

Adaptation to the host environment reduces *Pseudomonas aeruginosa* susceptibility to antibiotics

Dr Simon Michael Stoneham

A Thesis for the Degree of Doctor of Philosophy

January 2025

Department of Infectious Diseases
Imperial College London

Statement of Originality

I confirm that the work presented in this thesis is entirely my own. Where others have contributed to this work, they have been appropriately credited, acknowledged or referenced.

Copyright Declaration

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-Non Commercial 4.0 International Licence (CC BY-NC). Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose. When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe those changes. Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

Acknowledgements

First of all, thank you to my family. None of what you are about to read would have been possible without a supportive family. Coming to a PhD later in life presents its own challenges. Time management has had to be fierce and without the love and support of my wife Becca none of this could have been achieved. Friends and Grandparents too have all joined in the chaos to support my two young children and allow me the space to think, explore my ideas and present this thesis.

I would like to thank my supervisors, Dr Andrew Edwards and Prof Jane Davies have helped to steer and guide me over the past 3 years. Andy has been a constant source of practical and constructive advice and has helped me keep perspective. He is also always ready to set the world to rights in the lab. Jane has always been a source of enthusiasm as well as pragmatic advice in addition to asking interesting questions of my data. I would like to thank Dr Luke Allsopp for teaching me to manipulate *P. aeruginosa* genetically, and for always asking interesting and thought-provoking questions. I would like to thank Dr Gerald Larrouy-Maumus for performing Maldi-ToF on my samples and teaching me to interpret the resulting spectra. I would like to thank Dr Alex McCarthy and Dr Erin Catton for help re-establishing my rusty tissue culture skills and providing me with tips, advice and cell stock. Thank you to my progress review panel: Prof Shiranee Sriskandan and Prof Alain Filloux for supporting my progress over the past 3 years.

A huge thank you to the whole of the Edwards group (and extended Edwards group!): Lizzie, Amber, Akshay, Brigitte, Anna, Ed, Annie. Thank you for welcoming me and sharing your experience, ideas, enthusiasm (Amber) and really, really bad jokes (Akshay). Thank you to all the scientists on the 5th floor, I've enjoyed getting to know you and learning about your work. Thank you for taking the time to chat over coffee and for tolerating my steady stream of dad jokes.

Thank you to my students for all being brilliant: Mark, Amelie, Simran and Lucy.

Erin, Lindsey, Chryso, Lizzie and Andy thank you for rescuing my experiments when my boys were unwell or Southern rail conspired to ruin my time-points by storing my work safely.

Thank you to the scientists at the CBRB for welcoming me and sharing your ideas and discoveries.

Thank you to the MRC for funding this work and my colleagues in the NHS for allowing me the time to leave clinical medicine for a while and become a better scientist.

Abstract

Pseudomonas aeruginosa chronic lung infection is a major cause of morbidity and mortality in people with Cystic Fibrosis (pwCF). Although frequently treated with the polymyxin antibiotic colistin, chronic infection is often impossible to eradicate despite low rates of colistin resistance. In this work it was hypothesised that bacterial adaptation to the lung environment reduces susceptibility to colistin.

The established synthetic sputum model SCFM-2 was modified to investigate the impact of sputum composition on *P. aeruginosa* susceptibility to antimicrobials, using the well characterised strain PA14 and relevant CF clinical isolates. Synthetic sputum significantly reduced susceptibility to colistin in both PA14 and clinical isolates.

Extracellular DNA (eDNA) was found to be the key component responsible and was sufficient to reduce susceptibility to colistin in a dose-dependent manner. MALDI-ToF lipidomic analysis of lipid A revealed that eDNA induced LPS modification with phosphoethanolamine (pEtN). Mutants lacking either *eptA* or *arnC* confirmed modification of LPS with pEtN and 4-amino-4-deoxy L-arabinose (L-ara4N) were important for eDNA-induced reductions in colistin susceptibility. Reduced susceptibility was shown to occur via multiple two-component systems: ColRS, PmrAB and CprRS. Next, it was found that eDNA also induced resistance to the novel antimicrobial murepavadin. However, eDNA-induced resistance was circumvented by exposing bacteria to colistin and murepavadin in combination. Bacteria could also be sensitised to colistin or murepavadin by targeted normalisation of pH in the presence of eDNA by NaHCO₃ confirming environmental pH as an important trigger of reduced susceptibility.

This work demonstrates a previously unrecognised role for endogenous pEtN modification of LPS in host-triggered colistin resistance in *P. aeruginosa*. Both NaHCO₃ and the novel antimicrobial murepavadin enhanced colistin-mediated killing in synthetic sputum indicating their potential value as therapeutics in pwCF.

Contents

Statement of Originality	2
Copyright Declaration	2
Acknowledgements	3
Abstract.....	4
Contents	5
List of Abbreviations.....	9
List of Figures.....	12
List of Tables.....	14
Chapter 1: Introduction	15
1.1 <i>Pseudomonas aeruginosa</i>	15
1.1.1 General characteristics.....	15
1.1.2 <i>P. aeruginosa</i> as an opportunistic pathogen	16
1.1.3 Antimicrobial susceptibility and resistance in <i>P. aeruginosa</i>	18
1.2 Cystic Fibrosis	21
1.2.1 Pathogenesis.....	21
1.2.2 Lung-Disease in Cystic Fibrosis	22
1.2.3 <i>P. aeruginosa</i> in Cystic Fibrosis	24
1.2.4 Treatment of Cystic Fibrosis Lung Disease	29
1.3 Colistin.....	32
1.3.1 Polymyxin antibiotics	32
1.3.2 Structure and pharmacology	32
1.3.4 Mechanisms of resistance	36
1.4 The CF lung environment promotes <i>P. aeruginosa</i> persistence	38
1.4.1 <i>ASL composition</i>	39
1.4.2 Modelling CF lung disease	42
1.5 Hypothesis	47
1.4.1 Aims	48
Chapter 2: Materials and Methods	49
2.1 Bacterial strains and culture.....	49
2.1.1 Bacterial Strains and Plasmids.....	49
2.1.2 Growth Conditions	49
2.1.3 Artificial sputum media.....	51
2.2 Bacterial growth and survival assays.....	53

2.2.1 Determination of bacterial growth	53
2.2.2 Determination of Minimum Inhibitory Concentrations (MICs)	53
2.2.3 Determination of bactericidal activity of antibiotics	54
2.2.4 Determination of colistin activity in media	54
2.3 Cell culture and DNA extraction.....	55
2.3.1 Cell culture	55
2.3.2 DNA purification	55
2.4 Measurement of outer membrane integrity.....	55
2.5 Lipidomics.....	56
2.6 Genetic techniques.....	57
2.6.1 Bacterial DNA purification.....	57
2.6.2 Plasmid DNA purification	57
2.6.3 Polymerase chain reaction	57
2.6.4 Agarose gel electrophoresis	58
2.6.5 Gel purification.....	60
2.6.6 Restriction digestion	60
2.6.7 Gibson assembly	60
2.5.8 Preparation of chemically competent <i>E. coli</i>	61
2.5.9 Transformation of chemically competent <i>E. coli</i>	61
2.5.9 Three-Partner conjugation of <i>P. aeruginosa</i>	61
2.5.10 DNA sequencing.....	62
2.6 Statistical analysis	62
Chapter 3: The Host environment in CF modulates <i>P. aeruginosa</i> susceptibility to colistin.	63
3.1 Introduction	63
3.1.2 <i>P. aeruginosa</i> adaptation to the lung environment	64
3.1.3 Synthetic sputum models.....	64
3.2 Results	66
3.2.1 Synthetic sputum modulates the growth of <i>P. aeruginosa</i>	66
3.2.3 Synthetic sputum does not significantly alter colistin activity	72
3.2.4 Individual SCFM-2 macromolecules differentially modulate susceptibility of Pa to colistin	73
3.2.5 eDNA enhances growth but is inhibitory at a high concentrations.	76
3.2.7 Physiological concentrations of eDNA induce tolerance to supra-therapeutic concentrations of colistin.....	80
3.3 Discussion.....	81

Chapter 4: Extracellular DNA triggers LPS modifications associated with reduced susceptibility to colistin.	84
4.1 Introduction	84
4.1.1 Colistin resistance in <i>P. aeruginosa</i>	84
4.1.2 <i>P. aeruginosa</i> possesses multiple two-component systems linked to colistin resistance.....	85
4.1.3 eDNA reduces susceptibility to colistin	86
4.2 Results	87
4.2.1 Validation of Salmon sperm DNA as a proxy for Human DNA.	87
4.2.2 eDNA does not significantly sequester colistin in SCFM-1	87
4.2.2 eDNA disrupts the outer membrane of <i>P. aeruginosa</i>	88
4.2.4 excess magnesium prevents membrane damage but does not restore susceptibility to colistin	90
4.2.5 Acidification of SCFM by eDNA is partially responsible for colistin resistance.....	93
4.2.6 eDNA triggers LPS modifications	95
4.2.7 Multiple two-component systems contribute to DNA-induced colistin resistance. .	102
4.3 Discussion.....	106
Chapter 5: Developing Strategies to increase colistin susceptibility in synthetic sputum.....	111
5.1 Introduction.....	111
5.1.1 Targeting eDNA.....	111
5.1.2 Targeting pH homeostasis	111
5.1.3 Targeting LPS transport with murepavadin	112
5.2 Results	112
5.2.1 DNase treatment does not restore susceptibility to colistin.....	112
5.2.2 eDNA reduces susceptibility to tobramycin	114
5.2.3 eDNA reduces susceptibility to murepavadin.....	114
5.2.4 The mechanism of reduced murepavadin susceptibility diverges from that of colistin.	116
5.2.5 Murepavadin synergises with colistin in the presence of eDNA	117
5.2.5 Clinical isolates show decreased susceptibility to murepavadin in synthetic sputum	118
5.2.6 NaHCO ₃ restores susceptibility to murepavadin in the presence of eDNA	120
5.3 Discussion.....	123
Chapter 6: Discussion	126
6.1 The lung environment reduces <i>P. aeruginosa</i> susceptibility to colistin	126
6.2 eDNA triggers LPS modification through multiple two-component systems.....	127
6.3 Targeting the CF lung environment reduces bacterial survival	130

6.4 Thesis Summary	133
6.5 Future Work	134
6.5.1 Increasing understanding of <i>P. aeruginosa</i> susceptibility to colistin.	134
6.5.2 Increasing our understanding of the host environment.	136
6.5.3 Optimising treatment in based on the host environment.	138
References	140

List of Abbreviations

A	
āā	Amino acid
ALI	Air-liquid interface
ANOVA	Analysis of variance
<i>arn</i>	<i>arnBCEATEF</i>
ASL	Airway surface liquid
ASM	Artificial sputum medium
B	
BAL	Bronchoalveolar lavage
Bp	Base pairs
C	
CA-MHB	Cation adjusted muller hinton broth
CAMPs	Cationic antimicrobial peptides
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
CMS	Colistimethate sodium
D	
Dab	Diaminobutyric acid
DNA	Deoxyribonucleic acid
DOPC	Dioleoylphosphatidylcholine
DTPA	Diethylenetriaminepentaacetic acid
DTR	Difficult-to-treat resistance
E	
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
ENaC	Epithelial sodium channel
EPS	Extracellular polymeric substances
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
EVPL	Ex vivo pig lung
F	
Fad	Fatty acid degradation
FEV₁	Forced expiratory volume in 1 second
FVC	Forced vital capacity
G	
GlcNAc	N-acetylglucosamine
H	
HAE	Human airway epithelial
hCFTR	Human CFTR
hDNA	Human DNA
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
I	
IDSA	Infectious disease society of America
K	
KO	Knock out

L	
L-ara4N	4-amino-4-deoxy L-arabinose
LB	Lysogeny broth
LBA	LB agar
LCI	Lung clearance index
LPS	Lipopolysaccharide
M	
MALDI-ToF	Matrix-assisted laser-desorption ionisation time-of-flight
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
MCR	Mobile colistin resistance
MDR	Multi-drug resistant
MHRA	Medicines and Healthcare Regulatory Agency
MIC	Minimum inhibitory concentration
N	
NADPH	nicotinamide adenine dinucleotide phosphate
NEB	New England Biolabs
NETs	Neutrophil extracellular traps
NICE	National institute of Clinical Excellence
NPN	N-phenyl-1-naphthylamine
O	
OD	Optical density
oriT	origin of transfer
P	
PBP	Penicillin-binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pEtN	phosphoethanolamine
PIA	Pseudomonas isolation agar
PMB	Polymyxin B
PRRs	Pattern recognition receptors
pwCF	People with cystic fibrosis
R	
rhDNase	Recombinant human DNase
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RWV	Rotating wall vessel
S	
SCFM	Synthetic cystic fibrosis medium
SCV	Small colony variant
ssDNA	Salmon sperm DNA
T	
TAE	Tris, acetic acid, EDTA
TcR	tetracycline resistance
TCS	Two-component system
U	
UTI	Urinary tract infection
V	

VAP		Ventilator associated pneumonia
	W	
WT		Wild-type
	X	
XDR		Extensively drug resistant

List of Figures

1.1	Clinical manifestations of <i>P. aeruginosa</i> infection.	p17
1.2	Model of Cystic Fibrosis Lung Disease	p24
1.3	Structures of polymyxin B and colistin	p33
1.4	Primary mechanism of action of colistin	p35
1.5	Covalent modification terminal phosphates of lipid A results in colistin resistance.	p37
3.1	Synthetic sputum modulates bacterial growth	p67
3.2	Individual macromolecules exhibit strain dependent differences in growth	p68
3.3	Synthetic sputum modulates susceptibility to colistin	p70
3.4	SCFM-1 has a minimal effect on colistin susceptibility relative to MHB	p71
3.5	SCFM-2 Enhances recovery from colistin exposure	p72
3.6	Synthetic sputum does not significantly alter colistin activity	p73
3.7	Sputum macromolecules differentially effect colistin sensitivity	p74
3.8	N-acetyl glucosamine (GlcNAc) and 1,2-dioly-phosphatidylcholine (DOPC) have minimal effect on colistin susceptibility	p75
3.9	Extracellular DNA (eDNA) reduces susceptibility to colistin	p76
3.10	Extracellular DNA inhibits growth at high concentrations	p77
3.11	Clinical strains demonstrate varied responses to extracellular DNA	p78
3.12	Increasing concentrations of eDNA reduces colistin activity	p79
3.13	High concentrations of eDNA induce tolerance to colistin	p80
4.1	Both low molecular weight salmon sperm DNA and human DNA reduce susceptibility to colistin	p87
4.2	eDNA does not significantly reduce colistin activity	p88
4.3	eDNA disrupts the outer membrane of <i>P. aeruginosa</i>	p89
4.4	Magnesium supplementation protects the outer membrane from damage	p90
4.5	Magnesium supplementation does not restore susceptibility to colistin in the presence of eDNA	p92

4.6	Media acidification contributes to membrane damage and colistin resistance	p94
4.7	Representative MALDI-ToF traces	p96
4.8	Extracellular DNA induces lipid A modifications associated with colistin resistance	p97
4.9	<i>eptA</i> and <i>arnC</i> are required for high level resistance to colistin in the presence of eDNA	p99
4.10	Complementation of <i>eptA</i> reduces susceptibility to colistin in the presence of eDNA	p101
4.11	Multiple two-component systems are responsible for high level colistin resistance induced by eDNA	p103
4.12	Complementation of <i>colRS</i> reduces susceptibility to colistin in the presence of eDNA	p105
4.13	Proposed model for how eDNA reduces colistin susceptibility of eDNA in sputum	p110
5.1	Dnase treatment does not restore susceptibility to colistin in synthetic sputum	p113
5.2	eDNA reduces <i>P. aeruginosa</i> susceptibility to Tobramycin in SCFM-1	p114
5.3	eDNA reduces <i>P. aeruginosa</i> susceptibility to murepavadin in SCFM-1	p115
5.4	Disruption of <i>lpxT</i> and not <i>arnC</i> or <i>eptA</i> increases susceptibility to murepavadin in the presence of eDNA	p116
5.5	Colistin and murepavadin synergise to overcome eDNA induced resistance	p117
5.6	Increasing the concentration of murepavadin restores killing and synergy	p118
5.7	Clinical <i>P. aeruginosa</i> isolates from CF show decreased susceptibility to murepavadin	p119
5.8	NaHCO₃ restores susceptibility to murepavadin and increases killing in the presence of murepavadin and colistin	p121
5.9	The presence of sputum macromolecules further reduces susceptibility to Colistin in PA14	p122
6.1	Proposed model for how the CF lung environment reduces susceptibility to colistin	p133

List of Tables

2.1	Plasmids used in this study	p49
2.2	Bacterial Strains used in this study	p50
2.3	SCFM-1 Base	p51
2.4	Stock solutions for SCFM-1 base	p51
2.5	Same-day amino acids for SCFM-1 base	p52
2.6	Same-day reagents for SCFM-1	p52
2.7	SCFM-2 macromolecules	p53
2.8	Lysis Buffer	p55
2.9	PCR components	p58
2.10	PCR conditions	p58
2.11	PCR primers	p59
2.12	Restriction Digest components	p60
4.1	Two-component systems associated with decreased colistin susceptibility in <i>P. aeruginosa</i>	p86

Chapter 1: Introduction

1.1 *Pseudomonas aeruginosa*

1.1.1 General characteristics

Pseudomonas aeruginosa is a Gram-negative, non-lactose fermenting, oxidase-positive, catalase-positive, rod-shaped bacterium (1). First described by the French military surgeon Charles-Emmanuel Sédillot in 1850, *P. aeruginosa* was noted to cause a sweet odour and blue-green discolouration of bandages around wounds (2). Subsequently, the bacterium was isolated by the pharmacist Carle Gessard in 1882 (3). The blue-green colouration arises from the production of pigments pyoverdine and pyocyanin, synthesised by many strains, and remains a hallmark of *P. aeruginosa* infection (4,5). *P. aeruginosa* is ubiquitous in the environment and is often found in water or wet environments (6). This can be particularly problematic in hospital water supplies where colonisation of sinks and damaged water pipes has been associated with outbreaks of invasive disease (7). *P. aeruginosa* causes a wide spectrum of disease in humans and, whilst it rarely causes severe disease in the immunocompetent, is a significant source of morbidity and mortality in immunocompromised individuals and people with cystic fibrosis (pwCF) (8).

P. aeruginosa is an extremely versatile and adaptable pathogen. This versatility is, in part, conveyed by a relatively large genome (5.5-7 Mb), which allows for pleiotropic metabolic responses depending on nutrient availability (9). Freshi et al estimated the core genome to represent only 665 genes, or 1% of the entire genome (10). The relatively large accessory genome allows *P. aeruginosa* to adapt to a range of environments, including on and within humans, and a diverse range of animal species, in addition to persisting in the external environment (11). Coupled with the large genome are numerous two-component systems (TCSs) which allow *P. aeruginosa* to sense and respond to a wide variety of external stimuli. For example, *P. aeruginosa* strain PAO1 encodes 63 different sensor kinases, approximately double the number for *E. coli* (12). Although *P. aeruginosa* is commonly considered an aerobic organism, under anaerobic conditions it can utilise denitrification to produce energy in the presence of nitrite or nitrate, providing additional nutritional flexibility (13). Additionally, arginine or pyruvate can be used for fermentation (14). *P. aeruginosa* can utilise an array of carbon sources for aerobic respiration including amino acids, DNA, glucose, succinate, lactate and N-acetyl-glucosamine many of which are available during infection (15).

P. aeruginosa possesses a range of virulence factors that facilitate human infection. Quorum sensing is a system by which bacteria use small molecules to communicate with one another about population size (16). As the diffusible small molecules accumulate, they activate transcriptional pathways within different cells. *P. aeruginosa* possesses at least 3 known quorum sensing circuits: LasI/LasR, RhlI/RhlR and PQS (16). Whilst there is crosstalk between these three systems, an additional fourth circuit, IQS, modulates the behaviour of PQS and Rhl genes in low phosphate conditions (17). Collectively these systems regulate many virulence phenotypes including biofilm formation and pyocyanin production (18). *P. aeruginosa* is able to switch between planktonic and biofilm modes of growth (19). Biofilms are communities of bacteria encased in extracellular polymeric substances (EPS) such as secreted exopolysaccharides, extracellular DNA (eDNA) and proteins (19). Biofilms facilitate persistence in both the environment and in infection, including the CF lung, and reduce bacterial susceptibility to both antimicrobials and host immune effectors (20,21). Biofilm formation is also associated with medical devices including urinary catheters and endotracheal tubes contributing to urinary tract infection (UTI) or ventilator-associated pneumonia (VAP) respectively (22,23). With a plethora of virulence factors, metabolic and regulatory plasticity *P. aeruginosa* is readily able to adapt to the human host.

1.1.2 *P. aeruginosa* as an opportunistic pathogen

Being versatile, *P. aeruginosa* can infect a range of host tissues including skin and soft tissue, the lung, the urinary tract and the bloodstream (summarised in Fig 1.1). Skin and soft tissue infections are particularly prevalent in burn patients (24). The severity of skin infection can vary from relatively superficial colonisation of leg ulcers to an aggressive infection of the external pinna which progresses to invasive skull-base osteomyelitis in a condition called ‘malignant otitis externa’ (25,26). Malignant here refers to the invasive nature of the disease and not a neoplastic process. The most severe manifestations of *P. aeruginosa* are seen in patients with underlying immunocompromise, with malignant otitis externa being far more common in patients with diabetes, for example (25). *P. aeruginosa* bacteraemia is much more prevalent in patients with neutropenia than the general population and *P. aeruginosa* is the most frequently isolated pathogen from the lungs of pwCF (27,28)

Severe infections in immunocompetent individuals are seen in conjunction with plastic medical devices and are promoted by the ability of *P. aeruginosa* to form biofilms. For example, *P. aeruginosa* is common cause of catheter associated urinary tract infection (UTI) and the most frequent cause of ventilator associated pneumonia (VAP) (22,23). *P. aeruginosa* is also associated with severe forms of keratitis (infection of the cornea) linked to contact lens use (29).

Aside from medical devices *P. aeruginosa* is increasingly recognised as a cause of infective exacerbations of chronic obstructive pulmonary disease and non-CF bronchiectasis (30).

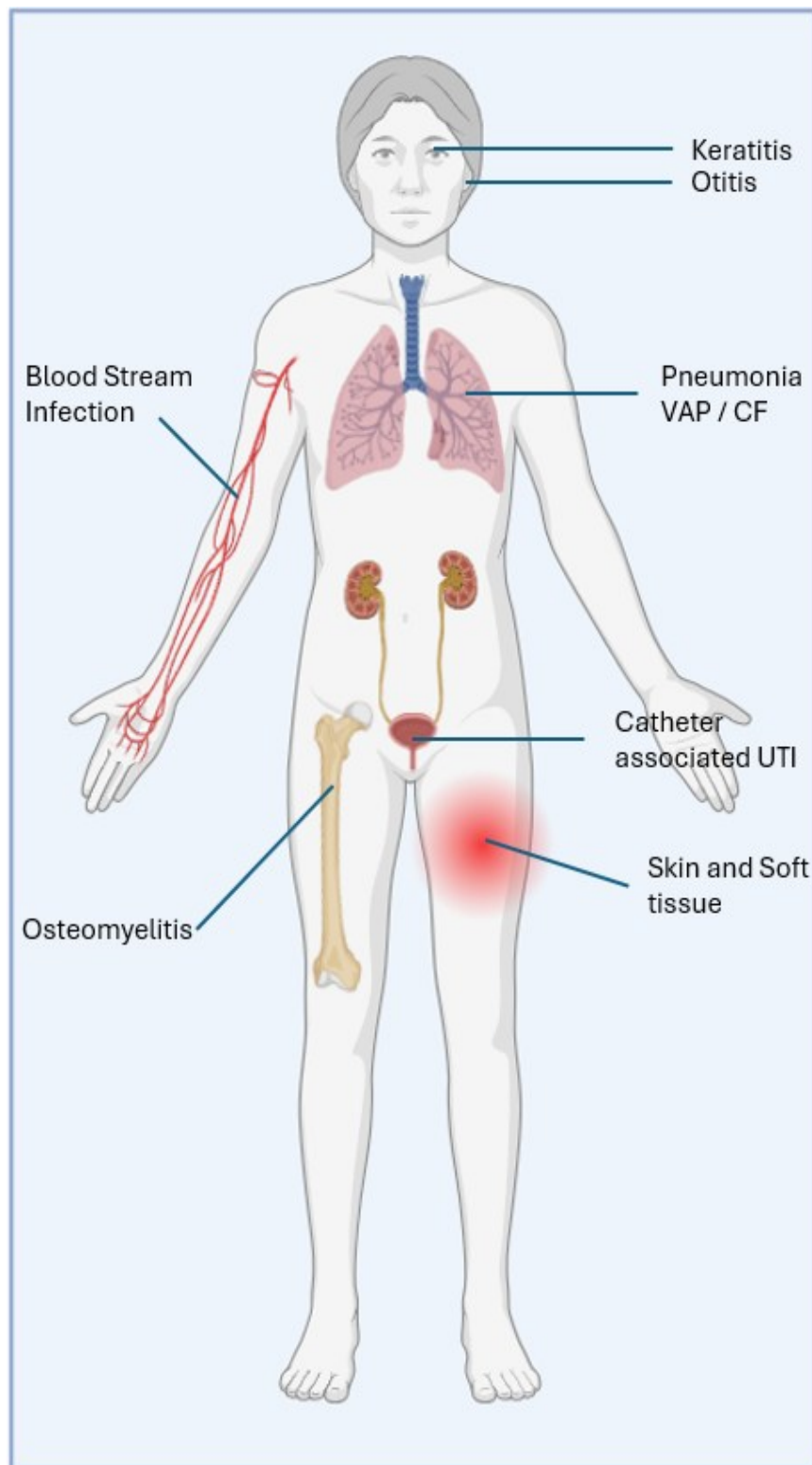


Figure 1.1 Clinical manifestations of *P. aeruginosa* infection. UTI – urinary tract infection. VAP – ventilator associated pneumonia. CF – Cystic fibrosis

1.1.3 Antimicrobial susceptibility and resistance in *P. aeruginosa*

One of the major challenges in treating *P. aeruginosa* infections is that the organism possesses intrinsic resistance to multiple classes of antibiotics. This intrinsic resistance arises from multiple mechanisms including: a relative impermeability of the outer membrane, alongside chromosomally encoded multi-drug efflux pumps MexXY, MexAB, MexCD, MexEF, and an AmpC β -lactamase(31–33). Together, these mechanisms confer resistance to many antibiotics routinely used to treat other Gram-negative infections, including: penicillin and amoxicillin with clavulanic acid; many cephalosporins; tetracyclines; macrolides; chloramphenicol and ertapenem (34). In addition to these intrinsic mechanisms, *P. aeruginosa* has a propensity for acquiring resistance via mutations that arise during infection, as well as via horizontal gene transfer (35). For these reasons *P. aeruginosa* is recognised as a WHO priority pathogen for which new treatments are urgently needed (36).

Drug resistance in *P. aeruginosa* is particularly problematic due to the already limited repertoire of antibiotics available for treatment. Multiple studies have demonstrated that infection with antibiotic-resistant isolates is associated with a high excess mortality relative to susceptible strains (37–39). The reasons for this are likely complex, however, resistance to many first line agents coupled with a delay in effective antibiotic therapy and a particularly vulnerable susceptible population, likely contribute. Because of high levels of intrinsic resistance, it has been difficult to classify *P. aeruginosa* resistance in the same manner as other enterobacterales because acquisition of resistance to the very few classes of antibiotic available rapidly introduces significant therapeutic challenges. As such, the Infectious Disease Society of America (IDSA) has adopted a novel classification of resistance: Difficult-to-treat resistance (DTR) (40). Whereas multi-drug resistance (MDR) is defined as non-susceptibility to at least 1 drug in 3 classes to which *P. aeruginosa* would be expected to be susceptible, DTR is defined by the IDSA as non-susceptibility to piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, meropenem, imipenem-cilastatin, ciprofloxacin and levofloxacin (40). This list encompasses many of the first line agents used against *P. aeruginosa*. The remaining drug options are either novel compounds or older antimicrobials with an unfavourable side-effect profile. The currently available treatment options for *P. aeruginosa* infection are outlined below.

β -lactams

β -lactam antibiotics are a group of generally bactericidal compounds that block bacterial cell wall synthesis by inhibiting penicillin binding proteins (PBPs) which cross-link the

peptidoglycan in the cell wall (41). β -lactams enter the bacterial cell through porins in the outer membrane (42). Due to their broad spectrum of activity and relatively mild side-effect profile β -lactams remain first line therapy for many infections. The combination of β -lactams with β -lactamase inhibitors further increases their spectrum of activity (41). This group of antibiotics includes penicillins, cephalosporins, carbapenems and monobactams (aztreonam). Due in part to a chromosomally encoded AmpC β -lactamase, which can be readily upregulated in response to antibiotic exposure, *P. aeruginosa* is intrinsically resistant to many penicillins and cephalosporins however, the β -lactam β -lactamase combination piperacillin-tazobactam and cephalosporin ceftazidime are commonly used in the UK as first line therapy for severe infections where there is a low risk of pre-existing resistance (35,43,44). Second line therapy commonly involves the use of a carbapenem although it should be noted that *P. aeruginosa* has intrinsic resistance to ertapenem, and imipenem is recommended against due to the high risk of acquiring resistance during treatment (43). For resistant isolates, a range of new agents has become available recently, including the β -lactamase inhibitor combinations ceftazidime-avibactam and ceftolozane-tazobactam both now licensed in the UK for severe MDR infection (45,46). The novel cephalosporin / siderophore cefiderocol has also recently become available (47). Carbapenem resistance is an ongoing challenge and can arise from both the acquisition of carbapenemases and the upregulation of drug efflux pumps (48).

Concerningly, resistance is emerging to newer cephalosporins. Ceftolozane-tazobactam is not effective against strains with metallo- β -lactamases (MBLs) or carbapenemases but appears effective against *P. aeruginosa* with upregulated drug efflux or de-repressed AmpC production (43). Similarly, ceftazidime-avibactam is not active against MBLs but does retain some activity against KPC and possibly OXA-48 carbapenemases(43). Rates of resistance to cefiderocol appear to be low even amongst carbapenemase producing isolates (49). Reduced susceptibility has been associated with mutations in regulators of the TonB iron transporter pathway, mutations in *ampC* in strains possessing NDM carbapenemases (50).

Aminoglycosides

Aminoglycosides are bactericidal antibiotics which inhibit the 30s ribosomal subunit of bacteria and cause aberrant peptide production (51), resulting in death due to misfolded proteins and an associated loss in membrane barrier formation. Cationic aminoglycosides promote their own uptake by binding to negatively charged components of bacterial membranes increasing permeability (51,52). Further entry into the cell is dependent on the electron transport chain and the resulting membrane potential this generates (52). Gentamicin, amikacin and tobramycin are the most widely used parenteral agents in the UK (53). Recently European Committee on

Antimicrobial Susceptibility Testing (EUCAST) has recommended against the use of aminoglycosides as monotherapy in severe infections and against the use of gentamicin for *P. aeruginosa* in general, due to the high risk of treatment failure (54). However, whilst monotherapy appears to be associated with worse outcomes than combination therapy in bacteraemia, it can be used for urinary tract infection (55). Tobramycin is the preferred agent for treating *P. aeruginosa* (56). Resistance to aminoglycosides arises from lipopolysaccharide (LPS) modification, target site modification, upregulation of drug efflux pumps, particularly MexXY, and can be acquired by antibiotic modifying enzymes (57,58). Due to the role of membrane potential in aminoglycoside uptake, the availability of carbon sources can also play a role in aminoglycoside susceptibility, though the significance of this mechanism is much less well defined (59,60). Aminoglycosides are available as intravenous, inhaled or topical agents but cannot be given orally. Systemic use can be associated with renal- and oto-toxicity (61).

Quinolones

Quinolones act by inhibiting DNA gyrase and topoisomerase IV, blocking DNA synthesis (62). In Gram-negative organisms, they are taken up into the bacterial cell via porins in the outer membrane (63). Quinolones represent the only oral antibiotics available to treat *P. aeruginosa* and have the additional benefit of extremely high bioavailability. As such, ciprofloxacin and levofloxacin are commonly used for the treatment of infections caused by *P. aeruginosa*. In recent years there has been increasing concern over the use of quinolones due to a broad range of toxic side-effects including QT-prolongation, which can be associated with fatal cardiac arrhythmias, and effects on connective tissue, including increased risk of aneurysmal rupture and tendonitis (64). Consequently, in the UK, the Medicines and Healthcare products Regulatory Agency (MHRA) has recommended against use of quinolone antibiotics if a commonly used alternative antimicrobial is available (65). Resistance most commonly arises due to target site modification and reduction of intracellular levels of drug either through the upregulation of drug efflux pumps or reduced expression of porins (63).

Polymyxins

Polymyxins are a group of cationic antimicrobial peptides with activity against a range of Gram-negative organisms including *P. aeruginosa* (66,67). Though systemic use is limited by significant toxicity, predominantly neurological and renal, they remain important last-line agents against antibiotic resistant isolates (68). By contrast, as an inhaled agent, colistin is first line treatment for chronic *P. aeruginosa* infection in CF in the UK and is also commonly used alongside intravenous antibiotics for the elimination of acute infection and to treat pulmonary

exacerbations (69). As the polymyxin colistin will form the main focus of this work, it will be discussed in more detail in section 1.3.

1.2 Cystic Fibrosis

1.2.1 Pathogenesis

Cystic fibrosis (CF) was first described in 1938 by Dorothy Anderson (70). The name arises from a pathological description of pancreatic tissue as, in that period, the condition was associated with death in early childhood due to nutritional deficiency resulting from defective exocrine pancreatic function (71). In the modern period, CF is recognised as a complex multi-organ disease affecting the sino-pulmonary, gastrointestinal, pancreatic and reproductive systems. In 1989, mutations in the cystic fibrosis transmembrane conductance regulator (*cftr*) gene were identified as the underlying cause of the disease (72–74). Since that time over 2000 different mutations have been identified (75–77). CFTR is an anion channel, predominantly at the apical surface of epithelia, and mutations in *cftr* typically result in an inability to secrete Cl^- and HCO_3^- ions from epithelial cells. Dysfunction of CFTR results in upregulation of the epithelial Na channel (ENaC) giving rise to increased Na^+ reabsorption, which in turn drives water reabsorption due to the resulting osmotic gradient resulting in increased viscosity of secretions (78,79).

Different *cftr* mutations are associated with differing severity of disease, although the data linking specific genotypes and phenotypes is incomplete. Mutations are classified I – VI, according to the way in which protein production or function is affected (80). Class I mutations are associated with premature termination codons often as a result of frame shifts or deletions resulting in the production of almost no functional protein. Class II mutations act at the level of the endoplasmic reticulum, often resulting in protein misfolding and premature degradation. Class III mutations affect channel function by reducing the open probability of the channel. Class IV mutations alter the channel conductance. Class V mutations do not alter the structure of the channel but alter the abundance of the channel at the membrane, often resulting from changes in the promoter sequence or splice variants. Class VI mutations reduce expression at the plasma membrane by increasing turnover (80,81). Broadly, Class I, II and III mutations result in a more severe phenotype than IV, V or VI, because of the level of channel dysfunction. Recently it has been proposed to include a class VII which recognises mutations which do not respond to *cftr* modulator therapy (discussed below) due to the absent production of mRNA (82). It is recognised that other risk factors will contribute to severity of disease, including innate immune function, nutrition, gender as well as the genetic background of the affected individual (83). F508del is the most common CFTR defect, with approximately 90% of pwCF in the Caucasian population

carrying at least one F508del causing genetic variant (84). CF is most frequent amongst northern European Caucasian populations, although the condition is distributed globally (84). Neonatal screening for CF has allowed for accurate estimates of the disease incidence where programs are in place. CF is inherited as an autosomal recessive trait with approximately 11,000 affected individuals in the UK and an incidence of 1:2500 (27). Although CF affects multiple organ systems the greatest contribution to morbidity and mortality arises from lung disease (27,75,85).

1.2.2 Lung-Disease in Cystic Fibrosis

Lung disease in CF is typified by repeated episodes of infection, both symptomatic and asymptomatic, leading to excessive inflammation, mucus hypersecretion and destruction of lung tissue. Damage to lung tissue begins early in cystic fibrosis, with evidence of structural damage apparent in approximately 20% of pre-school children (86,87). Recurrent infection is the result of impaired mucociliary clearance and an ineffective, excessive immune response.

Impaired mucociliary clearance is thought to arise from dehydration of respiratory mucus both as a consequence of increased Na^+ absorption (78,79), which promotes the uptake of water by osmotic pressure, and a failure to secrete HCO_3^- leading to defective mucin maturation (88). The resulting thick secretions compress the ciliated epithelium reducing the frequency of ciliary beating resulting in mucus stasis, which in turn results in ineffective pathogen and foreign body clearance allowing opportunistic infection, which drives further inflammation and associated destruction of lung tissue(89,90).

Innate immune responses also appear to be dysfunctional in CF. Under normal conditions innate immune pattern recognition receptors (PRRs) expressed on epithelial cells and tissue resident immune cells, recognise pathogen associated molecular patterns (PAMPs) leading to the production of pro-inflammatory cytokines and the recruitment of innate immune effector cells and production of innate immune effectors (91). Inflammation in the lung is tightly regulated such that pathogens are cleared whilst bystander tissue damage, for example that caused by secreted proteases, is minimised. Inflammation in the CF lung is characterised by extensive neutrophilic infiltration and the production of pro-inflammatory cytokines. Defective CFTR function appears to directly impair the normal function of phagocytes, both neutrophils and macrophages, by reducing their ability to kill bacteria (92,93). CFTR appears to be important for the generation of intracellular chloride and oxidative mediators involved in lysosomal killing (93). Failure to effectively clear pathogens results in further immune cell recruitment. Activated neutrophils release neutrophil-extracellular traps (NETs), a combination of DNA, histones and enzymes, which, though cytotoxic, can contribute to biofilm formation by *P. aeruginosa* (94).

Local acidification by DNA release can impair the function of endogenous antimicrobial peptides (95).

Acute deteriorations in respiratory function, often associated with infection, are referred to as pulmonary exacerbations. These exacerbations can be associated with a sudden, potentially irreversible, decrease in lung function (96,97) and frequent exacerbations are a poor prognostic indicator. Over time, repeated episodes of infection lead to a decline in lung function and the development of bronchiectasis, which describes the abnormal widening of the bronchi, and is associated with excessive mucus secretion, purulent sputum production, and ineffective airway clearance(85). Over time recurrent infection and inflammation results in lung damage leading to respiratory failure and the need for lung transplantation. Because of the concern that functional tissue, once lost during an exacerbation, may not be subsequently recovered, pulmonary exacerbations are managed aggressively, usually with a combination of inhaled and either intravenous or oral antibiotics (97–99).

Pulmonary function is typically monitored with spirometry and much of our understanding of disease progression and the impact of treatment comes from observed changes in pulmonary function tests. The two most commonly assessed parameters are the forced expiratory volume in 1 second (FEV₁) and the forced vital capacity (FVC) (100). The FEV₁ is the maximum volume of air an individual can exhale in the 1st second following maximal inspiration. It is dependent on airflow in the lungs and is therefore reduced when there is airflow obstruction (100). FVC is the maximum volume of exhaled air that can be expelled with a maximal effort following full inspiration (100). Pulmonary function tests can indicate the rate of lung function decline but may also give an indication of pulmonary exacerbation. However, they may be relatively insensitive to lung damage in the distal airways as many individuals with bronchiectasis on cross-sectional imaging may have preserved pulmonary function tests (101,102). Results will also vary within the same individual based upon technique. Furthermore, they require a degree of compliance that is not achievable in very young children. Other markers such as lung clearance index (LCI) are being investigated, particularly for use in young patients (103).

During the lifetime of people with CF (pwCF) particular pathogens tend to predominate. In early life the Gram-positive bacterium *S. aureus* is the most frequently isolated respiratory pathogen and was isolated in post-mortem samples during Anderson's original description (70). There is considerable uncertainty around the management of *S. aureus* in CF, although the bacterium if frequently isolated, it is unclear how far isolation contributes to infection and

disease. CF-START is an ongoing clinical trial examining the role of anti-staphylococcal prophylaxis in CF (104). There is particular concern that over-treatment of *S. aureus* with prophylaxis leads to early acquisition of *P. aeruginosa* (105,106). In the later stages of the disease, whilst *P. aeruginosa* is still present, other opportunistic pathogens may invade including *Burkholderia cepacia* and atypical (environmental) mycobacteria such as *Mycobacterium avium* (107). Fungal pathogens such as *Aspergillus fumigatus* may also emerge at this stage opportunistically colonising lung cavities (107).

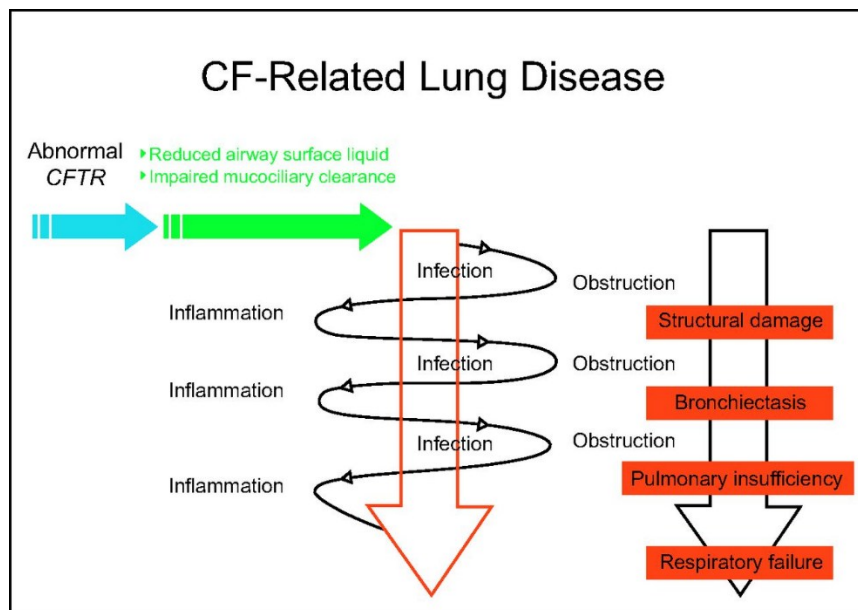


Figure 1.2 Model of Cystic Fibrosis Lung Disease (from (85))

1.2.3 *P. aeruginosa* in Cystic Fibrosis

P. aeruginosa is a major cause of morbidity and mortality in pwCF (108) and is the most frequently isolated pathogen from the lungs of pwCF in the UK (27) and second most common in the US (109). Chronic infection with *P. aeruginosa* is associated with a more rapid decline in lung function and early mortality (110–112). Initial infection occurs early in pwCF, with the most recent UK CF registry data reporting 2.8% of patients had *P. aeruginosa* detected in at least 1 respiratory sample before the age of 3 (27). Sampling bias from expectorated samples means that it can be difficult to know whether this reflects oropharyngeal colonisation or true airway infection. However, studies using bronchoalveolar lavage (BAL) for sampling, which identified *P. aeruginosa* within the lungs of young patients, support these findings. An Australian study that performed BAL screening annually in patients under 6 years old reported detection of pseudomonas in 28.4% of children with a median age of just 30 months (113). Similarly, another Australian study

of BAL directed therapy of 168 children under 5 reported mean age of acquisition of *P. aeruginosa* of 2.4 years (114). The study also reported high genetic diversity of *P. aeruginosa* between individuals, consistent with distinct acquisitions from the environment, rather than patient-to-patient transmission. Over time, acute and intermittent infection with wild-type *P. aeruginosa* gives way to chronic infection associated with the emergence of host-adapted phenotypes (108).

Initial Infection

Respiratory disease in CF is typified by repeated episodes of infection with characteristic organisms. In the first 3 years of life, *S. aureus* and *H. Influenzae* predominate (107). Over time, these give way to intermittent infection with *P. aeruginosa*. The precise mechanisms by which *P. aeruginosa* can establish infection in CF, but not in other healthy individuals, are incompletely defined. What is clear is that CF results in several important changes in the lung which favour bacterial colonisation. Thickened respiratory secretions contribute to impaired mucociliary clearance undermining the innate immunity of the airway (115). Evidence from post-mortem samples indicates that bacteria are localised to the mucus accumulated in the airway lumen and therefore it may be that impaired clearance of mucus is an adequate explanation by itself (116). Evidence from porcine models indicates that further defects of innate immunity are present, and that acidification or airway surface fluid (ASL) can impair the activity of innate immune effectors within the lung (117,118). Finally, pwCF may have impaired neutrophil killing through an inability to concentrate hypochlorous acid in phagolysosomes resulting in incomplete bacterial killing (93). Despite these immune defects, during acute infections it is still possible to eradicate *P. aeruginosa* with antibiotic therapy.

Chronic Infection

Over time, acute infection gives way to chronic infection where antibiotics are no longer effective in clearing bacteria. In the UK registry, chronic infection is defined as 3 or more isolates, from respiratory samples, of *P. aeruginosa* in a 12-month period (27). Longitudinal studies have revealed that individuals are often colonised by the same strain of *P. aeruginosa* for years (119–121). The rapid expansion in availability and affordable whole genome sequencing has allowed for several studies to reveal the process of adaptive evolution which occurs in many *P. aeruginosa* strains during the chronic infection process (119). Several phenotypes are frequently encountered in pwCF and will be discussed briefly below, they include: hypermutable strains; mucoid strains; *lasR* mutants; strong biofilm formers and strains with LPS modifications.

Hypermutable

Hypermutators are bacteria that show an increased rate of mutations, in some cases up to 1000-fold, compared to wild type isolates. Hypermutators are usually associated with loss of function in DNA repair or proof-reading enzymes (122). In *P. aeruginosa* mutations commonly arise in DNA mismatch repair enzymes *mutS* and *mutL* (123). Hypermutators are thought to occur with increased frequency in pwCF, possibly due to long-term colonisation (124,125). For example, one series published by Marvig et al, collected over 22 years, identified hypermutators in 48% of patients (125), whilst Waine et al identified hypermutators in 16/32 patients (126). The phenotype was more frequently seen in patients with worse lung function and mucoid phenotypes. Intuitively there would appear to be an advantage in increased adaptability in the 'hostile' lung environment where bacteria will frequently encounter immune effectors, compete with other colonising bacteria, and be exposed to antimicrobials. However, many mutations will be deleterious and so adaptability comes at a cost.

Quorum Sensing

As discussed earlier, the quorum sensing systems of *P. aeruginosa* allow the bacterium to regulate the expression of virulence genes in association with its population size and density. Whilst this may be important in early infection, chronic infection appears to select for loss of function mutations in *lasR* (127–129), although the selection pressure for this is unclear. One hypothesis is that it relates to the hierarchical structure of quorum sensing in *P. aeruginosa* with some products produced by Rhl or PQS negatively regulated by LasR conveying an advantage within the lung (130). Alternatively, D'argenio and colleagues observed enhanced growth in the presence of amino acids and increased production of β -lactamase in both clinical isolates and engineered *lasR* mutants (131).

