens **5:**e1000668.
186. **Petrova OE, Schurr JR, Schurr MJ, Sauer K.** 2011. The novel *Pseudomonas aeruginosa* two-component regulator BfmR controls bacteriophage-mediated lysis and DNA release during biofilm development through PhdA. Molecular microbiology **81:**767-783.
187. **Rice SA, Tan CH, Mikkelsen PJ, Kung V, Woo J, Tay M, Hauser A, McDougald D, Webb JS, Kjelleberg S.** 2009. The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage. ISME Journal **3:**271-282.
188. **Kirov SM, Webb JS, O'May CY, Reid DW, Woo JKK, Rice SA, Kjelleberg S.** 2007. Biofilm differentiation and dispersal in mucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Microbiology **153:**3264-3274.
189. **Webb JS, Lau M, Kjelleberg S.** 2004. Bacteriophage and phenotypic variation in *Pseudomonas aeruginosa* biofilm development. Journal of bacteriology **186:**8066-8073.
190. **Wang X, Kim Y, Ma Q, Hong SH, Pokusaeva K, Sturino JM, Wood TK.** 2010. Cryptic prophages help bacteria cope with adverse environments. Nature communications **1:**147.
191. **Baharoglu Z, Mazel D.** 2014. SOS, the formidable strategy of bacteria against aggressions. FEMS microbiology reviews **38:**1126-1145.

192. **Erill I, Campoy S, Barbe J.** 2007. Aeons of distress: an evolutionary perspective on the bacterial SOS response. FEMS microbiology reviews **31:**637-656.
193. **Brazas MD, Hancock RE.** 2005. Ciprofloxacin induction of a susceptibility determinant in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy **49:**3222-3227.
194. **Cirz RT, O'Neill BM, Hammond JA, Head SR, Romesberg FE.** 2006. Defining the *Pseudomonas aeruginosa* SOS response and its role in the global response to the antibiotic ciprofloxacin. Journal of bacteriology **188:**7101-7110.
195. **Penterman J, Singh PK, Walker GC.** 2014. Biological cost of pyocin production during the SOS response in *Pseudomonas aeruginosa*. Journal of bacteriology **196:**3351-3359.
196. **Nanda AM, Heyer A, Kramer C, Grunberger A, Kohlheyer D, Frunzke J.** 2014. Analysis of SOS-induced spontaneous prophage induction in *Corynebacterium glutamicum* at the single-cell level. Journal of bacteriology **196:**180-188.
197. **McCool JD, Long E, Petrosino JF, Sandler HA, Rosenberg SM, Sandler SJ.** 2004. Measurement of SOS expression in individual *Escherichia coli* K-12 cells using fluorescence microscopy. Molecular microbiology **53:**1343-1357.
198. **Pennington JM, Rosenberg SM.** 2007. Spontaneous DNA breakage in single living *Escherichia coli* cells. Nature Genetics **39:**797-802.
199. **Lwoff A.** 1953. Lysogeny. Bacteriological Reviews **17:**269-332.
200. **Radman M.** 1975. Phenomenology of an inducible mutagenic DNA repair pathway in *Escherichia coli*: SOS repair pichulein hypothesis. Basic Life Sciences **5A:**355-367.
201. **Simmons LA, Cohen SE, Foti JJ, Walker GC.** 2008. The SOS Regulatory Network. EcoSal Plus **3**.
202. **Broussard Gregory W, Oldfield Lauren M, Villanueva Valerie M, Lunt Bryce L, Shine Emilee E, Hatfull Graham F.** 2013. Integration-dependent bacteriophage immunity provides insights into the evolution of genetic switches. Molecular cell **49:**237-248.
203. **Roberts JW, Roberts CW.** 1975. Proteolytic cleavage of bacteriophage lambda repressor in induciton. Proceedings of the National Academy of Sciences **72:**147-151.
204. **Tomasz M.** 1995. Mitomycin C: small, fast and deadly (but very selective). Chemistry and Biology **2:**575-579.
205. **Piddock LJV, Wise R.** 1987. Induction of the SOS response in *Escherichia coli* by 4-quinolone antimicrobial agents. FEMS Microbiology Letters **41:**289-294.
206. **Wagner PL, Neely MN, Zhang X, Acheson DWK, Waldor MK, Friedman DI.** 2001. Role for a phage in shiga toxin 2 expression from a pathogenic *Escherichia coli* strain. Journal of bacteriology **183:**2081-2085.