Exopolysaccharide production

Mucoid colonies arise from over production of the exopolysaccharide alginate, which is synthesised by the products of a 12 gene operon *algD-algA* (132). Expression of the operon requires the activity of the sigma factor AlgT (aka AlgU), which also regulates an array of other genes involved in the envelope stress response (133,134). Under normal conditions AlgT is sequestered by the anti-sigma factor MucA (135). Commonly, in mucoid strains mutation in the gene encoding MucA gives rise to a truncated, non-functional protein and release of AlgT leading to the expression of its downstream effectors(136,137). Some of the downstream effectors of AlgT are known to repress several other virulence factors, including Psl and the type III secretion system(138,139). Mucoid *P. aeruginosa* typically emerges later in disease, with one longitudinal study from the US reporting median age of isolation of *P. aeruginosa* at 1 year but median isolation

of mucoid variants at 13 years (140). Consistent with this the emergence of mucoid variants is also associated with more severe lung disease (141,142). *In vitro*, oxidative stress selects for the emergence of mucoid variants, and it has been hypothesised mucoidy represents an adaptation to the host immune response (143–145). Although mucoid strains are associated with more severe disease, controversy remains over the selective advantage of mucoidy over wild-type (WT) strains. The best evidence supports a role in innate immune evasion and biofilm maturation (143,144,146–148). Mucoidy is thought to increase resistance to both antibiotics and host-derived antimicrobials (147,149)

Related to, but distinct from mucoidy, is the over production of exopolysaccharides Psl and Pel (145). This results in the formation of rugose small-colony variants (SCVs), which are hyper-aggregative when grown in liquid culture (150,151) and thus have been hypothesised to play a role in biofilm formation (132,152). They have also been demonstrated to have reduced susceptibility to host antimicrobial peptides and the aminoglycoside tobramycin (150,153). Phenotypically, rugose SCVs produce small, wrinkled colonies on solid media.

Biofilm

P. aeruginosa has become a paradigm for biofilm formation (154). Biofilms are bacterial communities encased in an extracellular matrix adherent to a surface. The biofilm mode of growth provides a high degree of protection from host immunity and antimicrobials. Biofilm has been hypothesised to represent a major proportion of colonising *P. aeruginosa* in CF lung with biofilms identified in post-mortem samples (155,156). Under *in vitro* conditions designed to mimic the CF lung environment, *P. aeruginosa* switches to a biofilm mode of growth (157). However, even in the late stages of CF, bacteria with features related to planktonic growth and acute infection are still isolated (158,159). The biofilm matrix contains a combination of exopolysaccharides, lipids, proteins and extracellular DNA (eDNA) that provide structural support to the community. eDNA is increasingly recognised as a vital component of biofilm so much so that, even though most of the eDNA in biofilm is host-derived, *P. aeruginosa* possesses an autolytic mechanism, under the regulation of the PQS quorum sensing system, to release its own DNA (160–162). eDNA is thought to contribute to biofilm acidification and, being negatively charged can restrict diffusion of aminoglycosides and cationic antimicrobial peptides (94,163,164). Whilst the matrix itself contributes to restricted access of antimicrobials to bacteria, metabolism and gene expression of bacteria within the biofilm is also altered, which contributes to altered susceptibility. Pamp and colleagues demonstrated that metabolically active cells are restricted to the outside of the biofilm and that different subpopulations had altered susceptibility to different antibiotics (165). Metabolic quiescence can reduce

susceptibility to antimicrobials including those that target peptidoglycan synthesis (beta-lactams), protein synthesis (aminoglycosides), and DNA synthesis (quinolones) (166).

Whilst a great deal of work and emphasis has been placed on strategies to eradicate biofilm, it is unclear whether eradication of established biofilms is possible. Even in immunocompetent hosts, treatment of biofilm requires surgical debridement and this is simply not possible in the lung. Current inhaled therapies therefore aim to suppress chronic infection rather than eradicate it. Recognising treatment recalcitrance in early CF infection and optimising *P. aeruginosa* clearance remains a reasonable strategy for preventing chronic infection. It is well recognised that the CF lung promotes biofilm formation and that biofilms reduce susceptibility to antimicrobials. What is less clear is whether the lung environment reduces susceptibility of bacteria to antimicrobials per se in the absence of biofilm and whether understanding the mechanisms of host-induced bacterial resistance may have wider implications for eliminating *P. aeruginosa* infection.

LPS modification

LPS is the major constituent of the outer leaflet of the outer membrane of Gram-negative bacteria where it is responsible for restricting access of extracellular substances to the periplasm (167). Additionally, LPS is a potent immunostimulant, recognised by the innate immune receptor TLR4 (167,168) and is thought to play an important role in activating the host immune system in Gram-negative sepsis (169). In response to the presence of bacteria, the host often produces antimicrobial peptides such as LL37 that target the LPS-containing membrane (66,170). LPS is also targeted by the polymyxin antibiotic colistin, which is a front-line treatment for *P. aeruginosa* infection in CF. As such, there is a strong selection pressure to modify LPS to alter these interactions and promote *P. aeruginosa* survival. Ernst and colleagues reported that particular lipid A structures were more common in *P. aeruginosa* isolated from the sputum of pwCF (171). In particular, modification of the lipid A moiety with aminoarabinose (L-ara4N) loss of lipid A deacylase (PagL) activity and the addition of palmitate moieties (171,172). The L-ara4N modification was reported in 24.3% of isolates and is associated with resistance to endogenous cationic peptides, polymyxins and aminoglycosides, though expression of this modification alone is not sufficient to convey high level colistin resistance (172,173). By contrast, increased acylation is associated with stronger inflammatory responses in host cells (174,175).

Whilst this list is by no means exhaustive, it highlights some of the more frequently encountered phenotypes of *P. aeruginosa* in CF and emphasises the key message that the CF lung environment exerts strong selection pressure on *P. aeruginosa* to adapt into a wide range of

phenotypes. Presently, work is being done to link our understanding of the diverse range of mutations identified in CF isolates and categorise them according to their functional impact on *P. aeruginosa* survival in CF lung. The genetic diversity of *P. aeruginosa* in addition to prolonged diversification within the CF lung means the impact of any given mutation will vary considerably between strains. Thus, linking single mutations or groups of mutations with functional outcomes can be challenging.

1.2.4 Treatment of Cystic Fibrosis Lung Disease

Cystic fibrosis management has seen remarkable progress in the last few years with the introduction of CFTR modulator therapy. The first cohort of patients treated with these remarkable drugs is aging and the long-term disease course of CF for individuals on treatment is only just beginning to be elucidated (176). Much of the data for this section comes from the pre-CFTR modulator era. Where relevant I have highlighted where disease processes are changing, and I discuss CFTR modulators in more detail. Whilst there is much optimism, CFTR modulators are not curative and they do not treat all CFTR mutations. Furthermore, they will not reverse already-established structural lung disease. CF lung disease remains a major cause of morbidity and mortality and opportunistic infections remain a major challenge in prolonging the life of pwCF.

Mucolytics

Treatment of CF lung disease aims to maintain lung function for as long as possible. Mechanistically this is achieved by clearing secretions and treating or preventing infection. Daily physical therapy helps clear secretions whilst inhaled mucolytics can reduce sputum viscosity. Recombinant human DNase (rhDNase) reduces sputum viscosity by shortening DNA molecules which accumulate in CF sputum (discussed below). Systematic review of RCT data demonstrates that regular use of nebulised rhDNase is associated with an increase in FEV₁ relative to placebo, and a reduction in pulmonary exacerbation frequency (177).

Mannitol and hypertonic saline are used as inhaled therapies to draw water into sputum and reduce sputum viscosity, although evidence supporting this practice is limited, with systematic reviews finding a small benefit to lung function with mannitol (178) but no clear evidence for hypertonic saline (179).

CFTR modulators

In the past few years, a novel class of drugs to treat CF have become available: CFTR modulators. These drugs act by restoring the native function of CFTR by compensating for defects at different levels of CFTR synthesis and transport. The drugs fall into two different classes:

potentiators and correctors. Ivacaftor was the first clinically available CFTR modulator specifically for the G551D substitution (180) and acts as a potentiator because it increases the open state probability of CFTR in the apical membrane (181) and therefore works for *cftr* mutations that do not block expression of CFTR at the apical membrane (182). Subsequently combination therapies were introduced including lumacaftor-ivacaftor and tezacaftor-ivacaftor. These combine a potentiator and a corrector. Correctors work by stabilising CFTR variants that are normally degraded before they reach the cell surface, such as the F508del variant (183). The clinical benefit of dual combination therapy was modest, however, with a 4% absolute increase in FEV₁ and a 35% reduction in exacerbation frequency relative to placebo in one phase III trial (184). The addition of a second corrector, elexacaftor improved the combination of tezacaftor and ivacaftor (EFl) resulted in an approximately a 10% increase in FEV₁ over tezacaftor-ivacaftor alone (185,186).

As the first cohort of patients treated with this combination ages, data on the wider impacts of CFTR modulator therapy are becoming available. Regarding pulmonary infection in individuals starting therapy with established lung disease, whilst there appears to be a reduction in pathogen burden, and associated inflammation, infections with *P. aeruginosa* appear to persist and may rebound after the first few years (187,188). There is an optimism that early initiation of therapy may alter the disease course substantially and clinical data is eagerly awaited. At present, however, there is still clearly a need to understand the pathogenesis of and develop strategies for eradication of *P. aeruginosa* in CF.

Antibiotics

Antibiotics are a mainstay of treatment in CF. A major component in the management of pwCF is regular surveillance to identify new acquisitions of *P. aeruginosa* and/or identify the early indications of exacerbation so that infection can be treated early and aggressively. Antibiotics are currently used as treatment for both acute infection and exacerbations, and as suppression for chronic infection. Additionally, macrolide azithromycin, though not directed against *P. aeruginosa*, is commonly used as an immunomodulator and is associated with improved outcomes(189). The use of prophylactic antibiotics, mainly directed against *S. aureus*, remains controversial and is the subject of ongoing randomised clinical trials.

One of the major challenges of antimicrobial treatment is that susceptibility *in vitro* is not always associated with clinical response to antibiotics (190). Current practice attempts to eradicate *P. aeruginosa*, when it is first identified, with a combination of intravenous or oral, and inhaled antibiotics, either tobramycin, colistin, aztreonam or levofloxacin. The TORPEDO-CF trial

identified that there was no benefit of utilising intravenous antibiotics over oral quinolones, in combination with inhaled colistin, for the eradication of acute *P. aeruginosa* infection (191). This finding is supported by a recent Cochrane review which found that use of antibiotics in the short term was associated with reduced isolation of *P. aeruginosa* compared to placebo, but existing trial data does not support any particular regime or route (99). The most recent NICE guidance reflects this, recommending treatment with an inhaled agent in combination with either an inhaled or oral agent (69). Despite this, the evidence that combination-therapy is more effective than mono-therapy is poor (192). Once chronic *P. aeruginosa* infection is established, antibiotics are used for suppression, most commonly, in the UK, with inhaled colistin .

Evidence for the benefit of long-term inhaled antibiotics is surprisingly limited. Both the current UK guideline and a recent Cochrane review highlight the high degree of heterogeneity between studies examining the role of inhaled antibiotics, making it difficult to draw firm conclusions (69,193), but the authors of a recent systematic review concluded long-term inhaled antibiotics probably reduce the rate of lung function decline and the frequency of pulmonary exacerbations (69,193). Specifically regarding colistin, a single randomised controlled trial of 40 patients compared inhaled colistin to placebo and found that following 90 days of treatment there was no statistically significant difference in FEV₁ in the placebo or the colistin groups but, a greater decline in FEV₁ was observed in the placebo group (194). By contrast, 4 trials examined the role of Tobramycin compared to placebo (195–198) and a pooled analysis by NICE reported a mean percent change in FEV₁ of 9.36 (5.01 – 13.7) at 1 to 3 months (69). Randomised trials comparing colistin to tobramycin found no statistically significant differences between the two agents (199,200). Overall, considering the majority of pwCF and chronic *P. aeruginosa* in the UK receive inhaled colistin therapy in some form, there is remarkably little data suggesting that it is helpful in the long term.

Although colistin is widely used, with 80% of *P. aeruginosa* colonised patients treated in the UK (27), the pathogen is rarely eradicated, despite apparently antibiotic sensitivity as determined by *in vitro* testing. Although colistin resistance can arise through horizontal gene transfer and spontaneous mutation, it is rarely encountered in CF with reported resistance rates at about 5% of isolates (201). Colistin resistance is easily achieved *in vitro* following exposure to colistin (202) leading to the hypothesis that in the CF lung there is not a strong selection pressure for colistin resistance or conversely, that there is strong selection pressure against resistance. Given the poor clinical response to colistin and low rates of resistance, **I hypothesise that the lung environment and *P. aeruginosa*'s adaptation to it, reduce susceptibility to colistin.**

In understanding why colistin treatment fails in cystic fibrosis it is important to consider colistin's mechanism of action and modes of resistance.

1.3 Colistin

1.3.1 Polymyxin antibiotics

Colistin (polymyxin E) is one of 2 polymyxin antibiotics in clinical use, the other being polymyxin B and both are cationic antimicrobial (lipo)peptides. Colistin was first discovered in Japan in 1949 and was isolated from *Paenibacillus polymyxa* var *colistinus* (203), although systemic use of the compound waned with the recognition of nephrotoxicity and the availability of alternative agents (68). Though colistin is frequently used as an inhaled agent in cystic fibrosis, systemic use has recently seen a resurgence due to the emergence of MDR and extensively drug resistant (XDR) Gram-negative infections(204). From a one health perspective, polymyxins have been used extensively in agriculture as growth enhancers (205,206), which has created a reservoir of colistin resistance.

1.3.2 Structure and pharmacology

The structures of colistin and polymyxin B (PMB) are very similar (Fig 1.3). They comprise a cationic decapeptide ring and a hydrophobic tail resulting in an amphipathic structure (67). Position 6 in PMB is occupied by D-Phe whereas in colistin it is occupied by D-Leu. Commercially available preparations are a mixture of PMB₁ and PMB₂ or colistin A and colistin B which are acylated by (S)-6-methyloctanoic acid and (S)-6-methylheptanoic acid respectively (67). Although colistin can be administered intravenously, its use is limited by significant toxicity. In CF this is overcome by local delivery to the lung via either nebulisation or via a dry powder inhaler. Colistin is administered in two forms: colistin sulphate or colistimethate sodium (CMS) (207). CMS is a pro-drug with no antimicrobial activity itself but is used to reduce systemic toxicity and is spontaneously hydrolysed to colistin in the lung (207). An additional benefit of delivering colistin directly to the lung is that higher concentrations can be achieved locally than would otherwise be possible with systemic delivery. Estimates on sputum concentrations vary. Yapa et al reported that following nebulised administration of CMS peak sputum concentrations of colistin lie in the range of 2-11 µg/mL following a nebulised dose of 2 million IU and 4-21 µg/mL following 4 million units (208). Similar results were obtained by Magréault and colleagues measured sputum concentrations of between 1 and 40 µg/mL after 6 days of twice daily administration of 2 million IU (209). These results conflict with an earlier study by Ratjen et al who estimated mean peak sputum concentrations of ~40 µg/mL following a single nebulised dose of 2 million IU (210). Yapa directly addresses this by suggesting the purification process used by

Ratjen may further promote the conversion of CMS to colistin whereas Ratjen acknowledges that higher concentrations were above the linear part of their assay and therefore unreliable. Complicating these estimates is also the observation that delivery of colistin by nebulisation is probably not uniform throughout the lung. This is supported by an animal model of *P. aeruginosa* lung infection in pigs, which demonstrated lower levels of colistin in more severely infected areas of lung following nebulised delivery (211).

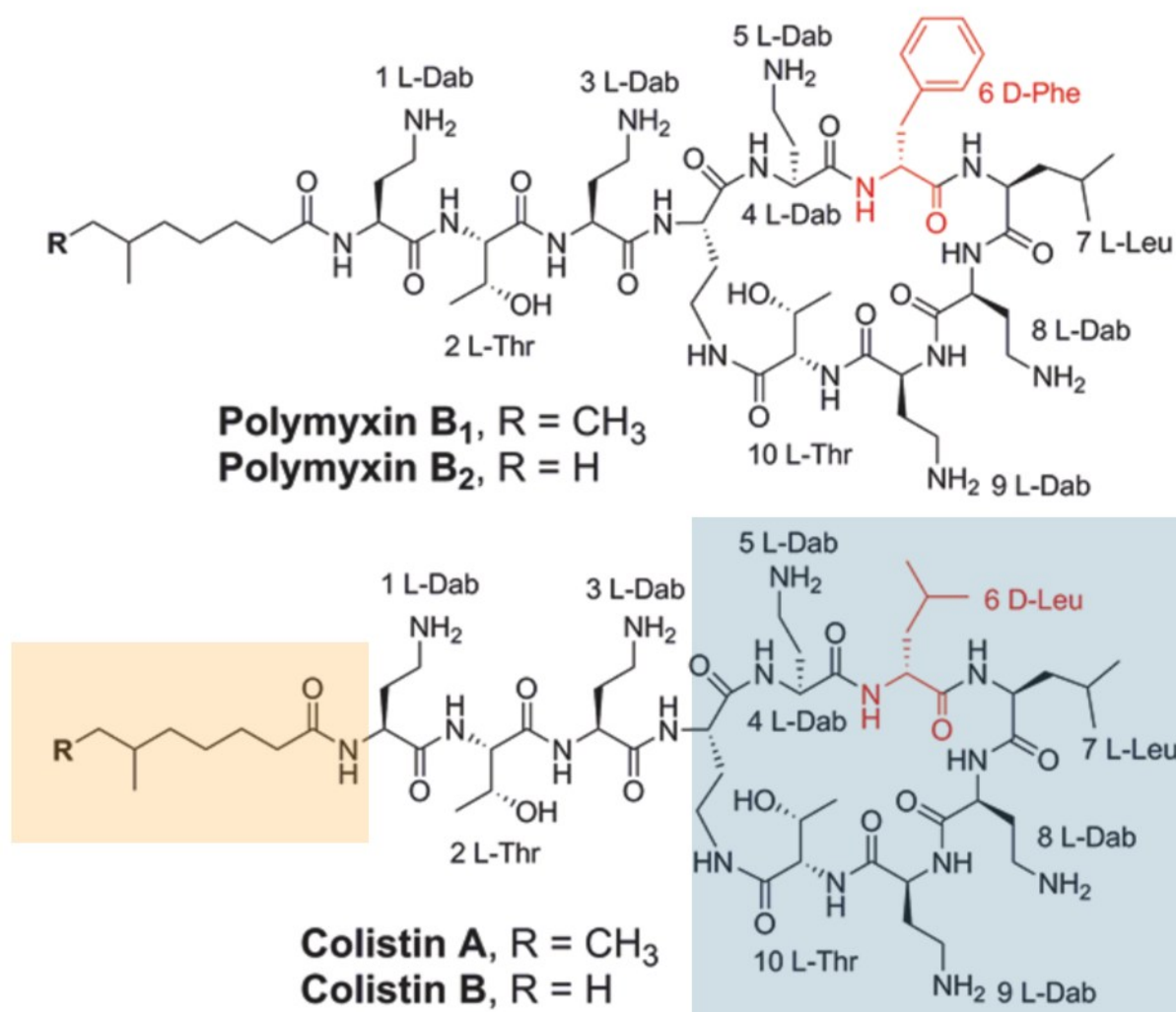


Figure 1.3: Structures of polymyxin B and colistin adapted from (212). Dab – diaminobutyric acid. For colistin key features have been highlighted including the hydrophobic tail (orange) and the cationic head group (blue).

1.3.3 Mechanism of action

Colistin is active against aerobic Gram-negative pathogens, notably *P. aeruginosa*, *A. baumannii* and Enterobacteriaceae (213). Much of the work to understand the mechanism of action has been done with polymyxin B, however given the overall similarity of structure the

mechanism is probably very similar. The primary target for colistin is the lipid A component of LPS (214–217), which is the most abundant component of the outer membrane of Gram-negative bacteria. LPS comprises 3 main components: The O-antigen, a core polysaccharide and lipid A, which acts as the hydrophobic anchor for LPS in the outer membrane. LPS is synthesised on the inner leaflet of the inner membrane, lipid A via the Raetz pathway, before the addition of the core carbohydrate (218,219). Lipid A with core carbohydrate is transported to the outer leaflet of the inner membrane via MsbA, before the O-antigen is attached (220). Newly synthesised LPS is then transported to the outer membrane by the Lpt complex (219).

The cationic head group of colistin (Fig 1.3 blue) is attracted to the terminal phosphate on lipid A (216) and this binding interaction disrupts divalent cation bridges between adjacent LPS molecules leading to permeabilisation of the outer membrane (217,221,222). Increasing the availability of divalent cations is sufficient to displace polymyxins from this site and ablate antibacterial activity (214,223). Studies utilising the modified polymyxin B nonapeptide, which contains the charged polymyxin head group but not the hydrophobic tail, have demonstrated that the head group is sufficient to cause outer membrane disruption, but insufficient to kill bacteria (223–226). The hydrophobic tail (Fig 1.3 orange) of colistin is thought to stabilise polymyxins in the outer membrane and promote their uptake (215). The hydrophobic tail region also appears to be responsible for stabilising the interaction with Lipid A (214,223). Permeabilisation of the outer membrane is thought to enable polymyxins to enter the periplasm and access the inner membrane via a process referred to as ‘self-directed uptake’ (213). Binding of polymyxins to LPS on the inner membrane is hypothesised to cause permeabilization, and subsequent leakage of cytosolic contents and cell death (227,228). The proposed model of action of colistin is summarised in (Fig 1.4) At present it is unclear exactly how colistin enters and crosses the periplasm and whether this is an active or passive process.

An alternative model of colistin activity has been proposed that hypothesises that colistin mediates fusion between the inner and outer membrane. Termed the vesicle-vesicle contact pathway evidence for this pathway arises from phospholipid exchange between the two membranes during colistin treatment (229,230).

Alongside their membrane targeting role, several authors have reported on the ability of polymyxins to inhibit cellular processes. Sampson and colleagues reported a hydroxyl radical death pathway whereby colistin triggered the production of reactive oxygen species within the cell leading to oxidative stress and ultimately cell death. This pathway was described in *A. baumannii* (231). The same could not be demonstrated in *P. aeruginosa* (232). Deris et al reported

on the ability of polymyxins to inhibit NADH-quinone oxidoreductases in *E. coli*, *K. pneumoniae* and *A. baumannii* but at high concentrations relative to MIC (233). These mechanisms hold some interest in that they partially explain the preference for aerobic Gram-negatives, and perhaps why de-nitrification offers some degree of protection against colistin in *P. aeruginosa*. It should be noted that they are under-explored in *P. aeruginosa*.

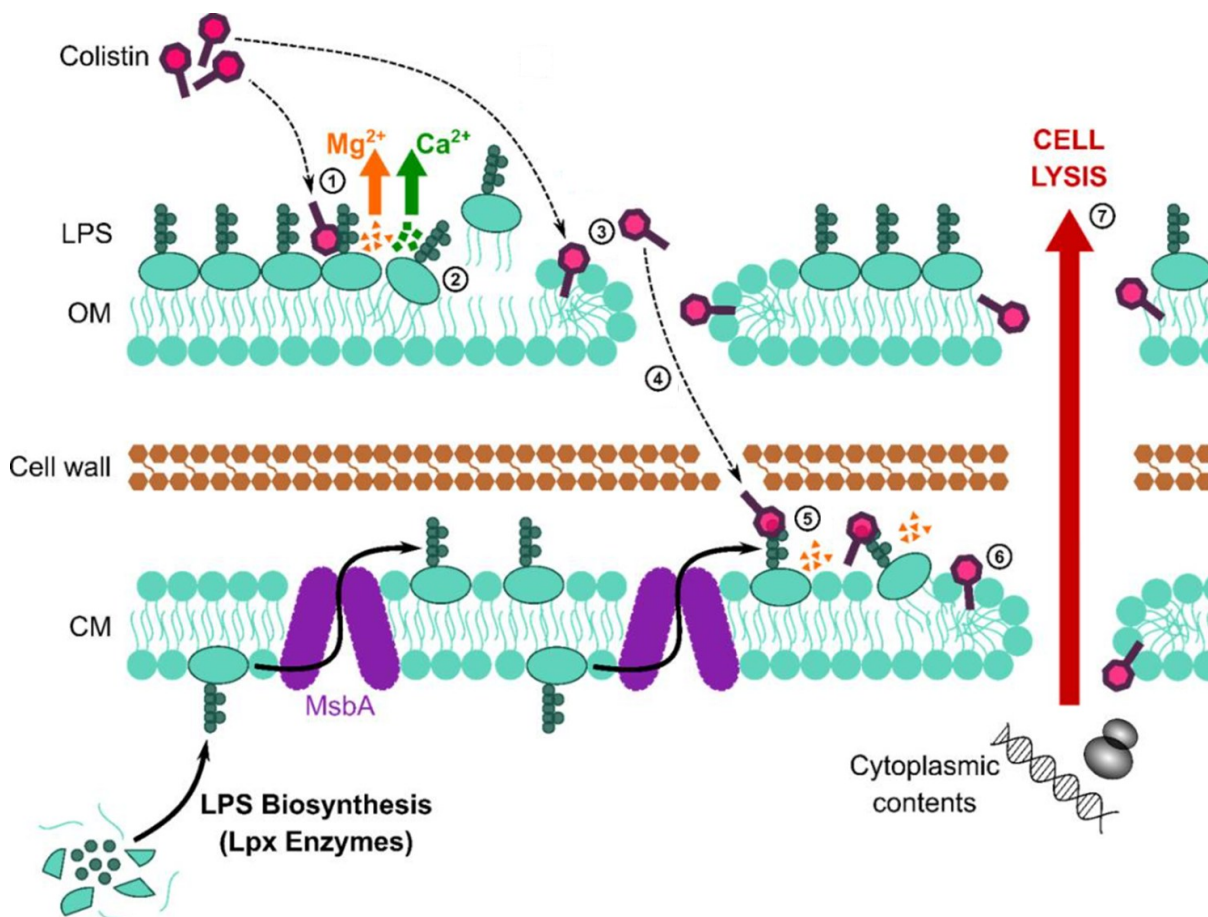


Figure 1.4: Primary mechanism of action of colistin. 1. Cationic colistin interacts with terminal phosphates on lipid A leading to 2. Disruption of divalent cation bridges between adjacent LPS molecules. This results in 3. Disruption of the bacterial outer membrane (OM). 4. Colistin enters the periplasm where it 5. interacts with colistin on the periplasmic face of the cytoplasmic membrane (CM) resulting in 6. Permeabilisation of the cytoplasmic membrane allowing 7. loss of cytoplasmic contents and cell death. Adapted from Sabnis et al (227)

In addition to its direct mechanism of action, colistin also demonstrates beneficial off-target effects. Permeabilisation of the outer membrane occurs at sub-lethal concentrations of colistin and can sensitise Gram-negative organisms to other antimicrobials in particular, beta-lactams and rifampicin(234,235). Rifampicin, in particular, is not normally active against Gram-negative organisms at physiological concentrations(236,237). In addition, it is well known that

LPS is a potent immunostimulatory molecule recognised by pattern recognition receptors of the innate immune system contributing to bacterial sepsis. Binding of colistin to LPS has been shown to reduce inflammatory cytokine production in human challenge studies (238).

1.3.4 Mechanisms of resistance

The best described mechanisms of colistin resistance arises from covalent modification of the terminal phosphates of lipid A with either 4-amino-4-deoxy L-arabinose (L-ara4N) or phosphoethanolamine (pEtN) (Fig 1.5) (239). These modifications are thought to mask the negative charge on the terminal phosphate residues reducing their affinity for colistin. Several Gram-negative organisms are intrinsically resistant to colistin due to their constitutive modification of lipid A including *Burkholderia cepacia*, *Proteus mirabilis*, and *Serratia marcescens* (66,240–242). Although other Gram-negative organisms utilise a combination of L-ara4N and pEtN, *P. aeruginosa* seems to rely predominantly on L-ara4N modification (243). Covalent modifications of lipid A are regulated by multiple two-component systems (TCS). To date 5 TCSs have been linked with polymyxin resistance: PmrAB (244,245), PhoPQ (58,246), CprRS (247,248), ParRS (247) and ColRS (248,249). PmrAB, PhoPQ, CprRS and ParRS all activate the *arnBCADTEF* operon (referred to as *arn*) leading to the synthesis and addition of L-ara4N to lipid A. It is only relatively recently that endogenous pEtN, synthesised and attached to lipid A by the phosphoethanolamine transferase EptA, has been identified in *P. aeruginosa*, triggered by ColRS (249). Cervoni and colleagues have questioned whether pEtN contributes to colistin resistance in *P. aeruginosa* at all (243,250). Mutational resistance to polymyxins in *P. aeruginosa* is, almost exclusively, linked to gain-of-function mutations in PmrAB and PhoPQ in colistin exposed isolates taken from pwCF (251–254). However, the overall rates of colistin resistance in *P. aeruginosa* isolates from CF remains low: approximately 5% in a recent meta-analysis and systematic review (201).

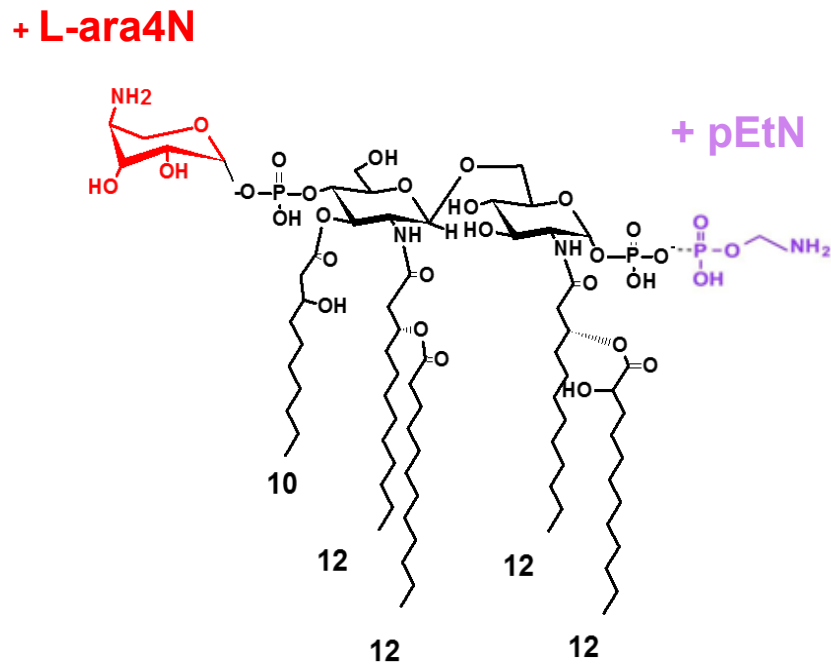


Figure 1.5 Covalent modification terminal phosphates of lipid A results in colistin resistance. Lipid A (black) can be modified with aminoarabinose (red) or phosphoethanolamine (purple).

Exogenous phosphoethanolamine transferases have been identified as a source of colistin resistance arising from horizontal gene transfer. These enzymes termed mobile colistin resistance genes (*mcr*) have been identified in multiple bacterial species, particularly in healthcare settings. Since *mcr-1* was identified in *E. coli* and *K. pneumoniae* isolates from China in 2016 (206) 8 further *mcr* genes have been identified (255). *Mcr-1* remains the most commonly identified variant with a global distribution, although these plasmids are very rarely encountered in *P. aeruginosa* (256).

Although lipid A modification is by far the most common resistance mechanism, alternatives have been reported. *LpxC* catalyses the first step of lipid A synthesis (219). In *A. baumannii* mutations in *lpxC* prevent LPS synthesis altogether, resulting in bacteria with no LPS expression that are highly resistant to polymyxins (257). *lpxC* mutations have been identified in colistin resistant *P. aeruginosa* but the phenotype was not confirmed (258,259). Other outer membrane proteins have been linked with polymyxin resistance in *P. aeruginosa*. The Drug-efflux pump MexXY-OprM was found to contribute to colistin resistance, but the precise mechanism is unclear because disruption of efflux can result in changes to the membrane (260). MexXY

appeared to be most important for resistance in the presence of both *arn* expression and expression of PA4773-74 which encode polyamine synthesis genes (260). Thus, MexXY may not be transporting colistin but may be involved in the export of polyamines or other LPS components (261). Similarly, OprH over-expression has been linked with reduced susceptibility to polymyxins but again the mechanism appears esoteric, with OprH potentially competing for divalent cations to bridge LPS molecules (246,262,263).

As mentioned above *P. aeruginosa* possesses its own polyamine synthesis pathways. Polyamines are primordial amino-acid derived polycations that subserve different functions across a range of organisms including prokaryotes and eukaryotes (264). The polyamines putrescine, spermidine, and spermine are derived from L-ornithine. The genes PA4773-4775 form an operon under the control of PmrAB (265) and ParRS (260) and have been implicated in *P. aeruginosa* polyamine synthesis. Johnson et al found disruption of PA4774 was associated with increased susceptibility to polymyxin B and endogenous antimicrobial peptides (265).

Being metabolically versatile, *P. aeruginosa* can grow under a range of different conditions and utilise an array of carbon sources. The effect of bacterial metabolism on susceptibility to polymyxins is relatively under-explored. However, it is clearly relevant to the CF lung environment. When amino acids were used as a sole carbon source in minimal media, susceptibility to colistin varied greatly, though the mechanism was not elucidated (266). Similarly, a more recent study by Sauvage et al identified that minimal media supplemented with glutamate increased the MIC of colistin relative to glucose (267). The effect was even greater when succinate or citrate were included instead of glucose (267), with proteomic analysis revealing that upregulation of proteins associated with lipid A modification: PhoP, PmrA, ArnA, ArnB and OprH. Anaerobic growth also appears to modulate polymyxin susceptibility. Hill and colleagues demonstrated reduced susceptibility of clinical isolates under anaerobic conditions in CA-MHB (268). By contrast, Kolpen demonstrated increased susceptibility to polymyxin in anaerobic conditions in the presence of 1mM nitrate (269). As previously discussed, *P. aeruginosa* can use de-nitrification to derive energy anaerobically however, excess nitrite can inhibit de-nitrification and biofilm formation itself, increasing susceptibility to polymyxins (270). Overall, it remains unclear whether, under physiological conditions, low oxygen tension increases or decreases susceptibility to polymyxins.

1.4 The CF lung environment promotes *P. aeruginosa* persistence

I have discussed how *P. aeruginosa* is a versatile and adaptive pathogen, adept at establishing chronic infection in challenging environments, particularly the lung environment in

CF. We have already seen that availability of oxygen, carbon and nitrate can alter susceptibility to polymyxins (Section 1.3.4). In this section I will discuss how specific features of the CF lung make eradication of *P. aeruginosa* with antibiotics a challenge. There are several features of the lung environment in CF that are recognised to either directly interact with antimicrobials or alter bacterial behaviour making thus reducing susceptibility to antimicrobials to antimicrobial treatment.

Airway Structure

During the course of CF, repeated cycles of infection and inflammation lead to the destruction of lung tissue and bronchiectasis. The distal airways are key for effective gas exchange. Microscopic examination of the airways from late CF reveal gross destruction of airway architecture, alveolar obstruction and collapse, increased septal thickening and over expression of glycoproteins (271). Over time, some areas of the lung become more severely affected than others and the distribution of air flow is altered. In turn, this can alter the distribution of inhaled antibiotics (272). Mucus trapping can also lead to areas of relative hypoxia within the lung (273). The airways of the lung are covered in a ciliated respiratory epithelium, which is covered in a layer of airway surface liquid (ASL) which comprises a thin periciliary layer and a thick mucus layer (89). Movement of the cilia wafts entrapped foreign particles and pathogens caudally where they can be swallowed or expectorated (274). Impaired secretion of Cl^- and HCO_3^- by defective CFTR function results in altered ASL composition, thick mucus and impaired clearance (75).

1.4.1 ASL composition

ASL in CF is altered from the composition of healthy lungs (for a useful summary see (275)). The ASL in CF contains high concentrations of amino acids (276,277), the availability of which has been recognised as an important cue for biofilm formation and quorum sensing (278–280) but can also dramatically alter susceptibility to antimicrobials when used as a sole carbon source (266,267). *P. aeruginosa* appears to metabolise amino acids in preference to sugars (281).

Mucin

Mucins are highly glycosylated glycoproteins secreted or tethered to the membrane at epithelial surfaces for lubrication and to help form a protective barrier against foreign bodies and pathogens (282). In CF, inflammation in the airway leads to the production of excess mucins both in steady state (90) and during pulmonary exacerbations (283). Healthy mucins appear to attenuate the virulence of *P. aeruginosa* by altering expression of quorum sensing and toxin production (284,285). However, the altered glycosylation seen in cystic fibrosis may promote bacterial adhesion and colonisation (286). Furthermore, mucins are anionic and have been

shown to bind to and reduce the activity of both cationic tobramycin and colistin (287–289). Butnarusu and colleagues demonstrated interactions with a range of antimicrobials from multiple classes though this wasn't linked to any functional assessment (290). Incorporation of mucin into artificial sputum media (ASM) also reduced susceptibility to imipenem and quinolones in addition to colistin and aminoglycosides (291).

DNA

CF sputum contains high concentrations of extracellular DNA which contributes to sputum viscosity. Estimates of eDNA concentration in sputum vary greatly from ~0.25 mg/mL to ~5 mg/mL (292–295) and likely vary with disease progression, inflammation and relative tissue destruction (89). Examination of sputum eDNA has revealed that the majority is host-derived likely arising from tissue destruction and NETosis (94,296,297). Aside from altering the rheological properties of sputum, eDNA is also a pro-inflammatory signal recognised by innate immune receptors. Unmethylated CpG motifs from bacterial DNA are recognised by TLR9 leading to the upregulation of proinflammatory cytokines (298). NETs can lead to bystander tissue damage and help drive the inflammatory process of CF. In addition, eDNA forms a major constituent of biofilm, thus contributing to antibiotic failure (160). Bacteria, including *P. aeruginosa*, are able to promote conformational changes withing eDNA, from the more common B-form to the high energy Z-form, which renders the biofilm matrix recalcitrant to nuclease destruction and reduces the lethality of NETs (299). Extracellular DNA can also chelate divalent cations and drive local acidification in the lung which act as signals driving LPS modification in *P. aeruginosa* reducing susceptibility to cationic antimicrobial peptides and aminoglycosides (164,300). Finally, *P. aeruginosa* can utilise eDNA as a nutrient source to promote growth (301).

Phospholipids

Estimates of phospholipid composition in ASL vary between 1% and 0.01% (w/v) and mainly consist of phosphatidylcholine (89,302,303). Interestingly, the total phospholipid content of CF sputum does not seem to be dramatically shifted from healthy individuals, but several authors have found changes in the relative abundance of different phospholipids (302–304). Relatively little data exists on the impact of exogenous phospholipids on susceptibility of *P. aeruginosa* to antibiotics. However, two studies identified that exogenous phospholipids could be incorporated into the membranes of *P. aeruginosa* (305,306). The absence of phospholipids from ASM dramatically increased the susceptibility of bacteria to the little used beta-lactam carbenicillin but did not alter susceptibility to gentamicin or colistin (305). Microarray analysis of *P. aeruginosa* isolated from the CF lung has demonstrated upregulation of fatty acid degradation

(*fad*) genes which allowed *P. aeruginosa* to grow in the presence of phosphatidylcholine as a sole carbon source (307). Furthermore, targeted mutation of *fad* genes was associated with altered virulence factor production (308). Taken together it is clear that phospholipid availability within the lung is likely to have an impact on *P. aeruginosa* virulence but it is unclear whether there is a significant effect on antimicrobial susceptibility.

Polyamines

In addition to the endogenous polyamines produced by *P. aeruginosa*, host-derived polyamines are also produced in the human lung. For example, Grasemann et al identified that spermine was elevated in the sputum of pwCF compared to healthy controls (309), whilst putrescine, spermidine and spermine were elevated during pulmonary exacerbation compared to healthy controls (309). This was confirmed by Twoney and colleagues, who found elevation of polyamines during pulmonary exacerbation (310). The impact of polyamines on antimicrobial susceptibility appears to be complex. Kwon reported the addition of exogenous spermidine and spermine reduced *P. aeruginosa* susceptibility to aminoglycosides, polymyxins and quinolones and the authors hypothesised that the effect was in part mediated by the induction of the PhoPQ two-component system (311). Meanwhile, Johnson and colleagues identified that the addition of exogenous spermidine, localised to the cell surface of the bacteria, could reduce membrane permeabilisation induced by polymyxin B (265). More recently, Hasan et al found that a polymyxin susceptible *P. aeruginosa* strain with a loss of function mutation in *pmrB*, could be protected by the addition of exogenous spermidine (312). The authors proposed that loss of L-ara4N expression resulted in the coating of PmrB deficient bacteria in positively charged spermidine, shielding the bacteria from similarly cationic colistin (312).

Collectively, these data highlight the importance of individual components of ASL in modulating antimicrobial susceptibility. However, relatively little work has been done to examine the overall effect in the lung environment. Many studies examined the role of individual components in minimal-media and it is therefore unclear the role many of these biomolecules have in the complex milieu of the CF lung. The study by Hasan et al provides an excellent example whereby mutations in *pmrB* result in increased susceptibility of *P. aeruginosa* to polymyxins *in vitro* this was not the case *in vivo* (312). Similarly, Hunt et al identified that the combination of DNA and mucin was less effective at sequestering tobramycin than DNA and mucin treated with DNase (289) highlighting the possibility of macromolecular interactions. Complex models of the CF lung are required to elucidate these interactions and the net effect on antimicrobial susceptibility.

1.4.2 Modelling CF lung disease

Animal Models

One of the persistent challenges in studying the effect of the CF lung environment is the limited availability of models for pre-clinical study and how well / poorly they recapitulate the condition in humans. As outlined above severe CF is a complex, destructive disease altering both the macroscopic and microscopic properties of human physiology and progressing over years. No model can hope to recapitulate such complexity, but a lack of complexity in conventional tools can miss important interactions. Models from CF vary from genetic knockouts in animal systems, tissue explants and cell lines to synthetic sputum.

A range of animal models exist for the study of cystic fibrosis. None recapitulate the human disease pattern but offer limited benefits in particular domains. Murine models were established soon after the identification of CFTR in 1989. CFTR in the mouse has a 78% amino acid (āā) homology with the human protein (313). Initial germline knock-outs (KOs) were developed in the 1990s (314,315) and these were followed by models mimicking human substitutions, including F508del (316) and G551D (317). The severity of the phenotype broadly correlates with the amount of CFTR mRNA produced and each model has slightly different features depending on the presence of various modifier genes (318,319). The phenotype of disease in mice also differs from humans with a high rate of neonatal lethality in mice due to intestinal obstruction (319,320). Whilst meconium ileus is observed in around 20% of newborn human infants with CF (321), this is clearly a more dramatic difference.

Another key difference between the murine model and human disease is the poor reflection of lung disease probably relating to intrinsic differences between human and murine CFTR function and differences in cellular architecture (322). Human disease is thought to be driven, to some extent, by sodium reabsorption via ENaC leading to increased mucus viscosity, however in mice this channel does not appear to be upregulated in response to CFTR dysfunction as it is in humans (322). None of the CF mice develop spontaneous lung disease. Lung infections are mimicked by inoculation of bacteria into the lungs. Susceptibility to *P. aeruginosa* was demonstrated by this method thought, to achieve infection, a slurry of beads was inoculated into the lungs (323). A modicum of lung disease was achieved when KO mice were back-crossed into a C57BL/6J background (324). These mice developed spontaneously impaired mucociliary clearance, increased interstitial inflammation and fibrosis in early life (324) and progressive multi-organ features later (325). They also demonstrate increased susceptibility to *P. aeruginosa*

after direct inoculation, with enhanced inflammatory responses (326). As an alternative approach to mimicking the muco-occlusive disease seen in human CF lung pathology, researchers have over-expressed the beta-subunit of ENaC (β ENaC) in mice (327). In human CF CFTR regulates ENaC leading to excessive reabsorption of Na^+ ions driving fluid depletion in ASL. Similarly, the β ENaC mouse develops chronic inflammatory lung disease characterised by mucus obstruction and inflammatory infiltration with a high mortality (328,329). Crucially, these mice have normal CFTR function and expression of β ENaC is restricted to the lungs. Murine CFTR shows varied responses to CFTR modulator drugs again highlighting a key difference from humans (330,331). Most recently a *cftr* KO expressing human *cftr* (*hCFTR*) as a platform for investigation of CFTR modulator drugs has been developed (332).

Rat models provide a larger animal, allowing for greater tissue samples, whilst still being fast to rear in large numbers. Rat CFTR has a 76% homology to the human $\alpha\alpha$ sequence (333). Tuggle and colleagues developed a *cftr* KO rat but again the animals showed an extremely high neonatal mortality from intestinal obstruction (~70%) and failed to develop spontaneous lung disease (334). Crispr/Cas9 has been used to generate F508del rats, but again GI pathology is dominant (335,336). Birket and colleagues reported that obstructive pathology developed as the rats aged and defects such as ASL acidification could be identified (337). Reyne and colleagues also report quantifiable differences in lung function parameters with healthy rats, although these could not be restored by the combination of elexacaftor, tezacaftor and ivacaftor whereas nasal potential difference was restored (338).

Due to the poor characterisation of lung disease afforded by smaller mammals, larger animals have also been used. Ferret CFTR has a 91% $\alpha\alpha$ homology to humans and their lung architecture is closer to humans than mice or rats (339). The first *cftr* KO ferrets were developed by Sun and colleagues (340). The majority of kits (~80%) died soon after birth with meconium ileus or gastro-intestinal obstruction (341). For those that survived, histological evidence of spontaneous lung infection was identified in the neonatal period. Older animals only survived with antibiotic treatment but went on to develop recurrent respiratory infection (342) and demonstrate an impaired ability to clear directly inoculated *P. aeruginosa* and elevated cytokine production (343). More recently, the ferret model has been adapted to include the G551D substitution allowing the CFTR modulator Ivacaftor to be used (344). Interestingly administration of Ivacaftor to pregnant mothers and neonates reduced incidence of pulmonary infection and reduced the attrition rate from meconium ileus (344).

Perhaps the longest established large animal model of cystic fibrosis is the pig. Porcine CFTR has a 93% homology with human (345). There are also structural and immunological similarities between humans and pigs that make them a favourable animal model (346,347). The first *cfr* KO and F508del variant were generated by Rogers and Welsh (348,349). Newborn-pigs spontaneously developed lung infection, displayed features of inflammation, impaired mucociliary clearance, and an impaired ability to clear a *S. aureus* inoculum (350). However, all newborn piglets developed meconium ileus and were only enabled to survive a few months by the performance of ileostomy (350). This significantly hampers the utility of the model. Being larger animals, non-invasive imaging tools can be used in a fashion similar to humans (351).

Overall animal models to date have had limited utility in recapitulating cystic fibrosis lung disease. Whilst all species generated exhibit severe GI pathology and to some extent there is evidence of immunodeficiency and hyperinflammation, there remains a large gap between human physiology and animal models, therefore any animal use should be limited in the study of CF and conclusions interpreted with caution.