207. **Lee KM, Park Y, Bari W, Yoon MY, Go J, Kim SC, Lee Hi, Yoon SS.** 2012. Activation of cholera toxin production by anaerobic respiration of trimethylamine N-oxide in *Vibrio cholerae*. Journal of Biological Chemistry **287:**39742-39752.

208. **Figueroa-Bossi N, Bossi L.** 1999. Inducible prophages contribute to *Salmonella* virulence in mice. Molecular microbiology **33:**167-176.
209. **Beall B, López E, Domenech A, Ferrándiz M-J, Frias MJ, Ardanuy C, Ramirez M, García E, Liñares J, de la Campa AG.** 2014. Induction of prophages by fluoroquinolones in *Streptococcus pneumoniae*: implications for emergence of resistance in genetically-related clones. PloS one **9:**e94358.
210. **Prudhomme M, Attaiech L, Sanchez G, Martin B, Claverys JP.** 2006. Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. Science **313:**89-92.
211. **Martin B, Garcia P, Castanie MP, Claverys JP.** 1995. The *recA* gene of *Streptococcus pneumoniae* is part of a competence-induced operon and controls lysogenic induction. Molecular microbiology **15:**367-379.
212. **Lunde M, Aastveit AH, Blatny JM, Nes IF.** 2005. Effects of diverse environmental conditions on LC3 prophage stability in *Lactococcus lactis*. Applied and environmental microbiology **71:**721-727.
213. **Alves de Matos AP, Lehours P, Timóteo A, Roxo-Rosa M, Vale FF.** 2013. Comparison of induction of B45 Helicobacter pylori prophage by acid and UV radiation. Microscopy and Microanalysis **19:**27-28.
214. **Tanouchi Y, Pai A, Buchler NE, You L.** 2012. Programming stress-induced altruistic death in engineered bacteria. Molecular systems biology **8:**626.
215. **Goodwin K, Levin RE, Doggett RG.** 1972. Autosensitivity of *Pseudomonas aeruginosa* to its own pyocin. Infection and immunity **6:**889-892.
216. **Gill JJ, Young RF.** 2011. Therapeutic Applications of Phage Biology: History, Practice and Recommendations. *In* Miller AA, Miller PF (ed.), Emerging Trends in Antibacterial Discovery: Answering the Call to Arms. Caister Academic Press, Norfolk, UK.
217. **Sun Z, Shi J, Liu C, Jin Y, Li K, Chen R, Jin S, Wu W.** 2014. PrtR homeostasis contributes to *Pseudomonas aeruginosa* pathogenesis and resistance aganist ciprofloxacin. Infection and immunity **82:**1638-1647.
218. **Jacob F.** 1954. Biosynthèse induite et mode d'action d'une pyocine, antibiotque de *Pseudomonas pyocyanea*. Annales de l'Institut Pasteur **86:**149-160.
219. **Shinomiya T.** 1972. Studies on biosynthesis and morphogenesis of R-pyocins of *Pseudomonas aeruginosa*. Journal of Biochemistry **72:**39-48.
220. **Kageyama M, Egami F.** 1962. On the purification and some proporties of a pyocin, a bacteriocin produced by *Pseudmonas aeruginosa*. Life Sciences **9:**471-476.
221. **Sano Y, Kageyama M.** 1987. The sequence and function of the *recA* gene and its protein in *Pseudomonas aeruginosa* PAO. Molecular Genetics and Genomics **208:**412-419.
222. **Michel-Briand Y, Baysse C.** 2002. The pyocins of *Pseudomonas aeruginosa*. Biochimie **84:**499-510.
223. **Matsui H, Sano Y, Ishihara H, Shinomiya T.** 1993. Regulation of pyocin genes in *Pseudomonas aeruginosa* by positive (prtN) and negative (prtR) regulatory genes. Journal of bacteriology **175:**1257-1263.

224. **Boles BR, Singh PK.** 2008. Endogenous oxidative stress produces diversity and adaptability in biofilm communities. Proceedings of the National Academy of Sciences **105:**12503-12508.
225. **Chang W, Small DA, Toghrol F, Bentley WE.** 2005. Microarray analysis of *Pseudomonas aeruginosa* reveals induction of pyocin genes in response to hydrogen peroxide. BMC genomics **6:**115.
226. **Fisher LM, Lawerence JM, Josty IC, Hopewell R, Margerrison EEC, Cullen ME.** 1989. Ciprofloxacin and the fluoroquinolones: New concepts on the mechanism of action and resistance. The American Journal of Medicine **87:**S2-S8.
227. **McFarland KA, Dolben EL, LeRoux M, Kambara TK, Ramsey KM, Kirkpatrick RL, Mougous JD, Hogan DA, Dove SL.** 2015. A self-lysis pathway that enhances the virulence of a pathogenic bacterium. Proceedings of the National Academy of Sciences **112:**8433-8438.
228. **Berry J, Rajaure M, Pang T, Young R.** 2012. The spanin complex is essential for lambda lysis. Journal of bacteriology **194:**5667-5674.
229. **Steiner M, Lubitz W, Bläsi U.** 1993. The missing link in phage lysis of Gram-positive bacteria: gene *14* of *Bacillus subtilis* phage 29 encodes the functional homolog of lambda S protein. Journal of bacteriology **175:**1038-1042.
230. **Frank SA.** 1998. Foundations of Social Evolution. Princeton University Press, Princeton.
231. **Ackermann M, Stecher B, Freed NE, Songhet P, Hardt WD, Doebeli M.** 2008. Self-destructive cooperation mediated by phenotypic noise. Nature **454:**987-990.
232. **Cascales E, Buchanan SK, Duche D, Kleanthous C, Lloubes R, Postle K, Riley M, Slatin S, Cavard D.** 2007. Colicin biology. Microbiology and Molecular Biology Reviews **71:**158-229.
233. **Bensing BA, Siboo IR, Sullam PM.** 2001. Proteins PblA and PblB of *Streptococcus mitis*, which promote binding to human platelets, are encoded within a lysogenic bacteriophage. Infection and immunity **69:**6186-6192.
234. **Mitchell J, Siboo IR, Takamatsu D, Chambers HF, Sullam PM.** 2007. Mechanism of cell surface expression of the *Streptococcus mitis* platelet binding proteins PblA and PblB. Molecular microbiology **64:**844-857.
235. **Seo HS, Xiong YQ, Mitchell J, Seepersaud R, Bayer AS, Sullam PM.** 2010. Bacteriophage lysin mediates the binding of *Streptococcus mitis* to human platelets through interaction with fibrinogen. PLoS pathogens **6:**e1001047.
236. **Hughes D, Matos RC, Lapaque N, Rigottier-Gois L, Debarbieux L, Meylheuc T, Gonzalez-Zorn B, Repoila F, Lopes MdF, Serror P.** 2013. *Enterococcus faecalis* prophage dynamics and contributions to pathogenic traits. PLoS Genetics **9:**e1003539.
237. **Beveridge TJ.** 1999. Structures of Gram-negative cell walls and their derived membrane vesicles. Journal of bacteriology **181:**4725-4733.
238. **Bomberger JM, Maceachran DP, Coutermarsh BA, Ye S, O'Toole GA, Stanton BA.** 2009. Long-distance delivery of bacterial virulence factors by *Pseudomonas aeruginosa* outer membrane vesicles. PLoS pathogens **5:**e1000382.

239. **Li Z, Clarke AJ, Beveridge TJ.** 1998. Gram-negative bacteria produce membrane vesicles which are capable of killing other bacteria. Journal of bacteriology **180:**5478-5483.
240. **Toyofuku M, Roschitzki B, Riedel K, Eberl L.** 2012. Identification of proteins associated with the *Pseudomonas aeruginosa* biofilm extracellular matrix. Journal of proteome research **11:**4906-4915.
241. **Mashburn LM, Whiteley M.** 2005. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nature **437:**422-425.
242. **Mashburn-Warren L, McLean RJ, Whiteley M.** 2008. Gram-negative outer membrane vesicles: beyond the cell surface. Geobiology **6:**214-219.
243. **Toyofuku M, Zhou S, Sawada I, Takaya N, Uchiyama H, Nomura N.** 2013. Membrane vesicle formation is associated with pyocin production under denitrifying conditions in *Pseudomonas aeruginosa* PAO1. Environmental microbiology **16:**2927-2938.