In vitro models

Given the limited utility of animal models, and their high cost, there is an increasing interest in creating better *in vitro* models for cystic fibrosis. To that end, considerable progress has been made in the last few years in generating 3D culture systems that mimic, to some extent, the architecture of the human airway. Traditional 2D cell culture systems have used immortalised cell lines or even primary human cells to examine disease processes. An immortalised trachea-bronchial cell line expressing F508del CFTR, CFBE41o-, has been developed (352). A range of other immortalised CF cell lines have been used: CFTE29o-, IB3, CFT1-F508del and CuFi-1 being the most common (353). There is a good deal of experimental heterogeneity surrounding the use of these cells that can make generalisable conclusions difficult (353). The representativeness of immortalised cells, in particular, has long been debated. Whilst there is clearly a role for these systems due to their availability and relative simplicity more complex systems afford the opportunity to investigate CF / Pathogen / Drug interactions in more depth.

Air liquid interface (ALI) models have been developed that allow the formation of a polarised epithelium on a transwell membrane such that the basal part of the cells is in contact with culture medium and the apical surface is in contact with atmospheric air (354). Both cell lines and primary human airway epithelial (HAE) cells can be used to generate ALI cultures (355). Primary human cells grown in this way demonstrate similar transcriptional profiles to cells obtained from endo-bronchial brushings (356). However, the use of primary human cells is

limited by passage number. Recently, a method for prolonging the life of these cultures by 'conditional reprogramming' was developed (357) further increasing their utility. Furthermore, the model can be adapted to include immune cells and endothelial cells increasing the physiological relevance (358). ALI systems have been adapted to co-culture bacterial pathogens including *P. aeruginosa* and have demonstrated reduced susceptibility to aminoglycosides (359,360). These models have also been used to test novel antimicrobials (361). ALIs using the CFBE41o- cell line increased the reproducibility, but the ASL produced lacks mucus production (362). To address this issue Murgia and colleagues were able to stably supplement growth medium with normal human mucus (362). Lewin et al have gone even further supplementing cells with artificial sputum medium (363).

An alternative approach to generating a polarised epithelial culture is the use of rotating wall vessel (RWV) bioreactors. RWVs culture cells in on extra-cellular matrix coated micro carrier beads in a low shear stress environment to produce in vivo like 3D cellular structures (364). The model has been adapted to examine the interaction between *P. aeruginosa*, A549 respiratory epithelial cells and antimicrobials (365,366). The system produces cells that are comparable to ALI models and can be adapted to CF cell lines (367). Validation by comparison to in vivo or ex vivo data is currently lacking.

Microfluidic devices are increasingly being used to create *in vitro* culture systems, the so-called 'organ on a chip'. These systems contain a cell-lined architecture perfused with medium and, in the case of lung mimicking systems, air (368). One of the first developed systems was able to generate an epithelial surface in contact with air separated by a semi-permeable membrane from an endothelial surface in contact with flowing medium. The system was able to recruit neutrophils when they were added to the culture fluid in response to bacteria or nanoparticles (369). A model of CF lung has been created in a similar fashion using primary cells from CF donors (370). Compared to chips seeded with healthy donor cells, the CF chip demonstrated enhanced mucus production, increased cilia density, increased cytokine production. *P. aeruginosa* was more readily able to proliferate in the CF chip and produced a more profound inflammatory response. This study represents the first attempt to recapitulate CF lung infection in such a model. It remains to be seen whether such systems more accurately reflect in vivo conditions.

Lung organoids are an alternative approach to recapitulating the host environment. Organoids are 3D culture systems derived from stem cells that are differentiated into tissue structures (371). The utilisation of stem cells allows for complex structures containing multiple cell types to be differentiated. The overall structure of organoids is spherical with the apical or

luminal surface at the centre. This provides a particular challenge for investigation of infection dynamics as microinjection is required to introduce organisms to the luminal surface (372). However, the dynamics of infection with *P. aeruginosa* are beginning to be investigated in such models (373). Recently, terminally differentiated cells from CF donors were converted to induced pluripotent stem cells (iPSCs) and used to generate lung organoids that were used to assess CFTR function (374,375).

Ex vivo models hybridise animal models with tissue culture. With regard to the respiratory system, isolated perfused lungs or precision cut slices have been used to examine host-pathogen interactions or drug efficacy (376). There is a clear benefit to these models in terms of the cellular complexity and 3D architecture they maintain (376). Ex vivo models have been adapted to examine host-pathogen interactions in the CF lung. Harrison and colleagues combine ex vivo pig lung (EVPL) with artificial sputum medium (ASM) to produce a CF-like environment (377,378). The model has been further adapted to investigate antimicrobial susceptibility (379). Most recently a transcriptomic analysis demonstrated that the transcriptome of *P. aeruginosa* isolated from the EVPL model was similar to that reported for isolates direct from human sputum (380,381). Notably, there was upregulation of *pmrA* and *arnA* relative to ASM at 24 hours of culture. Both genes are associated with resistance to cationic antimicrobial peptides (381).

Artificial sputum media

There has been significant progress in recapitulating the composition of CF airway surface liquid *in vitro* using artificial sputum media (ASM) based on the composition of sputum from pwCF. Broadly, there are two groups of ASM: Those based on the work of Ghani and Soothill (382) and those based on the work of Palmer et al (276). Ghani and Soothill set out to develop a medium to recapitulate CF sputum, promote biofilm formation and reflect antimicrobial susceptibility in the CF lung. Ghani and Soothill's medium contained DNA, mucin, NaCl, KCl, egg yolk emulsion, as a source of lipids, and the iron chelator diethylenetriaminepentaacetic acid (DTPA) (382). The medium has been subsequently modified with the addition of α -lactalbumin (278,383–386) albumin (383–385) or ferritin (384,385) and various authors have changed concentrations of different components (for review see (387)). In general, these models promote biofilm formation from *P. aeruginosa* and show differences in antimicrobial susceptibility against standard laboratory media but crucially lacked an external validation against actual CF sputum.

Palmer and colleagues set out to produce a chemically-defined medium (referred to here as SCFM-1) that would more precisely mimic the nutritional environment of CF sputum based on measurements from 12 adult non-exacerbating CF patients (276). SCFM-1 defined precise

concentrations of amino acids, FeSO₄, MgCl₂, CaCl₂, lactate and glucose. SCFM-1 was validated against CF sputum by transcriptional profiling and whilst it was similar with respect to carbon utilisation SCFM-1 lacked sputum macromolecules. SCFM-1 was modified by Turner et al to include DNA, mucin, dioleoylphosphatidylcholine (DOPC) and N-acetylglucosamine (GlcNAc) to produce SCFM-2 based on published observations of these macromolecules (388). The medium was initially validated by Tn-Seq to define essential genes for growth in SCFM-2 and CF-sputum (388). The authors identified several components that were more abundant in CF sputum than SCFM-2: thiamine, NAD, purines, folate, branched-chain amino acids, and tryptophan. More recently SCFM-2 has been combined with an ALI interface model to improve the representativeness relative to human infection (363). Metatranscriptomic data identified that the expression level of around 86% of genes from PAO1 grown in SCFM-2 were expressed within 2 standard deviations of genes from wild-type *P. aeruginosa* in expectorated sputum (AS₂). This ‘accuracy’ score was not significantly improved by the addition of the ALI model (363). Interestingly the model was improved by the addition of calprotectin to chelate exogenous zinc which had entered the SCFM-2 as a contaminant. In previous work Cornforth et al identified that random internal samples compared to the whole CF-sputum data set had an AS₂ score of 97% (389).

Recently, Ruhluel et al have developed more complex chemically defined media to reflect both healthy and CF lung and sinus fluid (275). The model incorporates the amino acid concentrations identified by Palmer et al but also includes lysozyme, lactoferrin, polyamines, albumin, bile salts, succinate, galactose, ZnCl₂ CuCl₂ but not phospholipids (275). The model appears to show a subset of genes differentially expressed between SCFM-2 and the novel media but a direct comparison was not performed.

Overall, ASM provides a tractable model for examining drug susceptibility and pathogenesis of bacteria in CF lung. A major limitation is the lack of a cellular component, though when this has been addressed by combining with an ALI model, whilst a different subset of genes were expressed, the model was not more accurate (363). As synthetic sputum models form a major part of this work they will be discussed in more detail in chapter 3.

1.5 Hypothesis

Although Colistin is widely used in cystic fibrosis as an inhaled antibiotic in the UK, it rarely sterilises chronic infection with *P. aeruginosa*. Furthermore, *in vitro* susceptibility to colistin is a poor predictor of clinical response to antibiotics despite resistance rates to colistin remaining low. Many of the components of cystic fibrosis sputum have individually been implicated in

altering susceptibility to polymyxins though the relative importance of these individual molecules in physiological conditions is unknown. Therefore, I hypothesised that:

The lung environment in Cystic Fibrosis impairs the ability of colistin to eliminate chronic *P. aeruginosa* infection by promoting adaptive changes in the bacterium.

1.4.1 Aims

The aims of this project were:

1. To investigate the effect of the host microenvironment on susceptibility of *P. aeruginosa* to colistin using a physiologically relevant *in vitro* model and representative clinical isolates.

Based on the observations of this first chapter:

2. To determine the mechanism by which eDNA reduces susceptibility of *P. aeruginosa* to colistin in synthetic sputum.
3. To investigate strategies to enhance the antimicrobial activity of colistin under physiological conditions.

Chapter 2: Materials and Methods

2.1 Bacterial strains and culture

2.1.1 Bacterial Strains and Plasmids

The Plasmids used in this study are listed in table 2.1. The bacterial strains used in this study are listed in table 2.2. Clinical *P. aeruginosa* strains were provided by Prof Jane Davies (Imperial College London). Transposon mutants, *E. coli* strains and the miniCTX-1 plasmid were provided by Dr Luke Allsopp (Imperial College London). Plasmids used in this study are listed in table 2.2.

2.1.2 Growth Conditions

All bacteria were grown to stationary phase over 18 hours in Lysogeny broth (LB) (Thermo Fisher Scientific) cation adjusted Muller Hinton Broth (CA-MHB) or SCFM-1 (see section 2.1.3) in a shaking incubator at 37°C and 180rpm. Where required, selection to maintain transposon mutants or plasmids was provided by supplementing media with 15 µg/mL gentamicin (Gibco), 15 or 150 µg/mL tetracycline (Sigma) or 50 µg/mL Kanamycin (Sigma). Enumeration of colony forming units (CFU) was performed by generating and plating (10 µL) of 10-fold serial dilutions of samples in phosphate-buffered saline (PBS) on to LB supplemented with 1.5% agar (LBA; BD biosciences).

Plasmid	Description	Reference
pMiniCTX-1	Mobilisable self-integrating plasmid containing <i>oriT</i> , <i>int</i> , <i>attP</i> , <i>TcR</i> .	(390)
pMiniCTX-1-eptA	MiniCTX-1 carrying eptA under its native promoter	This Study
pMiniCTX-1 colRS	MiniCTX-1 carrying the ColRS operon under its native promoter	This Study
pRK2013	Conjugation competent plasmid containing <i>oriT</i> , <i>tra</i> , <i>mob</i> , <i>KmR</i>	(391)

Table 2.1 Plasmids used in this study. *oriT* = origin of transfer; *int* = integrase; *attB* = chromosomal integration targeting sequence; *TcR* = tetracycline resistance gene; *tra* = transfer locus; *mob* = mobilisation locus; *KmR* = Kanamycin resistance gene.

Strain	Characteristics	Reference
PA14	<i>P. aeruginosa</i> . Wild-type, reference strain, isolated from burn wound. Normal colony phenotype.	(392)
AK3	<i>P. aeruginosa</i> . Clinical isolate from sputum of cystic fibrosis patient. Small colony phenotype.	(227)
AK10	<i>P. aeruginosa</i> . Clinical isolate from sputum of cystic fibrosis patient. Normal colony phenotype.	(227)
AK20	<i>P. aeruginosa</i> . Clinical isolate from sputum of cystic fibrosis patient. Normal colony phenotype.	(227)
AK8	<i>P. aeruginosa</i> . Clinical isolate from sputum of cystic fibrosis patient. Muroid colony phenotype.	(227)
AK11	<i>P. aeruginosa</i> . Clinical isolate from sputum of cystic fibrosis patient. Muroid colony phenotype.	(227)
AK13	<i>P. aeruginosa</i> . Clinical isolate from sputum of cystic fibrosis patient. Normal colony phenotype.	(227)
AK16	<i>P. aeruginosa</i> . Clinical isolate from sputum of cystic fibrosis patient. Normal colony phenotype.	This Study
AK18	<i>P. aeruginosa</i> . Clinical isolate from sputum of cystic fibrosis patient. Normal colony phenotype.	(227)
AK1	<i>P. aeruginosa</i> . Clinical isolate from sputum of cystic fibrosis patient. Normal colony phenotype. Colistin Resistant	This Study
AK24	<i>P. aeruginosa</i> . Clinical isolate from sputum of cystic fibrosis patient. Normal colony phenotype. Colistin Resistant.	This Study
PA14 <i>arnC</i>::Tn	PA14 transposon mutant with disrupted <i>arnC</i> gene	(393)
PA14 <i>eptA</i>::Tn	PA14 transposon mutant with disrupted <i>eptA</i> gene	(393)
PA14 <i>pagL</i>::Tn	PA14 transposon mutant with disrupted <i>pagL</i> gene	(393)
PA14 <i>pagP</i>::Tn	PA14 transposon mutant with disrupted <i>pagP</i> gene	(393)
PA14 <i>lpxT</i>::Tn	PA14 transposon mutant with disrupted <i>lpxT</i> gene	(393)
PA14 <i>pmrA</i>::Tn	PA14 transposon mutant with disrupted <i>pmrA</i> gene	(393)
PA14 <i>pmrB</i>::Tn	PA14 transposon mutant with disrupted <i>pmrB</i> gene	(393)
PA14 <i>phoP</i>::Tn	PA14 transposon mutant with disrupted <i>phoP</i> gene	(393)
PA14 <i>phoQ</i>::Tn	PA14 transposon mutant with disrupted <i>phoQ</i> gene	(393)
PA14 <i>colR</i>::Tn	PA14 transposon mutant with disrupted <i>colR</i> gene	(393)
PA14 <i>colS</i>::Tn	PA14 transposon mutant with disrupted <i>colS</i> gene	(393)
PA14 <i>parR</i>::Tn	PA14 transposon mutant with disrupted <i>parR</i> gene	(393)
PA14 <i>parS</i>::Tn	PA14 transposon mutant with disrupted <i>parS</i> gene	(393)
PA14 <i>cprR</i>::Tn	PA14 transposon mutant with disrupted <i>cprR</i> gene	(393)
PA14 <i>cprS</i>::Tn	PA14 transposon mutant with disrupted <i>cprS</i> gene	(393)
<i>E. coli</i> 1047 + pRK2013	Helper strain used for 3 partner conjugation carrying the pRK2013 plasmid.	(394)
<i>E. coli</i> CC118λPIR	Conjugation competent donor strain used for 3 partner conjugation.	(395)
PA14 <i>eptA</i>::Tn pEmpty	PA14 <i>eptA</i> ::Tn containing the empty pMiniCTX-1	This Study
PA14 <i>eptA</i>::Tn PeptA	PA14 <i>eptA</i> ::Tn containing the pMiniCTX-1-eptA	This Study
PA14 <i>colS</i>::Tn pEmpty	PA14 <i>colS</i> ::Tn containing the empty pMiniCTX-1	This Study
PA14 <i>colS</i>::Tn PcolRS	PA14 <i>colS</i> ::Tn containing the pMiniCTX-1-colRS	This Study

Table 2.2 Bacterial Strains used in this study.

2.1.3 Artificial sputum media

SCFM-1 and 2 were prepared as described previously (276,388). The SCFM buffered base was prepared in 250mL aliquots and stored at 4°C for up to 2 weeks.

The following reagents were dissolved in 190mL of nanopure water:

Chemical	Mass added (g)	Final concentration (mM)
NH₄Cl	0.031	2.2808
KCl	0.279	14.943
NaCl	0.758	51.848
MOPS	0.523	10

Table 2.3 SCFM-1 Base.

Stock solutions of the following reagents were prepared in advance in nanopure water (unless indicated), filter sterilised with a 0.2µm filter and stored at 4°C before adding to the mixture at the indicated volumes. Tryptophan and aspartate were prepared in 0.2M and 0.5M sodium hydroxide (NaOH) respectively.

Chemical	Stock concentration (M)	Volume added (mL)	Final concentration (mM)
NaH₂PO₄	0.2	1.625	1.3
Na₂HPO₄	0.2	1.563	1.25
KNO₃	1	0.087	0.348
K₂SO₄	0.25	0.271	0.271
Serine	0.1	3.615	1.446
Sodium glutamate	0.1	3.873	1.549
Proline	0.1	4.153	1.661
Glycine	0.1	3.008	1.203
Alanine	0.1	4.450	1.78
Valine	0.1	2.793	1.117
Methionine	0.1	1.583	0.633
Isoleucine	0.1	2.803	1.121
Leucine	0.1	4.023	1.609
Ornithine monohydrochloride	0.1	1.690	0.676
Lysine monohydrochloride	0.1	5.320	2.128
Arginine monohydrochloride	0.1	0.765	0.306
Tryptophan	0.1	0.033	0.013
Aspartate	0.1	2.068	0.827

Table 2.4 Stock solutions for SCFM-1 base

Stock solutions of the following reagents were prepared and filter sterilised in nanopure water on the same day as the buffered base was made except tyrosine which was dissolved in 1M NaOH.

Chemical	Stock concentration (M)	Volume added (mL)	Final concentration (mM)
Tyrosine	0.1	2.005	0.802
Threonine	0.1	2.680	1.072
Cysteine monohydrochloride	0.1	0.400	0.16
Phenylalanine	0.1	1.325	0.53
Histidine monochloride monohydrate	0.1	1.298	0.519

Table 2.5 Same-day amino acids for SCFM-1 base.

Following addition of the above reagents the pH of the solution was adjusted to 6.8 or 5.5 with concentrated hydrochloric acid, the total volume adjusted to 250mL, and the solution filter sterilised with a 0.2µm filter.

On the day of each experiment 50mL of SCFM-1 was prepared by the addition of the following reagents:

Chemical	Stock concentration (M)	Volume added per 50mL (µL)	Final concentration (mM)
Dextrose	1	150	3
L-lactic acid	1	465	9.3
CaCl₂	1	87.7	1.754
MgCl₂	1	30.3	0.606
FeSO₄	0.0036	50	0.0036

Table 2.6 Same-day reagents for SCFM-1

Stock solutions were prepared and stored at 4°C except FeSO₄ which was prepared fresh on the day of each experiment. Laboratory stocks of FeSO₄ heptahydrate (Sigma) were replaced every 6 months or when there was visual evidence of colour change (green to white). The L-lactic acid stock was pH adjusted to 7.0 with NaOH. All solutions were filter sterilised with a 0.2µm filter.

To Prepare SCFM-2, On the day before each experiment, bovine maxillary mucin (Sigma) and Salmon sperm DNA (Sigma) was incubated at 50°C overnight to ensure sterility and this was confirmed by plating for CFU.

On the day of each experiment 50mL of SCFM-2 was prepared by the addition of the following reagents to SCFM-1:

Chemical	Mass added to 50mL (g)	Final concentration (mM)
Bovine maxillary mucin (Sigma)	250mg	5mg/mL
Salmon Sperm DNA (Sigma)	30mg	0.6mg/mL

Chemical	Stock concentration	Volume added per 50mL (μL)	Final concentration
N-acetylglucosamine (Sigma)	0.25M	60	0.3
1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Fisher)	250mg/mL dissolved in chloroform	20	100 μg/mL

Table 2.7 SCFM-2 macromolecules.

After addition of DOPC (Fisher) chloroform was allowed to evaporate for at least 30 minutes at 37°C prior to each experiment.

For experiments where eDNA was pre-digested recombinant human DNase (rhDNase; Pulmozyme, Genentech) was added to SCFM-1 and SCFM-1 supplemented with eDNA to a final concentration of 3μg/mL and incubated for 1 hour prior to the addition of bacteria.

For experiments where the acidity of eDNA was neutralised, 1mL of 1M NaOH was used to restore the pH of 30mL SCFM-1 containing 2.4mg/mL eDNA to pH 6.8. Neutralisation was performed immediately prior to each experiment.

2.2 Bacterial growth and survival assays

2.2.1 Determination of bacterial growth

Stationary phase bacteria were washed twice by centrifugation (13,000rpm 3 minutes) and diluted 1:50,000 to approximately 1×10^5 CFU/mL in a volume of 200μL in sterile 96 well microtiter plates in the indicated media. Plates were incubated for at least 18 hours at 37°C with shaking at 180rpm on a Tecan Infinite 200 Pro multi-well plate reader. Optical density (OD) at 600nm was recorded at 15-minute intervals. Background OD was determined using sterile media and subtracted from bacterial growth OD readings.

2.2.2 Determination of Minimum Inhibitory Concentrations (MICs)

MICs were determined by microbroth dilution in CA-MHB (Sigma), SCFM-1 and SCFM-2 according to EUCAST methodology (396). Stationary phase bacteria were washed twice by centrifugation (13,000 rpm 3 minutes) in CA-MHB where MICs were performed in CA-MHB, or

SCFM-1 where MICs were performed in SCFM-1 or SCFM-2, and resuspension in the final medium, before dilution 10,000-fold to approximately 5×10^5 CFU/mL then added to prepared 96-well microtiter plates containing either colistin (Sigma), tobramycin (Sigma), murepavadin (DC Chemicals, China) or Rifampicin (Sigma). Inoculum size was confirmed by enumeration of CFU counts as described above. 96-well plates were prepared immediately prior to testing by 2-fold serial dilution of antibiotics in triplicate or duplicate. Following inoculation, plates were incubated statically at 37°C for 20 hours in air. The MIC was read as the lowest concentration of antibiotic at which no visible growth occurred. For some experiments, bacterial growth was quantified by reading absorbance at 595nm using a Bio-Rad iMark microplate absorbance reader (Bio-Rad Laboratories, USA).

2.2.3 Determination of bactericidal activity of antibiotics

The minimum bactericidal concentration (MBC) of an antibiotic was defined as the minimal antimicrobial concentration required to eliminate 99.9% of the bacterial inoculum (5×10^5). This was determined by the broth microdilution method in tandem with the MIC outlined above (Section 2.2.2) according to CLSI methodology (397). Following incubation of MIC assays, a 10 μ L sample from all wells that contained no visible growth was plated on to LBA and CFUs enumerated after 24 hours incubation.

For time-kill assays, stationary phase cultures were washed twice by centrifugation (13,000rpm 3 minutes) and diluted 1:10, to approximately 3×10^8 CFU/mL in appropriate media containing indicated concentrations of colistin, murepavadin or tobramycin and incubated at 37 °C and 180rpm on a shaking incubator. 20 μ L aliquots were taken at indicated time points and serially diluted in 10-fold increments in PBS. 10 μ L of each dilution was plated onto LB agar and CFUs enumerated after 18 to 24 hours static incubation at 37 °C in air.

2.2.4 Determination of colistin activity in media

The activity of colistin and tobramycin in specified media was determined using a zone of inhibition assay as described by Pader et al (398) 100 μ L of approximately 1×10^7 CFU/mL PA14 was spread over the surface of CA-MHB agar and allowed to dry in air. 8mm circular wells were formed in the plate to receive media. Colistin or tobramycin was diluted to 8 and 16 μ g/mL in PBS, SCFM-1 or SCFM-1 supplemented with 0.6 or 2.6mg/mL eDNA. 130 μ L of media at each concentration was inoculated in to triplicate wells. Zones of inhibited growth were measured after 18 hours of static incubation at 37 °C in air. Zone sizes were measured using EUCAST criteria (399).

2.3 Cell culture and DNA extraction

2.3.1 Cell culture

The respiratory epithelial cell line A549 (400) was donated by Dr Alex McCarthy (Imperial College London) and was cultivated as a source of human DNA to investigate the effect of DNA source on colistin susceptibility. Cells were grown in RPMI 1640 (Gibco) supplemented with 10% heat inactivated bovine serum (Gibco) at 37°C with 5% CO₂. Penicillin and streptomycin (Sigma) were added to the growth medium to prevent bacterial contamination, at a final concentration of 100 U/mL and 100 µg/mL respectively. Cells were grown to confluence in T175 vented flasks (Sarstedt) and split 1:2 approximately twice per week. To split, cells were washed twice in Dulbecco's phosphate buffered saline (Gibco) before incubating for 2 mins 37°C 5% CO₂ with 0.25% trypsin-EDTA (Sigma). Following trypsinisation, cells were detached by gentle agitation and tapping of the flask before cells were resuspended in RPMI-1640 to deactivate trypsin. Cells were again washed in RPMI-1640 and resuspended to the desired concentration. Cells were harvested for DNA extraction when they reached a density of approximately 5x10⁶ cells / mL. Cells were washed and resuspended in 3mL PBS (Sigma) and stored at -20°C prior to DNA purification.

2.3.2 DNA purification

A549 cells were lysed with SDS and proteinase K in a method outlined by Qamar et al (401). Cells were resuspended in a lysis buffer (table 2.8) for 2 hours at 50°C in a water bath with occasional agitation. Following incubation, the lysate was allowed to cool to room temperature before the addition of 3mL of 7.5M Ammonium acetate (Sigma) followed by 31mL of ice-cold ethanol. DNA was allowed to precipitate overnight at -20°C. Following precipitation, DNA was pelleted at 3750 rpm for 30 mins before washing twice in 70% ethanol. Purified DNA was allowed to dry before it was resuspended in nanopure water. DNA concentration was determined NanoVue Plus spectrophotometer (GE Healthcare) and stored at -20°C.

Component	Concentration	Volume
A549 cells		3mL
Tris-HCl pH8.0 (Sigma)	20mM	9mL
+ NaCl	25mM	
SDS (Sigma)	10%	3mL
Proteinase K in PBS (Sigma)	10mg/mL	75µL

Table 2.8 Lysis Buffer

2.4 Measurement of outer membrane integrity

To assess the integrity of the outer membrane of *P. aeruginosa*, the NPN uptake assay was used as described by Helander and Mattila-Sandholm (402). Stationary phase cultures of *P.*

aeruginosa in CA-MHB were washed twice in HEPES (5mM pH 7.2 or pH 4.4 Sigma) and diluted to an OD₆₀₀ of 0.5. Bacteria were then immediately added to a pre-prepared clear-bottomed black 96-well microtiter plate containing colistin or DNA dissolved in HEPES buffer with the fluorescent dye N-phenyl-1-naphthylamine (NPN; Acros organics USA) to a final concentration of 10µM. The dye is normally excluded by the presence of LPS in the outer membrane of Gram-negative bacteria but fluoresces on contact with phospholipids. Fluorescence was immediately determined with a Tecan Infinite 200 pro multi-well plate reader (Tecan Group Ltd; Switzerland) with an excitation wavelength of 355nm and an emission wavelength of 405nm. Readings were taken every 30 seconds for 10 minutes and the NPN uptake was calculated with the following formula:

$$\frac{\text{Fluorescence of sample with NPN} - \text{Fluorescence of sample without NPN}}{\text{Fluorescence of HEPES buffer with NPN} - \text{Fluorescence of HEPES buffer without NPN}}$$

2.5 Lipidomics

Mass-spectroscopy-based lipidomic analysis was used to examine lipid A for chemical modifications in response to environmental conditions as described in (403). Stationary phase cultures were washed twice by centrifugation (13,000rpm 3 minutes) and diluted 1:10, to approximately 3x10⁸ CFU/mL in 3mL of SCFM-1 or SCFM-1 containing 2.4 mg/mL DNA. Every 2 hours 200µL aliquots were taken and washed 3 times in nanopure water before being subjected to mild acid hydrolysis with 2% acetic acid at 100°C for 30 minutes to remove carbohydrate moieties, before being washed again, then resuspended in 50µL nanopure water. Lipidomic analysis was performed by Matrix-assisted laser desorption time-of-flight microscopy (MALDI-ToF) in collaboration with Dr Gerald Larrouy-Maumus (Imperial College London). 0.5µL of the cell suspension was spread on to the target plate and overlaid with 1.2µL of 9H-Pyrido[3,4-B]indole (Norharmane; Sigma) dissolved in 90:10 (v/v) chloroform-methanol (final concentration 10mg/mL) and allowed to dry at room temperature. Once dried, samples were analysed on a MALDI biotyper Sirius system (Bruker Daltonics, USA) in negative ion mode. Lipid A peaks were identified manually from the resulting spectra. The relative abundance of Lipid A species was determined by dividing the area under the modified Lipid A peaks by the area under the unmodified native peak (m/z 1447).

2.6 Genetic techniques

2.6.1 Bacterial DNA purification

Genomic DNA was purified from *P. aeruginosa* using the GenElute Bacterial genomic DNA kit (Sigma) according to the manufacturer's instructions. Stationary phase bacteria were lysed with a chaotropic salt buffer before binding to a silica-based column and repeated washes to remove contaminants. Genomic DNA was eluted into nanopure water. Purified DNA was quantified using a NanoVue Plus spectrophotometer (GE Healthcare) and stored at -20°C.

2.6.2 Plasmid DNA purification

Plasmid DNA was purified using the Qiaprep spin miniprep kit according to the manufacturer's instructions. Stationary phase bacteria were lysed with a chaotropic salt buffer before removal of genomic DNA and contaminants by precipitation. Plasmid DNA is bound to a silica-based column and repeated washes to remove contaminants. Plasmid DNA was eluted into nanopure water. Purified DNA was quantified using a NanoVue Plus spectrophotometer (GE Healthcare) and stored at -20°C.

2.6.3 Polymerase chain reaction

PCR was used to confirm that transposons had been inserted into the correct genes and for cloning purposes. Reactions were performed using 100ng of purified genomic DNA from PA14 or the appropriate transposon mutant. The high-fidelity Phusion polymerase (Thermo Fisher) was used. The composition of specific PCR reactions is described in table 2.8. Reactions were carried out in 200µL PCR tubes in a thermocycler (Applied Biosystems) using the conditions in table 2.9. Primers were designed using Snapgene viewer (Snapgene; USA) and were synthesized by Sigma. Annealing temperatures were optimised for each primer pair beginning at 5°C below the lowest estimated melting point of that pair. All primers used in this study are listed in table 2.10.

Component	Volume (μL)
Phusion Buffer (5x)	5
dNTPs (10mM)	0.5
Forward primer (10μM)	1.25
Reverse primer (10μM)	1.25
Template DNA	Volume corresponding to 100ng
Phusion polymerase	0.5
Nanopure H ₂ O	Volume required to make 25μL
Total	25

Table 2.9 PCR components

Cycle Stage	Temperature °C	Time	Cycles
First Denaturation	95	2 mins	1
Denaturation	95	30s	30
Annealing	55-65	30s	30
Extension	72	30s /kb + 30s	30
Final Extension	72	5 min	1

Table 2.10 PCR conditions

2.6.4 Agarose gel electrophoresis

To analyse PCR products, DNA was separated by agarose gel electrophoresis. A 1% gel (w/v) was prepared by dissolving the required mass of agarose powder (Sigma) in TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA) and heating via microwave. 1:10,000 SYBR safe (Thermo Fisher) was added to the cooled mixture before it was poured into an appropriately sized mould with a comb. Once gels were set the combs were removed and the gel was submerged in a tank containing TAE buffer. To visualise DNA samples were mixed with 6x DNA loading dye (NEB) before loading into wells. Size was confirmed using a 2-log DNA Ladder (0.1-10kB; NEB). Gels were run between 100 and 120V until bands had separated.

Oligonucleotide	Sequence 5' > 3'
For confirmation of transposon insertion	
PA14_pmrA_Fw	GGAATCACTGAAACGAGGCTG
PA14_pmrA_Rv	GCGAGAGGTGGTAGGTCAG
PA14_pmrB_Fw	GAAAACCGCCTACCGGAGTC
PA14_pmrB_Rv	CTATCCCTTTCCCGGCCAATG
PA14_phoP_Fw	CTGCTCGATGGTGTCTCCAG
PA14_phoP_Rv	GGGCGCCAACTACAAGTTC
PA14_phoQ_Fw	GCGGAAACACCTCAGACGTAG
PA14_phoQ_Rv	CTTCAAGCCGATCGATACGGTG
PA14_colR_Fw	CTTATACTCCATTCCGGCTCC
PA14_colR_Rv	CCGGTGAAGAATCCGTAAATG
PA14_colS_Fw	GTATGAATCCAGCCGTCAAG
PA14_colS_Rv	GAGGAGCCGAATGGAGTATAAG
PA14_parR_Fw	GATTCCGCTCCGACCGATG
PA14_parR_Rv	GAACAGGCGCAGCATTGAG
PA14_parS_Fw	CTGAATGCTGCGCCTGTTC
PA14_parS_Rv	GAAAGATTGTTAAGCCTCCGCTG
PA14_cprR_Fw	CCGTAGTTGCCGAGCATG
PA14_cprR_Rv	CGACCCAGGCCATGCATATC
PA14_cprS_Fw	GCTTCAGTCCGGTGTGGC
PA14_cprS_Rv	CTCAACGTGCACATGCACC
PA14_arnC_Fw	GAAGACGACGGCTTTCGAGG
PA14_arnC_Rv	GGACATCCTGGAGAAACGTCG
PA14_eptA_Fw	GAAATAGTCCTTTCGCCGGG
PA14_eptA_Rv	GAAGTCAGTGAAGATCCGTGC
PA14_pagL_Fw	GCCGATGGTCGACCTGAAG
PA14_pagL_Rv	GCAACTTTCGAAAAGGACGTTTC
PA14_pagP_Fw	CCTAGACCAGCCAGCGTTC
PA14_pagP_Rv	GTCTTGCCCTCCTTACCTGGC
PA14_lpxT_Fw	GTGACAGTCGTACGCCGAC
PA14_lpxT_Rv	GCGGCCAGGACTGAGAAG
For construction of colRS:colS::Tn	
PA14_ColRSpromgib_Fw	CCGGGCTGCAGGAATTCGATGTATGAATCCAGCCGTCAAG
PA14_ColRSpromgib_Rv	ACGGTATCGATAAGCTTGATCGGTCGTGCAGGATGTTC
For construction of eptA:eptA::Tn	
PA14_eptApromgib_Fw	CCGGGCTGCAGGAATTCGATGAAATAGTCCTTTCGCCGG
PA14_eptApromgib_Rv	ACGGTATCGATAAGCTTGATCTCGAGGAAATTCACAGAAG

Table 2.11 PCR primers

2.6.5 Gel purification

Prior to Gibson assembly, cloned PCR products and linearised plasmid were separated on an agarose gel as described above. The band of the correct size was excised with a clean scalpel and the DNA was extracted using the Gel extraction kit (Qiagen) according to the manufacturer's instructions. Purified DNA was quantified using a NanoVue Plus spectrophotometer (GE Healthcare) and stored at -20°C.

2.6.6 Restriction digestion

Before assembly of newly cloned fragments into the miniCTX-1 plasmid, the plasmid was linearised with the restriction enzyme EcoRV-HF (NEB). Reagents were combined as described in table 2.11. The digest was allowed to proceed at 37°C overnight. Following overnight incubation linearised plasmid was phosphatase treated to prevent re-circularisation by adding 1µL of the Quick CIP (NEB) enzyme for 1 hour at 37°C. Linearised plasmid was gel purified as described above.

Component	Volume / Mass
Purified Mini-CTX1	3µg
Cutsmart Buffer (NEB)	2µL
EcoRV-HF	1µL
H2O	3µL
Total	20µL

Table 2.12 Restriction Digest components.

2.6.7 Gibson assembly

Cloned inserts were incorporated into the miniCTX-1 plasmid by Gibson assembly. In this method PCR primers (listed in table 2.10) were designed using the NEBuilder tool to contain sequences homologous to the plasmid which, through the action of a T5 exonuclease, allow the insert to anneal into the plasmid before the action of a high fidelity DNA polymerase fills any gaps in the annealed fragment and a ligase covalently links the fragments in a single isothermal reaction. Prepared fragments were assembled using the NEBuilder HiFi DNA Assembly cloning kit (NEB) according to the manufacturer's instructions. Vector and insert were combined at a 1:1 molar ratio. Given the low concentration of the vector, reaction mixtures were made up to 24µL with HiFi DNA assembly master mix in 200µL PCR tubes and incubated at 50°C in a thermocycler (Applied Biosystems) for 1 hour. 2.4µL of this reaction mixture was transformed directly in to chemically competent CC118λPIR *E. coli* as described in section 2.5.8.

2.5.8 Preparation of chemically competent *E. coli*

E. coli CC118λPIR, donated by Dr Luke Allsopp, was grown overnight in LB then 200μL was inoculated into 200mL of LB and grown on the shaking incubator at 37°C 180rpm to an OD_{600nm} of 0.5. Cells were transferred to falcon tubes and centrifuged at 3750rpm at 4°C for 10 minutes. Cells were resuspended in 40mL of ice cold 50mM CaCl₂ with 15% glycerol. Cells were left at 4°C overnight before a second spin (3750rpm, 10min, 4°C) and resuspension in 2mL 50mM CaCl₂ with 15% glycerol. Cells were then aliquoted into 50uL and flash frozen with liquid nitrogen before being stored at -80°C.

2.5.9 Transformation of chemically competent *E. coli*

Assembled constructs and empty plasmids were directly transformed into chemically competent CC118λPIR. 50μL Aliquots were thawed on ice before DNA was added (2.4μL of Gibson reactions) and allowed to rest for 30 minutes. Cells were then heat shocked at 42°C for 30s in a water bath then allowed to rest on ice for 5 minutes. 500μL of LB was added and cells were allowed to recover for 2 hours at 37°C. Cells were then plated on to LBA containing 15μg ml tetracycline and incubated statically for 16 hours at 37°C. Positive clones were confirmed by DNA sequencing.

2.5.9 Three-Partner conjugation of *P. aeruginosa*

Once constructs were confirmed in *E. coli*, they were transferred into *P. aeruginosa* by conjugation. Recipient strains were grown to stationary phase overnight in LB supplemented with 15μg/mL gentamicin to maintain the transposon in the target disrupted gene. 100 μL of the recipient strain was incubated for 2 hours at 43°C in a water bath to inactivate the *P. aeruginosa* restriction modification system. Helper strain *E. coli* 1047 containing the pRK2013 plasmid was grown overnight to stationary phase in LB supplemented with 50μg/mL Kanamycin. Donor strain CC118λPIR containing empty plasmid or constructs was grown overnight to stationary phase in LB supplemented with 15μg/mL tetracycline. 20 μL aliquots containing the donor and helper strain mixture were inoculated onto LBA and incubated for 2 hours at 37°C. After 2 hours 40 μL of the recipient strain was also added to the plate and it was incubated for 4 hours at 37°C. After 4 hours the patch containing conjugated bacteria as well as control patches for donor, recipient and helper strains were suspended in LB and plated on PIA containing 15 μg/mL gentamicin and 150 μg/mL tetracycline. Success was determined by the absence of growth on control plates and the presence of colonies on the plate corresponding to the conjugated strain. Approximately 4 colonies of each conjugated strain were grown separately overnight in LB containing 15μg/mL gentamicin and 150 μg/mL tetracycline before being frozen in aliquots of 1mL at -80°C.

2.5.10 DNA sequencing

Constructs were purified from overnight cultures of CC118λPIR grown in LB containing 15 µg/mL tetracycline as per section 2.5.2. Purified plasmids were diluted to 50 ng/µL in nuclease free water and sent to Full Circle Labs (London; UK) for whole plasmid sequencing.

2.6 Statistical analysis

Statistical analysis was performed using Prism 11 (Graphpad, USA). Most data are shown as means with error bars indicating the standard deviation except MICs are demonstrated as median with error bars indicating the range. For multiple comparisons at a single time point 1-way analysis of variance was used (ANOVA). for multiple comparisons across multiple time points, concentrations, or drugs, 2-way ANOVA was used. For multiple individual comparisons appropriate post-hoc tests were used as described in the figure legend. Statistical significance was taken as $P < 0.05$ and denoted with an asterisk as described in the figure legend.

Chapter 3: The Host environment in CF modulates *P. aeruginosa* susceptibility to colistin.

3.1 Introduction

3.1.1 The CF lung environment and its effect on antibiotic activity

In the lung, the respiratory epithelium is coated in a layer of airway surface liquid (ASL) whose primary function is as an innate immune barrier entrapping foreign particles and bacteria and directing them caudally for expulsion (89). In CF, CFTR dysfunction and chronic inflammation result in changes to ASL composition, relative to unaffected individuals, resulting in a reduced ability to clear bacterial pathogens and favouring *P. aeruginosa* colonisation (89). Additionally, many of the components of ASL have been demonstrated to alter the activity of colistin impairing its ability to clear infecting bacteria. For example, amino acids can act as carbon sources for *P. aeruginosa* (404,405) and when acting as sole carbon sources, susceptibility to colistin could be both increased or decreased depending on individual amino acids, relative to CA-MHB (266). CF ASL is a rich source of amino acids (276), contains high concentrations of mucin and DNA and is frequently acidified, relative to unaffected individuals (132). Glucose concentrations in ASL are generally lower than in blood but appear elevated in CF (406,407).

Chronic inflammation coupled with increased fluid resorption in CF lung results in high concentrations of mucin, producing a particularly thick mucus and impairing mucociliary clearance (408). Airway acidification can alter the rheological properties of mucin further increasing sputum viscosity (409). In addition to reducing clearance of microbes, mucin has been shown to bind and reduce the activity of colistin in synthetic sputum models (287,410).

Another consequence of chronic inflammation is the recruitment of neutrophils (411), leading to tissue destruction and accumulation of large quantities of extracellular DNA (eDNA) which contributes to sputum viscosity, biofilm formation, and environmental acidification (164,412,413). Evidence for the degree of ASL acidification outside of CF pulmonary exacerbation is conflicting, however it would appear ASL is acidified in the presence of infection relative to unaffected controls (414,415). In addition to eDNA, impaired bicarbonate secretion in CF is thought to contribute to acidification as well as a failure to mature mucins (88). Furthermore, reduced pH has been associated with changes in antimicrobial susceptibility via effects on bacteria and/or the drug itself (416). As lung disease progresses, tissue destruction leads to

devitalised areas of lung with variable oxygen tension which in turn can further alter bacterial susceptibility to antimicrobials (269,270).

3.1.2 *P. aeruginosa* adaptation to the lung environment

Longitudinal sequencing data has demonstrated that pwCF are often colonised with the same strain of *P. aeruginosa* for several years, resulting in adaption to the lung niche (417). Whilst there appears to be high genetic diversity in *P. aeruginosa* isolates between and even within individuals, there is growing evidence that many of the adaptive changes represent convergent evolution (121,418–420). For example, particularly in late colonising strains, diverse mutations result in similar functional consequences resulting in frequent isolation of strains with certain phenotypes, including over-production of exo-polysaccharides resulting in either rugose small-colony variants (RSCV) or mucoid strains (152,421), attenuation of virulence factors, often through loss of function of quorum sensing (422), favouring biofilm formation over planktonic growth (154), and reduced phenazine production (423).

Although complete resistance to colistin remains rare in *P. aeruginosa* isolated from pwCF (201) decreased susceptibility to anti-microbials, including colistin, has been observed in long-term colonising strains. For example, Ernst and colleagues identified an increased abundance of LPS modified with aminoarabinose (L-ara4N) in isolates from pwCF, relative to environmental or non-CF strains (171,424). Though modification of LPS with L-ara4N is commonly seen in resistant isolates, the presence of this modification alone does not convey high level resistance to colistin (173,425).

3.1.3 Synthetic sputum models

To investigate the effects of the CF lung environment many groups have used artificial sputum media (ASM), of which several different formulations have been reported (426). Although these models define particular methods of preparation, frequently individual publications will make small modifications to preparation that may impact the reproducibility and translatability of findings. ASM aims to recapitulate the key features of the CF lung environment by including amino acids, salts and macromolecules at more physiologically relevant concentrations than standard laboratory media (426). Large shifts in antimicrobial susceptibility can be seen when more physiologically relevant media are used to culture bacteria. For example, Belanger and colleagues found that MICs to commonly used antibiotics varied greatly in a diverse panel of bacterial pathogens, including *P. aeruginosa*, when lung- or serum-mimicking media were used, relative to CA-MHB (39).

Ghani and Soothill developed one of the earliest ASM, to investigate the susceptibility of antimicrobial activity of *P. aeruginosa* biofilm (382). Whilst this medium contained salts, mucin, DNA, lipids, from egg yolk emulsion and an iron chelator, Ghani and Soothill's medium did not contain supplemental amino acids (382). Sriramulu and colleagues modified that medium with the addition of 20 amino acids at fixed concentrations (278) and examined biofilm formation. The presence of amino acids promoted microcolony and biofilm formation (278). Following the inclusion of amino acids, several derivatives have been created with altered buffering capacity (386), altered mucin and DNA concentrations and the addition of albumin (383). Hare and colleagues made further changes by chelating iron with ferritin, and replaced the standardised amino acids with casamino acids, a collection of all naturally occurring amino acids resulting from the acid hydrolysis of casein, to better reflect conditions *in vivo* (384). Further changes to amino acid and mucin concentrations were subsequently made by Quinn and colleagues to increase sputum consistency when examining complex bacterial populations in a column-based system (385). What these media have in common are mucin, DNA, lipids, iron and most contain amino acids. They are all based on published concentrations found in human CF sputum but crucially, in terms of validation, made little comparison between bacterial phenotype in artificial sputum and human sputum other than the ability to form biofilm and how this might be regulated.

Palmer and colleagues took the most rigorous approach to replicating the CF lung environment by creating a more strictly chemically defined medium, with respect to a wider range of constituents, containing specific concentrations of individual amino acids, Fe^{2+} , Ca^{2+} , Mg^{2+} , lactate and glucose in a buffered MOPS solution to create SCFM-1 (276). Although Palmer found that the relative abundance of amino acids varied between CF sputum samples, the ratios of concentrations were relatively consistent. SCFM-1 was further modified through the addition of relevant sputum macromolecules: DNA, mucin, 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and N-acetylglucosamine (GlcNAc) to create SCFM-2 (388). Crucially the phenotype of *P. aeruginosa* in SCFM-2 was validated by high throughput transposon sequencing and compared relative abundance of transposon mutants in SCFM-2 and MOPS buffered CF-sputum from human donors. Although there was close correlation between SCFM-2 and CF sputum there were several mutants that still had a survival disadvantage in SCFM-2, with defects in pathways for synthesis of thiamine, folate, purines, branched chain amino acids and tryptophan indicating relatively higher abundance of these metabolites in CF sputum. Although the authors demonstrated that addition of individual substrates P-amino benzoic acid, purine nucleotides, and nicotinamide-adenine dinucleotide, in excess, could enhance growth of specific transposon mutants, they did not go as far as to formulate SCFM-3 or define physiological concentrations of

these substrates (388). More recently SCFM-2 has been incorporated into an ALI model which includes a cellular component. Interestingly when a metatranscriptomic analysis was performed the model was not more accurate than SCFM-2 alone (363). Lewin and colleagues confirmed previous findings that this model is fairly comparable to CF sputum with respect to the transcriptome of *P. aeruginosa* with 86% of genes expressed within 2 standard deviations of *P. aeruginosa* grown in expectorated sputum of pwCF who were not receiving CFTR modulator therapy (363).