244. **Beveridge TJ, Makin SA, Kadurugamuwa JL, Li Z.** 1997. Interactions between biofilms and the environment. FEMS microbiology reviews **20:**291-303.
245. **Bauman SJ, Kuehn MJ.** 2009. *Pseudomonas aeruginosa* vesicles associate with and are internalized by human lung epithelial cells. BMC microbiology **9:**26.
246. **Kadurugamuwa JL, Beveridge TJ.** 1996. Bacteriolytic effect of membrane vesicles from *Pseudomonas aeruginosa* on other bacteria including pathogens: conceptually new antibiotics. Journal of bacteriology **178:**2767-2774.
247. **Turnbull L, Toyofuku M, Hynen AL, Kurosawa M, Pessi G, Petty NK, Osvath SR, Carcamo-Oyarce G, Gloag ES, Shimoni R, Omasits U, Ito S, Yap X, Monahan LG, Cavaliere R, Ahrens CH, Charles IG, Nomura N, Eberl L, Whitchurch CB.** 2016. Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. Nature communications **7:**11220.
248. **Pang X, Moussa SH, Targy NM, Bose JL, George NM, Gries C, Lopez H, Zhang L, Bayles KW, Young R, Luo X.** 2011. Active Bax and Bak are functional holins. Genes and Development **25:**2278-2290.
249. **Rice KC, Bayles KW.** 2003. Death's toolbox: examining the molecular components of bacterial programmed cell death. Molecular microbiology **50:**729-738.
250. **Tashiro Y, Ichikawa S, Shimizu M, Toyofuku M, Takaya N, Nakajima-Kambe T, Uchiyama H, Nomura N.** 2010. Variation of physiochemical properties and cell association activity of membrane vesicles with growth phase in *Pseudomonas aeruginosa*. Applied and environmental microbiology **76:**3732-3739.
251. **Simon R, O'Connell M, Labes M, Pühler A.** 1986. Plasmid vector for the genetic analysis and manipulation of rhizobia and other gram-negative bacteria. Methods in Enzymology **118:**640-659.
252. **Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, Ausubel FM.** 1995. Common virulence factors for bacterial pathogenicity in plants and animals. Science **268:**1899-1902.

253. **Liu PV.** 1966. The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. III. Identity of the lethal toxins produced *in vitro* and *in vivo*. Journal of Infectious Diseases **116:**481-489.
254. **Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, Villanueva J, Wei T, Ausubel FM.** 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proceedings of the National Academy of Sciences **103:**2833-2838.
255. **Lindner AB, Madden R, Demarez A, Stewart EJ, Taddei F.** 2008. Asymmetric segregation of protein aggregates is associated with cellular aging and rejuvenation. Proceedings of the National Academy of Sciences **105:**3076-3081.
256. **Newman JR, Fuqua C.** 1999. Broad-host-range expressions vectors that carry the L-arabinose-inducible *Escherichia coli ara BAD* promoter and the *araC* regulator. Gene **1999:**197-203.
257. **Alm RA, Mattick JS.** 1996. Identification of two genes with prepilin-like leader sequences involved in type fimbrial biogenesis in *Pseudomonas aeruginosa*. Journal of bacteriology **178:**3809-3817.
258. **Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP.** 1998. A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77-86.
259. **West SEH, Schweizer HP, Dall C, Sample AK, Runyen-Janecky LJ.** 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and the sequence of the region required for their replication in *Pseudomonas aeruginosa*. Gene **128:**81-86.
260. **Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A.** 2012. Fiji: an open-source platform for biological-image analysis. Nature methods **9:**676-682.
261. **O'Toole GA.** 2011. Microtiter dish biofilm formation assay. Journal of Visualised Experiments **47:**e2437-e2437.
262. **Filloux A, Ramos JL.** 2014. *Pseudomonas* Methods and Protocols, vol. 1149. Humana Press, NY, USA.
263. **Harmsen M, Lappann M, Knochel S, Molin S.** 2010. Role of extracellular DNA during biofilm formation by *Listeria monocytogenes*. Applied and environmental microbiology **76:**2271-2279.
264. **Allocati N, Masulli M, Di Ilio C, De Laurenzi V.** 2015. Die for the community: an overview of programmed cell death in bacteria. Cell Death and Disease **6:**e1609.
265. **Grundling A, Manson MD, Young R.** 2001. Holins kill without warning. Proceedings of the National Academy of Sciences **98:**9348-9352.