In the present work I have chosen to use the SCFM model as the best-validated and most representative model CF sputum available. It provides a reproducible way of examining antimicrobial susceptibility in a more physiologically relevant model system than laboratory media. I have also chosen to use the *P. aeruginosa* strain PA14 as a well validated and characterised strain isolated from human infection (392). Although the PA14 strain was not isolated from CF, PA14 clones are frequently isolated from in pwCF (417). The use of PA14 also provided access to an ordered transposon library and a range of other genetic resources (393). Alongside *P. aeruginosa* PA14, a range of *P. aeruginosa* clinical isolates from CF patients were selected to confirm the validity of my findings: AK3, AK10 and AK20. The isolates were selected as they demonstrate evidence of adaptation to the CF lung: AK3 is a small colony variant, whilst AK10 and AK20 had lost phenazine production. As described earlier, many chronic CF isolates demonstrate adaptation to the host niche and it is unclear how this process may alter their response to the sputum model. Having used a range of isolates at an early stage, more general conclusions about the behaviour of *P. aeruginosa* in CF can be drawn. This combination of model and strains enabled me to address my first aim:

Aim: To investigate the effect of the host microenvironment on susceptibility of *P. aeruginosa* to colistin using a physiologically relevant *in vitro* model and representative clinical isolates.

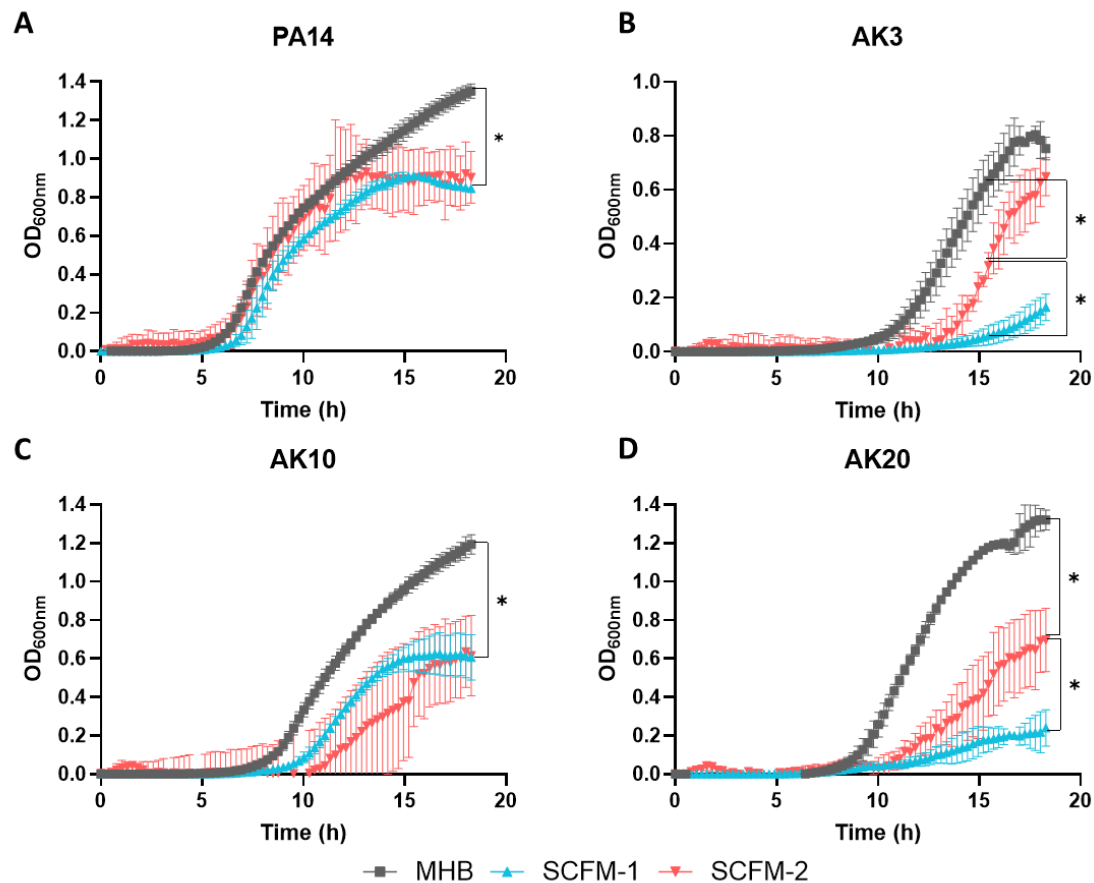
3.2 Results

3.2.1 Synthetic sputum modulates the growth of *P. aeruginosa*

As a starting point to test whether synthetic sputum medium altered *P. aeruginosa* susceptibility to colistin, *P. aeruginosa* strain PA14 and clinical isolates AK3, AK10, and AK20 were grown in cation adjusted Muller-Hinton broth (CA-MHB), SCFM-1 and SCFM-2. Growth analysis was important because the inability of strains to grow under the conditions used would make susceptibility testing difficult. Furthermore, understanding growth is an important precursor to understanding survival following antibiotic exposure as, over time, the size of the surviving

bacterial population will be determined by both the death rate and the growth rate. CA-MHB was included as the laboratory medium as it is the standard broth used for antimicrobial susceptibility testing of *P. aeruginosa* and therefore provided a useful benchmark.

Growth was estimated from optical density readings at 600nm (OD_{600nm}) over time at 37°C with shaking. All strains demonstrated strong growth in CA-MHB, with the exception of *P. aeruginosa* AK3, which is an SCV (Fig 3.1). Growth was reduced in synthetic sputum for all strains (Fig 3.1). However, this was more pronounced for clinical isolates compared to *P. aeruginosa* PA14 (Fig 3.1B-D). For *P. aeruginosa* PA14 and AK10 there was little difference between SCFM-1 and SCFM-2 (Fig3.1A and C). Both AK3 and AK20 isolates demonstrated marked impairment of growth in SCFM-1 relative to SCFM-2, implying that these strains used the macromolecules in SCFM-2 as



nutrient sources (3.1B, 3.1D).

Figure 3.1: Synthetic sputum modulates bacterial growth. *P. aeruginosa* strains PA14 (A) AK3 (B) AK10 (C) and AK20 (D) were inoculated into CA-MHB, synthetic sputum without (SCFM-1) or with (SCFM-2) macro molecules and incubated at 37°C over 18 hours. Growth was determined by measuring optical density at 600nm. Data points represent the mean of 3 independent experiments with error bars indicating the standard deviation. Data were analysed by two-way ANOVA with Tukey's post hoc test. * $P < 0.05$.

To assess the impact of individual macromolecules on growth, each strain was inoculated into SCFM-1 supplemented with each macromolecule separately (Fig 3.2). Extracellular DNA (eDNA) enhanced growth in *P. aeruginosa* PA14, AK10 and AK20 relative to SCFM-1 (Fig 3.2A, C

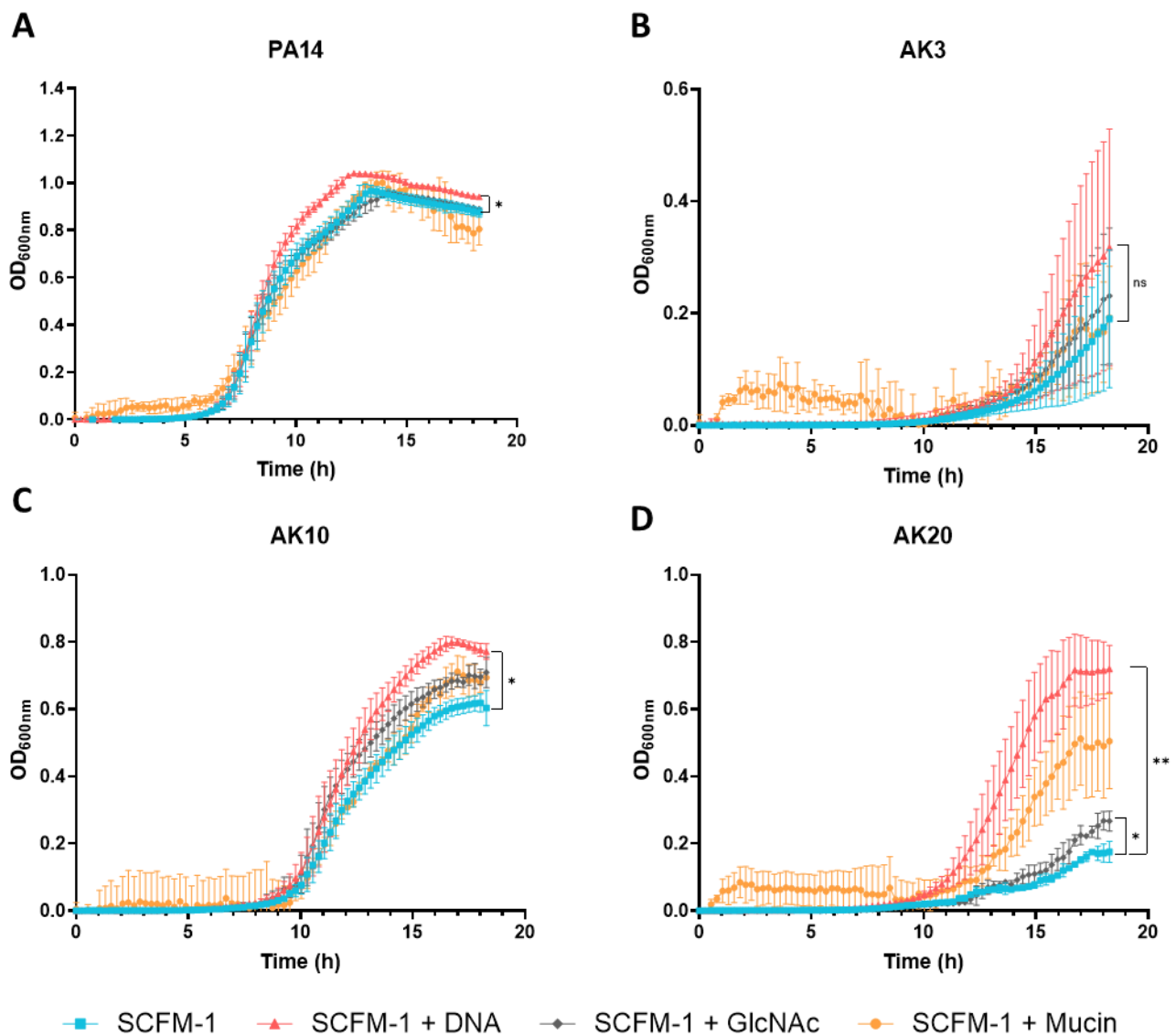


Figure 3.2: Individual macromolecules exhibit strain dependent differences in growth. *P. aeruginosa* strains PA14 (A) AK3 (B) AK10 (C) and AK20 (D) were inoculated into CA-MHB, synthetic sputum without (SCFM-1) or with individual macro molecules (DNA, *GlcNAc*, Mucin) and incubated at 37°C over 18 hours. Growth was determined by measuring optical density at 600nm. Data points represent the mean of 3 independent experiments with error bars indicating the standard deviation. Data were analysed by two-way ANOVA with Dunnett's post hoc test. *P<0.05 **P<0.01 (relative to SCFM-1)

and D). Whilst there was a trend for the same in *P. aeruginosa* AK3 interpretation of data for AK3 was limited by marked inter-assay variability and lower overall growth (Fig 3.2B). Mucin did not significantly alter growth for any of the strains however there was a trend for enhanced growth in AK20 (Fig 2D P=0.1). GlcNAc also demonstrated a small but consistent enhancement of growth in AK20 (Fig 2D).

3.2.2 Synthetic sputum modulates susceptibility of *P. aeruginosa* to colistin

Next, the impact of ASM on colistin susceptibility was determined using two different approaches. Firstly, broth microdilution was used to measure the minimum inhibitory concentration (MIC). This is the recommended standard susceptibility test for antibiotics including colistin *in vitro* and provides a useful reference to compare clinical data. Secondly, time-kill assays were used to measure bacterial survival over time following exposure to clinically relevant concentrations of colistin (208). The use of both assays allows effects on both bacterial growth (MIC) and bacterial killing (time-kill) to be measured accurately.

Across all four strains SCFM-1 resulted in 2 to 4-fold increases in MIC relative to CA-MHB (Fig 3.3). The presence of macromolecules in SCFM-2 further increased the colistin MIC 2-fold for *P. aeruginosa* PA14 and AK20 but had no effect on AK3. The colistin MIC for AK10 was highest in SCFM-1, with SCFM-2 and MHB resulting in the same MIC.

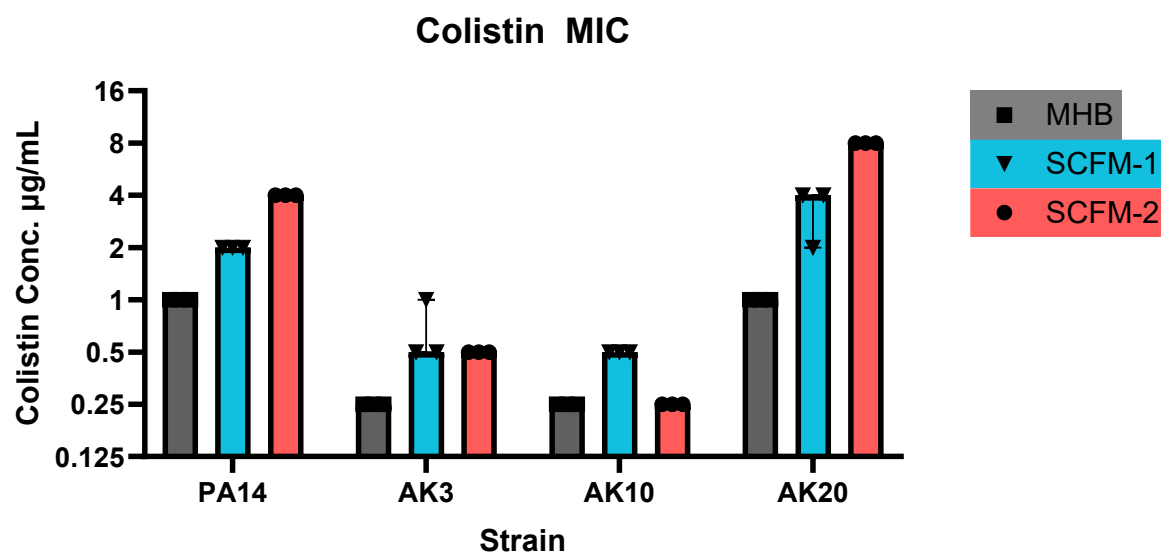


Figure 3.3: Synthetic sputum modulates susceptibility to colistin. MICs to colistin were determined by broth microdilution for *P. aeruginosa* strains PA14, AK3, AK10, and AK20. Strains were inoculated into CA-MHB, synthetic sputum without (SCFM-1) or with (SCFM-2) macro molecules and incubated at 37°C for 20 hours. Growth was determined by measuring optical density at 600nm. Data points represent the result of 3 independent experiments with coloured bars indicating the median and error bars indicating the range.

For Time-kill assays an initial concentration of 4 µg/mL was selected as twice the MIC in SCFM-1 for PA14 and therefore was expected to be high enough to achieve killing whilst also being in the physiological range (208) and close enough to the MIC that even small differences in survival would become evident. For *P. aeruginosa* PA14, there was relatively little difference in killing (the reduction in CFU following colistin exposure) in CA-MHB versus SCFM-1, with a single log₁₀ difference apparent for PA14 at 2 and 6 hours (Fig 3.4A). However, there were no significant differences observed between CA-MHB and SCFM-1 for any of the clinical isolates (Fig 3.4 B-D). Much greater differences were observed in SCFM-2 relative to CA-MHB (Fig 3.5), with 97% of *P. aeruginosa* PA14 (Fig 3.5A) and 99% of AK20 (Fig 3.5D) killed after 2 hours of exposure to colistin, but both strains had begun to recover, as demonstrated by increasing CFUs, by 4 hours and fully recover by 6 hours. By contrast, there were no statistically significant differences in colistin-

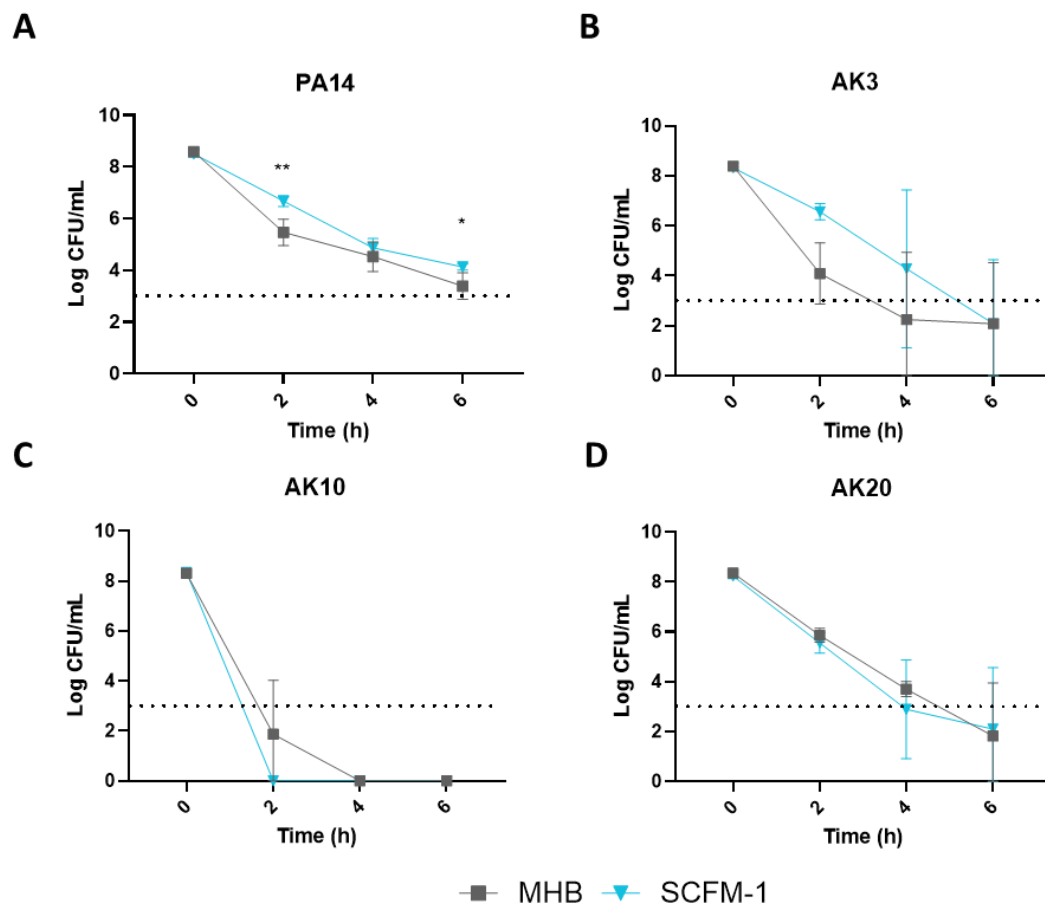


Figure 3.4: SCFM-1 has a minimal effect on colistin susceptibility relative to MHB. Stationary phase *P. aeruginosa* strains PA14 (A) AK3 (B) AK10 (C) and AK20 (D) were inoculated into CA-MHB, or SCFM-1 containing 4 µg/mL colistin and bacterial survival enumerated over 6 hours. Data points represent the mean of 4 independent experiments with error bars indicating the standard deviation. Data were analysed by 2-way ANOVA with Sidak's post-hoc test. *P<0.05 **P<0.01. The dashed line indicates the threshold of detection.

mediated killing observed for AK3 (3.5B) or AK10 (3.5C), between CA-MHB and SCFM-2, with both strains being eliminated after 6 hours of antibiotic exposure.

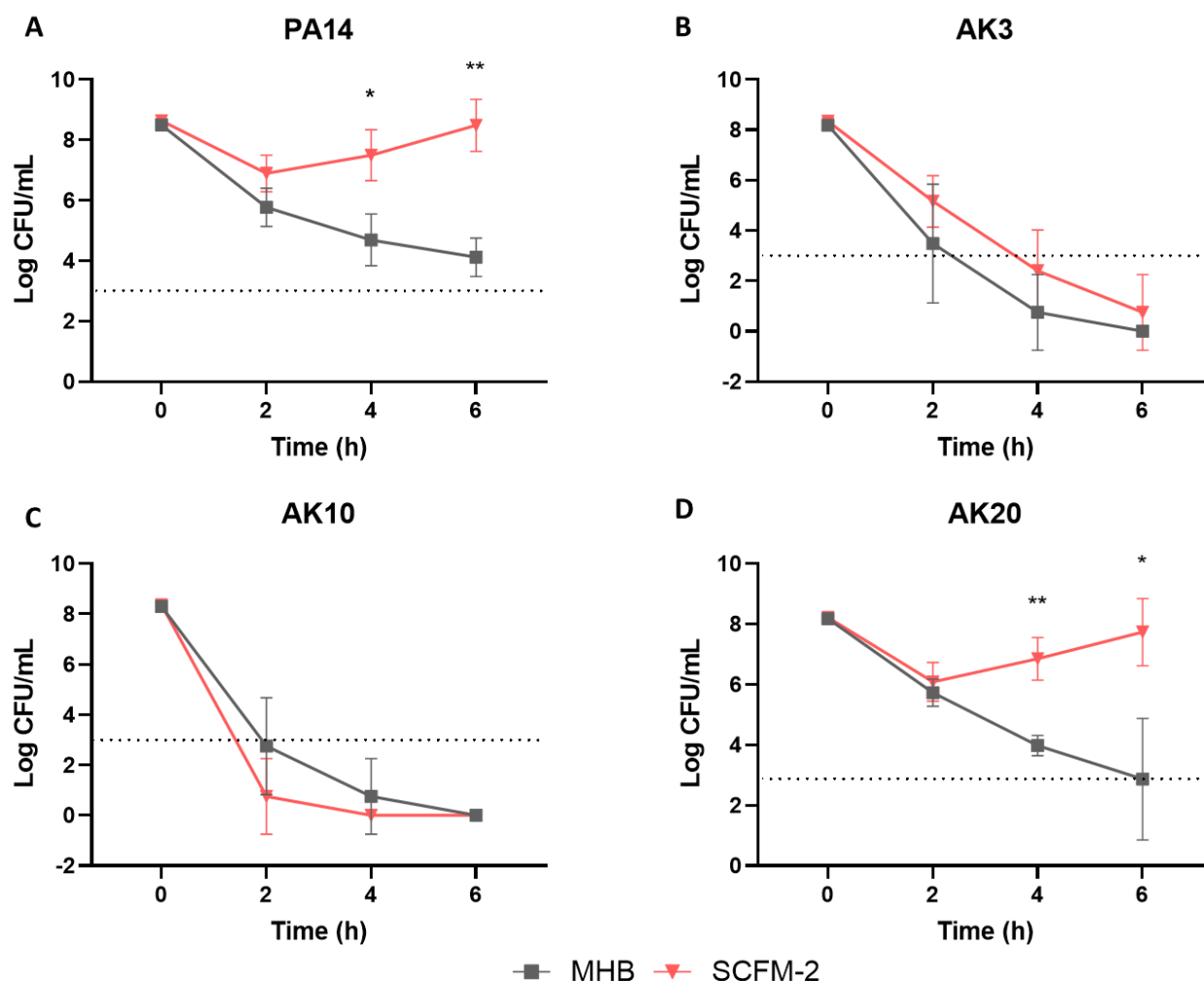


Figure 3.5: SCFM-2 Enhances recovery from colistin exposure. *P. aeruginosa* strains PA14 (A) AK3 (B) AK10 (C) and AK20 (D) were exposed to 4µg/mL of colistin in CA-MHB or SCFM-2 and bacterial survival measured over time. Data points represent the mean of 4 independent experiments. Error bars represent the standard deviation. Data were analysed by 2-way ANOVA with Sidak's post-hoc test. *P<0.05 **P<0.01. The dashed line indicates the threshold of detection.

3.2.3 Synthetic sputum does not significantly alter colistin activity

Cationic antimicrobial peptides have been reported to bind to several components of synthetic sputum namely, mucin (287) and eDNA (427). To investigate whether there was a significant effect of synthetic sputum on colistin activity a zone of inhibition assay was used. This approach has been used previously to show that the phospholipid phosphatidylglycerol specifically bound and inhibited activity of the lipopeptide antibiotic daptomycin (398). Briefly, liquid media containing antibiotics is inoculated into a well on an agar plate spread with *P.*

aeruginosa. The size of the resulting zone of inhibition around the well (Fig 3.6A) is determined by the ability of the antibiotic to diffuse through the agar, as well as the susceptibility of the organism. In this experiment colistin concentrations were increased to 8 (Fig 3.6B) and 16 (Fig 3.6C) $\mu\text{g/mL}$ to produce large enough zone sizes for accurate measurement, as 4 $\mu\text{g/mL}$ colistin resulted in a zone of only 1-2mm. There were no significant differences in zone sizes between either sputum formulation or MHB making significant sequestration of colistin unlikely in this model.

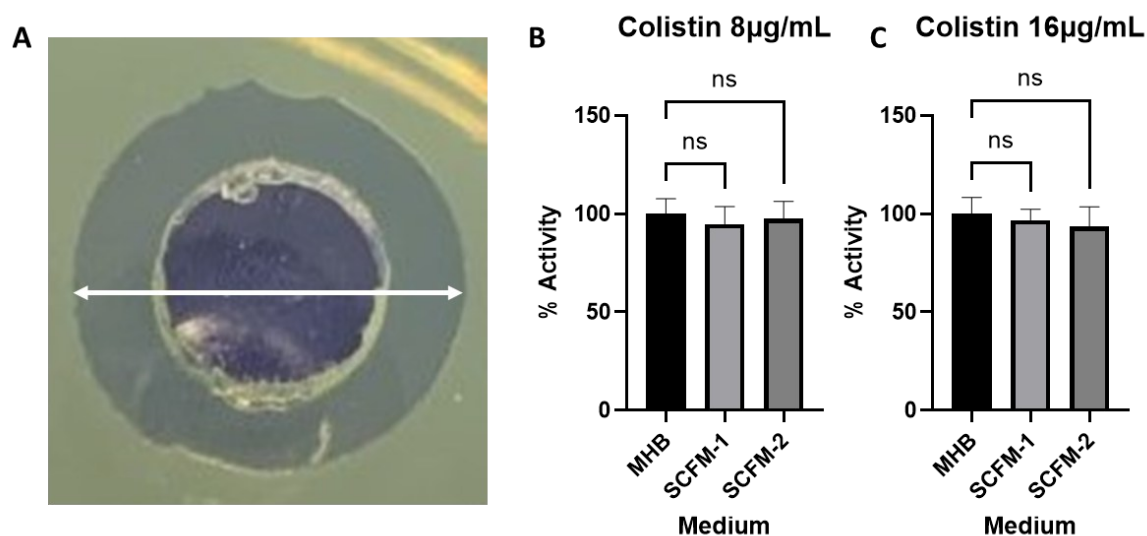


Figure 3.6: Synthetic sputum does not significantly alter colistin activity. MHB agar plates were spread with a lawn of PA14 and indicated media containing colistin at 8 $\mu\text{g/mL}$ (B) or 16 $\mu\text{g/mL}$ (C) was inoculated into wells. Zone diameters were measured around wells at symmetrical points (A). Data were normalised to MHB. Each bar represents the mean of 3 independent experiments with each condition performed in triplicate. Error bars represent the standard deviation. Data were analysed by 1-way ANOVA with Dunnett's post hoc test using MHB as the control.

3.2.4 Individual SCFM-2 macromolecules differentially modulate susceptibility of Pa to colistin

Since SCFM-2 reduced susceptibility to colistin relative to SCFM-1 for most strains, it was next investigated which macromolecules were responsible (Fig 3.7). In these experiments SCFM-1 was supplemented with each of the macromolecules and colistin MIC determined.

For *P. aeruginosa* PA14 the presence of DNA or mucin resulted in 2-fold increases in MIC whereas there was no effect of GlcNAc or DOPC. For the AK3 isolate, neither DNA nor GlcNAc altered the colistin MIC but Mucin and DOPC each resulted in a 2-fold decrease from 0.5 to 0.25 $\mu\text{g/mL}$. A similar picture was seen for *P. aeruginosa* AK10, with mucin resulting in a 4-fold

decrease in MIC and DOPC a 2-fold decrease from 0.5 to 0.25 $\mu\text{g/mL}$. For *P. aeruginosa* AK20, DOPC resulted in a 2-fold decrease in colistin MIC whereas there was no change for other macromolecules.

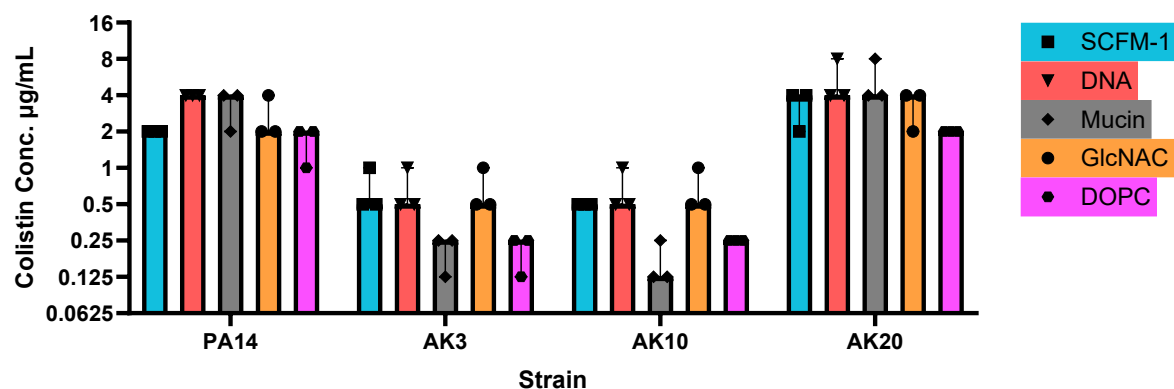


Figure 3.7: Sputum macromolecules differentially effect colistin sensitivity. MICs to colistin were determined by broth microdilution for *P. aeruginosa* strains PA14, AK3, AK10, and AK20. Strains were inoculated into SCFM-1 alone or SCFM-1 supplemented with individual macromolecules at the same concentration they are found in SCFM-2. bacteria were incubated at 37°C for 20 hours. Growth was determined by measuring optical density at 600nm. Data points represent the result of 3 independent experiments with coloured bars indicating the median and error bars indicating the range.

Next, the effect of individual macromolecules on colistin-mediated killing was investigated by time-kill assay. For *P. aeruginosa* PA14, DOPC resulted in a 2 $\log_{10}$ increase in killing relative to SCFM-1 alone by 4 hours, which was maintained by 6 hours (Fig 3.8A). By contrast, the presence of DOPC did not affect the survival of any of the clinical isolates relative to SCFM-1 alone (Fig 3.8B-D). The presence of GlcNAc had not significant effect on the killing of any of the *P. aeruginosa* strains relative to SCFM-1 (Fig 3.8)

The addition of mucin to SCFM-1 did not increase the survival of *P. aeruginosa* PA14, AK3 or AK10 relative to SCFM-1 alone (Fig 3.9 A-C). By contrast, the presence of mucin enhanced the recovery of *P. aeruginosa* AK20, resulting in a 3-log increase in survival of this isolate at 6 hours relative to SCFM-1 alone (Fig 3.9D). The addition of eDNA reduced killing and increased recovery in *P. aeruginosa* PA14, a 2-log difference at 6 hours, AK3, a 6-log difference at 6 hours, and AK20, a 3-log difference at 6 hours (Fig 3.9A, 3.9B and 3.9D). Although there is a trend for increased survival in AK10 this was not statistically significant (Fig 3.9C). Overall, eDNA resulted in the greatest increase in bacterial survival across most strains and was therefore selected for further investigation.

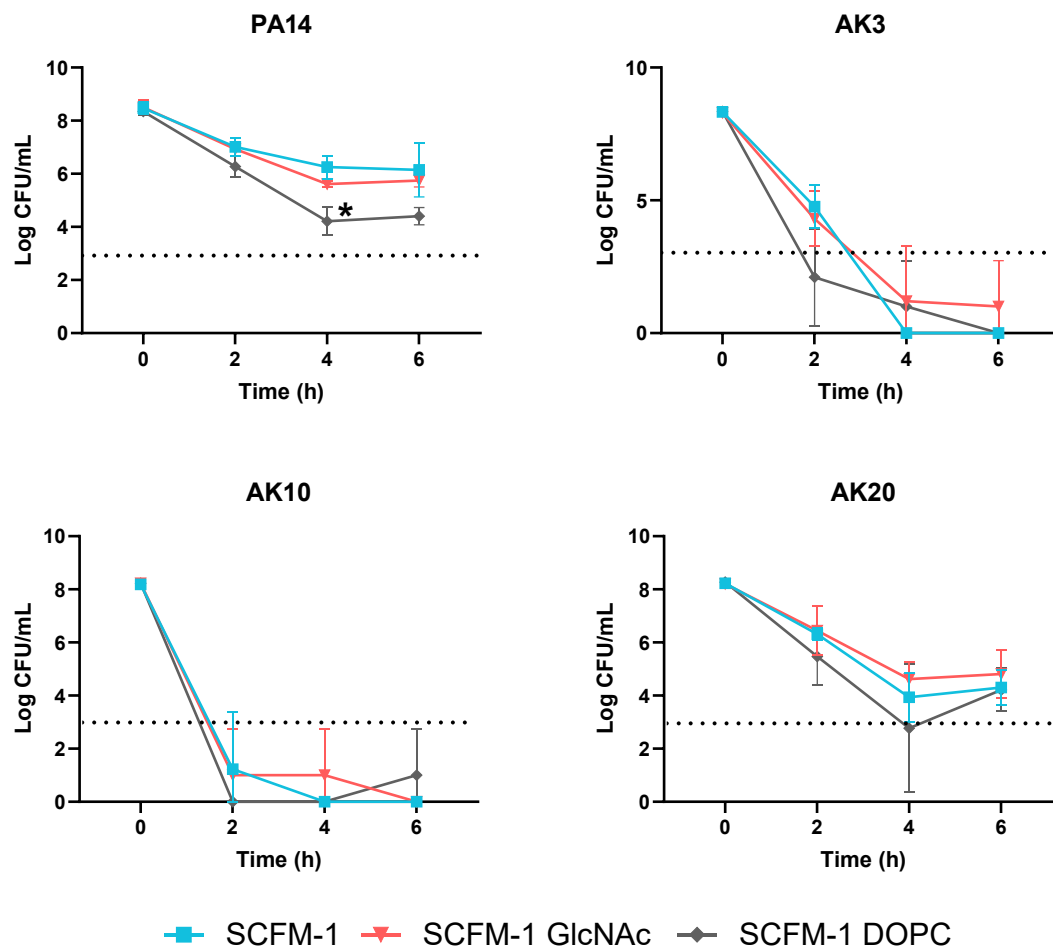


Figure 3.8: N-acetyl glucosamine (GlcNAc) and 1,2-dioly-phosphatidylcholine (DOPC) have minimal effect on colistin susceptibility. Stationary phase cultures of *P. aeruginosa* strains PA14 (A) AK3 (B) AK10 (C) and AK20 (D) were exposed to 4 μ g/mL of colistin in SCFM-1, or SCFM-1 supplemented with 0.3mM GlcNAc or 100 μ g/mL DOPC bacterial survival measured over 6 hours. Data points represent the mean of 4 independent experiments and error bars represent the standard deviation. Data were analysed by 2-way ANOVA with Dunnett's post-hoc test. *P<0.05 (relative to SCFM-1). The dashed line indicates the threshold of detection.

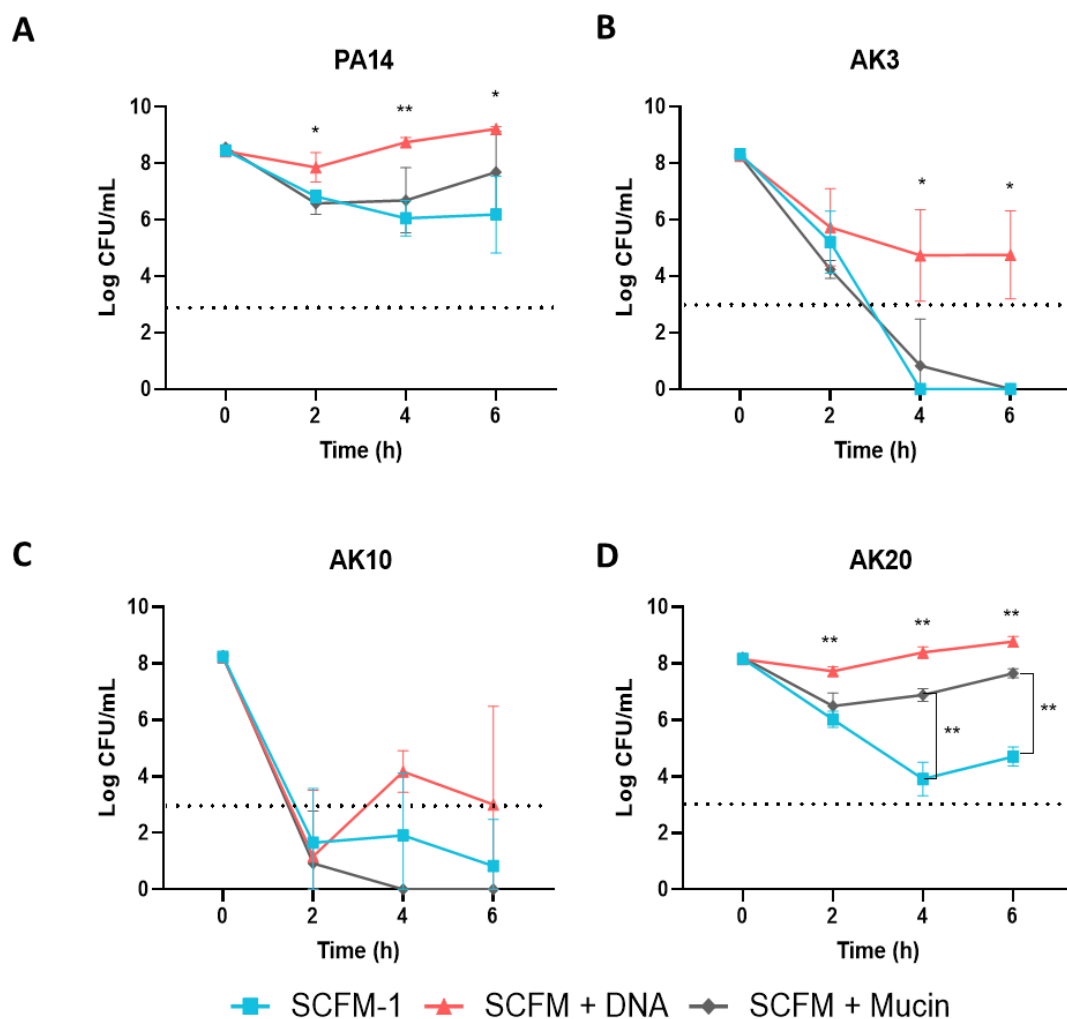


Figure 3.9: Extracellular DNA (eDNA) reduces susceptibility to colistin. Stationary phase cultures of *P. aeruginosa* strains PA14 (A) AK3 (B) AK10 (C) and AK20 (D) were exposed to 4µg/mL of colistin in SCFM-1, or SCFM-1 supplemented with 0.6 mg/mL DNA or 5 mg/mL Mucin and bacterial survival measured over 6 hours. Data points represent the mean of 4 independent experiments and error bars represent the standard deviation. Data were analysed by 2-way ANOVA with Dunnett's post-hoc test. *P<0.05 **P<0.01 (relative to SCFM-1). The dashed line indicates the threshold of detection.

3.2.5 eDNA enhances growth but is inhibitory at a high concentrations.

Estimates for the concentration of eDNA in CF sputum vary within the literature from 0.25 mg/mL to 5 mg/mL (292–295). As SCFM-2 contains only 0.6 mg/mL eDNA, it was decided to investigate the effect of higher concentrations on bacterial growth and colistin susceptibility. This revealed that eDNA enhanced the growth of all strains at intermediate concentrations (0.6 and 1.2 mg/mL), relative to SCFM-1 (Fig 3.10). At 2.4mg/mL, eDNA further enhanced growth of *P. aeruginosa* PA14 (Fig 3.10A), but inhibited the growth of each of the clinical isolates (Fig 3.10B, 3.10C, 3.10D).

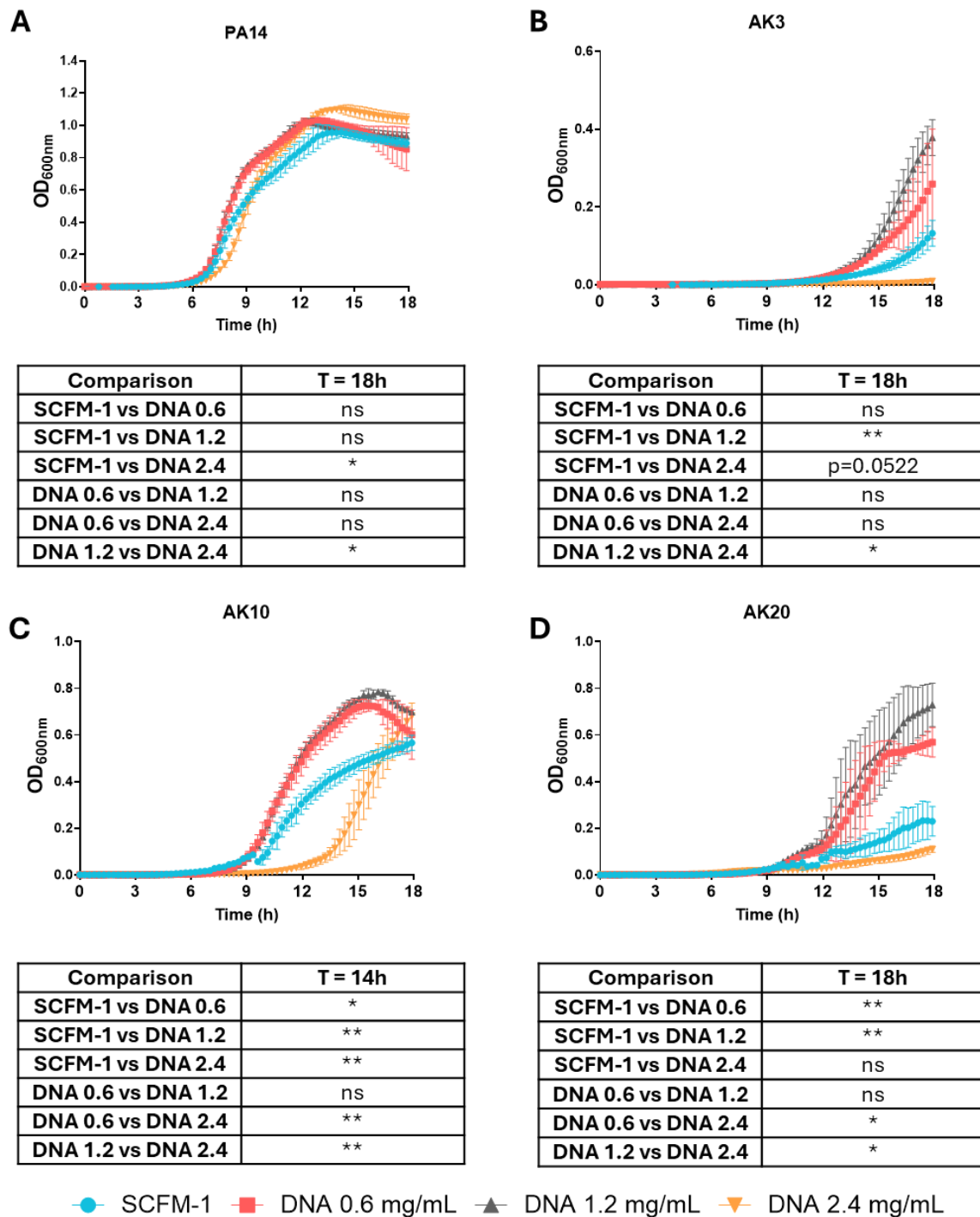


Figure 3.10: Extracellular DNA inhibits growth at high concentrations. *P. aeruginosa* strains PA14 (A) AK3 (B) AK10 (C) and AK20 (D) were inoculated into SCFM-1 containing increasing concentrations of eDNA (0, 0.6, 1.2, 2.4 mg/mL) and incubated at 37°C over 18 hours. Growth was determined by measuring optical density at 600nm. Data points represent the mean of 3 independent experiments with error bars indicating the standard deviation. Data were analysed by two-way ANOVA with Tukey's post hoc test. *P<0.05 **P<0.01. Comparisons at appropriate time points are summarised in the table below each graph.

3.2.6 eDNA reduces susceptibility of *P. aeruginosa* to colistin in SCFM-1

Next, to understand the whether the effect of eDNA on colistin susceptibility was conserved across *P. aeruginosa*, I measured the colistin MIC of a wider panel of clinical CF isolates grown in SCFM-1 supplemented, or not, with 1.2mg/mL eDNA (Fig 3.11). This panel was selected to include a wider range of phenotypic variants including colistin resistant (AK1 and AK24) and mucoid variants (AK8 and AK11). The higher concentration of eDNA was chosen to enhance the eDNA effect without being inhibitory, as seen for 2.4 mg/mL.

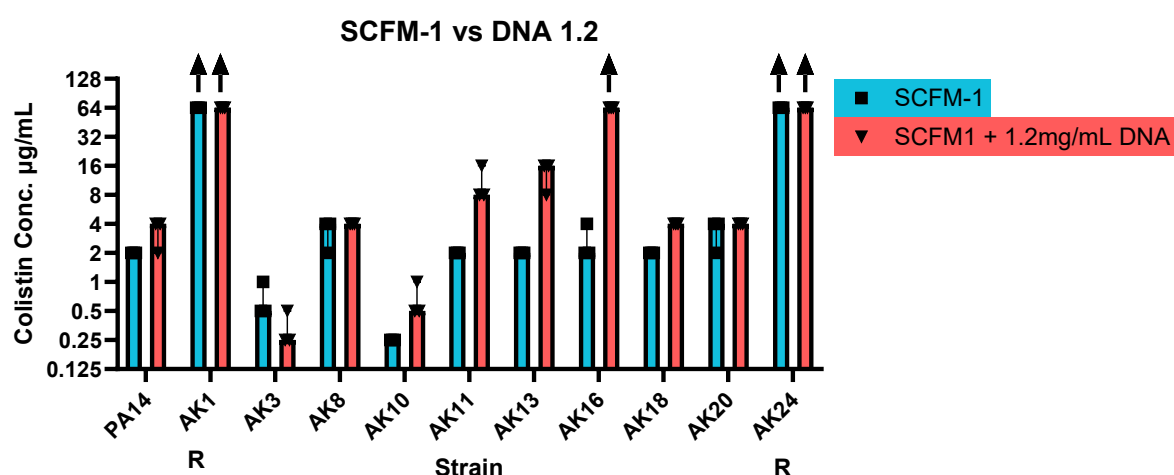


Figure 3.11: Clinical strains demonstrate varied responses to extracellular DNA. MICs to colistin were determined by broth microdilution for *P. aeruginosa* and a range of clinical strains isolated from CF patients. Strains were inoculated into SCFM-1 with and without 1.2mg/mL eDNA. bacteria were incubated at 37°C for 20 hours. Growth was determined by measuring optical density at 600nm. Data points represent the result of 3 independent experiments with coloured bars indicating the median and error bars indicating the range. Arrows indicate where the MIC is above the maximum concentration tested (64µg/mL). Strains known to be resistant to colistin are indicated by the letter R.