266. **Smith AW, Hirst PH, Hughes K, Gensberg K, Govan JRW.** 1992. The pyocin Sa receptor of *Pseudomonas aeruginosa* is associated with ferripyoverdin uptake. Journal of bacteriology **174:**4847-4849.
267. **Ameisen JC.** 2002. On the origin, evolution, and nature of programmed cell death: a timeline of four billion years. Cell Death and Differentiation **9:**367-393.

268. **Bayles KW.** 2014. Bacterial programmed cell death: making sense of a paradox. Nature Reviews Microbiology **12:**63-69.
269. **Nedelcu AM, Driscoll WW, Durand PM, Herron MD, Rashidi A.** 2011. On the paradigm of altruistic suicide in the unicellular world. Evolution; international journal of organic evolution **65:**3-20.
270. **Aertsen A, Michiels CW.** 2004. Stress and how bacteria cope with death and survival. Critical reviews in microbiology **30:**263-273.
271. **Wagner PL, Waldor MK.** 2002. Bacteriophage control of bacterial virulence. Infection and immunity **70:**3985-3993.
272. **Nanda AM, Thormann K, Frunzke J.** 2015. Impact of spontaneous prophage induction on the fitness of bacterial populations and host-microbe interactions. Journal of bacteriology **197:**410-419.
273. **Shinomiya T, Shiga S, Kageyama M.** 1983. Genetic determinant of pyocin R2 in *Pseudomonas aeruginosa* PAO. Molecular and General Genetics MGG **189:**375-381.
274. **Maredia R, Devineni N, Lentz P, Dallo SF, Yu J, Guentzel N, Chambers J, Arulanandam B, Haskins WE, Weitao T.** 2012. Vesiculation from Pseudomonas aeruginosa under SOS. Scientific World Journal **2012:**402919.
275. **Govan JR.** 1986. *In vivo* significance of bacteriocins and bacteriocin receptors. Scandinavian Journal of Infectious Diseases **49:**31-37.
276. **Mogk A, Deuerling E, Vorderwülbecke S, Vierling E, Bukau B.** 2003. Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. Molecular microbiology **50:**585-595.
277. **Modrof J, Lymperopoulos K, Roy P.** 2005. Phosphorylation of bluetongue virus nonstructural protein 2 is essential for formation of viral inclusion bodies. Journal of Virology **79:**10023-10031.
278. **Hui JG, Mai-Prochnow A, Kjelleberg S, McDougald D, Rice SA.** 2014. Environmental cues and genes involved in establishment of the superinfective Pf4 phage of *Pseudomonas aeruginosa*. Frontiers in microbiology **5:**654.
279. **Knezevic P, Voet M, Lavigne R.** 2015. Prevalence of Pf1-like (pro)phage genetic elements among *Pseudomonas aeruginosa* isolates. Virology **483:**64-71.
280. **Garcia-Fruitos E, Gonzalez-Montalban N, Morell M, Vera A, Ferraz RM, Aris A, Ventura S, Villaverde A.** 2005. Aggregation as bacterial inclusion bodies does not imply inactivation of enzymes and fluorescent proteins. Microbial Cell Factories **4:**27.
281. **Dobson CM.** 2001. The structural basis of protein folding and its links with human disease. Philosophical Transactions of the Royal Society B: Biological Sciences **356:**133-145.
282. **Harding JJ.** 2002. Viewing molecular mechanisms of ageing through a lens. Ageing Research Reviews **1:**465-479.
283. **Muchowski PJ, Wacker JL.** 2005. Modulation of neurodegeneration by molecular chaperones. Nature Reviews Neuroscience **6:**11-22.
284. **Speed MA, Wang DIC, King J.** 1996. Specific aggregation of partially folded polypeptide chains: The molecular basis of inclusion body composition. Nature biotechnology **14:**1283-1287.

285. **Mitraki A, Fane B, Haase-Pettinggell C, Sturtevant J, King J.** 1991. Global suppression of protein folding defects and inclusion body formation. Science **253**.
286. **Allen SP, Polazzi JO, Gierse JK, Easton AM.** 1992. Two novel heat shock genes encoding proteins produced in response to heterologous protein expression in *Escherichia coli*. Journal of bacteriology **174:**6938-3947.