The presence of eDNA resulted in 2-fold increases in colistin MIC for *P. aeruginosa* strains PA14, AK10 and AK18 and ≥ 4 -fold increases were seen for *P. aeruginosa* strains AK11, AK13 and AK16. No consistent increase in MIC was observed for *P. aeruginosa* isolates AK8 or AK20, whereas AK3 became more susceptible to colistin at this higher DNA concentration, with a fall in MIC from 0.5 to 0.25 µg/mL. The two resistant isolates (AK1 and AK24) had MICs greater than 64 µg/mL.

Next, to better understand the relationship between eDNA concentration and colistin susceptibility, the survival of *P. aeruginosa* PA14 was measured over time in SCFM-1

supplemented with increasing concentrations of eDNA (0.0375 – 2.4mg/mL) and 4 µg/mL (Fig 3.12A) or 8 µg/mL (Fig 3.12B) colistin. Increasing eDNA concentrations were associated with decreased susceptibility to both concentrations of colistin, with killing by 8 µg/mL colistin completely abrogated at 1.2 and 2.4mg/mL eDNA. Notably, even a low concentration of 0.0375 mg/mL eDNA appeared sufficient to enable *P. aeruginosa* replication in the presence of 4 µg/mL of colistin, after an initial fall in CFU counts. As such, eDNA efficiently induces decreased colistin susceptibility in a dose-dependent manner.

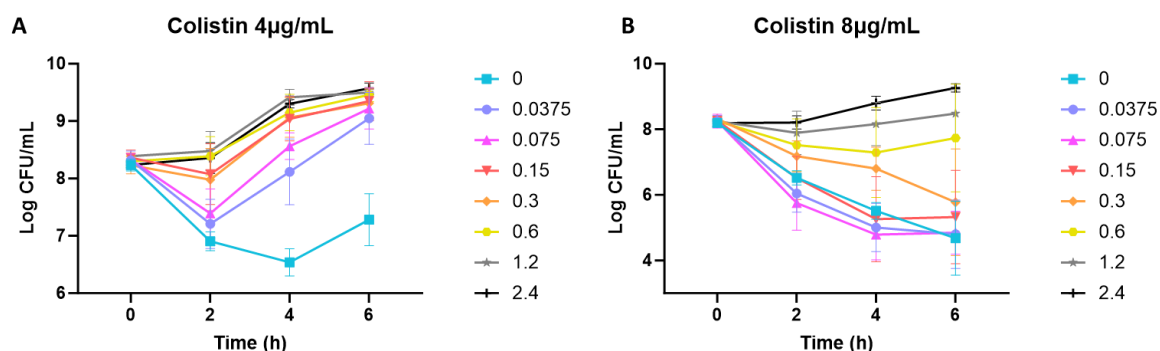


Figure 3.12: Increasing concentrations of eDNA reduces colistin activity. Stationary phase cultures of *P. aeruginosa* PA14 were exposed to 4µg/mL (A) or 8µg/mL (B) of colistin in SCFM-1, or SCFM-1 supplemented with indicated concentrations of DNA (mg/mL). Bacterial survival was measured over time. Data points represent the mean of 4 independent experiments and error bars represent the standard deviation.

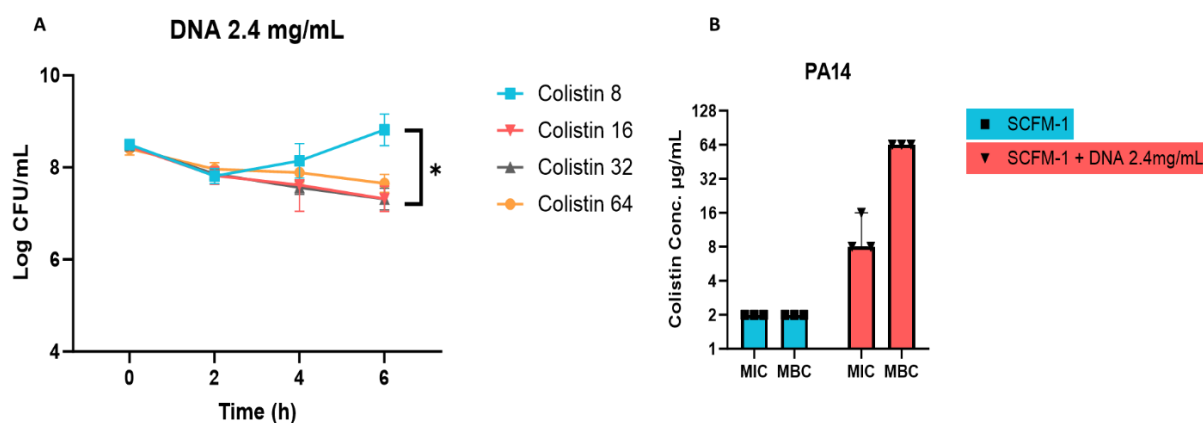


Figure 3.13: High concentrations of eDNA induce tolerance to colistin. (A) Stationary phase cultures of *P. aeruginosa* PA14 were exposed to indicated concentrations of colistin in SCFM-1 supplemented with 2.4mg/mL eDNA. Bacterial survival was measured over time. Data points represent the mean of 4 independent experiments and error bars represent the standard deviation. Data were analysed by 2-way ANOVA with Sidak's post-hoc test * $P < 0.05$. (B) MIC and MBCs were determined for *P. aeruginosa* PA14 in SCFM-1 or SCFM-1 supplemented with 2.4 mg/mL eDNA. Data points represent individual experiments with bars the median and error bars the range. Data were analysed by 2-way ANOVA with Tukey's post-hoc test * $P < 0.05$.

3.2.7 Physiological concentrations of eDNA induce tolerance to supra-therapeutic concentrations of colistin

To examine the apparent discrepancy between only minor shifts in MIC but much more dramatic reductions in killing, time-kill assays were performed for *P. aeruginosa* PA14 using higher concentrations of colistin in the presence of the highest concentration of eDNA, 2.4 mg/mL. As before, bacteria were able to grow in the presence of 8 µg/mL of colistin when eDNA was present (Fig 3.13A). Although eDNA did not enable growth at colistin concentrations up to 64 µg/mL, it did confer colistin tolerance with only a single $\log_{10}$ reduction in CFU between 0 and 6 hours (Fig 3.13A).

To further explore the effect of eDNA on colistin resistance and tolerance, the colistin MIC and MBC were subsequently determined for *P. aeruginosa* PA14 in both SCFM-1 and SCFM-1 supplemented with 2.4mg/mL eDNA (Fig 3.13B). Although the MIC and MBC were identical in SCFM-1 at 2 µg/mL, in the presence of eDNA, though growth was inhibited at 8 µg/mL (MIC), bacteria were not effectively killed until 64 µg/mL (MBC) (Fig 3.13B). The ability of bacteria to survive in the time-kill but not MBC assay likely reflects the larger inoculum used in the former assay.

In summary the presence of eDNA resulted in a dose dependent reduction in *P. aeruginosa* killing by colistin but relatively small increases in colistin MIC.

3.3 Discussion

Colistin is the most widely used inhaled antibiotic in CF, in the UK, but is usually unable to eradicate infection. The aim of this chapter was to investigate whether- and how- the host microenvironment alters susceptibility of *P. aeruginosa* to colistin using a clinically relevant *in vitro* model.

Data from this chapter demonstrated that SCFM-1 and SCFM-2 do not support bacterial growth to the same extent as standard laboratory media (CA-MHB) and the effect is most marked in isolates from CF patients, perhaps reflecting the tendency of CF isolates to develop auxotrophy over time (404,405). Given that *P. aeruginosa* can utilise amino acids as a carbon source (266) it is perhaps a little surprising that we see limited growth in SCFM-1. Furthermore, extracellular DNA supported the growth of all 4 strains until it reached inhibitory concentrations. Data of the growth enhancing effects of eDNA is extremely limited. It is well recognised that eDNA forms an important component of *P. aeruginosa* biofilm (160,428) but, wider effects on planktonic growth have not been reported. Kumar and colleagues isolated a purine auxotroph that was dependent on eDNA for growth (429). Mulcahy and colleagues demonstrated *P. aeruginosa* is capable of utilising DNA as a nutrient source with expression of a deoxyribonuclease to facilitate this (301). By contrast, DNA is known to be cytotoxic to cells and is an important component of neutrophil extracellular traps (NETs) (430). Growth in the presence of mucin and its breakdown product GlcNAc as an energy source have also been reported (431,432). Taken together these observations support the prevailing model that *P. aeruginosa* is metabolically versatile though becomes niche-adapted with chronic infection in the CF-lung.

It was clear from both antibiotic susceptibility assays used that both SCFM-1 and SCFM-2 modulate bacterial susceptibility to colistin, with the greatest effect observed in SCFM-2, albeit with marked variability between strains. In SCFM-1, although there was a consistent 2-fold increase in MIC relative to MHB this was not associated with a significant decrease in killing in the time-kill assays for the clinical strains. Potentially, this could be attributed to an inoculum effect as much fewer bacteria are exposed to colistin in an MIC assay than in the time-kill (10^5 vs 10^8). Sweeney and colleagues identified much larger shifts in MIC between MHB and SCFM-1 when they used a modified SCFM-1 which did not contain glucose (433) and as previously noted, the relative availability of carbon sources can have dramatic effects on MIC (266). In SCFM-2, there was a 4-fold increase in MIC and an increase in survival in *P. aeruginosa* strains

PA14 and AK20 in time-kill assays. There was no change in MIC or killing for strain AK3, and *P. aeruginosa* AK10 demonstrated a decrease in MIC in SCFM-2 (relative to SCFM-1). Such variability in response to ASM has been reported in the literature when a range of clinical isolates were exposed to polymyxin antibiotics, including colistin, in the presence of sputum macromolecules (410). Both AK3, and AK10 were rapidly killed by colistin at 4 µg/mL due to their high susceptibility, and statistically significant differences may become apparent at lower colistin concentrations, although these may be lower than colistin levels achieved in CF lung (208).

When the effects of individual macromolecules were examined, a range of bacterial responses were observed. In general, there was a trend for DOPC to increase bacterial susceptibility to colistin. Deschamps and colleagues found a similar pattern where the addition of DOPC lowered the MIC of strain PAK to colistin, compared to colistin in SCFM-1 alone (305).

Mucin caused various changes in susceptibility to colistin. Susceptibility of strains PA14 and AK20 decreased whereas, susceptibility of AK3 and AK10 increased. Mucin has been reported to bind and sequester colistin, decreasing its activity (287) as well as the endogenous cationic peptide LL-37 (434). However, it should be noted that Huang and colleagues used higher concentrations of mucin to demonstrate restricted diffusion of colistin (12.5% vs 0.5% used here) and examined mucin in PBS or non-cation-adjusted MHB (287). This is important because electrolyte concentrations are likely to significantly alter the physico-chemical properties of mucin (435) and cation availability is crucial for determining colistin susceptibility (436). Therefore, the translatability between MHB, PBS and SCFM is likely to be limited. When the effect of synthetic sputum on colistin activity was examined, there was no evidence for significant sequestration in SCFM-2 relative to MHB. Finally, Huang et al. used porcine gastric mucin, a more widely used form of mucin than the bovine maxillary mucin employed in SCFM-2. Porcine gastric mucin was previously identified by Neve and colleagues as a significant source of contamination in synthetic sputum formulations in that commercial preparations contained additional iron and aromatic amino acids (426). Aside from direct interactions between mucin and colistin, mucin has been shown to alter the phenotype of *P. aeruginosa* and this may, in part, explain why increased susceptibility is seen in some strains (437). Taken together it is difficult to compare our work to these publications given significant methodological differences. However, colistin binding does not appear to be an important modulator of bacterial susceptibility in synthetic sputum given the variability of its impact on MIC and time-kill data reported here.

In contrast to other sputum macromolecules modulation of colistin susceptibility with eDNA appeared greater in magnitude and more consistent across bacterial strains. Though large

shifts in MIC were not observed at 0.6mg/mL eDNA, when a limited panel of isolates was investigated, decreased killing was seen for strains PA14, AK3 and AK20, with a trend for the same in AK10. Additionally, larger colistin MIC shifts were seen at higher DNA concentrations across a wider clinical panel and a dose-dependent reduction in killing observed for *P. aeruginosa* PA14 with both 4 and 8 µg/mL of the polymyxin antibiotic. Furthermore, tolerance to colistin was observed in strain PA14 up to 64 µg/mL in the presence of 2.4mg/mL eDNA, indicative of a significant reduction in bacterial susceptibility.

The physiological range of peak colistin concentrations, following inhaled administration of colistimethate sodium (CMS) are 2-11 µg/mL following a 2 million IU dose and 4-21 µg/mL following 4 million IU administration (48)(438). Thus, even at supra physiological concentrations the bactericidal activity of colistin would be expected to be abrogated in the presence of high levels of eDNA.

Previous work by Mulcahy and colleagues examined the effect of extracellular DNA on *P. aeruginosa* growth and colistin susceptibility (300). Concordant with the findings presented here, they identified high concentrations of eDNA were cytotoxic but sublethal concentrations induced colistin resistance when dissolved in LB or minimal medium. They used a reporter system to demonstrate activation of the *arn* operon, known to be responsible for the L-ara4N LPS modification, which could be abrogated by the addition of divalent cations. Further work suggested eDNA could also induce the production of polyamines (439) and have wider metabolic consequences for bacteria (440). However, none of these interactions were studied in the context of a sputum-like environment.

In addition to colistin, eDNA has also been shown to trigger decreased bacterial susceptibility to several cationic antimicrobial peptides (410) in the context of an alternative ASM model (386). This suggests that the CF lung environment may reduce the potency of the host immune response, further contributing to the establishment of persistent infections.

In summary, the data presented here highlights the key role of eDNA in modulating colistin susceptibility in the context of synthetic sputum, although further work will be required to confirm whether the mechanism of action is similar to that observed by Mulchay et al. Understanding how eDNA in ASM triggers decreased colistin susceptibility of *P. aeruginosa* will be explored in the next chapter.

Chapter 4: Extracellular DNA triggers LPS modifications associated with reduced susceptibility to colistin.

4.1 Introduction

Colistin treatment fails to eradicate *P. aeruginosa* from the CF lung. The previous chapter demonstrated that eDNA reduced susceptibility to colistin in a dose dependent fashion in artificial sputum. The aim of this chapter was to explore how eDNA reduces susceptibility to colistin.

4.1.1 Colistin resistance in *P. aeruginosa*

The primary mode of action of colistin is binding to the terminal phosphates of lipid A disrupting cation bridges between adjacent LPS molecules resulting in permeabilisation of bacterial membranes (213,222). Permeabilisation of the outer membrane allows colistin to access the inner membrane where disruption leads to cell death (227,228). Colistin resistance in *P. aeruginosa* arises through multiple mechanisms, the most common of which are the covalent modification of the terminal phosphates of lipid A with either pEtN or L-ara4N, reducing their negative charge and therefore affinity for colistin (218). Though Lipid A can be modified by either L-ara4N or pEtN the former appears more common in *P. aeruginosa* relative to other Gram-negative bacteria (425,441,442). Colistin resistance in *P. aeruginosa* from CF is rare (443), but has most commonly been associated with gain of function mutations in two-component systems regulating the *arnBCEATEF* (*arn*) operon responsible for the synthesis and transfer of L-ara4N to lipid A (251,254,442). Indeed, targeted disruption of the *arn* operon leads to a dramatic reduction in spontaneous colistin resistance (425).

Although *P. aeruginosa* encodes the genes responsible for pEtN modification of lipid A, this appears to be a rare contributor to mutational polymyxin resistance in this bacterium (243). This is in contrast to many other bacteria where pEtN modification can occur via the acquisition of spontaneous resistance or mobile colistin resistance genes (MCR) which encode for phosphoethanolamine transferases, typically located on plasmids that are not readily acquired by *P. aeruginosa* (256).

Aside from covalent modifications to lipid A, colistin resistance is associated with metabolic changes (441), mutations in the genes encoding the LPS synthesis pathway (258), upregulation of drug efflux pumps (260,262), and polyamine synthesis (265,312). Data surrounding efflux pumps and polyamine synthesis remains contradictory with some authors

reporting no effect on polymyxin susceptibility, and this may be due to the impact of efflux disruption on expression of the *arn* operon (260). Alternatively, the methods used to determine bacterial susceptibility vary in the literature with MICs appearing largely unaffected in some studies, whereas bacterial survival and outer membrane permeability assays due to polymyxin exposure appear more sensitive (260,265). In this chapter I have used a combination of these approaches to determine bacterial susceptibility.

4.1.2 *P. aeruginosa* possesses multiple two-component systems linked to colistin resistance

P. aeruginosa is a prevalent and versatile environmental organism, able to colonise a range of niches. As such, it has a wide range of two-component systems to respond to changing conditions, with 64 reported to date (444). Two-component systems are a common way for bacteria to respond to their environment and are typically composed of a sensor-histidine kinase which phosphorylates a response regulator following activation or de-repression by a particular stimulus (445). The response regulator then goes on to affect a change within the cell via transcriptional activation or repression of specific genes. Five two-component systems have been directly linked to colistin resistance in *P. aeruginosa* (Table 2.1): PmrAB, PhoPQ, ColRS, CprRS and ParRS. Most systems are linked to the activation of *arn*, with the exception of ColRS which activates *eptA* (249). Importantly, these systems and in particular, PmrAB and PhoPQ were initially described and characterised in *E. coli* and *S. enterica* and inferences to their function in *P. aeruginosa* are often made with little supporting evidence. Furthermore, there are key differences in structure and function of these systems in *P. aeruginosa* relative to *E. coli* (245,446).

Both systems are activated by low concentrations of divalent cations (244,246) and low pH (164) PmrAB has been linked to activation by CAMPs themselves (244). CprRS and ParRS are likewise activated by CAMPS (247,447). ColRS appears to respond to transition metals (448). Relatively recently, Wiesman and colleagues identified that susceptibility to iron appeared to be ColRS dependent and could be enhanced by lowering the pH (449).

Gain of function mutations, particularly in *phoPQ* and *pmrAB* have been reported in clinical *P. aeruginosa* isolates from CF and elsewhere (442,450). These result in constitutive activation and thus modification of lipid A with L-ara4N, conferring polymyxin resistance.

Two-component system	Gene locus in PA14	Environmental triggers	Relevant Downstream effects
PhoP PhoQ	PA14_49180 PA14_49170	Divalent cations (246), pH (164)	L-ara4N modification of lipid A (451)
PmrA PmrB	PA14_63150 PA14_63160	Divalent cations (244), pH (164), CAMPs (244)	L-ara4N modification of lipid A (451) Polyamine synthesis increased (451) MexXY efflux pump upregulation(451)
ColR ColS	PA14_56950 PA14_56940	Transition metals (448,449) (pH dependent)	pEtN modification of lipid A (249)
ParR ParS	PA14_41260 PA14_41270	CAMPs (447)	L-ara4N modification of lipid A (447) Polyamine synthesis increased(260) MexXY efflux pump upregulation (260)
CprR CprS	PA14_24350 PA14_24340	CAMPs (247)	L-ara4N modification of lipid A (247)

Table 4.1 Two-component systems associated with decreased colistin susceptibility in *P. aeruginosa*. Key references are highlighted in parentheses.

4.1.3 eDNA reduces susceptibility to colistin

eDNA has been recognised as a key component of synthetic sputum, forming a part of biofilm matrix as well as reducing susceptibility to antibiotics. Mulcahy and colleagues recognised that eDNA could act both as a nutrient source (301) and that it could reduce susceptibility to polymyxins and aminoglycosides through activation of the *arn* operon (300). Additionally, eDNA has been demonstrated to bind and thus remove the activity of the cationic antimicrobial peptide LL-37 (427), as well as the similarly cationic tobramycin (289) raising the possibility that it could have a similar effect on polymyxins. However, the mechanism(s) by which eDNA modulates colistin susceptibility of *P. aeruginosa* in sputum is unknown. Therefore, the aim of this chapter was:

Aim: To determine the mechanism by which eDNA reduces susceptibility of *P.aeruginosa* to colistin in synthetic sputum.

4.2 Results

4.2.1 Validation of Salmon sperm DNA as a proxy for Human DNA.

Sequencing experiments have confirmed that the most abundant extracellular DNA (eDNA) in CF sputum is of human origin (94,452). However, most artificial sputum media, including that used in chapter 3, utilise salmon sperm DNA (ssDNA) because it is readily available. To exclude a non-specific effect mediated by ssDNA or contamination during the commercial purification process, human DNA (hDNA) was purified from the respiratory epithelial cell line A549 (400). When added to SCFM-1, both ssDNA and hDNA at 0.6mg/mL inhibited killing of *P. aeruginosa* PA14 by 4 µg/mL colistin to comparable levels, resulting in a 2-log increase in survival by 4 hours relative to that seen for bacteria in SCFM-1 without eDNA (Fig 4.1). Therefore, ssDNA was deemed to be a suitable replacement for human DNA.

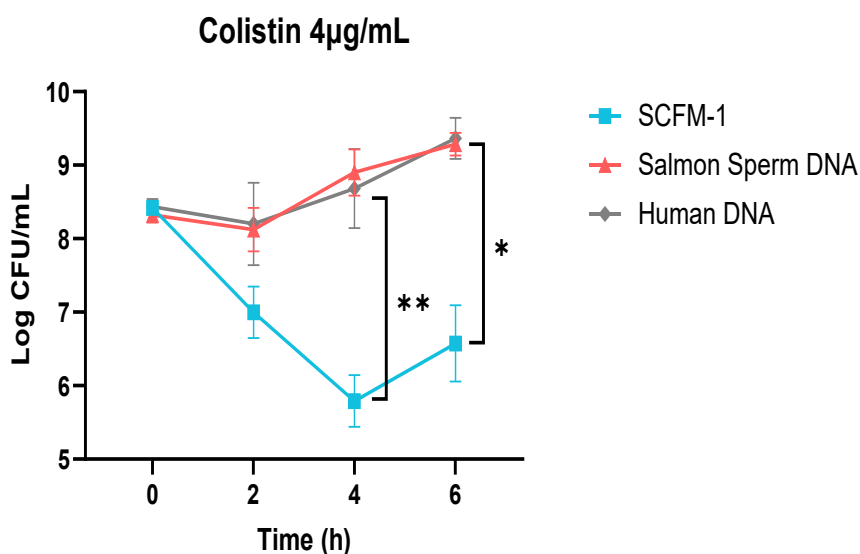


Figure 4.1: Both low molecular weight salmon sperm DNA and human DNA reduce susceptibility to colistin. *P. aeruginosa* PA14 was exposed to 4µg/mL colistin in SCFM-1 or SCFM-1 supplemented with commercially available low molecular weight salmon sperm DNA (0.6mg/mL) or SCFM-1 supplemented with human DNA purified from cultured A549 respiratory epithelial cells (0.6 mg/mL). Data points represent the mean of 3 independent experiments and error bars represent the standard deviation. Data were analysed by 2-way ANOVA with Tukey's post-hoc test. *P<0.05 **P<0.01

4.2.2 eDNA does not significantly sequester colistin in SCFM-1

eDNA has been reported to directly interact with cationic antimicrobial peptides, reducing their antibacterial activity (289,427). To investigate whether a significant interaction between eDNA and colistin occurred in synthetic sputum, a zone of inhibition assay was used as

described in chapter 3. Given there was no effect on colistin in SCFM-2, when the effect of eDNA alone was investigated, tobramycin was included as a positive control, given reports that eDNA sequesters aminoglycosides (289). For colistin there was no difference in zone size (Fig 2A) between phosphate buffered saline, SCFM-1 alone or SCFM-1 containing 0.6 (Fig 4.2B) or 2.4mg/mL (Fig 4.2C) eDNA. By contrast there was a 10% reduction in zone size for tobramycin at 2.4mg/mL of eDNA relative to PBS or SCFM-1 at both 8 (Fig 4.2D) and 16 μ g/mL (Fig4.2E). This supports previous data showing that eDNA sequesters aminoglycosides, but rules out a similar mechanism for colistin, at least in the context of SCFM-1.

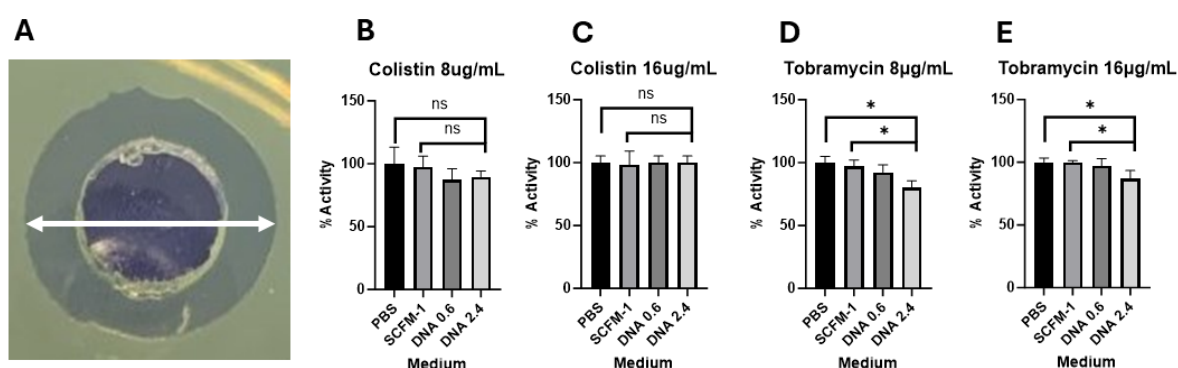


Figure 4.2: eDNA does not significantly reduce colistin activity. MHB agar plates were spread with a lawn of PA14 and indicated media containing either colistin (**B** and **C**) or tobramycin (**D** and **E**) at indicated concentrations was inoculated into wells. Zones diameters were measured around wells at symmetrical points (A). Data were normalised to MHB. Each bar represents the mean on 3 independent experiments with each condition performed in triplicate. Error bars represent the standard deviation. Data were analysed by 1-way ANOVA with Tukey's post hoc test. *P<0.05

4.2.2 eDNA disrupts the outer membrane of *P. aeruginosa*

Mulcahy and colleagues identified that high concentrations of extracellular DNA resulted in disruption of both the inner and outer membranes of *P. aeruginosa* leading to propidium iodide uptake (300). Therefore, I hypothesised that sublethal concentrations of eDNA would be capable of disrupting the outer membrane of *P. aeruginosa*, triggering outer membrane modification that confers polymyxin resistance. To determine the integrity of the outer membrane the hydrophobic dye NPN was used (453). NPN is normally excluded from the outer leaflet of Gram-negative bacteria by LPS. Loss of integrity of the LPS monolayer allows NPN to bind phospholipids and produce a fluorescent signal (453).

NPN was unstable in SCFM-1 and therefore the assay was performed in HEPES buffer as previously described (453). Stationary phase PA14 was diluted to 0.5 OD_{600nm} and exposed to

eDNA at increasing concentrations. Colistin was included as a positive control. There was no significant difference in NPN signal between HEPES alone and HEPES containing 0.6mg/mL eDNA (Fig 3A). However, 2.4mg/mL of eDNA resulted in a 32-fold increase in fluorescence, comparable to colistin at 2 µg/mL, consistent with disruption of the outer membrane (Fig 3A).

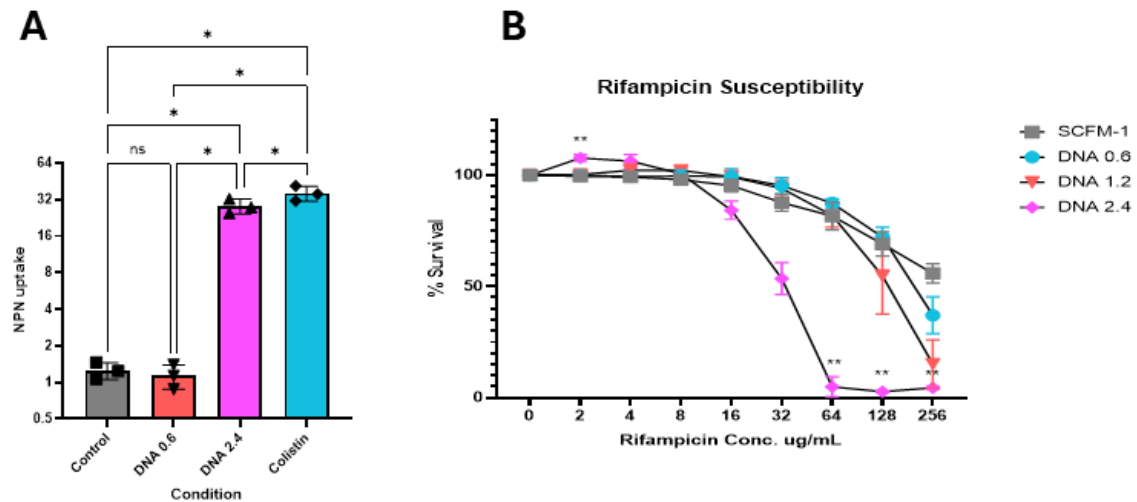


Figure 4.3: eDNA disrupts the outer membrane of *P. aeruginosa*. (A) The hydrophobic dye NPN was used to assess outer membrane permeability. Stationary phase bacteria challenged with DNA at indicated concentrations (mg/mL) or colistin at 2 µg/mL in HEPES buffer. HEPES buffer alone acted as the negative control. Data points represent the mean of 3 independent experiments with 3 technical replicates. Error bars represent the standard deviation. Data were analysed by one-way ANOVA with Tukey's post hoc test. (B) Susceptibility to Rifampicin was determined by broth microdilution in SCFM-1 in the presence of indicated concentrations of DNA (mg/mL). Bacterial growth was determined by OD_{595nm} and normalised to bacterial growth media containing no antibiotics. Data points represent the mean of 3 independent experiments. Error bars represent the standard deviation. Data were analysed by 2-way ANOVA with Dunnett's post hoc test. Significant differences between 2.4 mg/mL DNA and SCFM-1 alone are highlighted for clarity. *P<0.05 **<0.01.

To confirm the functional integrity of the outer membrane had been compromised by eDNA, the rifamycin antibiotic rifampicin was used. Rifampicin is commonly used to treat infections caused by Gram-positive organisms as it is excluded from the cytosol of Gram-negative bacteria by LPS within the outer membrane (454). As such, susceptibility to rifampicin is increased by disruption of the outer membrane. Broth microdilution was used to measure the susceptibility of PA14 to rifampicin in the presence of increasing concentrations of eDNA (Fig 3B). Bacterial growth was quantified by measuring absorbance at 595nm. In SCFM-1, *P. aeruginosa* PA14 was able to grow at 256 µg/mL rifampicin, however, as the concentration of eDNA was increased, growth at 256mg/mL was first inhibited (~10% growth at 0.6mg/mL, 40% at 1.2mg/mL)

and then the MIC shifted to 64 $\mu\text{g/mL}$ at 2.4mg/mL DNA in keeping with increasing susceptibility to rifampicin. Both rifampicin susceptibility and NPN uptake support the conclusion that high concentrations of eDNA damage the outer membrane of *P. aeruginosa*.

4.2.4 Excess magnesium prevents membrane damage but does not restore susceptibility to colistin

It has been suggested that eDNA damages membranes by chelating cations such as magnesium that form bridges between LPS molecules (455), triggering the *arn* operon and giving rise to colistin resistance (300). In support of this, membrane disruption by eDNA can be prevented by the addition of excess divalent cations. Therefore, I hypothesised that the addition of excess magnesium could prevent outer membrane damage and restore colistin susceptibility by saturating the cation binding capacity of eDNA.

To understand the effect of Mg^{2+} on eDNA induced membrane disruption, NPN uptake was measured in the presence of 2.4 mg/mL eDNA with the addition of increasing concentrations of extracellular Mg^{2+} in HEPES buffer (0,1, 5 and 10-fold the base concentration of SCFM-1), with colistin used as a control (Fig 4.4). As expected, the addition of Mg^{2+} reduced outer membrane disruption by both colistin and eDNA. The effect of magnesium on colistin was not significant

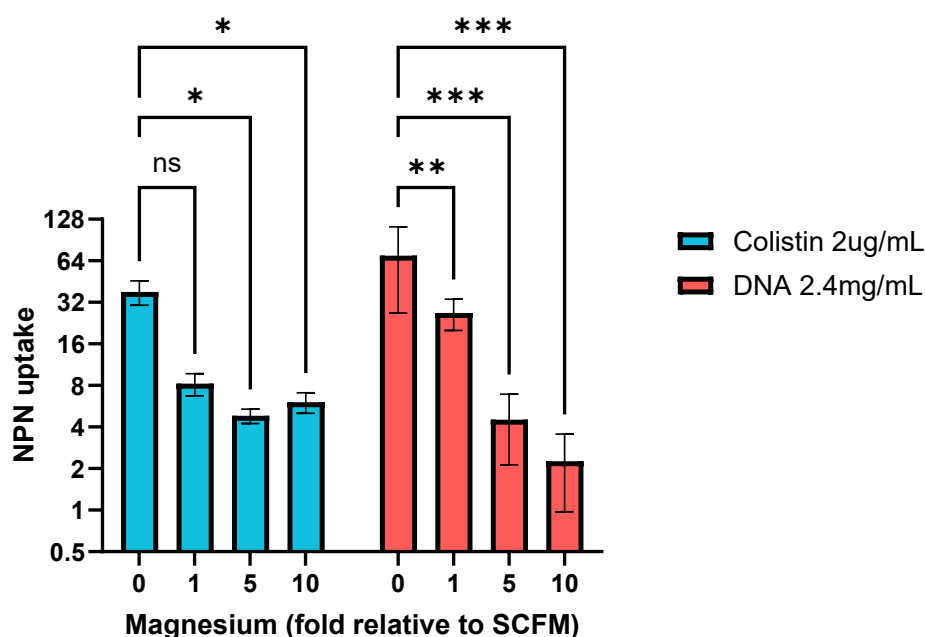
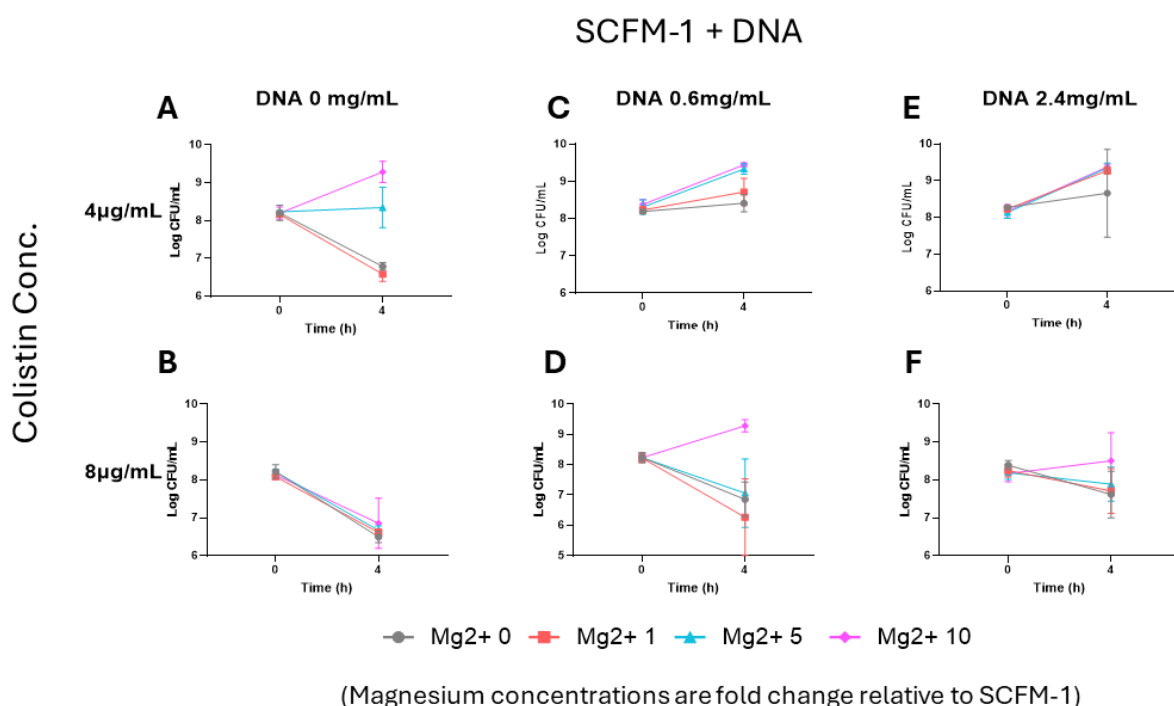


Figure 4.4: Magnesium supplementation protects the outer membrane from damage. The hydrophobic dye NPN was used to assess outer membrane permeability. Stationary phase bacteria challenged with 2.4 mg/mL DNA or colistin at 2 $\mu\text{g/mL}$ in HEPES buffer supplemented with addition magnesium (relative to SCFM). Bars represent the mean of 3 independent experiments with 3 technical replicates. Error bars represent the standard deviation. Data were analysed by 2-way ANOVA with Dunnett's post hoc test. *P<0.05 **P<0.01 ***<0.001.

between 0 and 1-fold magnesium although $p=0.06$. At 5- and 10-fold Mg^{2+} there was significant 8-fold reduction in NPN fluorescence. By contrast increasing magnesium concentrations resulted in decreasing NPN fluorescence in the presence of 2.4 mg/mL eDNA.

Next, to understand how Mg^{2+} affected polymyxin-mediated killing of *P. aeruginosa* in the presence or absence of eDNA, magnesium chloride was added to SCFM-1 at 0, 1, 5 and 10-fold the base concentration of SCFM-1, and bacteria were challenged with 4 or 8 $\mu\text{g/mL}$ colistin in SCFM-1 alone or SCFM-1 supplemented with 0.6 or 2.4 mg/mL of eDNA before bacterial survival was enumerated over 4 hours (Fig 4.5).

Because excess magnesium can reduce polymyxin-mediated killing independently of eDNA (217,221,222), it was first necessary to understand the impact of this cation in SCFM-1 without eDNA. In the absence of eDNA, at 4 $\mu\text{g/mL}$ colistin, the addition of Mg^{2+} resulted in a dose dependent reduction in killing (Fig 4.5A), which was not apparent when 8 $\mu\text{g/mL}$ colistin was used (Fig 4.5B). In the presence of 0.6 mg/mL of eDNA at 4 $\mu\text{g/mL}$, killing was abrogated at all concentrations of Mg^{2+} and growth was enhanced 5 or 10-fold (Fig 4.5C). At 8 $\mu\text{g/mL}$ colistin killing was restored at 1-fold and 5-fold Mg^{2+} but abrogated at 10-fold (Fig 4.5D). Finally in the presence of 2.4 mg/mL eDNA, 4 $\mu\text{g/mL}$ of colistin was insufficient to kill *P. aeruginosa* and the addition of magnesium did not restore killing (Fig 4.5E). At 8 $\mu\text{g/mL}$ colistin killing was observed at 0, 1 and 5-fold magnesium but abrogated at 10-fold (Fig 4.5F). Taken together there appears to be an additive effect whereby eDNA reduced susceptibility to colistin and magnesium further reduced susceptibility. Therefore, whilst eDNA is capable of sequestering divalent cations resulting in membrane disruption, this alone does not completely explain the reduced susceptibility to colistin observed in SCFM-1. Therefore, it was important to investigate other potential resistance mechanisms induced by eDNA.



Colistin Conc.	Mg ²⁺ comparison	DNA Concentration mg/mL		
		0	0.6	2.4
4 µg/mL	0 vs 1	ns	ns	ns
	0 vs 5	**	**	ns
	0 vs 10	**	**	ns
	1 vs 5	**	**	ns
	1 vs 10	**	**	ns
	5 vs 10	**	ns	ns
8 µg/mL	0 vs 1	ns	ns	ns
	0 vs 5	ns	ns	ns
	0 vs 10	ns	**	*
	1 vs 5	ns	ns	ns
	1 vs 10	ns	**	ns
	5 vs 10	ns	**	ns

Figure 4.5: Magnesium supplementation does not restore susceptibility to colistin in the presence of eDNA. Stationary phase cultures of *P. aeruginosa* PA14 were exposed to 4µg/mL (A, C, E) or 8µg/mL (B, D, F) colistin in SCFM-1 alone (A and B) or SCFM-1 supplemented with 0.6mg/mL (C and D) or 2.4mg/mL (E and F) DNA with increasing concentrations of magnesium. Bacterial survival was measured at 0 and 4 hours. Data points represent the mean of 4 independent experiments. Error bars represent the standard deviation. Pairwise comparisons are demonstrated in the table below. Data were analysed by 2-way ANOVA with Tukey's post hoc test. *P<0.05 **P<0.01.

4.2.5 Acidification of SCFM by eDNA is partially responsible for colistin resistance.

The phosphate backbone of DNA acts as a strong acid in solution and a key feature of the CF lung is that the pH is often lower than in unaffected individuals due to impaired physiological buffering (414,415). Since media acidification is known to modulate susceptibility to antibiotics, including colistin, it was hypothesised that eDNA may reduce polymyxin susceptibility via acidification of the medium (456). As a first step towards testing this, the pH of SCFM-1 containing eDNA or not was measured (Fig 6A). Basal SCFM-1 had a pH of 6.79. However, with the addition of 0.6mg/mL or 2.4mg/mL eDNA, the pH fell to 6.55 and 5.42 respectively (Fig 4.6A). When 2.4mg/mL of eDNA was added to HEPES buffer, which was used for the NPN assay, the pH fell even further to 4.40. To examine the role of pH on *P. aeruginosa* susceptibility to colistin, time-kill experiments were performed with *P. aeruginosa* PA14. The role of pH was examined in two ways: firstly, SCFM-1 containing 2.4mg/mL eDNA was neutralised with NaHCO₃ (33mM) (Fig 4.6B), which is the main physiological buffer in the lung. Secondly, SCFM-1 was acidified in the absence of eDNA to pH 5.5 to establish whether this reproduced the phenotype seen with eDNA (Fig 4.6C). *P. aeruginosa* survival was then measured over time under these conditions in the presence of 8µg/mL colistin.

The addition of NaHCO₃ largely ablated the effect of eDNA during the first two hours of the assay, compared to SCFM-1 without eDNA (Fig 4.6B). However, bacteria were still able to recover from colistin exposure faster than in SCFM-1 alone (Fig 4.6B). Acidification of SCFM-1 in the absence of eDNA, was sufficient to abrogate killing to the same extent as the addition of 2.4mg/mL extracellular DNA against 8µg/mL colistin (Fig 4.6C). As such, the acidification of SCFM-1 by eDNA appeared to make an important contribution to the reduction in polymyxin susceptibility.

To determine whether pH was sufficient to explain the membrane disruption observed in the presence of eDNA, NPN fluorescence was quantified in the presence of eDNA in HEPES, or in HEPES adjusted to pH 4.4 with HCl (Fig 4.6D). Lowering the pH with HCl resulted in increased NPN fluorescence to a greater extent than that seen with 1.2mg/mL eDNA, but not to the same extent as was seen with 2.4mg/mL eDNA. Together these results supported the hypothesis that acidification due to the presence of eDNA contributes to membrane damage and subsequent colistin resistance but did not explain the full extent of resistance observed.

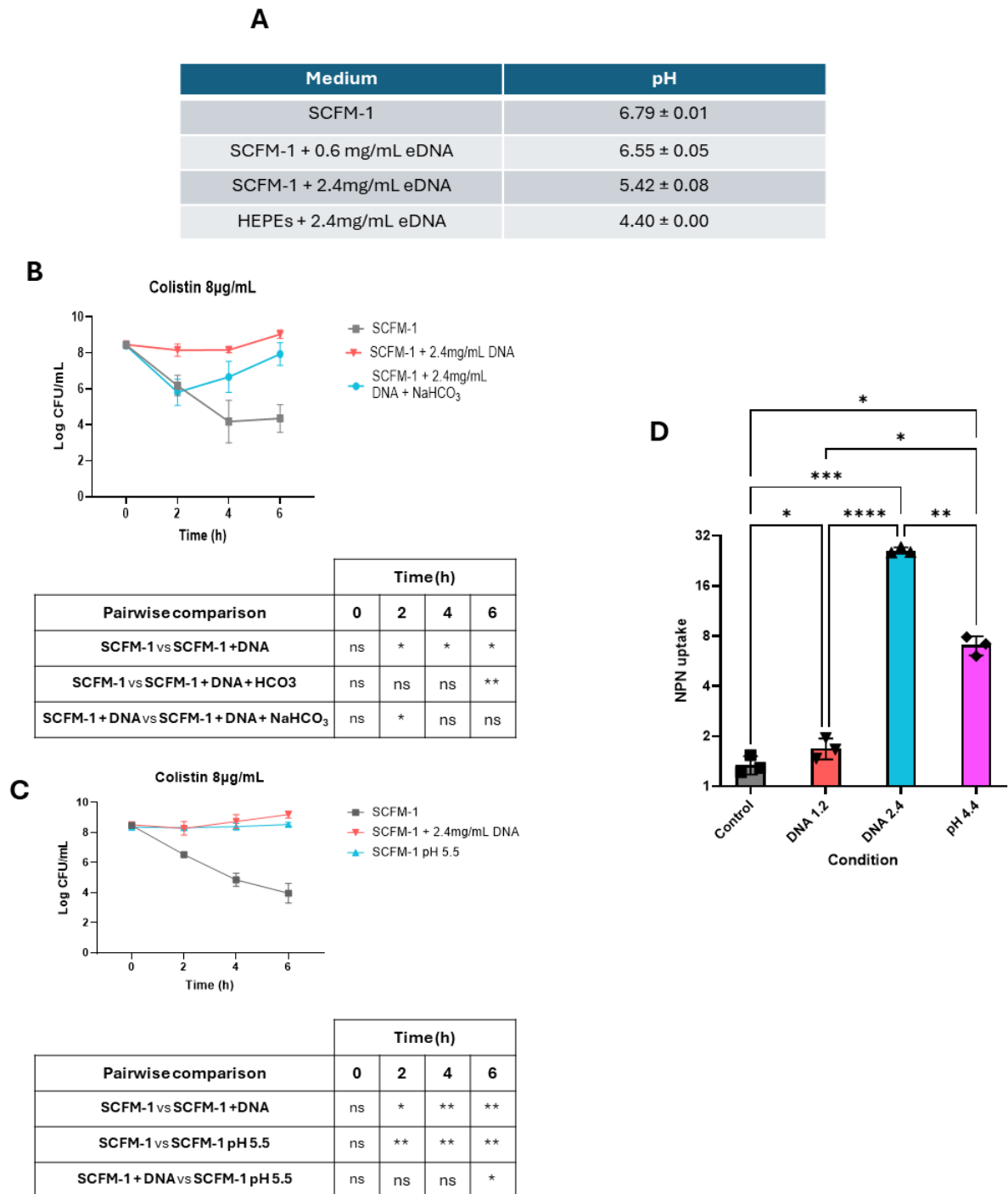


Figure 4.6: Media acidification contributes to membrane damage and colistin resistance. The pH of experimental media used in this section is summarised in table (A). Values are the mean of 3 independent experiments with the standard deviation shown. Stationary phase cultures of *P. aeruginosa* PA14 were exposed to 8 µg/mL (B and C) of colistin in SCFM-1 or SCFM-1 supplemented with 2.4mg/mL eDNA. NaHCO₃ was added to restore the pH of SCFM-1 with DNA to 6.8 (B) or SCFM-1 was acidified to pH 5.5 (C) to mimic the addition of DNA. Bacterial survival was measured over time. Data points represent the mean of 3 independent experiments. Error bars represent the standard deviation. Data were analysed by 2-way ANOVA with Tukey's post hoc test. Pairwise comparisons are summarised in the tables below each graph. NPN was used to assess outer membrane permeability (D). Stationary phase bacteria challenged with DNA at indicated concentrations (mg/mL) or HEPES buffer acidified to pH 4.4. HEPES alone was the negative control. Bars represent the mean of 3 independent experiments with 3 technical replicates. Error bars represent the standard deviation. Data were analysed by 1-way ANOVA with Tukey's post hoc test. *P<0.05 **P<0.01 *** P<0.001 ****P<0.0001.

4.2.6 eDNA triggers LPS modifications

Colistin resistance in *P. aeruginosa* arises due to covalent modification of the terminal phosphate groups of lipid A. To investigate whether eDNA gives rise to colistin resistance by triggering LPS modification, negative-ion matrix-assisted laser-desorption time of flight spectroscopy (MALDI-ToF) was used to determine the structure of LPS expressed by bacteria exposed to eDNA (403).

P. aeruginosa PA14 was grown in SCFM-1 in the presence or absence of 2.4mg/mL eDNA. Bacteria were analysed at 2 h (Fig. 4.7A and B) and 6 h (C and D) in the presence (B and D) or absence (A and C) of eDNA in SCFM-1, since these were the most relevant conditions (peak bacterial killing/increased growth). Representative traces are shown in Fig 4.7 and reveal the presence of various lipid A variants. Since the absolute abundance of each peak varied between different measurements, the area under each peak, corresponding to each lipid A species, was normalised for each condition, with reference to the most abundant peak at baseline m/z 1447 representing penta-acylated lipid A (Fig 4.8A). After 2 h growth in SCFM-1, either with or without eDNA, hexa-acylated lipid A (m/z 1617) was the most abundant lipid A species (Fig 4.8B). In *P. aeruginosa*, lipid A is synthesised as a hexa-acylated form and then converted to the penta-acylated form by the action of the lipid A de-acylase PagL (424,457) (Fig 4.8D). At baseline there were no differences between PA14 grown in the presence or absence of eDNA (Fig 4.8A). Following 2 hours of growth in the presence of eDNA (Fig 4.8B), there was a relative increase in lipid A with a m/z of 1819, corresponding to hexa-acylated lipid A modified with an additional phosphate and pEtN compared to SCFM-1 (Fig 4.8B). By six hours, the relative abundance of m/z 1617 hexa-acylated lipid A (Fig 4.8C) had decreased regardless of the presence of eDNA. However, the presence of eDNA resulted in a relative increase in hexa-acylated tri-phosphorylated pEtN modified lipid A (m/z 1819) compared to SCFM-1 (Fig 4.8C). Additionally, there was also an increase in tri-phosphorylated penta-acylated lipid A at m/z 1649 (Fig 4.8C) likely arising from the activity of PagL (424). Lastly eDNA resulted in an increase in penta-acylated mono-phosphorylated lipid A m/z 1367 (Fig 4.8C). In summary, eDNA triggers changes to lipid A that could feasibly confer polymyxin resistance, particularly the addition of the pEtN moiety to lipid A.

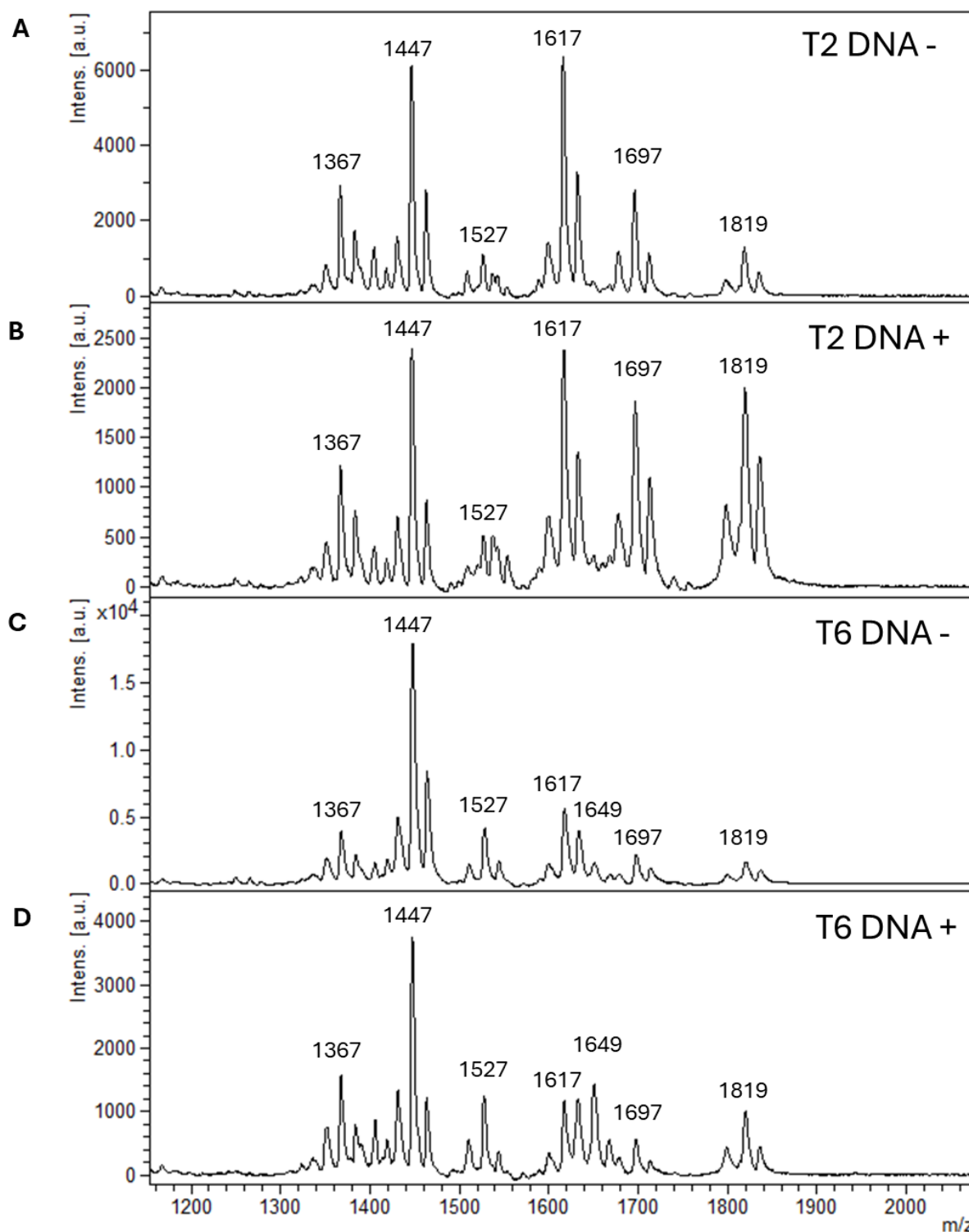


Figure 4.7: Representative MALDI-ToF traces. Stationary phase *P. aeruginosa* was grown in SCFM-1 alone (**A** and **C**) or SCFM-1 supplemented with 2.4mg/mL eDNA (**B** and **D**) for 6 hours. Samples were taken at 2 (**A** and **B**) and 6 hours (**C** and **D**) and analysed by MALDI-ToF. 4 representative traces are shown from a single experiment. Dominant peaks are labelled with m/z . unlabelled minor peaks represent variation in hydroxylation ($m/z \pm 16$).

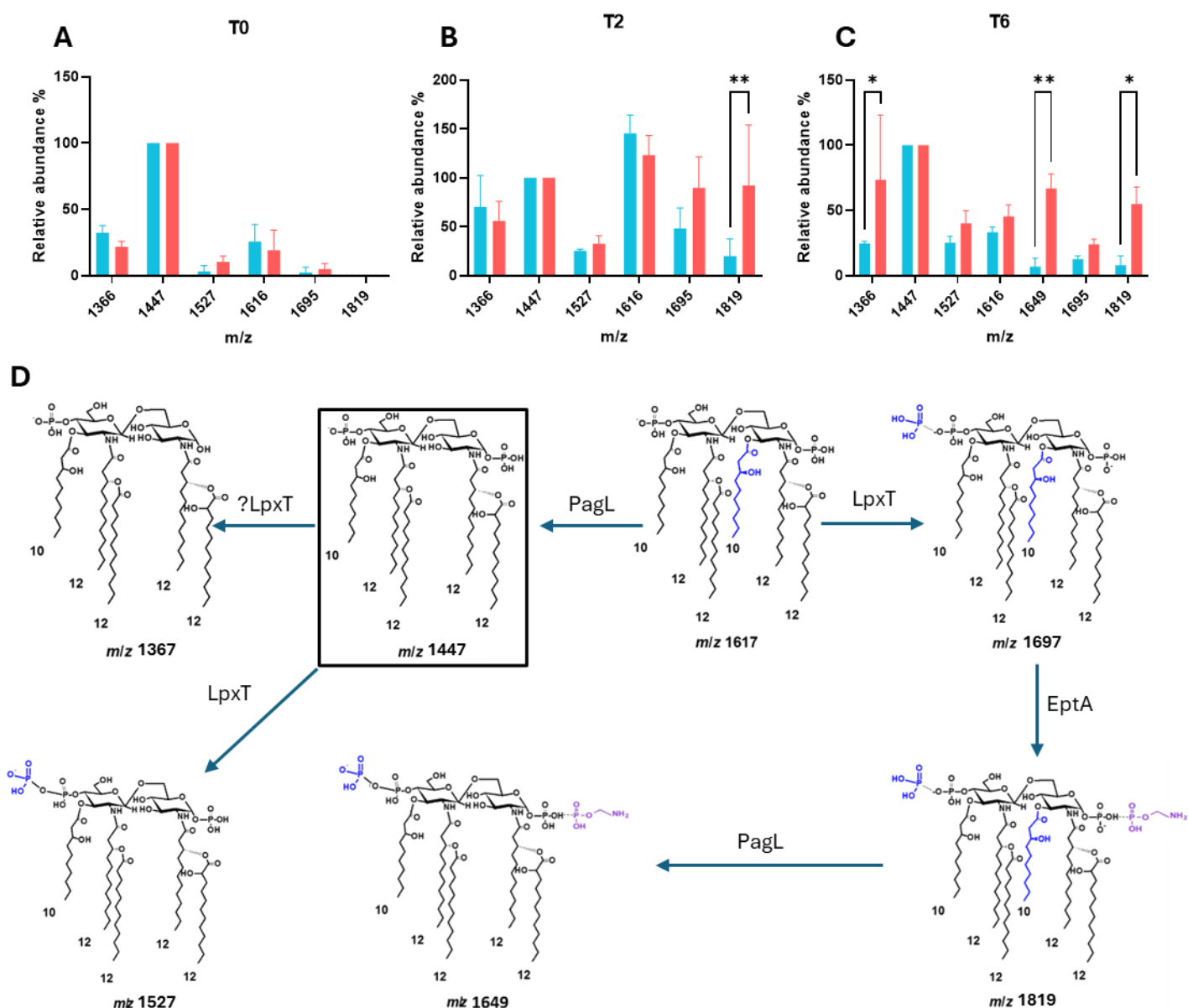


Figure 4.8: Extracellular DNA induces lipid A modifications associated with colistin resistance.

Stationary phase cultures of PA14 were exposed to SCFM-1 or SCFM-1 supplemented with 2.4mg/mL DNA. Samples were taken for MALDI-TOF analysis in negative ion mode at 0 (A) 2 (B) and 6 hours (C) of exposure. The most abundant lipid A species at each time point are shown. The area under each peak was normalised to the most prevalent lipid A species at baseline T0 [m/z 1447]. Data points represent the mean of 3 independent experiments. Error bars represent the standard deviation. Peaks in m/z were identified +/- 5. Data were analysed by 2-way ANOVA with Sidak's post-hoc test. *P<0.05 **P<0.01. Corresponding lipid A structures, their predicted mass / charge and key enzymes are shown (D): Penta-acylated mono-phosphorylated lipid A [m/z 1367]; Penta-acetylated lipid A [m/z 1447]; Hexa-acylated lipid A [m/z 1617]; Hexa-acylated tri-phosphorylated lipid A; penta-acylated mono-phosphorylated lipid A; penta-acylated tri-phosphorylated lipid A modified with phosphoethanolamine [m/z 1649]; hexa-acylated tri-phosphorylated lipid A modified with phosphoethanolamine [m/z 1819].

Next, to investigate the functional consequences of lipid A modification, a *P. aeruginosa* PA14 transposon library was used (393). Mutants defective for major pathways associated with lipid A modification were examined including those lacking functional copies of genes required for lipid A acylation: *pagL* (424) and *pagP* (175) as well as, and the lipid A phosphatase *lpxT* (458). LpxT was included due to its role in phosphorylation of lipid A which was always observed in conjunction with phosphoethanolamine (Fig 4.8). Although there was significant levels of mono-phosphorylated lipid A detected at 6 hours, the enzyme responsible for this process has not been identified in *P. aeruginosa*. Nowicki et al, when they described the activity of LpxT in *P. aeruginosa*, identified that a knock-out of *lpxT* resulted in a significant reduction of monophosphorylated lipid A (458). Therefore, it is possible that LpxT has dual kinase / phosphatase activity. A mutant lacking *eptA* was also included based on the lipidomics detection of pEtN modified lipid A. Although L-ara4N was not clearly present in the lipidomics data, it is possible that low abundance species could be missed as L-ara4N differs in m/z from pEtN by 10. As previously noted, L-ara4N is well recognised to play a major role in reducing colistin susceptibility in *P. aeruginosa*, and has reported to be induced by eDNA (300). Although L-ara4N is synthesised and applied to lipid A by the *arn* operon previous authors have successfully disrupted this process by disruption of the Undecaprenyl-phosphate 4-deoxy-4-formamido-L-arabinose transferase *arnC* (300), therefore the *arnC::Tn* mutant also examined.

Initially, the colistin MICs for all transposon mutants were determined by broth microdilution. To assess whether any of the mutants demonstrated altered susceptibility to colistin in the absence of eDNA, MICs were first measured in SCFM-1 (Fig 4.9A). None of the mutants demonstrated a different MIC from the *P. aeruginosa* PA14 (Fig 9A). As expected, the wild-type MIC increased 8-fold in the presence of eDNA relative to SCFM-1 alone (Fig 9B). However, the MIC of the *eptA::Tn* mutant only increased 2-fold in the presence of eDNA, indicating that the pEtN modification of LPS contributes to eDNA-induced polymyxin resistance. The colistin MICs of the *arnC::Tn* and *pagP::Tn* mutants increased 4-fold, suggesting a minor contribution to eDNA induced polymyxin resistance. The *pagL::Tn* mutant had the same MIC as the wild type in the presence of eDNA, whereas the *lpxT::Tn* mutant had MICs that were at least 4-fold higher than wild type.

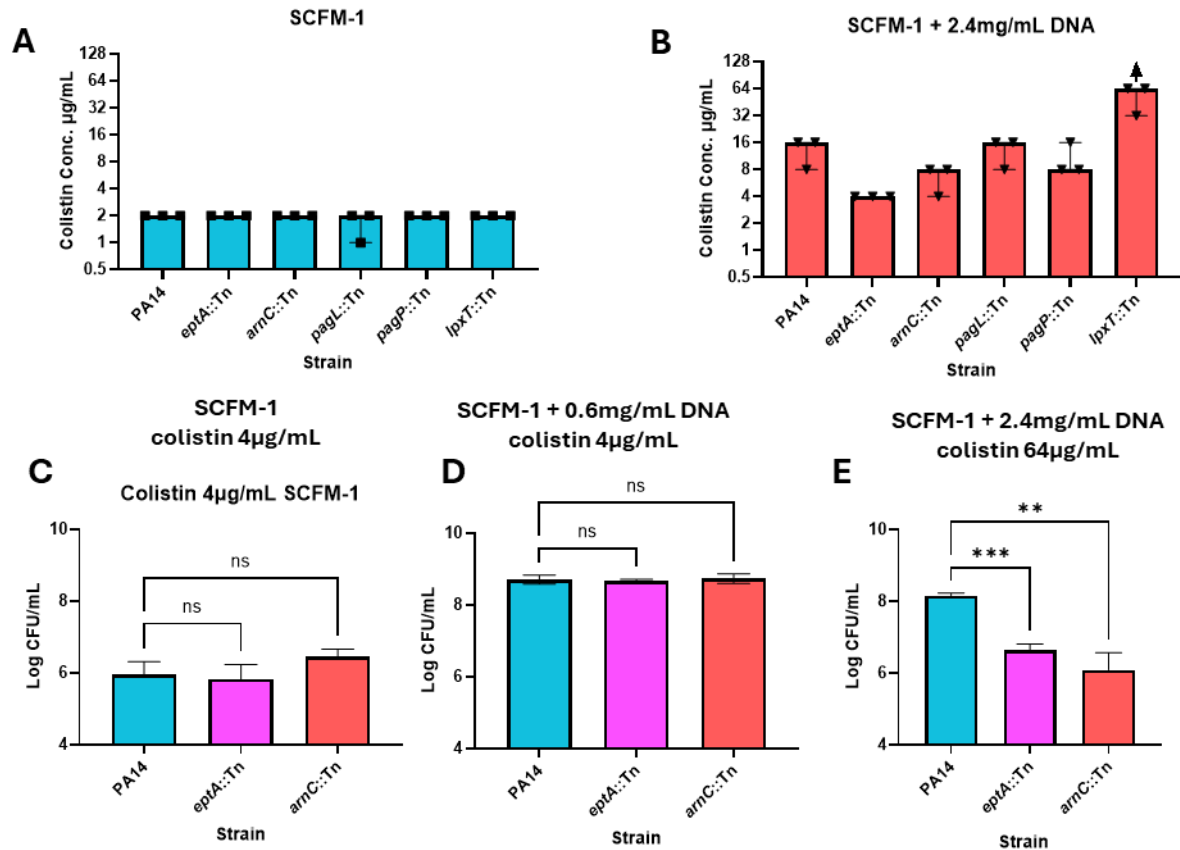


Figure 4.9: *eptA* and *arnC* are required for high level resistance to colistin in the presence of eDNA. MICs to colistin were determined by broth microdilution for *P. aeruginosa* strains PA14 and PA14 transposon mutants in key lipid A modifying enzymes in either SCFM-1 (A) or SCFM-1 supplemented with 2.4 mg/mL eDNA (B). Bacteria were incubated at 37°C for 20 hours. Growth was determined by measuring optical density at 600nm. Data points represent the result of 3 independent experiments with coloured bars indicating the median and error bars indicating the range. Arrows indicated where the MIC was above the range tested (0.05-64µg/mL). Stationary phase PA14, *eptA::Tn* and *arnC::Tn* were exposed to colistin at indicated concentrations in SCFM-1 (C) or SCFM-1 supplemented with 0.6mg/mL (D) or 2.4 mg/mL eDNA (E). Bacterial survival was enumerated at 4 hours. Bars represent the mean of 4 independent experiments. Data were analysed by one-way ANOVA with Dunnett's post hoc test *P<0.05 **P<0.01 ***P<0.001.

Having demonstrated that *eptA::Tn* and *arnC::Tn* demonstrated lower MICs to colistin relative to PA14 in the presence of eDNA susceptibility was also examined by time-kill assay to confirm that the lower MIC was associated with increased killing over time. In keeping with the MIC data, there was no difference in susceptibility between wild type and mutants to 4 µg/mL colistin in SCFM-1 without eDNA at 4 hours (Fig 4.9C).

Likewise, there was no difference in susceptibility observed in the presence of 0.6 mg/mL eDNA at 4 µg/mL (Fig 4.9D). Hypothesising that there may be redundancy between the *arn* and *eptA* systems both the DNA and colistin concentrations were increased. When *P. aeruginosa* wild-type and mutants were incubated in SCFM-1 containing 2.4 mg/mL eDNA together with colistin (64 µg/mL), though the wild type strain survived at a high level, viability of both the *eptA::Tn* and *arnC::Tn* mutants was decreased ~50-fold relative to the inoculum (10^8 CFU/mL).

Given the previously very limited recognition of a role for pEtN LPS modification to polymyxin resistance in *P. aeruginosa* (243,249), the *eptA::Tn* mutant was complemented with a wild-type copy of the gene, to confirm its role, using the miniCTX-1 system. *eptA* was cloned, along with its endogenous promoter, by amplifying both the gene and a 500bp upstream region. Gibson assembly was used to incorporate the cloned fragment into the miniCTX-1 vector. MiniCTX-1 was used as firstly, it is competent for conjugation and therefore improves transformation efficiency in *P. aeruginosa*. Secondly, miniCTX-1 stably incorporates cloned fragment into a target region of the *P. aeruginosa* genome, resulting in a stable single gene copy complementation (51). Successful assembly of the construct was confirmed by sequencing the entire plasmid miniCTX-*eptA*. The annotated sequence alignment is demonstrated in Fig 4.10A. Plasmids were transformed into the conjugation competent *E. coli* strain CC118λPIR by heat shock, then conjugation with the helper strain *E. coli* 1047 containing pRK2013 plasmid was used to transform both the empty miniCTX-1 alongside miniCTX-1-*eptA* into the *eptA::Tn* background (394). The presence of the 997bp MAR2xT7 transposon in the native *eptA* gene was confirmed by PCR (Fig 4.10B) Successful incorporation of the plasmid was confirmed by tetracycline resistance. As before, wild type *P. aeruginosa* survived at a high level but CFU counts of the *eptA::Tn* mutant were ~50-fold reduced. Complementation of the *eptA::Tn* mutant restored survival to wild type levels, whereas the miniCTX-1 vector without *eptA* had no effect on survival.

The data presented in this section supports the existing literature that eDNA can reduce colistin susceptibility by triggering the *arn* operon (300). However, this section also provides strong evidence that under conditions mimicking the CF lung, eDNA also induces *eptA* to modify lipid A with pEtN which plays a previously unrecognised role in reducing colistin susceptibility in *P. aeruginosa*. In the next section the two-component systems responsible for activating both *arn* and *eptA* were examined.

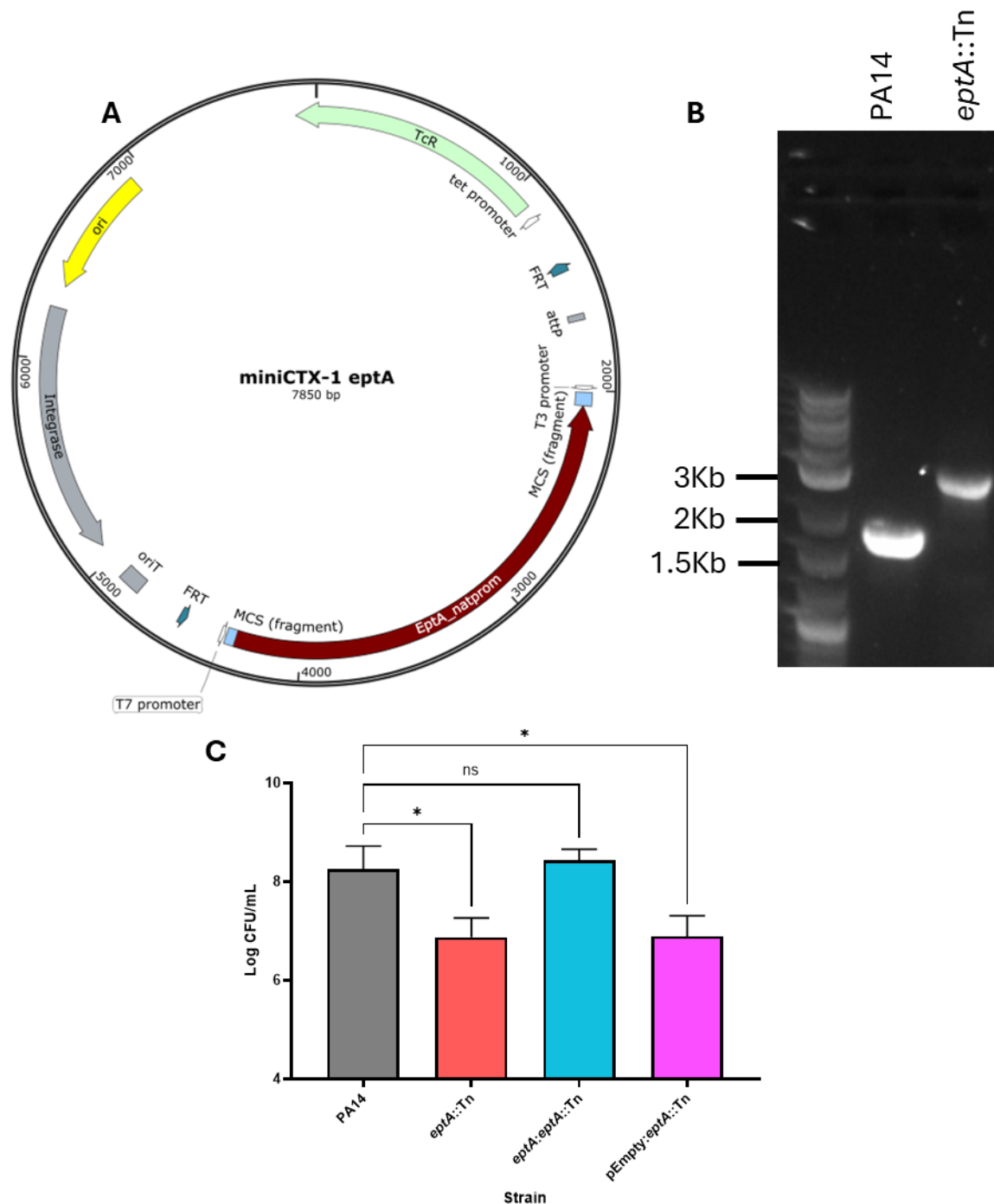


Figure 4.10: Complementation of *eptA* reduces susceptibility to colistin in the presence of eDNA. (A) an annotated vector map generated from plasmid sequencing data for miniCTX-1-eptA containing *eptA* with its native promoter (EptA_natprom). The plasmid also contains a functional tetracycline resistance gene (TcR), origin of transfer (oriT) integrase and targeting sequence for chromosomal integration (attP). (B) Gel electrophoresis of PCR product using primers directed against the *eptA* gene confirming the presence of the MAR2xT7 transposon in *eptA*::Tn. (C) Mini-CTX1-eptA was transformed into *eptA*::Tn alongside the empty mini-CTX-1 vector. Stationary phase bacteria were exposed to 64µg/mL colistin in SCFM-1 containing 2.4mg/mL eDNA and survival was enumerated at 4 hours. Bars represent the mean of 4 independent experiments. Data were analysed by one-way ANOVA with Dunnett's post hoc test *P<0.05.

4.2.7 Multiple two-component systems contribute to DNA-induced colistin resistance

Multiple two-component systems have been associated with LPS modification in *P. aeruginosa* (summarised in table 4.1). Most activate the *arn* operon, although ColRS is a known trigger of *eptA* (249). To understand which, if any, of these systems were involved in eDNA-triggered colistin resistance, a panel of mutants was assembled from the *P. aeruginosa* PA14 transposon library (393). Mutants in both the sensor histidine-kinase and response regulator for each of the two-component systems were selected: PhoPQ, PmrAB, ColRS, CprRS and ParRS.

Section 4.2.2 demonstrated that exposure to eDNA damages the bacterial outer membrane. To ensure that changes in bacterial survival, observed with any of the transposon mutants were due to changes in susceptibility to colistin and not to direct cellular effects of eDNA alone, time-kill assays were used to compare the survival of each of the transposon mutants to PA14 in the presence of SCFM-1 supplemented with 2.4 mg/mL eDNA in the absence of colistin (Fig 4.11A). None of the mutants demonstrated reduced survival relative to PA14.

Next, time-kill assays were used to investigate susceptibility changes associated with transposon mutants in each of the identified TCSs. After 4 h incubation in SCFM-1 supplemented with 2.4mg/mL eDNA and 64µg/mL colistin *colR*::Tn, *colS*::Tn, *cprR*::Tn and *cprS*::Tn mutants had a 2-3-log decreased level of survival CFU relative to wild type bacteria, similar to that seen for the *eptA* and *arnC*::Tn mutants (Fig 4.9E; Fig 4.11B). The *parR*::Tn and *parS*::Tn mutants survived at similar levels to the wild type. There was also no survival defect of the *phoP*::Tn or *phoQ*::Tn mutants, but both *pmrA*::Tn and *pmrB*::Tn were associated with a 2-log increase in killing relative to the wild type strain (Fig 4.11C).

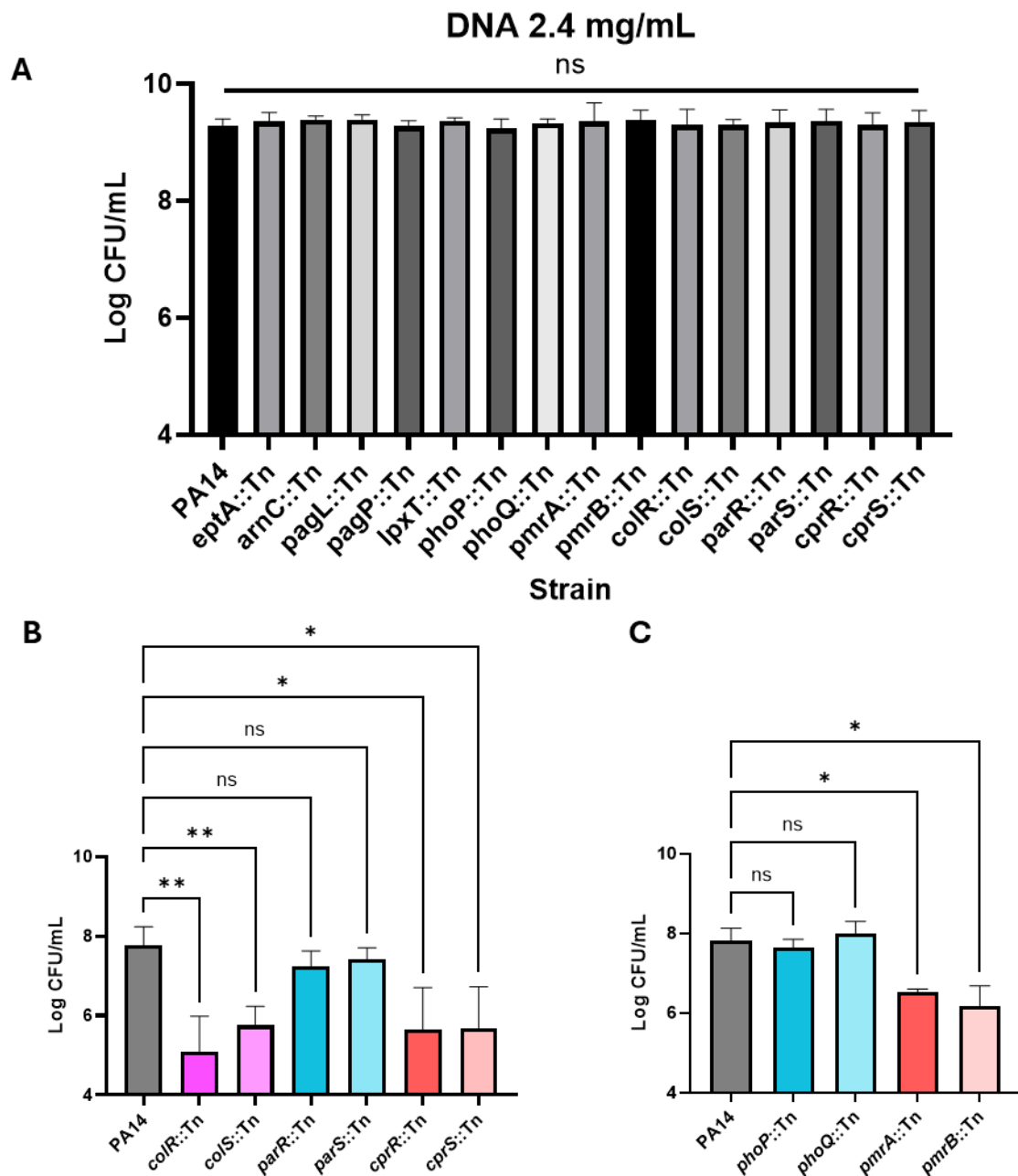


Figure 4.11: Multiple two-component systems are responsible for high level colistin resistance induced by eDNA. Stationary phase PA14 and all transposon mutants were exposed to SCFM-1 supplemented with 2.4mg/mL eDNA and survival enumerated at 4 hours (**A**). PA14 and transposon mutants for all two-component systems linked with colistin resistance were exposed to colistin at 64µg/mL in SCFM-1 supplemented with 2.4 mg/mL eDNA. Bacterial survival was enumerated at 4 hours (**B** and **C**). Bars represent the mean of 5 (**B**) or 3 (**A** and **C**) independent experiments. Data were analysed by one-way ANOVA with Dunnett's post hoc test *P<0.05 **P<0.01.

Since ColRS was required for eDNA induced polymyxin resistance (Fig 4.11B), and it has previously been demonstrated to induce *eptA* (249), it was decided to complement the *colS* mutant. Although complementation of either *colR* or *colS* would have been a viable strategy. PCR of *colS* confirmed the location of the transposon within this gene whereas PCR of *colR* resulted in 2 bands consistent with both the transposon insertion and the WT gene (Fig 4.12A), therefore the decision was made to complement *colS*::Tn alone. *colR* and *colS* are expressed as part of a 2 gene operon and therefore, for complementation, both genes and a 500bp upstream region were cloned as a single 2500bp fragment. As described previously (section 4.2.6) Gibson assembly was used to create the construct (miniCTX-1-colRS) and success was confirmed by sequencing (Fig 4.12B). As above, the *colS*::Tn mutant survived significantly less well than wild type in the presence of eDNA and colistin. Complementation of the *colS*::Tn mutant with the intact *colRS* operon restored survival to wild type levels, whereas the miniCTX-1 vector alone had no effect (Fig 4.12C).

Combined, these data reveal that multiple pathways are required for eDNA triggered polymyxin resistance, most likely via their roles in regulating pEtN and /or L-ara4N modification of LPS.

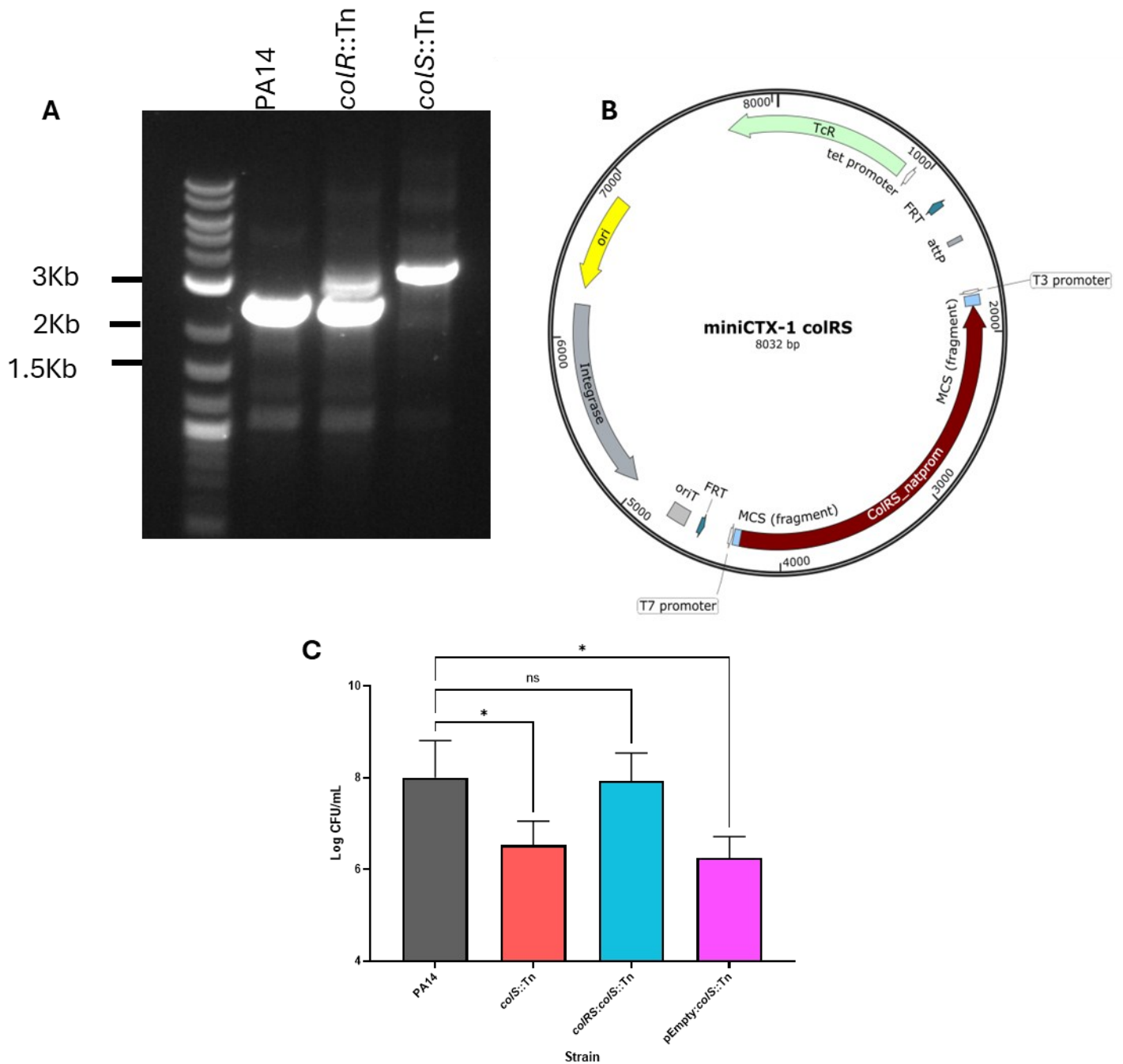


Figure 4.12: Complementation of *colRS* reduces susceptibility to colistin in the presence of eDNA. (A) Gel electrophoresis of PCR product using primers directed against the *colRS* operon confirming the presence of the MAR2xT7 transposon in *colS*::Tn but native gene in *colR*::Tn. (B) an annotated vector map generated from plasmid sequencing data for miniCTX-1-*colRS* containing the *colRS* operon with its native promoter (*ColRS_natprom*). The plasmid also contains a functional tetracycline resistance gene (*TcR*), origin of transfer (*oriT*) integrase and targeting sequence for chromosomal integration (*attP*). (C) Mini-CTX1-*colRS* was transformed into *colS*::Tn alongside the empty miniCTX-1 vector. Stationary phase bacteria were exposed to 64µg/mL colistin in SCFM-1 containing 2.4mg/mL eDNA and survival was enumerated at 4 hours. Bars represent the mean of 3 independent experiments. Data were analysed by one-way ANOVA with Dunnett's post hoc test * $P < 0.05$.

4.3 Discussion

Having established in the previous chapter that eDNA reduces colistin susceptibility in diverse CF-associated *P. aeruginosa* strains, the work in this chapter investigated the mechanism responsible. The data described in this chapter demonstrates that under conditions mimicking the CF lung eDNA resulted in modification of Lipid A with pEtN, that both *eptA* and *arnC* contribute to reduced susceptibility to colistin in the presence of eDNA and that multiple two-component systems are required for eDNA induced polymyxin resistance. Furthermore, eDNA appears to trigger polymyxin resistance, at least partly, via acidification.

Previously, eDNA was shown to reduce susceptibility to polymyxins in a synthetic sputum model by Schneider-Futschik and colleagues (410) although no work was done to elucidate the mechanism. Previous work has suggested that sputum macromolecules can reduce polymyxin activity by sequestering the lipopeptide (427). However, whilst reduced activity was observed this for tobramycin in the presence of eDNA, there was no effect on colistin activity in our assay. This led to the hypothesis that eDNA triggered changes to bacterial physiology that reduced susceptibility to colistin.

In *P. aeruginosa* modification of lipid A with L-ara4N is thought to be central to the development of colistin resistance (173,243,425). The work presented in this chapter is the first strong evidence that, in a more physiologically relevant model system, eDNA can trigger colistin resistance via lipid A modification with pEtN, as a result of *eptA* induction, in addition to L-ara4N. Covalent modification of lipid A with pEtN has been linked to colistin resistance in other Gram negative bacteria and can either be chromosomally encoded (*eptA*) or acquired through horizontal gene transfer through the MCR genes (459). Both endogenous (*eptA*) and exogenous (Mcr-1) phosphoethanolamine transferases were found to result in reduced colistin susceptibility in *P. aeruginosa* when expressed on a plasmid under an IPTG promoter (250). However, this is the first report of eDNA induced *eptA* expression in *P. aeruginosa*.

Mulcahy and colleagues demonstrated that eDNA, in standard laboratory media or minimal media, led to induction of the *arn* operon and decreased susceptibility to polymyxins as determined by MIC and minimum biofilm eradication concentration (MBEC) assays (300). Expression of an *arn* reporter could be abrogated by the addition of divalent cations, leading to the conclusion that eDNA acted by chelating cations and thus triggering expression of *arn* (300). However, the relationship between DNA and divalent cations is complex and highly dependent on the local pH and availability of other cations (460–462). For example, Gao et al demonstrated that the concentration of divalent cations required to neutralise the charge on λ phage DNA, and

trigger condensation, as observed by atomic force microscopy, was decreased from 100mM to 3mM when the pH was shifted from 8 to 3 (461). The behaviour of eDNA is likely to be altered in complex media relative to minimal media. The data in this chapter supports the conclusion that eDNA can induce the *arn* operon, having demonstrated both a reduction in colistin MIC and reduced survival of *P. aeruginosa* in the absence of *arnC* in the presence of eDNA. However, the data in this chapter also indicates the interaction between *P. aeruginosa* and eDNA is likely to be more complex than cation sequestration alone in complex media.

Arn can be triggered by multiple two-component systems in *P. aeruginosa*. PhoPQ and PmrAB are perhaps the best described although controversy exists as to the independence of these two systems in *P. aeruginosa*. Both have been reported to respond to low Mg^{2+} (58,244,451) and hypothesised to respond to low pH, although the evidence supporting this is less clear (164). Wilton and colleagues demonstrated that low pH could induce downstream effectors of both PmrAB and PhoPQ (164) but both were suppressed by the addition of excess Mg^{2+} . Additionally, the induction of PhoPQ dependent genes was much lower than that of PmrAB dependent genes. This is in keeping with the finding of this work: that PmrAB was of greater importance for colistin resistance in the presence of eDNA than PhoPQ.

In addition to PhoPQ and PmrAB ParRS and CprRS were also examined. Both have been linked to *arn* induction but are less well described than PmrAB and PhoPQ. Both ParRS and CprRS appear to respond to the presence of cationic antimicrobial peptides (247,447,463). Fernandez found that a preference for ParRS or CprRS could be peptide specific or concentration specific (247). Thus, although a role for CprRS in survival at 64µg/mL of colistin was identified in this chapter, it does not preclude a role for ParRS at lower concentrations. Furthermore, Gutu et al identified that, in a Δ phoQ background which constitutively activates *arn* through PhoP, loss of CprRS resulted in increased susceptibility to polymyxin B, supporting the hypothesis that multiple two-component systems are required for high-level resistance to polymyxins (248). Interestingly Gutu also identified synergy between PhoPQ and ColRS and hypothesised that ColRS activated additional downstream effectors other than *arn*.

The ColRS two-component system is able to trigger expression of *eptA* in response to transition metals (249,448,449). Nowicki and colleagues linked ColRS to *eptA* induction in the presence of excess Zinc but stated that this did not result in colistin resistance, although data supporting this assertion was not shown (249). The data presented in this chapter strongly refute that supposition by linking both ColRS and EptA to reduced colistin susceptibility in the presence of eDNA. Wiesmann et al identified that ColRS was essential for surviving exposure to

excess iron particularly at low pH in *P. aeruginosa* (449) which could be relevant to this model as SCFM-1 contains 3.6 μ M of FeSO₄. In *S. enterica* EptA also been linked to iron tolerance (464,465). It is possible that the combination of low pH and media acidification by eDNA are the trigger for ColRS in SCFM although future work will be required to investigate the role of FeSO₄ in this process. Additionally, it is possible that pH links activation of both PmrAB and ColRS to induce high level colistin resistance through both *arn* and *eptA*. The data in this chapter has shown that SCFM-1 at pH 5.5 can mimic the addition of 2.4mg/mL eDNA and that neutralisation of eDNA can partially abrogate its effect on colistin susceptibility.

The results of lipidomics presented here strongly support a role for pEtN but provide little evidence of L-ara4N despite the decreased survival of *arnC::Tn*. A possible explanation for this is the relative importance of LPS modification at the inner membrane compared to the outer membrane. Sabnis et al established that, for bacterial killing by polymyxins, LPS at the inner membrane was the primary target (227). Indeed, expression of MCR-1 did not protect the outer membrane from permeabilisation by colistin (227). Penta-acylated lipid A modified with an additional C16 (through the action of PagP) and L-ara4N has a predicted m/z of 1816. LPS at the inner membrane makes up a small proportion of total bacterial LPS and therefore L-ara4N modification may not be detected in the presence of abundant pEtN modified Lipid A at m/z 1819. Unfortunately, there is no evidence of intermediaries (lipid A modified with C16 or L-ara4N alone) in this process to support this hypothesis.

In addition to conventional colistin resistance through L-ara4N and pEtN, the lipidomics data highlighted several other lipid A modifications of uncertain functional significance with respect to colistin susceptibility. pEtN modification was only found in the presence of tri-phosphorylated lipid A. Phosphorylation of lipid A is mediated by the enzyme LpxT (458). In *E. coli*, LpxT appears to be reciprocally regulated with EptA through PmrA and competes for lipid A modification sites, with loss of LpxT associated with decreased susceptibility to polymyxins (466). Interestingly, Nowicki and colleagues found that hypomagnesaemia also inhibited LpxT activity in *P. aeruginosa* although it is unclear whether this was mediated by PmrA (458). In keeping with these findings this chapter demonstrates that loss of LpxT was associated with decreased susceptibility to colistin. The increase in abundance and significance of mono-phosphorylated LPS, following eDNA exposure, is harder to explain. When they described the native LpxT in *P. aeruginosa*, Nowicki and colleagues noted an increase in mono-phosphorylated LPS in Δ *lpxT* which they attributed to an artefact of the acid hydrolysis used to prepare the samples. It is notable that there is far less mono-phosphorylated LPS in the Δ *lpxT* mutant raising the possibility of dual phosphatase / kinase activity. It may also be that, if the

terminal phosphate is prone to hydrolysis at low pH, acidification by eDNA itself may result in phosphate loss and therefore upregulation of LpxT, as evidenced by the increase in tri-phosphorylated Lipid A, may represent a physiological response to acidic conditions. Finally, other Gram-negative bacteria including *H. pylori* (467), possess phosphatases which specifically modify LPS and are linked with resistance to cationic antimicrobial peptides, though no such enzyme has yet been identified in *P. aeruginosa*.