287. **Lethanh H, Neubauer P, Hoffmann F.** 2005. The small heat-shock proteins IbpA and IbpB reduce the stress load of recombinant *Escherichia coli* and delay degradation of inclusion bodies. Microbial Cell Factories **4:**6.
288. **Hancock RE.** 1981. Aminoglycoside uptake and mode of action - with special reference to streptomycin and gentamicin: I. Antagonists and mutants. Journal of Antimicrobial Chemotherapy **8:**249-276.
289. **Koyama Y, Kurosasa A, Tsuchiya A, Takakuta K.** 1950. A new anitbiotic "colistin" produced by spore-forming soil bacteria. Journal of Antibiotics **3:**457-458.
290. **Koike M, Iida K, Matsuo T.** 1969. Electron microscopic studies on mode of action of polymyxin. Journal of bacteriology **97:**448-452.
291. **Schindler PRG, Teuber M.** 1975. Action of polymyxin B on bacterial membranes: morphological changes in the cytoplasm and in the outer membrane of *Salmonella typhimurium* and *Escherichia coli* B. Antimicrobial Agents and Chemotherapy **8:**95-104.
292. **Ciofu O, Tolker-Nielsen T.** 2010. Antibiotic tolerance and resistance in biofilms. Biofilm Infections**:**215-229.
293. **Linares JF, Gustafsson I, Baquero F, Martinez JL.** 2006. Antibiotics as intermicrobial signaling agents instead of weapons. Proceedings of the National Academy of Sciences **103:**19484-19489.
294. **Schadow K, H., Simpson WA, Christiansen GD.** 1988. Characteristics of adherence to plastic tissue culture plates of coagulase-negative Staphylococci exposed to subinhibitory concentrations of antimicrobial agents. Journal of Infectious Diseases **157:**71-77.
295. **Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI.** 2005. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature **436:**1171-1175.
296. **Bagge N, Schuster M, Hentzer M, Ciofu O, Givskov M, Greenberg EP, Hoiby N.** 2004. *Pseudomonas aeruginosa* Biofilms Exposed to Imipenem Exhibit Changes in Global Gene Expression and -Lactamase and Alginate Production. Antimicrobial Agents and Chemotherapy **48:**1175-1187.
297. **Boehm A, Steiner S, Zaehringer F, Casanova A, Hamburger F, Ritz D, Keck W, Ackermann M, Schirmer T, Jenal U.** 2009. Second messenger signalling governs *Escherichia coli* biofilm induction upon ribosomal stress. Molecular microbiology **72:**1500-1516.
298. **Ahmed NA, Petersen FC, Scheie AA.** 2009. AI-2/LuxS is involved in increased biofilm formation by *Streptococcus intermedius* in the presence of antibiotics. Antimicrobial Agents and Chemotherapy **53:**4258-4263.
299. **Wang Q, Sun FJ, Liu Y, Xiong LR, Xie LL, Xia PY.** 2010. Enhancement of biofilm formation by subinhibitory concentrations of macrolides in

icaADBC-positive and -negative clinical isolates of *Staphylococcus epidermidis*. Antimicrobial Agents and Chemotherapy **54:**2707-2711.
300. **Justice SS, Hunstad DA, Cegelski L, Hultgren SJ.** 2008. Morphological plasticity as a bacterial survival strategy. Nature Reviews Microbiology **6:**162-168.
301. **Becerra MC, Albesa I.** 2002. Oxidative stress induced by ciprofloxacin in *Staphylococcus aureus*. Biochemical and Biophysical Resaerch Communications **297:**1003-1007.
302. **Albesa I, Becerra MC, Battan PC, Paez PL.** 2004. Oxidative stress involved in the antibacterial action of different antibiotics. Biochemical and Biophysical Research Communications **317:**605-609.
303. **Goswami M, Mangoli SH, Jawali N.** 2006. Involvement of reactive oxygen species in the action of ciprofloxacin against *Escherichia coli*. Antimicrobial Agents and Chemotherapy **50:**949-954.
304. **Fang FC.** 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nature Reviews Microbiology **2:**820-832.
305. **Kaplan JB.** 2011. Antibiotic-induced biofilm formation. International Journal of Artificial Organs **34:**737-751.
306. **Gotoh H, Kasaraneni N, Devineni N, Dallo SF, Weitao T.** 2010. SOS involvement in stress-inducible biofilm formation. Biofouling **26:**603-611.