Under the current model of colistin activity binding of colistin to lipid A disrupts cation bridges between adjacent LPS molecules resulting in membrane permeabilisation. Whilst L-ara4N and pEtN reduce the charge on the terminal phosphates, reducing affinity for cationic antimicrobials, there is little consideration of how integrity between adjacent LPS molecules is maintained. Simulations suggest L-ara4N modified LPS molecules can bind to each other and maintain membrane integrity (468) whereas pEtN subsumes the role of the divalent cation (469). However, Cervoni et al demonstrated that whilst low level expression of *eptA* reduced membrane permeability in *P. aeruginosa*, high level expression increased permeability (250) underscoring the importance of regulated of *eptA* expression. Therefore, it is interesting to speculate that polar lipid-A structures expressing dual phosphates on one pole and pEtN help maintain membrane integrity which cannot be achieved by *eptA* alone beyond a certain point.

The impact of lipid A acylation was also investigated using two mutants *pagL*::Tn, and *pagP*::Tn. PagL catalyses the conversion of hexa-acylated lipid A to the penta-acylated form (424). Mutations are commonly seen in cystic fibrosis (424,470). Loss of PagL does not appear to alter the susceptibility of wild-type *P. aeruginosa* to polymyxin B but could increase susceptibility of *pmrB* mutants constitutively expressing L-ara4N (471). I found no difference in MIC in the *pagL*::Tn mutant though time-kill assays were not performed. The palmitoyltransferase PagP is also responsible for the addition of C16 acyl chains to lipid A and was associated with resistance to the CAMP C18G but not polymyxins in a $\Delta phoQ$ background (175). A small (2-fold), variable shift in colistin MIC was found in the presence of 2.4mg/mL of eDNA the significance of this finding is unclear. The work presented in this chapter provides little evidence for the role of acylation of lipid A in colistin susceptibility though a more detailed examination utilising time-kill assays would provide more sensitive data.

Overall, this chapter makes a significant contribution to our understanding of how eDNA in sputum triggers colistin resistance by demonstrating a central role for ColRS and EptA and supporting previous evidence that PmrAB and the *arn* operon likely play a role. This work also

raises the possibility that several other eDNA induced changes to lipid A modulate polymyxin susceptibility, including phosphorylation by LpxT.

These findings, together with the literature help to build a model of how the CF lung environment modulates bacterial signalling systems and physiology to change antibiotic susceptibility: PmrAB and ColRS are activated by acidification and magnesium sequestration (in the case of PmrAB) or iron toxicity exacerbated by that pH (in the case of ColRS) leading to the expression of both the *arn* operon and *eptA* and extensive modification of LPS. CprRS is also activated, likely through colistin itself and contributes to high level colistin resistance (summarised in Fig 4.13). These observations provide novel targets for therapeutic interventions aimed at preventing or reducing the impact of the CF environment on antibiotic susceptibility, which will be explored in the next chapter.

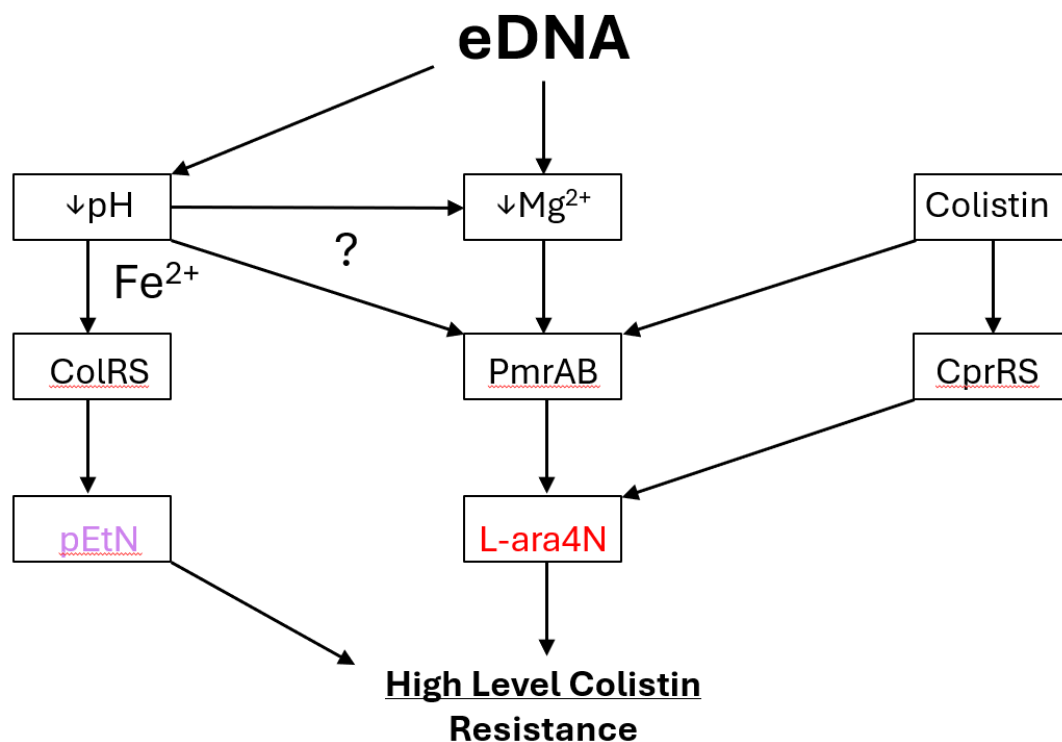


Figure 4.13: Proposed model for how eDNA reduces colistin susceptibility of eDNA in sputum. Extracellular DNA reduces free Mg^{2+} and reduces sputum pH triggering the PmrAB two-component system (TCS) which in turn triggers L-ara4N modification of LPS. Reduced pH also increases the susceptibility to Fe^{2+} which is sensed by the ColRS TCS and triggers pEtN modification of LPS. Reduced susceptibility to colistin allows survival of initial colistin exposure and sensing of colistin by the CprRS system, triggering further L-ara4N modification resulting in high level colistin resistance. Uncertainty remains on the effect of pH on the ability of eDNA to bind Mg^{2+} in the cation rich environment of the lung.

Chapter 5: Developing Strategies to increase colistin susceptibility in synthetic sputum

5.1 Introduction

In the third chapter, eDNA was identified as an important component of sputum that reduced susceptibility of *P. aeruginosa* to colistin. In the fourth chapter, multiple two-component systems were found to be required for LPS modification as a mechanism of resistance, with pH a putative environmental trigger. Based on this new insight, the aim of this chapter was to develop new therapeutic approaches to restore the antibacterial activity of colistin in synthetic sputum based on the pathways involved.

5.1.1 Targeting eDNA

Estimates of eDNA content of CF sputum vary from around 0.25 mg/mL (292,293) to 5 mg/mL (294,295) and will vary with progression of the disease and/or severity of disease (293,472). Purification and analysis of eDNA from CF sputum has confirmed that it is mostly of human origin (94,452). Since mucus viscosity is increased with length and abundance of eDNA, which impairs clearance (473), inhaled recombinant human DNase (rhDNase) was developed as a mucolytic. DNase use is associated with improvement in lung function in the short term, a reduction in pulmonary exacerbations in the longer term and is associated with a reduction in proinflammatory cytokines (177,474). Data on the effect of DNase treatment on microbial physiology and susceptibility to antibiotics is limited, however. Hunt et al. found that treatment of sputum with rhDNase reduced the activity of cationic tobramycin (289), whilst Halverson et al. identified that DNase treatment could abrogate the cytotoxic effect of DNA on bacteria (475). Since eDNA triggered colistin resistance, the first aim of this chapter was to determine the impact of DNase on colistin susceptibility of *P. aeruginosa* in the context of synthetic sputum.

5.1.2 Targeting pH homeostasis

Acid-base homeostasis is perturbed in cystic fibrosis, with mutations in the CFTR gene directly resulting in an inability to concentrate HCO_3^- in the extracellular space (476). This in turn contributes to airway acidification in the CF lung and impaired mucus hydration (477,478) reducing clearance of secretions. Acidification of CF sputum also contributes to innate immune dysfunction by reducing the activity of the endogenous antimicrobial peptides (479) and compromising neutrophil function (480). The addition of NaHCO_3 appears to restore these functions (476). Furthermore, adjunctive NaHCO_3 enhances killing by aminoglycosides as well as

exerting directly antibacterial effects and impairing biofilm formation *in vitro* (481–483). Several studies have examined NaHCO₃ as an adjunctive therapy in CF, with Garcia and colleagues showing that inhaled NaHCO₃ was well tolerated by human subjects in a small cohort (484). Therefore, the second aim of this chapter was to determine whether NaHCO₃ can neutralise eDNA-induced acidification and thereby restore antimicrobial susceptibility in *P. aeruginosa* in the context of synthetic sputum.

5.1.3 Targeting LPS transport with murepavadin

LPS transport and synthesis are promising targets for antibiotic development as they are conserved processes that only occur in Gram-negative bacteria. Murepavadin is a first in class LptD inhibitor with a narrow spectrum of activity targeted at *Pseudomonas* sp. (485). The Lpt complex transports LPS from the inner membrane (IM) to the outer membrane (486). Unfortunately, clinical trials of intravenous murepavadin were abandoned due to concerns around renal toxicity (487). However, murepavadin has been developed into an inhaled formulation, making it a potential therapy for CF, with a clinical trial due to be reported (488).

Sabnis et al demonstrated synergy between colistin and murepavadin and hypothesised this was due to murepavadin induced LPS accumulation at the inner membrane providing more targets for colistin (227). Murepavadin also synergises with aminoglycosides (489). Alone, murepavadin seems to be extremely effective against *P. aeruginosa*, even demonstrating activity against XDR isolates (490). However, the function of murepavadin in a sputum model has not yet been explored. It was hypothesised that murepavadin may be able to resensitise *P. aeruginosa* to colistin in the presence of eDNA by increasing the pool of unmodified LPS in the IM. The final aim of this chapter was to test this hypothesis using a panel of clinical isolates.

5.2 Results

5.2.1 DNase treatment does not restore susceptibility to colistin

Pulmozyme, a currently used brand of rhDNase was obtained from the Royal Brompton Hospital to mimic as closely as possible the activity of DNase in human lung. To test whether direct treatment with pulmozyme was sufficient to restore colistin susceptibility, SCFM-1 supplemented with 0.6 mg/mL eDNA and 4 µg/mL colistin was pre-treated with 3 µg/mL pulmozyme for 1 hour before bacteria were added, and survival measured over time. The concentration of pulmozyme used was based on the peak sputum concentration 15 minutes after inhalation of a standard dose (491). To control for off-target effects, pulmozyme was also supplemented into SCFM-1 alone. Since magnesium is an important co-factor of DNase I (492,493), and hypomagnesaemia has been associated with a clinical failure to respond to

DNase treatment (494), the impact of increasing magnesium concentration 5-fold was also investigated.

Pretreatment with pulmozyme did not alter susceptibility of *P. aeruginosa* PA14 to colistin in either SCFM-1 or SCFM-1 + DNA (Fig 5.1A). The supplementation of magnesium decreased susceptibility to colistin further but this was not affected by pulmozyme (Fig 5.1B). To ensure that the lack of effect of DNase was not due to a lack of enzymatic function, a sample of the DNase treated medium was run on an electrophoresis gel to examine digestion of eDNA. The addition of pulmozyme resulted in a visible reduction in DNA content (Fig 5.1 C). Therefore, digestion of eDNA using a clinically relevant DNase did not reduce the induction of colistin resistance in *P. aeruginosa*.

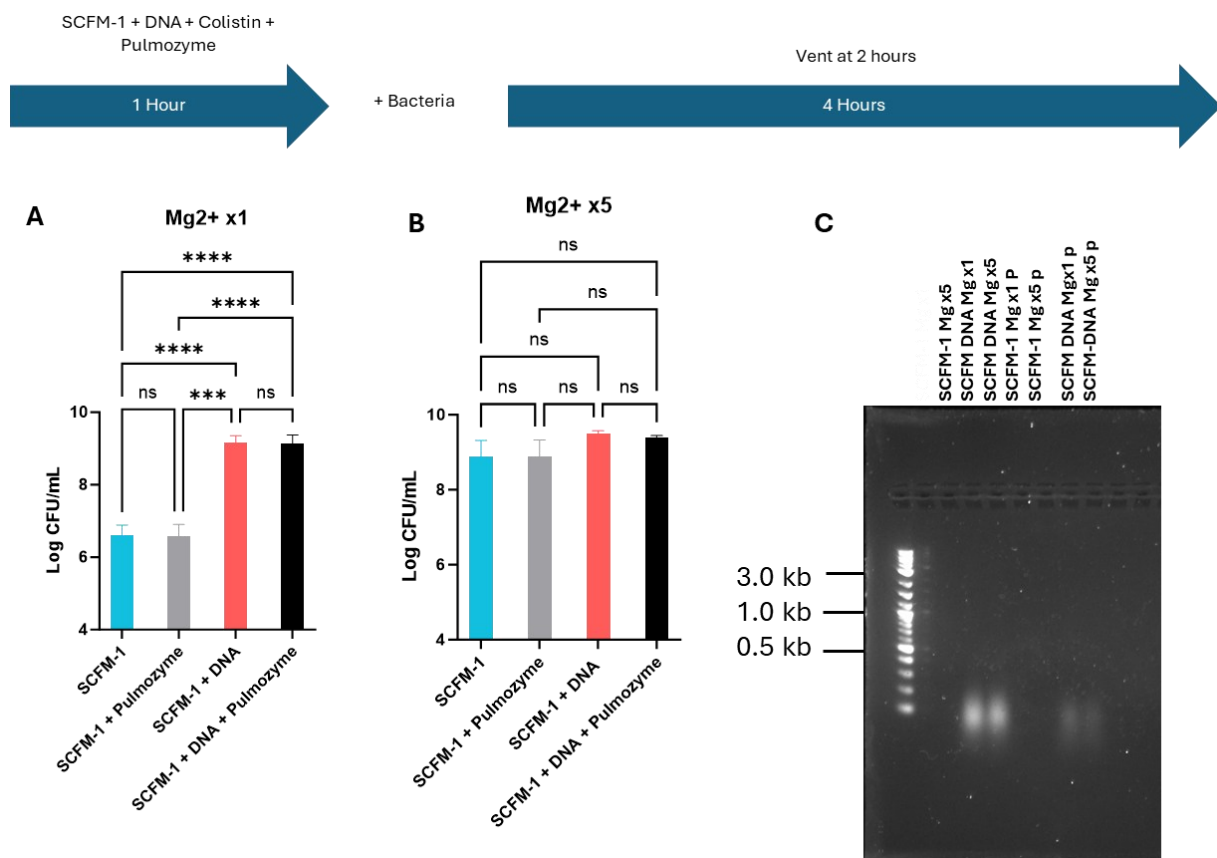


Figure 5.1: Dnase treatment does not restore susceptibility to colistin in synthetic sputum.

SCFM-1 or SCFM-1 containing 0.6mg/mL eDNA containing 4 µg/mL colistin was supplemented with rhDNase (pulmozyme) as indicated, in the presence of 1-fold (A) or 5-fold (B) magnesium and incubated for 1 hour at 37°C. DNA digestion was confirmed by agarose gel electrophoresis (C); p indicates the presence of pulmozyme. After 1 hour Stationary phase PA14 was added and bacterial survival enumerated after a further 4 hours. Bars represent the mean of 4 independent experiments. Error bars represent the standard deviation. Data were analysed by one-way ANOVA with Tukey' post hoc test *P<0.05 **P<0.01 ***P<0.001 ****P<0.0001. To reproduce the conditions of the time-kill assay tubes containing bacteria were opened at 2 hours.

5.2.2 eDNA reduces susceptibility to tobramycin

Second line inhaled therapy for *P. aeruginosa* is the aminoglycoside tobramycin (69). Given the evidence presented here supports the hypothesis that colistin is ineffective in the lung environment, examining the role of alternative antibiotics seemed prudent. In the previous chapter we saw how eDNA can bind and sequester tobramycin, restricting diffusion through agar consistent with previously published data (289). There is also evidence that lipid A modifications that confer polymyxin resistance might also convey resistance to cationic aminoglycosides (164,300). Increasing eDNA concentration resulted in increased tobramycin MICs 2-fold, 4-fold and greater than 64-fold in the presence of 0.6, 1.2 and 2.4 mg/mL eDNA respectively (Fig 5.2).

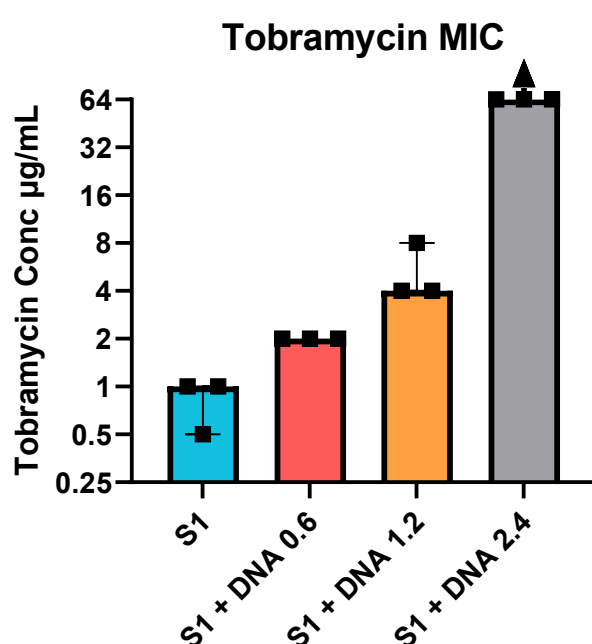


Figure 5.2: eDNA reduces *P. aeruginosa* susceptibility to Tobramycin in SCFM-1. MICs to tobramycin were determined by broth microdilution for *P. aeruginosa* strain PA14 in SCFM-1 supplemented with indicated concentrations of eDNA (mg/mL). bacteria were incubated at 37°C for 20 hours. Growth was determined by measuring optical density at 600nm. Data points represent the result of 3 independent experiments with coloured bars indicating the median and error bars indicating the range. Arrows indicated where the MIC was above the range tested (0.25-64µg/mL).

5.2.3 eDNA reduces susceptibility to murepavadin

In chapter 4, it was hypothesised that the relatively small change in MIC relative to MBC induced by eDNA in *P. aeruginosa* was due to the protective effect of LPS modifications at the IM rather than successful protection of the OM. Sabnis *et al.*, demonstrated that murepavadin treatment led to LPS accumulation at the inner membrane and so it was hypothesised here that this might also sensitise *P. aeruginosa* in the presence of eDNA by increasing the pool of unmodified LPS available for colistin to target.

First, murepavadin susceptibility of *P. aeruginosa* PA14 was determined in SCFM-1 with or without eDNA supplementation (Fig 5.3). MICs to murepavadin increased 4-fold with 0.6 and 1.2mg/mL of eDNA but were increased 16-fold by the presence of 2.4mg/mL eDNA relative to SCFM-1 alone (Fig 5.3A). When susceptibility was examined by time-kill assay 5 µg/mL of

murepavadin resulted in 2-logs of killing in 6 hours in SCFM-1 without eDNA (Fig 5.3B). There was no observable difference in survival with 0.6 mg/mL eDNA relative to SCFM-1. 1.2 mg/mL of eDNA reduced killing 10-fold at 6 hours, whilst killing was completely abrogated by 2.4mg/mL eDNA (Fig 5.3B). Together these results demonstrated a dose-dependent reduction in susceptibility to murepavadin induced by eDNA.

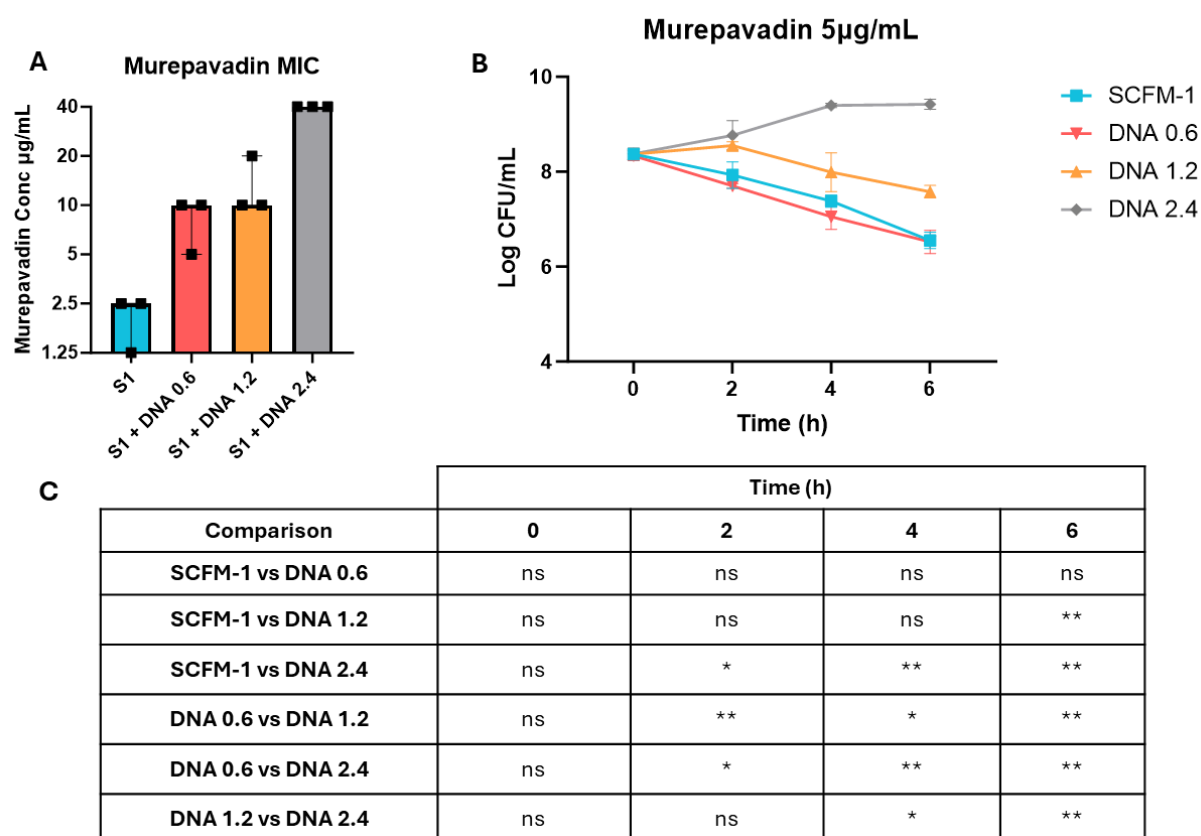


Figure 5.3: eDNA reduces *P. aeruginosa* susceptibility to murepavadin in SCFM-1. (A) MICs to murepavadin were determined by broth microdilution for *P. aeruginosa* strain PA14 in SCFM-1 supplemented with indicated concentrations of eDNA (mg/mL). bacteria were incubated at 37°C for 20 hours. Growth was determined by measuring optical density at 600nm. Data points represent the result of 3 independent experiments with coloured bars indicating the median and error bars indicating the range. (B) Stationary phase PA14 strain *P. aeruginosa* was exposed to 5µg/mL murepavadin in SCFM-1 supplemented with indicated concentrations of eDNA (mg/mL) and survival enumerated every 2 hours for 6 hours. Data points represent the mean of 4 independent experiments and error bars the standard deviation. Data from (B) were analysed by 2-way ANOVA with Tukey's post hoc test with results summarised in table (C) *P<0.05 **P<0.01.

5.2.4 The mechanism of reduced murepavadin susceptibility diverges from that of colistin.

Relatively little is known of the mechanisms of murepavadin resistance. Ghassani et al reported that mutations in *pmrB* were associated with decreased susceptibility to murepavadin, similar to colistin (495). However, the impact of sputum macromolecules on murepavadin susceptibility has not been reported at all. Based on the mechanisms of eDNA-induced LPS changes identified in the previous chapter, the susceptibility of transposon mutants in *eptA*, *arnC*, *pagL*, *pagP*, and *lpxT* to murepavadin in the presence of eDNA were examined. In contrast to colistin, there was no difference in susceptibility of *P. aeruginosa* PA14 wild type and transposon mutants in either *eptA* or *arnC* (Fig 5.4). However, susceptibility to 5 µg/mL murepavadin in the presence of 2.4mg/mL eDNA was increased by disruption of the *lpxT* gene (Fig 5.4).

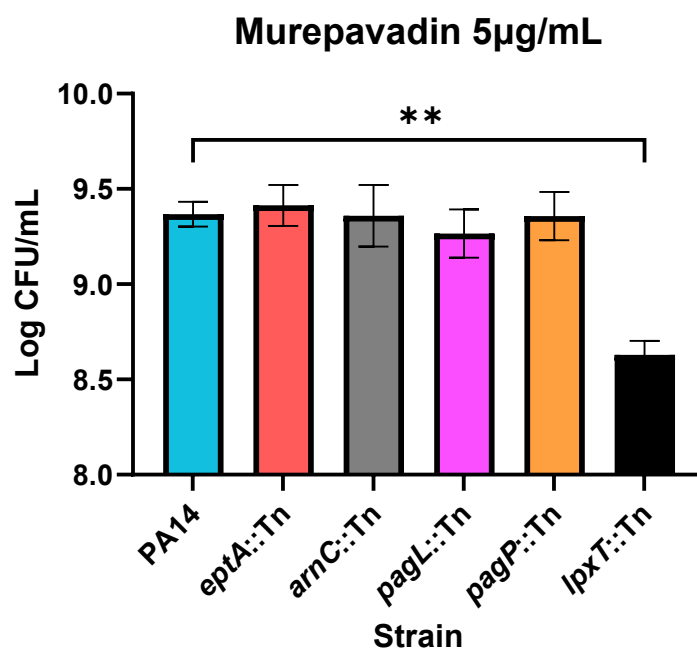


Figure 5.4: Disruption of *lpxT* and not *arnC* or *eptA* increases susceptibility to murepavadin in the presence of eDNA. Stationary phase PA14 strain *P. aeruginosa* and indicated PA14 transposon mutants were exposed to 5µg/mL murepavadin in SCFM-1 supplemented with 2.4 mg/mL eDNA and survival enumerated at 4 hours. Bars represent the mean of 4 independent experiments and error bars the standard deviation. Data were analysed by one-way ANOVA with Dunnett's post hoc test *P<0.05 **P<0.01.

5.2.5 Murepavadin synergises with colistin in the presence of eDNA

Sabnis et al demonstrated synergy between colistin and murepavadin killing when sub-inhibitory concentrations of each antibiotic were used (227). Therefore, even though killing by each compound was inhibited separately by the presence of eDNA it was decided to test whether synergy could still be achieved; both compounds were used at 2 x MIC that occurred in SCFM-1 (4 µg/mL for colistin and 5 µg/mL murepavadin). At these concentrations, when colistin and murepavadin were used in combination, killing was not more effective than colistin alone in SCFM-1 (Fig 5.5A). However, at 0.6mg/mL and 1.2mg/mL eDNA an additional 2-3 logs of killing were obtained by the combination over murepavadin alone (Fig 5.5B and C). At 2.4mg/mL eDNA killing by both compounds and the combination was completely abrogated (Fig 5.5D).

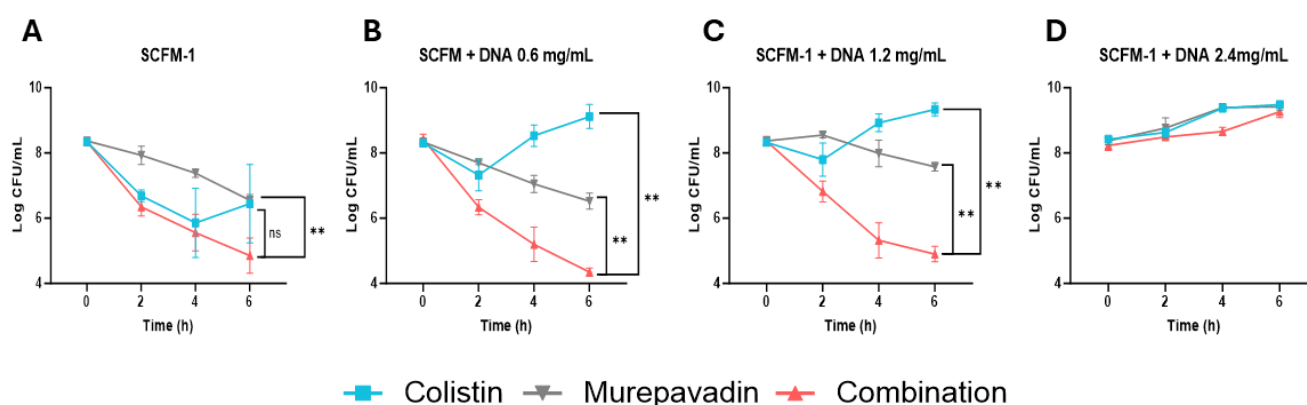


Figure 5.5: Colistin and murepavadin synergise to overcome eDNA induced resistance.

Stationary phase PA14 strain *P. aeruginosa* was exposed to colistin 4µg/mL colistin, 5µg/mL murepavadin or the combination of the two in SCFM-1 (A) or SCFM-1 supplemented with increasing concentrations of eDNA (B-D) and survival enumerated at every 2 hours for 6 hours. Data points represent the mean of 4 independent experiments and error bars the standard deviation. Data were analysed by two-way ANOVA with Dunnet's post hoc test with the combination as the reference condition *P<0.05 **P<0.01.

Next, it was investigated whether the failure of the colistin-murepavadin combination to kill in the presence of 2.4mg/mL eDNA could be overcome by increasing the concentrations of each compound individually. Consistent with the results presented in chapter 3, colistin concentrations above 8 µg/mL resulted in, at most, a single log of killing of *P. aeruginosa* PA14 by 6 hours exposure and increasing the concentration of colistin did not restore synergy with murepavadin (Fig 5.6A). Increasing the concentration of murepavadin restored *P. aeruginosa* killing at 10 µg/mL between 1 and 2-log at 6 hours but did not restore synergy. At 20 µg/mL and 40µg/mL of murepavadin, 2 to 3-logs of *P. aeruginosa* killing were achieved, although there was no significant increase associated with the addition of colistin (Fig 5.6B). At 40µg/mL of

murepavadin the presence of colistin did increase killing at 4 hours between 1 and 2-logs whilst there was a single log difference at 6 hours, although this was not statistically significant ($p=0.06$). In summary colistin and murepavadin demonstrate synergy up to 1.2mg/mL eDNA. At 2.4mg/mL eDNA, higher concentrations of murepavadin are sufficient to restore killing but synergy was only restored at the highest concentration of murepavadin examined.

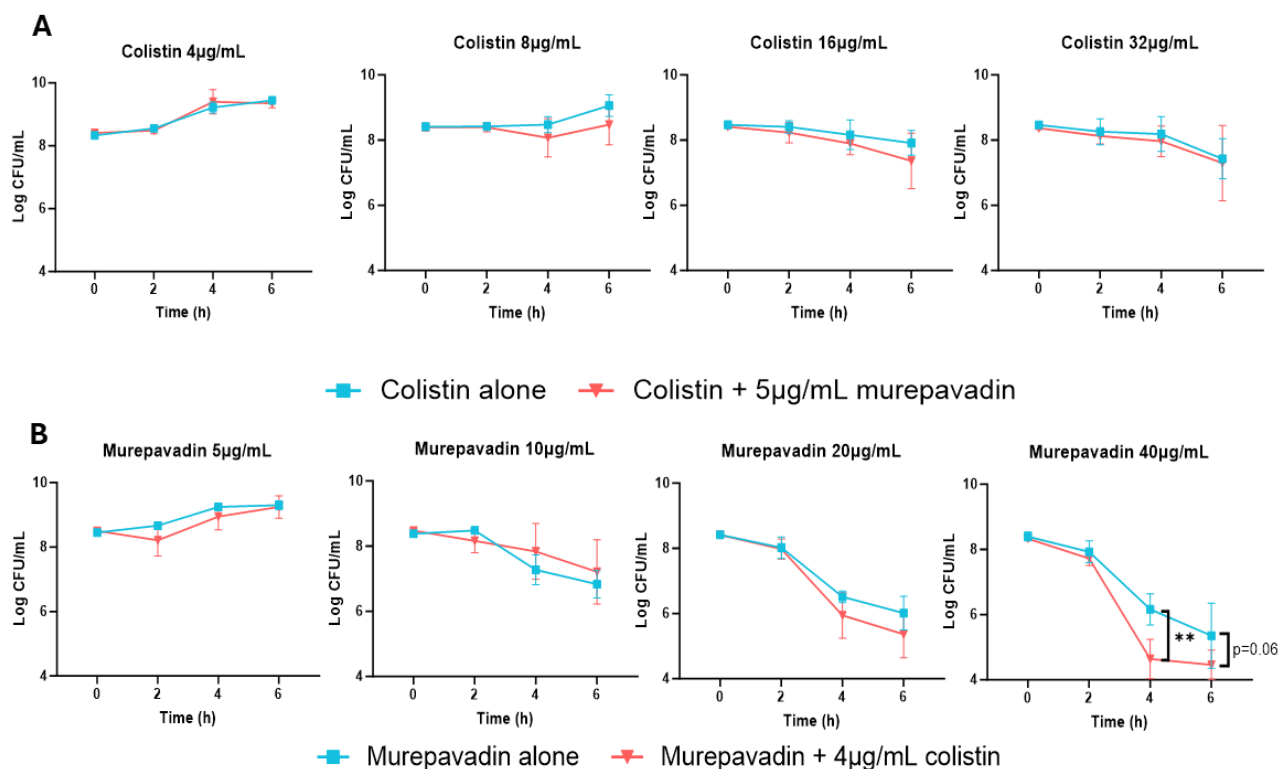


Figure 5.6: Increasing the concentration of murepavadin restores killing and synergy.

Stationary phase PA14 strain *P. aeruginosa* was exposed to indicated concentrations of murepavadin or colistin or the combination in SCFM-1 supplemented with 2.4 mg/mL eDNA. Bacterial survival was enumerated every 2 hours for 6 hours. Data points represent the mean of 4 independent experiments and error bars the standard deviation. Data were analysed by two-way ANOVA with Sidak's post hoc test * $P<0.05$ ** $P<0.01$.

5.2.5 Clinical isolates show decreased susceptibility to murepavadin in synthetic sputum

Having demonstrated that synergy between murepavadin and colistin could achieve killing in synthetic sputum, it was next examined whether this was also true for CF lung-adapted *P. aeruginosa* strains. When a panel of clinical isolates was tested for susceptibility to murepavadin in SCFM-1 without the presence of macromolecules, colistin MICs were 2-4 fold higher than PA14 for the 8 colistin susceptible strains (Fig 5.7A). The two colistin resistant strains, AK1 and AK24 had murepavadin MICs that were 8- and 16- fold higher than *P. aeruginosa* PA14 respectively.

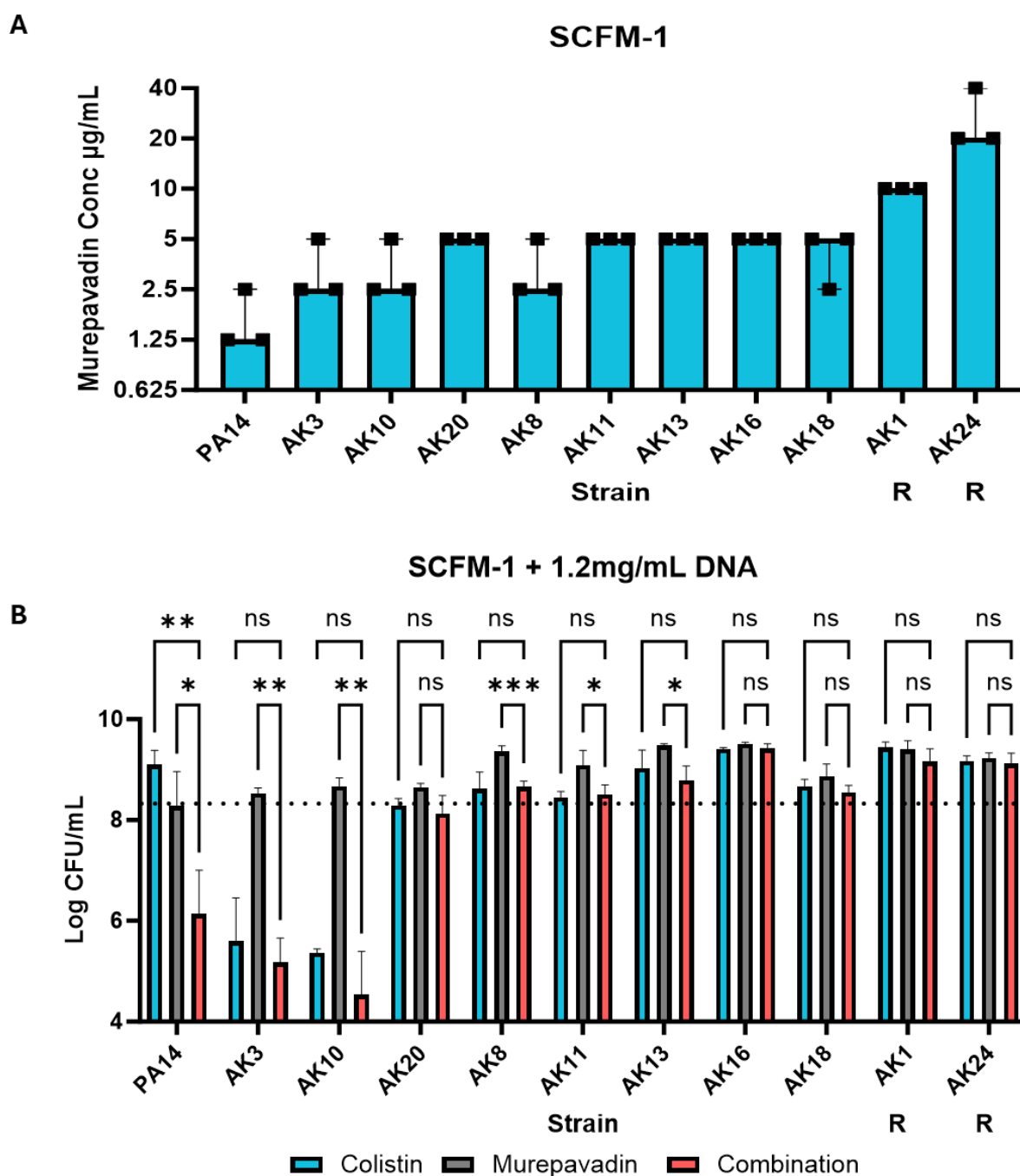


Figure 5.7: Clinical *P. aeruginosa* isolates from CF show decreased susceptibility to murepavadin. (A) MICs to murepavadin were determined by broth microdilution for *P. aeruginosa* strain PA14 and a panel of clinical isolates in SCFM-1. Bacteria were incubated at 37°C for 20 hours. Growth was determined by measuring optical density at 600nm. Data points represent the result of 3 independent experiments with coloured bars indicating the median and error bars indicating the range. (B) Stationary phase PA14 strain *P. aeruginosa* and a panel of clinical isolates were exposed to 4µg/mL colistin or 5µg/mL murepavadin or both in SCFM-1 supplemented with 1.2mg/mL eDNA and survival enumerated at 4 hours. Data points represent the mean of 4 independent experiments and error bars the standard deviation. The dotted line indicates the starting inoculum. Data were analysed by two-way ANOVA with Tukey's post hoc test. *P<0.05 **P<0.01

Time-kill assays were used to examine the extent of killing achieved by the two compounds, again at 4 µg/mL for colistin and 5 µg/mL for murepavadin (Fig 5.7B). In chapter 3, eDNA at 2.4 mg/mL inhibited growth of AK3, 10 and 20, and completely abrogated killing in PA14. Therefore, a DNA concentration of 1.2 mg/mL was used for these assays with clinical isolates. At this combination of concentrations, synergy was only achieved for PA14 (Fig 5.7B). Killing by colistin alone was observed for AK3 and AK10 and the presence of murepavadin did not further reduce viability. Despite a lack of killing, growth was inhibited for AK11, AK18 and AK20 with the colistin-murepavadin combination. Taken together these results demonstrate that this concentration of colistin and murepavadin does not provide synergistic killing of most clinical isolates in the context of synthetic sputum. Since synergy against *P. aeruginosa* PA14 at 1.2 mg/mL was achievable, it was hypothesised that targeting acidification of the lung environment may restore synergy.

5.2.6 NaHCO₃ restores susceptibility to murepavadin in the presence of eDNA

In the previous chapter, it was demonstrated that NaHCO₃ could partially restore susceptibility of PA14 to colistin in the presence of eDNA and that pH was therefore likely a significant environmental trigger of reduced polymyxin susceptibility. To investigate whether pH also contributed to reduced murepavadin susceptibility, a time-kill assay was performed comparing *P. aeruginosa* PA14 susceptibility to murepavadin in SCFM-1 alone versus SCFM-1 containing 2.4 mg/mL eDNA with or without NaHCO₃. As before, the presence of eDNA significantly reduced susceptibility to murepavadin (Fig 5.8A). However, when SCFM-1 containing eDNA was supplemented with 33mM NaHCO₃, susceptibility to murepavadin was restored, with 2-log of killing of *P. aeruginosa* PA14 (Fig 5.8A).

Next, the same assay was repeated on the panel of clinical isolates were exposed to 4 µg/mL colistin and 5 µg/mL of murepavadin in the presence of 1.2 mg/mL eDNA with or without NaHCO₃. As expected, in the absence of NaHCO₃, there was limited anti-pseudomonal activity of the antibiotic combination (Fig 5.8B). However, when NaHCO₃ was present, the growth of 7/8 clinical colistin susceptible strains was inhibited (Fig 5.8B) and killing was observed in 5 of these. Neither of the two colistin resistant strains, AK1 and AK24, were susceptible to the colistin-murepavadin combination in the presence of eDNA, even at pH 6.8.

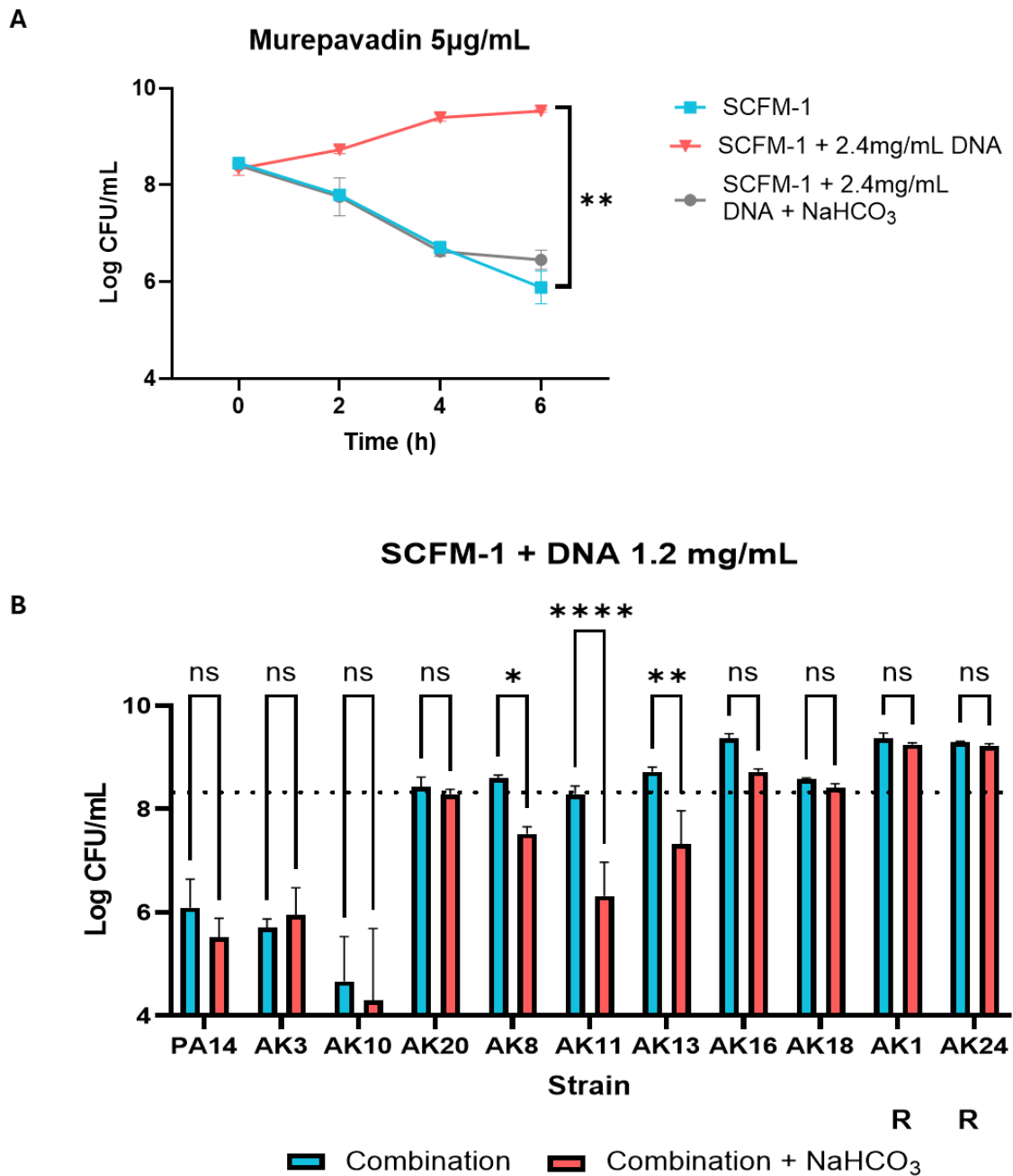
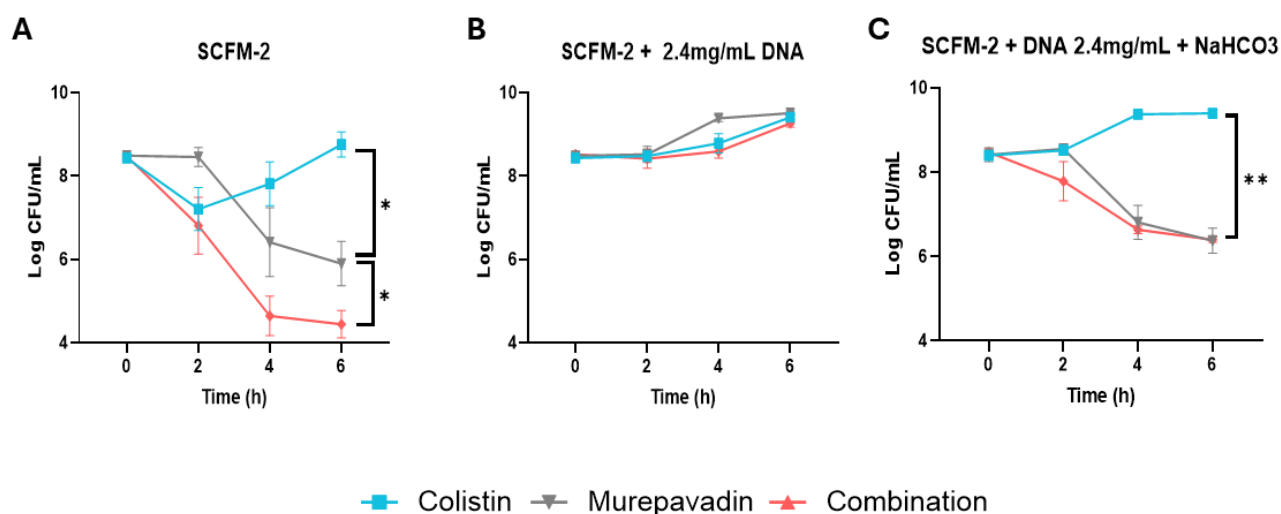


Figure 5.8: NaHCO₃ restores susceptibility to murepavadin and increases killing in the presence of murepavadin and colistin. (A) Stationary phase *P. aeruginosa* strain PA14 was exposed to 5µg/mL murepavadin in SCFM-1 or SCFM-1 supplemented with 2.4mg/mL eDNA or SCFM-1 supplemented with eDNA neutralised with NaHCO₃. Bacteria were enumerated every 2 hours for 6 hours. Data points represent the mean of 4 independent experiments error bars the standard deviation. Data were analysed by two-way ANOVA with Tukey's post hoc test *P<0.05 **P<0.01. (B) Stationary phase PA14 strain *P. aeruginosa* and a panel of clinical isolates were exposed to 4µg/mL colistin and 5µg/mL murepavadin in SCFM-1 supplemented with 1.2mg/mL eDNA with and without NaHCO₃ and bacterial survival enumerated at 4 hours. Colistin resistant strains are annotated with the letter **R**. Data points represent the mean of 3 independent experiments and error bars the standard deviation. The dotted line indicates the starting inoculum. Data were analysed by two-way ANOVA with Sidak's post hoc test. *P<0.05 **P<0.01 ****P<0.0001

Having studied the impact of eDNA on colistin-murepavadin susceptibility in clinical isolates, the final step was to study this combination in SCFM-2, with all macromolecules present. *P. aeruginosa* PA14 was inoculated into standard SCFM-2, SCFM-2 containing 2.4 mg/mL eDNA or SCFM-2 with supplemented with 2.4 mg/mL eDNA and NaHCO₃.