307. **Wagner PL, Acheson DWK, Waldor MK.** 2001. Human neutrophils and their products induce shiga toxin production by enterohemorrhagic *Escherichia coli*. Infection and immunity **69:**1934-1937.
308. **Zhang X, McDaniel AD, Wolf LE, Keusch GT, Waldor MK, Acheson DWK.** 2000. Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. Journal of Infectious Diseases **181:**664-670.
309. **Matsushiro A, Sato K, Miyamoto H, Yamamura T, Honda T.** 1999. Induction of prophages of enterohemorrhagic *Escherichia coli* O157:H7 with Norfloxacin. Journal of bacteriology **181:**2257-2260.
310. **Köhler B, Karch H, Schmidt H.** 2000. Antibacterials that are used as growth promoters in animal husbandry can affect the release of Shiga-toxin-2-converting bacteriophages and Shiga toxin 2 from *Escherichia coli* strains. Microbiology **146:**1085-1090.
311. **Kimmitt PT, Harwood CR, Barer MR.** 2000. Toxin gene expression by shiga toxin-producing *Escherichia coli*: the role of antibiotics and the bacterial SOS response. Emerging Infectious Diseases **6:**458-465.
312. **Carter AO, Borczyk AA, Carlson JAK, Harvey B, Hockin JC, Karmali MA, Krishnan C, Korn DA, Lior H.** 1987. A severe outbreak of *Escherichia coli* O157:H7-associated hemorrhagic colitis in a nursing home. The New England Journal of Medicine **317:**1496-1500.
313. **Butler T, Islam MR, Azad MAK, Jones PK.** 1987. Risk factors for development of hemolytic uremic syndrome during shigellosis. The Journal of Pediatrics **110:**894-897.
314. **D'Argenio DA, Calfee MW, Rainey PB, Pesci EC.** 2002. Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. Journal of bacteriology **184:**6481-6489.
315. **Goodman SD, Obergfell KP, Jurcisek JA, Novotny LA, Downey JS, Ayala EA, Tjokro N, Li B, Justice SS, Bakaletz LO.** 2011. Biofilms can

be dispersed by focusing the immune system on a common family of bacterial nucleoid-associated proteins. Mucosal immunology **4:**625-637.
316. **Brockson ME, Novotny LA, Mokrzan EM, Malhotra S, Jurcisek JA, Akbar R, Devaraj A, Goodman SD, Bakaletz LO.** 2014. Evaluation of the kinetics and mechanism of action of anti-integration host factor-mediated disruption of bacterial biofilms. Molecular microbiology **93:**1246-1258.
317. **Novotny LA, Jurcisek JA, Goodman SD, Bakaletz LO.** 2016. Monoclonal antibodies against DNA-binding tips of DNABII proteins disrupt biofilms in vitro and induce bacterial clearance in vivo. EBioMedicine **10:**33-44.
318. **Jurcisek JA, Bakaletz LO.** 2007. Biofilms formed by nontypeable *Haemophilus influenzae* in vivo contain both double-stranded DNA and type IV pilin protein. Journal of bacteriology **189:**3868-3875.
319. **Das PM, Ramachandran K, vanWert J, Singal R.** 2004. Chromatin immunoprecipitation assay. BioTechniques **38:**961-969.
320. **Gustave JE, Jurcisek JA, McCoy KS, Goodman SD, Bakaletz LO.** 2013. Targeting bacterial integration host factor to disrupt biofilms associated with cystic fibrosis. Journal of Cystic Fibrosis **12:**384-389.
321. **Pressler T.** 2008. Review of recombinant human deoxy ribonuclease (rhDNase) in the management of patients with cystic fibrosis. Biologics: Targets and Therapy **2:**611-617.
322. **McCoy K, Hamilton S, Johnson C.** 1996. Effects of 12-week administration of dornase alfa in patients with advanced cystic fibrosis lung disease. Pulmozyme Study Group. Chest **110:**889-895.
323. **Kaparakis-Liaskos M, Ferrero RL.** 2015. Immune modulation by bacterial outer membrane vesicles. Nature Reviews Immunology **15:**375-387.
324. **André J-B, Godelle B.** 2005. Multicellular organization in bacteria as a target for drug therapy. Ecology Letters **8:**800-810.