In SCFM-2 (which contains 0.6mg/mL eDNA), 4 µg/mL of colistin alone achieved a single log of killing by 2 hours but bacteria had completely recovered by 6 hours (Fig 5.9A). Although murepavadin alone (5 µg/mL) demonstrated little killing in the first 2 hours, 2 logs of killing were achieved by 6 hours (Fig 5.9A). The combination of colistin with murepavadin achieved an additional 2-logs of killing above murepavadin (4-log overall) at 4 and 6 hours, indicative of synergy.

When the DNA content of SCFM-2 was increased to 2.4mg/mL, killing by colistin or murepavadin alone was again completely abrogated and there was no evidence of synergy between murepavadin and colistin (Fig 5.8B). However, when eDNA and NaHCO₃ were present in SCFM-2, in contrast to SCFM-1, there was no restoration of killing by colistin, but there was a 2-



log reduction in *P. aeruginosa* PA14 CFU with murepavadin alone. NaHCO₃ was not sufficient to restore synergy between colistin and murepavadin, however. Overall, these experiments demonstrated that the combination of colistin, murepavadin and NaHCO₃ can be effective at overcoming eDNA-induced antibiotic resistance and tolerance in the context of synthetic sputum.

5.3 Discussion

The results described in this chapter demonstrate that eDNA-induced colistin resistance presents a particularly difficult challenge to overcome. However, whilst digestion of eDNA was unsuccessful in abrogating resistance, a combination of colistin and murepavadin, together with targeted normalisation of pH with NaHCO_3 could restore colistin susceptibility in PA14 and most of the clinical *P. aeruginosa* strains examined.

When eDNA was digested directly with rhDNase susceptibility to colistin was not restored. Mechanistically, rhDNase does not completely degrade DNA to single nucleotides but produces oligonucleotides (496). Halverson reported on the ability of DNase to ameliorate the cytotoxic effect of DNA on *P. aeruginosa* but at much higher concentrations of DNase than are achieved clinically in the CF lung (475). Additionally, dilution of DNase to levels still higher than in CF lung, rapidly abrogated the ameliorating effect, raising the possibility of off-target effects (475). In CF, DNase is predominantly used as a mucolytic by reducing the length of eDNA, which may have a limited effect on its ability to trigger bacterial adaptation. In the previous chapter, eDNA was identified as an inducer of colistin resistance, in part by lowering pH, thus it is perhaps not surprising that cleavage of DNA does not alter susceptibility, as the medium remains acidified.

There are no established reference standards for 'resistance' to murepavadin as no breakpoint has been defined. However, several groups have tested murepavadin against a range of *P. aeruginosa* isolates, including MDR and XDR isolates from CF. In one of these cases, murepavadin MIC_{50} was 0.12 $\mu\text{g/mL}$ and MIC_{90} 2.0 $\mu\text{g/mL}$ (443), which is similar to other studies (495,497). This is the first study examining the susceptibility of *P. aeruginosa* to murepavadin in liquid synthetic sputum and although a direct comparison was not made to MHB in this work, the

Figure 5.9: The presence of sputum macromolecules further reduces susceptibility to Colistin in PA14. Stationary phase *P. aeruginosa* strain PA14 was exposed to 4 $\mu\text{g/mL}$ colistin, 5 $\mu\text{g/mL}$ murepavadin or both in (A) SCFM-2 (B) SCFM-2 supplemented up to 2.4mg/mL eDNA or (C) SCFM-2 supplemented with 2.4mg/mL eDNA and neutralised with NaHCO_3 . Bacteria were enumerated every 2 hours for 6 hours. Data points represent the mean of 4(A and B) or 3 (C) independent experiments. Error bars represent the standard deviation. Data were analysed by two-way ANOVA with Tukey's post hoc test * $P < 0.05$ ** $P < 0.01$.

MICs in SCFM-1 are high compared to published literature.

Clinical trials of inhaled murepavadin are ongoing and it remains to be seen what murepavadin concentrations can be achieved in the lung. However, this treatment may not prove to be as effective *in vivo* as expected, if the impact of eDNA seen here holds true for the CF lung. It should be noted that Diez-Aguilar et al. used agar dilution with synthetic sputum and, whilst there was no difference in MIC between MHB and synthetic sputum on solid media, agar dilution alone resulted in up to a 16-fold increase in MIC relative to broth microdilution (497) limiting the value of this approach.

The presence of eDNA increased the MIC of *P. aeruginosa* PA14 for murepavadin in a dose-dependent fashion up to 16-fold higher than the MIC in SCFM-1 alone. Since murepavadin is a cationic peptide, it raises the possibility of binding to eDNA itself and/or of being excluded from the outer membrane by LPS modification. Consistent with this second hypothesis, previous work showed that gain of function mutations in *pmrB* resulted in a modest decrease in murepavadin susceptibility (498). However, in the work described above, the *arnC*::Tn mutation did not restore susceptibility at the murepavadin concentration tested. By contrast loss of *lpxT* function was associated with a modest inhibition of growth in the presence of murepavadin and eDNA. *lpxT* mutations have been reported in murepavadin resistant isolates but the precise mechanism is unknown (497). As such, the mechanism(s) by which eDNA trigger loss of murepavadin susceptibility may differ from those for colistin, although pH seems to play an important role in both.

Although effective synergy was observed for colistin-murepavadin against *P. aeruginosa* PA14 at DNA concentrations up to 1.2mg/mL, killing was completely abrogated at 2.4mg/mL. Increasing the concentration of murepavadin was able to restore killing at the highest eDNA concentration and killing was further enhanced in the presence of colistin. This is consistent with a model of activity whereby murepavadin sensitises bacteria to colistin by increasing LPS on the cytoplasmic membrane. Under this model susceptibility to murepavadin is required for synergy with colistin. Consistent with this hypothesis, I speculate that synergy was not achieved in the clinical isolates at 1.2 mg/mL of eDNA, in the absence of NaHCO₃ because all isolates demonstrated increased MICs to murepavadin relative to *P. aeruginosa* PA14 in SCFM-1 despite many being more susceptible to colistin.

Susceptibility to both murepavadin and colistin was dependent on pH in the presence of eDNA. Increasing pH to 6.8 with NaHCO₃ partially restored susceptibility to colistin and completely restored susceptibility to murepavadin at the tested concentrations. Although the combination of murepavadin and colistin did not achieve killing in the panel of clinical isolates,

murepavadin, colistin and NaHCO_3 achieved killing in the majority of susceptible strains (6/9) and inhibited the growth of 2 of the remaining 3 strains. Given that higher concentrations of murepavadin increase *P. aeruginosa* killing in the presence of eDNA, higher concentrations of murepavadin in combination with NaHCO_3 are predicted to have been more effective against the clinical isolates.

Synergy between colistin and murepavadin was effective against *P. aeruginosa* PA14 in SCFM-2 at an eDNA concentration of 0.6 mg/mL but was abrogated at 2.4 mg/mL eDNA. NaHCO_3 was able to restore susceptibility to murepavadin in SCFM-2 containing 2.4 mg/mL eDNA, but not colistin and no synergy was observed. Highlighting that although eDNA remains of central importance for determining murepavadin susceptibility, the role of other sputum macromolecules with respect to colistin requires further exploration. This result underscores the role of pH in murepavadin susceptibility.

Identifying optimal and achievable antibiotic combinations, together with adjunct therapies, in SCFM-2 should be a key focus of future work and the basis for exploring potential therapeutic combinations in CF which directly target bacteria in a physiological state that reflect the host context.

Chapter 6: Discussion

Though colistin is the most widely used inhaled antibiotic for CF in the UK (27), it is unable to eliminate chronic *P. aeruginosa* lung infection and long-term use is not associated with improved clinical outcomes (194). This is despite colistin resistance being a relatively rare phenotype in *P. aeruginosa* colonising the lungs of pwCF (201). One hypothesis to explain the lack of efficacy is that the CF lung environment reduces bacterial susceptibility to colistin. To test this, an artificial sputum model (ASM) was used. It was discovered that ASM dramatically reduces the susceptibility of *P. aeruginosa* to colistin, via a mechanism that involves sputum eDNA as a key trigger of LPS modification. This study also identified potential therapeutic routes to improving colistin efficacy in pwCF.

6.1 The lung environment reduces *P. aeruginosa* susceptibility to colistin

Inhaled colistin is a mainstay of suppression for chronic *P. aeruginosa* infection in pwCF. Despite this, *in vitro* susceptibility testing and clinical outcomes are very poorly correlated (190). A single randomised trial of colistin versus placebo for chronic *P. aeruginosa* infection found no improvement in lung function with colistin use (194). Despite frequent prolonged courses of inhaled colistin the emergence of resistance is rare from pwCF whereas it is easily induced *in vitro* following a few days of exposure to colistin (201,202). This work builds on the findings of previous work who have identified that individual sputum components can alter antimicrobial susceptibility individually and that the presence of biofilm in the lung renders *P. aeruginosa* recalcitrant to elimination by antibiotics (156,300,410).

The work presented in this thesis demonstrates that under conditions mimicking the CF-lung *P. aeruginosa* becomes far less susceptible to colistin relative to rich laboratory broth. Even in the absence of sputum macromolecules, SCFM-1 resulted in 2-4 fold increases in colistin MIC across PA14 and 3 clinical isolates, although this was not associated with significant differences in bacterial survival by time-kill assay. Previous work demonstrated nutrient availability can influence susceptibility to colistin with various amino acids both increasing and decreasing susceptibility when used as a sole carbon source (266,267). By contrast, data from studies exploring susceptibility in more complex media, that better mimics the host environment, is more limited. Sweeney and colleagues found larger shifts in colistin MIC, up to 32-fold, when SCFM-1 was modified by removing glucose (433) which is consistent with the findings of Sauvage and

colleagues, who identified that *P. aeruginosa* grown in the presence of glucose was more susceptible to colistin compared with other carbon sources (267).

The presence of sputum macromolecules in SCFM-2 had a large impact on susceptibility to colistin, with 4-8 fold increases in MIC and a significant reduction in killing in 2/4 strains compared to SCFM-1. Host-adapted phenotypes of *P. aeruginosa* are well described in the literature (499) and varied responses to individual sputum macromolecules have also been reported in another artificial sputum model (410). When individual macromolecules were supplemented into SCFM-1, though changes in susceptibility were observed across all four strains tested in the presence of mucin and DOPC, the most consistent effect was observed for the addition of eDNA, which resulted in a dose-dependent reduction in killing against PA14, reduced killing in 3/4 clinical isolates and increases in colistin MIC in 6/9 colistin susceptible clinical isolates.

eDNA is well recognised as a key component of *P. aeruginosa* biofilm (160,162,163) and has been demonstrated to reduce colistin susceptibility when supplemented into minimal media, where the mechanism was hypothesised to be dependent on sequestration of divalent cations (300). However, this work significantly adds to our understanding of the effect of eDNA under physiological conditions and across a range of concentrations found in the CF lung. For example, this is the first demonstration of eDNA induced tolerance to colistin and altered killing kinetics. Although the MIC of PA14 was raised to 8 µg/mL by the presence of 2.4 mg/mL eDNA, bacteria were able to survive exposure to much higher concentrations of colistin: up to 64 µg/mL, for 6 hours. This work highlights eDNA as a major barrier to colistin use in the CF lung.

More broadly, eDNA is found under a range of physiological conditions outside of the CF lung. Henke and colleagues reported eDNA levels in healthy volunteers to be approximately 0.96 mg/mL, still within our inhibitory range (283). eDNA levels have also been reported to be elevated in VAP where *P. aeruginosa* is a major pathogen (500). Therefore, this work has significant implications for colistin treatment outside of CF.

6.2 eDNA triggers LPS modification through multiple two-component systems.

Whilst eDNA can bind and sequester the endogenous cationic antimicrobial peptide LL-37 and similarly cationic tobramycin (289,427) I found no evidence of sequestration of colistin by eDNA. Mulcahy and colleagues reported that exposure of *P. aeruginosa* to eDNA triggered activation of the *arn* operon (300) and that high concentrations of eDNA were cytotoxic to bacteria. The work presented in chapter 4 confirmed that physiological concentrations of eDNA

can permeabilise the outer membrane of *P. aeruginosa*, allowing uptake of NPN and increasing susceptibility to Rifampicin. Mulcahy hypothesised that *arn* was activated by eDNA mediated sequestration of divalent cations which could, in turn, activate both PmrAB and PhoPQ (300). Crucially, in that publication, the pH effect of eDNA was not controlled for. Chapter 4 significantly increases our understanding of the mechanism by which eDNA decreases colistin susceptibility in *P. aeruginosa* by demonstrating that, in the context of synthetic sputum, the addition of divalent cations did not abrogate the eDNA effect on colistin susceptibility but instead augmented it. Furthermore, media acidification alone was sufficient to decrease susceptibility to colistin and gave rise to outer membrane disruption. Neutralisation of eDNA by NaHCO₃ was also successful in partially restoring susceptibility to colistin, underscoring the central, but not exclusive, role of reduced pH in eDNA induced colistin resistance. Further evidence supporting a range of environmental triggers was obtained from a PA14 transposon library which implicated ColRS, PmrAB and CprRS in decreasing *P. aeruginosa* susceptibility to colistin in SCFM-1 supplemented with eDNA.

Whilst PmrAB has been reported to act as a sensor for low divalent cation concentrations, it has also been shown to be activated by low pH and by the presence of cationic antimicrobial peptides (164,244). ColRS has been demonstrated to activate in the presence of excess metal ions, of which perhaps the best described is Zinc, which should not be present in SCFM to any significant level (249,388,448). However, Wiesmann and colleagues reported that ColRS was important for survival of *P. aeruginosa* at low pH in the presence of excess metal ions most notably FeSO₄ which is a major constituent of SCFM (449). Therefore, it is plausible that eDNA can trigger PmrAB directly, through cation sequestration, and through media acidification, and can trigger ColRS through media acidification in the presence of FeSO₄, although further work will be required to confirm the requirement of iron for this process.

Therefore, the work presented in this thesis adds to our understanding of how high-level colistin resistance can be achieved by *P. aeruginosa* in response to environmental pressure. In *P. aeruginosa*, expression of the *arn* operon and modification of lipid A with L-ara4N are thought to be central to mutational colistin resistance (173,243). However, it has been reported that expression of *arn* alone is insufficient to achieve high level colistin resistance, and that *arn* mediated resistance was also augmented by the addition of divalent cations to MHB (173). Taken together it is therefore unsurprising that the data in chapter 4 revealed that the disruption of *arnC* or *eptA* alone was insufficient to restore susceptibility at low concentrations of colistin and only partially achieved restored susceptibility at high concentrations in the presence of eDNA. Evolutionary studies of *P. aeruginosa* in CF have highlighted that, whilst mutations in *pmrB* can

give rise to moderate colistin resistance, additional mutations in independent pathways were necessary for high level resistance, particularly LPS transport, BamA, and phospholipid metabolism (501). In chapter 4, multiple two-component systems namely: ColRS, PmrAB and CprRS were found to be important for high level resistance in synthetic sputum, underscoring the complexity of the system and the tuning of the response to multiple environmental signals.

Relatively little work has been conducted investigating the contribution of multiple two-component systems in complex media. Gutu identified that polymyxin resistance due to deletion of PhoQ, leading to expression of the *arn* operon, could be abrogated by disruption of ColRS or CprRS (248) and again confirmed that the presence of L-ara4N modified Lipid A alone was insufficient to confer resistance. Lee et al reported that *in vitro* selected resistance to colistin in the presence of $\Delta pmrA$, $\Delta phoP$, $\Delta cprR$ or $\Delta parR$, involved compensatory mutations in the other TCSs that activate *arn* (502). In chapter 4, when LPS modifications, other than pEtN and L-ara4N, were investigated, acylation state, mediated by PagL or PagP, had a minimal impact on MIC, although disruption of the Lipid A phosphatase LpxT resulted in increased MICs to colistin in the presence of eDNA. Although I have primarily focused on LPS modification as a key downstream effector of these systems it is possible there are other effectors that contribute to resistance, for example PmrAB is known to activate expression of polyamines which in turn can modulate susceptibility to cationic antimicrobial peptides (265). To date much of our understanding of colistin resistance in *P. aeruginosa* has focused on L-ara4N and further work will be required to understand how this modification integrates into a wider framework of cellular changes arising in complex media.

To date, evidence supporting the role of pEtN in spontaneous colistin resistance in *P. aeruginosa* has been limited. Though pEtN is recognised as contributing to polymyxin resistance in enterobacteriales, both via spontaneous mutation and *mcr* gene acquisition (206,213,255,503), in *P. aeruginosa* there is strong evidence for the centrality of L-ara4N and the *arn* operon in colistin-selected resistance. Lo Sciuto et al. reported that *P. aeruginosa* strains from multiple backgrounds with $\Delta arnBCA$ were unable to acquire high level colistin resistance when selected for by increasing concentrations of the polymyxin antibiotic (425). Furthermore, when Nowicki and colleagues induced expression of *eptA* in *P. aeruginosa* with excess Zinc, they reported no change in colistin susceptibility (249). By contrast, when Cervoni et al expressed both endogenous *eptA* or *mcr-1* via an IPTG-inducible promoter, colistin susceptibility was decreased to levels comparable to L-ara4N expression (250). Chapter 4 represents the first strong evidence of a role for endogenous pEtN-based LPS modification in *P. aeruginosa* contributing to high level colistin resistance under physiological conditions. This was confirmed, in chapter 4, by

disruption of *eptA* with transposon insertion resulting in a 4-fold decrease in MIC to colistin in the presence of eDNA and a 2-log reduction in survival of the *eptA*::Tn mutant relative to the wild-type, which could be restored by complementation. Additionally, lipidomic analysis revealed an abundance of pEtN modified lipid A in the presence of eDNA. In contrast to data published by Cervoni et al., pEtN was only identified in the presence of tri-phosphorylated lipid A, raising the possibility of co-regulation of LpxT when the native *eptA* is expressed from its endogenous locus or that both modifications are responding to the same environmental trigger, rather than on a plasmid.

Whilst the regulation of *lpxT* is poorly understood in *P. aeruginosa*, in *E. coli*, this gene is reciprocally regulated with *eptA* by *PmrR* (466). Unlike in *E. coli*, *PmrAB* does not regulate *eptA* expression in *P. aeruginosa* but does regulate the *arn* operon (245). Additionally, EptA appears to be site specific in *P. aeruginosa* rather than in *E. coli* only phosphorylating the 4' phosphate of lipid A, whilst *lpxT* is able to phosphorylate wither the 4' or 1' position(249,458). Therefore, when both EptA and LpxT enzymes are activated, they will both compete for the 4' phosphate of lipid A but, where lipid A is pEtN modified it will tend to be phosphorylated at the 1' position.

Cervoni identified that high levels of *eptA* expression were associated with disruption of the outer membrane and, surprisingly, increased susceptibility to colistin, relative to low level expression (250). One possibility is that by phosphorylating the opposite pole to pEtN, membrane integrity is maintained in *P. aeruginosa* by increasing the electrostatic interactions between adjacent LPS molecules normally conferred by divalent cations. Further work will need to investigate the complex interaction between modified LPS molecules.

Taken together the work presented here emphasises a role for endogenous EptA in *P. aeruginosa* and raises the possibility that co-regulation with other lipid A modifications is important for colistin resistance in synthetic sputum.

6.3 Targeting the CF lung environment reduces bacterial survival

Having established that the CF lung-like environment of synthetic sputum, and particularly eDNA, reduced *P. aeruginosa* susceptibility to colistin, strategies were investigated to restore the activity of colistin.

Despite the important role of eDNA, treatment with DNase did not restore susceptibility. This is likely due to a combination of factors, namely that DNA is not completely degraded by DNase I (496), and that the addition of eDNA led to media acidification, which wouldn't be expected to be abrogated by hydrolysis. In related work, Hunt et al found that partial digestion of

DNA, in combination with mucin, increased the sequestration of cationic tobramycin rather than decreasing it the authors hypothesising that smaller fragments of eDNA had an increased binding capacity(289). The finding of eDNA driven media acidification is supported by the work of Wilton et al. who identified that eDNA biofilms of *P. aeruginosa* are locally acidified, leading to reduced susceptibility to aminoglycosides (164). Lewenza and colleagues demonstrated close similarities between the transcriptional profiles of *P. aeruginosa* grown in the presence of eDNA and bacteria grown at pH 5.5 (440). Whilst data is conflicting there is evidence that the airways of pwCF are acidified relative to unaffected individuals (415,504). Together, therefore, acidification of the airways in CF may represent a potential therapeutic target rather than digestion of eDNA itself.

Given the failure of DNase to restore colistin activity, I next investigated the ability of the novel antimicrobial murepavadin to restore susceptibility because previous work from our group has demonstrated the ability of sub-inhibitory concentrations of this antibiotic to synergise with colistin (227). The combination of colistin and murepavadin was effective at low concentrations of eDNA against PA14, but 2.4 mg/mL of eDNA was sufficient to abrogate killing by either murepavadin or colistin or both in combination.

This is the first study to examine the activity of murepavadin in synthetic sputum in the liquid phase. Previous work has examined murepavadin in standard laboratory media where *P. aeruginosa* appears to be extremely susceptible (443,495,497). When Diez-Aguilar and colleagues supplemented synthetic sputum into agar, although MICs were comparable to MHB agar, they were at least 8-fold higher than MHB in liquid phase (497). Although the combination of murepavadin and colistin was effective against *P. aeruginosa* PA14 up to 1.2 mg/mL eDNA, clinical isolates appeared to be less susceptible with only 3/9 colistin-susceptible strains killed and all strains growing in the presence of murepavadin alone.

Limited data exists around the resistance determinates for murepavadin. Cross-resistance has been reported between colistin and murepavadin arising through mutations in *pmrB*, *bamA* and *lpxI* (495,497). This is the first report of a significant effect of pH on murepavadin susceptibility. When susceptibility to murepavadin was examined in the panel of transposon mutants, disruption of neither *eptA* nor *arnC* restored susceptibility, although similarly high concentrations of murepavadin, as were used with colistin, were not examined. However, increased murepavadin susceptibility was observed when *lpxT* was disrupted. Interestingly, mutations in *lpxT* have been reported in laboratory-generated murepavadin resistance strains although further phenotypic work to understand the mechanistic relationship

was not undertaken (497). Further work will be required to understand the mechanism of eDNA-induced murepavadin resistance in *P. aeruginosa*.

As described above, ColRS and PmrAB have each been reported to be triggered by low pH (164,449). Additionally, CFTR dysfunction results in a failure to secrete HCO_3^- , which leads to reduced pH and has been linked to endogenous AMP dysfunction (95). This has led to the investigation of NaHCO_3 as a potential inhaled agent in CF with promising results (505). Therefore, I sought to supplement NaHCO_3 to restore pH to neutral in eDNA acidified SCFM. Whilst this was partially successful in restoring susceptibility to colistin, it was more successful in restoring susceptibility to murepavadin. When NaHCO_3 was added to the combination of colistin and murepavadin, increased susceptibility was observed in a further 3 bacterial strains increasing the total number of susceptible strains killed by the combination to 6/9. These findings raise the possibility that pH may be important for determining bacterial susceptibility to colistin and murepavadin in the CF lung. The pH of ASL in CF remains controversial, whilst multiple lines of evidence have supported the hypothesis that CFTR dysfunction leads directly to depletion of extracellular HCO_3^- and ASL acidification including: cultures of primary cells (506), measurements from the pig model (117,507), and exhaled breath (504), a recent study by Schultz and colleagues, which directly measured pH in the lungs of CF and non-CF children, found no evidence for acidification (508). Future work will be required to establish whether NaHCO_3 is a useful intervention alongside inhaled colistin.

6.4 Thesis Summary

The work presented in this thesis demonstrates that *P. aeruginosa* grown under conditions mimicking the CF lung environment has significantly reduced susceptibility to colistin. It identifies eDNA as a major environmental trigger which, in the presence of colistin itself, activates the TCSs PmrAB, ColRS and CprRS leading to LPS modification. Finally, this work demonstrates that targeting eDNA mediated acidification or LPS transport, with the novel anti-microbial murepavadin, can restore susceptibility to colistin in at least some clinical isolates (Figure 6.1).

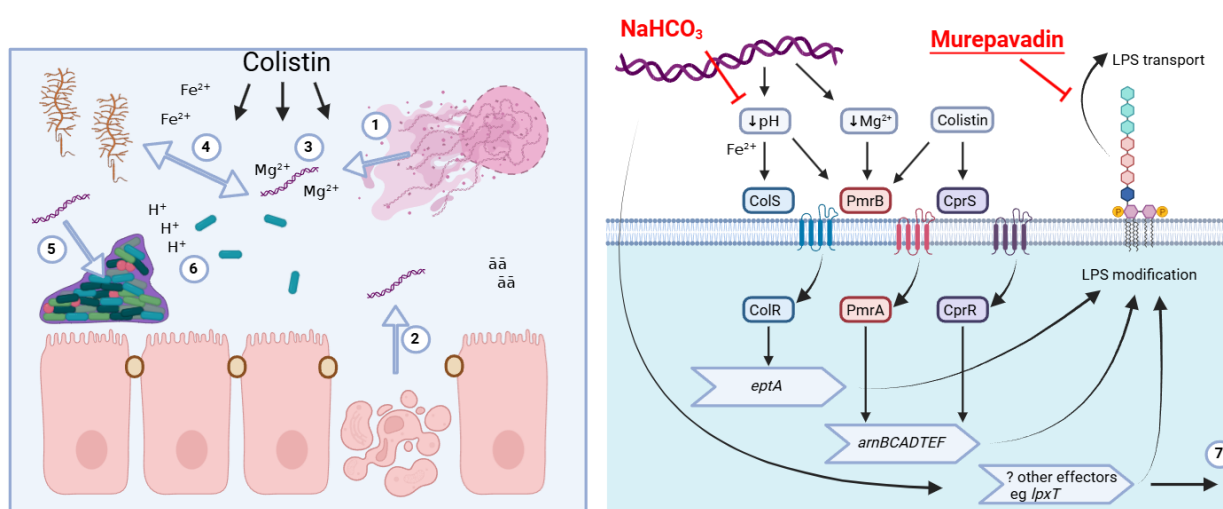


Figure 6.1 Proposed model for how the CF lung environment reduces susceptibility to colistin. The airway surface liquid (ASL) is a rich source of amino acids (aa) iron and salts and contains high concentrations of eDNA and other sputum macromolecules. DNA is released from neutrophils (1) and dying host cells (2). Extracellular DNA can sequester divalent cations (3) interact with other sputum macromolecules (4) and contribute to biofilm formation (5). Release of eDNA results in acidification of the ASL (6). The presence of eDNA in ASL results in activation of the two-component systems PmrAB and ColRS. Colistin itself also activates PmrAB and CprRS. All three two-component systems trigger the modification of LPS alongside the expression of unknown downstream effectors (7) resulting in decreased susceptibility to colistin. NaHCO₃ and murepavadin restore susceptibility by reversing media acidification and inhibiting LPS transport.

6.5 Future Work

6.5.1 Increasing understanding of *P. aeruginosa* susceptibility to colistin.

Although there is sufficient data here to construct a theoretical model of how the lung environment reduces colistin susceptibility in *P. aeruginosa* (Figure 6.1), there are several key questions that remain to be answered. I have hypothesised that the combination of media acidification and excess Fe^{2+} are responsible for triggering the ColRS TCS and downstream activation of EptA. To test this hypothesis the iron content of SCFM containing eDNA could be varied to assess the impact on bacterial growth and susceptibility to colistin. Additionally, iron chelators such as diethylnetriamine, which is widely used in other synthetic sputum models (426) could be supplemented into the media to ascertain whether this has an additional effect on colistin susceptibility. Alternatively, the more physiological ferritin or lactoferrin could be used as both have also been supplemented into existing models (275,384). To confirm that the result of both media acidification and iron excess is conferring altered susceptibility, the expression of *eptA* can be measured directly via Real-time (RT-) PCR and lipidomics could be performed on both SCFM-1 supplemented with eDNA and NaHCO_3 and media where the iron content has been modified to identify lipid A modification. As an alternative, reporter systems for both *eptA* and *arn* could be generated by cloning their cognate promoters into a GFP containing plasmid which could be transformed into *P. aeruginosa* PA14, or RNAseq analysis could be undertaken. The additional benefit of this approach would be that it would enable confirmation that both *arn* and *eptA* are activated in the presence of eDNA in SCFM excluding, polar effects of the transposon insertion process on *arn*. Additionally, the putative environmental triggers including Fe^{2+} , Mg^{2+} , pH and colistin could also be investigated for their impact on *PeptA*-GFP and *Parn*-GFP expression.

The work in this thesis raises some important questions into how high-level resistance to colistin is achieved *in vivo*. Previous work has supported the hypothesis that a range of cellular changes are required alongside L-ara4N to achieve high level resistance to polymyxins. The work presented here demonstrated that disruption of *eptA* or *arnC* alone were insufficient to restore susceptibility of PA14 to wild-type levels at low concentrations of colistin in the presence of high concentrations of eDNA. I hypothesise that in the complex lung environment activation of multiple TCSs achieves redundancy against disruption of either *eptA* or *arn* alone. To test this hypothesis, knock out mutants could be constructed using either homologous recombination or through use of the CRISPR/Cas9 system (509,510). Constructing a double knock-out of *arnC* and *eptA* would be useful to identify how much of the change in susceptibility is due to these two pathways *per se*, and how much is due to other down-stream effectors. Identifying further downstream effectors would be more challenging, however. Whilst several downstream effectors

are known e.g. MexXY (260) and polyamine synthesis (265), there may be as yet unidentified loci which contribute to colistin resistance induced by synthetic sputum. Therefore, a less selective approach using RNA sequencing to identify the relative abundance of gene transcripts for *P. aeruginosa* PA14 grown in MHB compared to SCFM-1 supplemented with 2.4mg/mL eDNA might be useful. This approach is likely to identify numerous metabolic pathways of little relevance to colistin resistance, but important for survival in SCFM. Therefore a more informative analysis is likely to arise comparing PA14 to *colR::Tn*, *pmrB::Tn* and *cprR::Tn* in SCFM supplemented with eDNA as this should give rise to differences relating specifically to each TCS's downstream effectors. Alternatively, a TnSeq or TraDIS approach could be used to identify all genes that contribute to colistin resistance in SCFM (511).

This work identified a key role for endogenous *eptA* in reducing susceptibility to colistin in *P. aeruginosa*. Interestingly, the lipidomics data identified pEtN modification in the presence of tri-phosphorylated lipid A, presumably mediated by LpxT, while the *lpxT::Tn* strain had dramatically increased MICs to colistin. The role of LpxT in this process could be confirmed by performing a lipidomic analysis of *lpxT::Tn* in the presence and absence of eDNA. It would be interesting to investigate the effect of disruption of both *eptA* and *lpxT* in tandem to see whether this enhances or decreases susceptibility to colistin, with the hypothesis that the two are working synergistically to maintain membrane integrity. This hypothesis could be further tested by comparing NPN uptake in response to low level colistin exposure and high level eDNA exposure in PA14, PA14Δ*lpxT*, PA14Δ*eptA*, and PA14Δ*lpxT*Δ*eptA*. Given the hypothesised redundancy between pEtN and L-ara4N modifications in this model, it will be necessary to perform a lipidomic analysis in parallel with these strains as I would hypothesise, in the absence of pEtN, the relative abundance of L-ara4N would increase, as they are competing for the same terminal phosphates on lipid A.

One of the key observations giving rise to the central hypothesis of this thesis: that the lung environment reduces susceptibility to colistin, was that despite prolonged therapy with inhaled colistin, mutational resistance rarely emerges. Given that the lung environment appears to reduce susceptibility to colistin there may be no strong selection pressure for mutational colistin resistance. To test this hypothesis PA14 could be grown in the presence of SCFM-1 supplemented with increasing concentrations of eDNA and challenged with increasing concentrations of colistin for prolonged periods. Any resistant mutants could be identified by screening cultures on solid media containing colistin. From these experiments, the rate at which colistin resistance emerges could be compared between synthetic sputum and standard

laboratory media. I would anticipate that, if the model is correct, spontaneous colistin resistance would arise at a much slower rate in the presence of higher eDNA concentrations.

6.5.2 Increasing our understanding of the host environment.

Defining optimal models for the investigation of the lung environment in CF is challenging. Animal models are limited in their utility due to the predominance of significant GI pathology and limited lung disease in smaller mammals (2). Throughout this work SCFM-1 and 2 have been used as a proxy for the lung environment, as one of the best validated models of CF lung, with the additional benefit that they were validated against the growth and survival of *P. aeruginosa* (276,388). When it was originally devised, SCFM-2 did not contain all of the essential components for growth of all *P. aeruginosa* strains tested (388). However, SCFM-2 was not inferior to an air-liquid interface model (363), highlighting that increasing complexity does not necessarily increase the benefits of a model. Broadly, ASM can be used in two ways to investigate antimicrobial susceptibility: 1) to mimic as closely as possible the host environment to better understand the pathogenesis of disease and potentially identify changes in bacteria that alter susceptibility 2) to define key components of the media to more reliably reflect antimicrobial susceptibility *in vivo*. Whilst similar, these two aims can be investigated quite separately: The work of Hasan et al highlights how the composition of synthetic sputum models can alter bacterial interactions with antibiotics where bacterial survival was dependent on the presence or absence of polyamines in that model (312). That work led to the development of more complex media closely mimicking the composition of ASL in CF (275). To test the conclusions of this thesis key findings could be recapitulated in this more complex model. In particular, determining the survival of a range of clinical isolates to colistin exposure. This could be further validated using the reporter strains described above to confirm that resistance mechanisms are similar in a yet more complex model.

It remains unclear what the key components of an *in vitro* model mimicking the CF lung should be. Coupled with the work outlined above (6.4.1) the key environmental triggers identified as determining colistin resistance eg eDNA, pH and Mg^{2+} could be used to produce a less complex medium. The benefits of this process would be that many of the biological components of ASM are purified from organic systems and have the potential to introduce contamination and/or vary from batch to batch. Neve et al highlighted that some commercial preparations of porcine gastric mucin were heavily contaminated with iron, undermining the conclusions that could be drawn from ASM produced using these products (426). Lewin and colleagues also identified Zinc as a potential contaminant in their sputum model, the source of which was unclear (363).

Naturally, there is always a trade-off between complexity and reproducibility in model systems, depending on the research question in mind. For example, a minimal, reproducible medium that reflects colistin susceptibility *in vivo* has a potential application for antimicrobial susceptibility testing. Ersoy and colleagues identified significant differences between susceptibility testing in MHB or host-mimicking medium but were able to modify MHB through the addition of NaHCO₃ to achieve similar results to host-mimicking medium (512). With respect to SCFM and other artificial sputum media a transcriptional analysis of *P. aeruginosa* strains grown in complex media, SCFM-1 supplemented with eDNA, MHB II supplemented with eDNA and ex vivo sputum could provide a useful indicator of whether there are significant differences in the bacterial response to these models and the likely significance of those differences alongside MIC and time-kill assays to compare the differences in colistin susceptibility.

The translatability of the current work to the *in vivo* setting was limited by recent developments in therapeutics in CF, which curtailed access to sputum samples. CFTR modulators have completely revolutionised the disease process in CF by partially correcting the underlying defect (513). What is clear from the evidence is that, although there are initial dramatic gains in lung function for pwCF started on these novel therapeutics, this still does not allow them to clear established *P. aeruginosa* infection (187,188). Furthermore, whilst there appears to be an initial benefit in lung function it remains unclear whether this will be maintained in the longer term. Whilst data are emerging that indicates the behaviour of bacteria may be different in pwCF on CFTR modulator therapy (514,515), there is little work identifying the compositional changes in CF sputum that arise from the use of these drugs. Furthermore, it is likely that there will be inter-individual differences between pwCF started on treatment with established lung disease compared to individuals started in early childhood (516). Measuring the difference in composition of CF sputum from these groups is likely to generate a more accurate model of the CF lung in the current and future cohorts of pwCF.

Current sputum models are based on measurements from multiple studies over many years. Recapitulating some of that work would be a major challenge, however in the first instance quantifying the key components of SCFM-2 would be more achievable. A combination of chromatography, mass spectrometry and enzymatic analysis was used to determine the concentrations of amino acids and salts comprising the basal medium SCFM-1 (276). The concentration of eDNA could be determined by colorimetric assay (292), mucin content could be determined by western blot analysis (283,517) and phospholipids by lipid extraction and chromatography (518,519). The concentration of GlcNAc could be determined by chromatography (520). Whilst this starting medium would not reflect the total complexity of

sputum it would provide a basis for comparison to SCFM-2 and a starting medium to investigate CF sputum in the post CFTR-modulator era. The new sputum model should be optimised with external validation in mind: RNAseq profiling of divergent strains of *P. aeruginosa* grown in expectorated sputum and compared to a synthetic sputum model could provide evidence to support the use of a new sputum model.

6.5.3 Optimising treatment in based on the host environment.

Murepavadin has shown promise as a potential new inhaled agent in CF. However, the work in this thesis raises some questions about the efficacy of murepavadin under physiological conditions, particularly against host-adapted strains. This should be investigated further by examining susceptibility against a broader panel of clinical isolates in SCFM-2 and against SCFM-2 with supplemental eDNA. At present, there is no published data on the achievable lung concentrations of murepavadin following nebulisation. However, given that killing by murepavadin against PA14 could be restored by increasing its concentration, higher concentrations of colistin and murepavadin should be investigated to identify whether synergy can be achieved against clinical isolates in the presence of eDNA with and without supplemental NaHCO_3 .

Whilst this work has predominantly focused on the effect of the lung environment on the growth of planktonic bacteria and the challenges of restoring susceptibility, there is strong evidence that *P. aeruginosa* biofilms exist throughout the cystic fibrosis lung (155,156). Having established a model of the CF lung and identified the central importance of eDNA with putative strategies to improve susceptibility to colistin, I propose investigating the efficacy of those strategies against *P. aeruginosa* biofilm. Haley and colleagues identified that, in an artificial sputum model, the availability of eDNA could have dramatic effects on biofilm structure with higher concentrations of eDNA associated with a failure to form defined biofilm structures (521). To investigate the effect of eDNA on colistin susceptibility in biofilm, simple biofilms could be grown using a Calgary device in SCFM containing increasing concentrations of eDNA. Bacterial survival could then be determined against colistin alone, colistin in the presence of murepavadin and colistin in the presence of murepavadin and NaHCO_3 . Moskowitz et al have proposed a reproducible method of generating biofilm using Calgary devices which has already been adapted to include the use of synthetic sputum (433,522)

The work presented in this thesis provides strong evidence that acidification of the lung environment local to bacteria acts as a trigger for bacterial adaptation, which reduces susceptibility to both colistin and murepavadin. Supplementation of NaHCO_3 was partially

successful in restoring susceptibility. What is less clear is whether this would be effective in humans. There has already been some interest in NaHCO_3 as an adjunct to therapy in cystic fibrosis for several reasons: CFTR dysfunction results in lower levels of HCO_3^- in secretions from cystic fibrosis than in unaffected individuals (523), this has been linked to a failure to mature mucins impairing mucociliary clearance, and to dysfunction of endogenous AMPs increasing susceptibility of pwCF to pseudomonas (117,477). NaHCO_3 itself may have some intrinsic antimicrobial activity, although it is difficult to separate this from its effects on pH (117). There has also been some interest in the use of NaHCO_3 as an anti-biofilm agent for the elimination of *P. aeruginosa* (482,483). Finally, NaHCO_3 has already been trialled as a therapeutic agent in cystic fibrosis (505). Although the reported study is small with only 12 patients completing the study, inhaled NaHCO_3 was well tolerated, with no adverse effects. Unfortunately, the study was not sufficiently powered to detect differences in infection or antimicrobial susceptibility. Increased sputum pH and decreased viscosity were identified (505). Finally, CFTR modulator therapy was only partially successful in restoring HCO_3^- secretion in an *ex vivo* epithelial culture model (524) raising the possibility that inhaled HCO_3^- therapy may be beneficial even in pwCF on CFTR modulators.

Taken together there appears to be good evidence that inhaled NaHCO_3 may be beneficial in pwCF, even in the presence of CFTR modulator therapy. There appear to be several potential benefits to all pwCF, not just those on inhaled colistin therapy or chronically infected with *P. aeruginosa*. A challenge in how to approach such a study lies in defining a clinical population most likely to demonstrate a benefit from inhaled NaHCO_3 , and the challenges of designing new interventional studies in pwCF already taking CFTR modulators. In the first instance investigating the effect on *P. aeruginosa* density in sputum in individuals already colonised with *P. aeruginosa* and receiving inhaled colistin would be a key target population. Ideally such a study could be designed to measure clearance of *P. aeruginosa*, however, it remains unclear whether this is achievable in chronic infection. Other important parameters to measure would include frequency of pulmonary exacerbation and lung function. Given the potential broader benefits of NaHCO_3 therapy I would envisage this study nested in a larger study of inhaled NaHCO_3 in CF as a mucolytic. Potentially this could also allow data to be collected on clearance and recurrence rates of individuals with acute *P. aeruginosa* infection as well as newly acquired *P. aeruginosa* infection.

References

1. UK Health Security Agency. Identification of *Pseudomonas* species and other Non-Glucose Fermenters. UK Standards for Microbiology Investigations. 2015.ID 17(3).
2. Wood SJ, Kuzel TM, Shafikhani SH. *Pseudomonas aeruginosa*: Infections, Animal Modeling, and Therapeutics. *Cells*. 2023.12(1):199.
3. Gessard C. On the blue and green coloration that appears on bandages. *Rev Infect Dis*. 1984.6 Suppl 3(Supplement_3):S775-6.
4. Meyer JM. Proverdines: Pigments, siderophores and potential taxonomic markers of fluorescent *pseudomonas* species. Vol. 174, *Archives of Microbiology*. 2000. p. 135–42.
5. Abdelaziz AA, Kamer AMA, Al-Monofy KB, Al-Madboly LA. *Pseudomonas aeruginosa*'s greenish-blue pigment pyocyanin: its production and biological activities. *Microb Cell Fact*. 2023.22(1):110.
6. Crone S, Vives-Flórez M, Kvich L, Saunders AM, Malone M, Nicolaisen MH, et al. The environmental occurrence of *Pseudomonas aeruginosa*. *APMIS*. 2020.128(3):220–31.
7. Loveday HP, Wilson JA, Kerr K, Pitchers R, Walker JT, Browne J. Association between healthcare water systems and *Pseudomonas aeruginosa* infections: a rapid systematic review. *J Hosp Infect*. 2014.86(1):7–15.
8. Kerr KG, Snelling AM. *Pseudomonas aeruginosa*: a formidable and ever-present adversary. *Journal of Hospital Infection*. 2009.73(4):338–44.
9. Klockgether J, Cramer N, Wiehlmann L, Davenport CF, Tümmler B. *Pseudomonas aeruginosa* Genomic Structure and Diversity. *Front Microbiol*. 2011.2.
10. Freschi L, Vincent AT, Jeukens J, Emond-Rheault JG, Kukavica-Ibrulj I, Dupont MJ, et al. The *Pseudomonas aeruginosa* Pan-Genome Provides New Insights on Its Population Structure, Horizontal Gene Transfer, and Pathogenicity. Martin B, editor. *Genome Biol Evol*. 2019.11(1):109–20.
11. Silby MW, Winstanley C, Godfrey SAC, Levy SB, Jackson RW. *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiol Rev*. 2011.35(4):652–80.
12. Sultan M, Arya R, Kim KK. Roles of Two-Component Systems in *Pseudomonas aeruginosa* Virulence. *Int J Mol Sci*. 2021.22(22):22.
13. Schobert M, Jahn D. Anaerobic physiology of *Pseudomonas aeruginosa* in the cystic fibrosis lung. *Int J Med Microbiol*. 2010.300(8):549–56.
14. Eschbach M, Schreiber K, Trunk K, Buer J, Jahn D, Schobert M. Long-term anaerobic survival of the opportunistic pathogen *Pseudomonas aeruginosa* via pyruvate fermentation. *J Bacteriol*. 2004.186(14):4596–604.
15. Palmer GC, Whiteley M. Metabolism and Pathogenicity of *Pseudomonas aeruginosa* Infections in the Lungs of Individuals with Cystic Fibrosis. *Microbiol Spectr*. 2015.3(4).

16. Turkina M V, Vikström E. Bacteria-Host Crosstalk: Sensing of the Quorum in the Context of *Pseudomonas aeruginosa* Infections. *J Innate Immun.* 2019.11(3):263–79.
17. Lee J, Wu J, Deng Y, Wang J, Wang C, Wang J, et al. A cell-cell communication signal integrates quorum sensing and stress response. *Nat Chem Biol.* 2013.9(5):339–43.
18. Ahator S Dela, Zhang L. Small Is Mighty-Chemical Communication Systems in *Pseudomonas aeruginosa*. *Annu Rev Microbiol.* 2019.73:559–78.
19. Thi MTT, Wibowo D, Rehm BHA. *Pseudomonas aeruginosa* Biofilms. *Int J Mol Sci.* 2020.21(22).
20. Pang Z, Raudonis R, Glick BR, Lin TJ, Cheng Z. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnol Adv.* 2019.37(1):177–92.
21. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science.* 1999.284(5418):1318–22.
22. Mittal R, Aggarwal S, Sharma S, Chhibber S, Harjai K. Urinary tract infections caused by *Pseudomonas aeruginosa*: a minireview. *J Infect Public Health.* 2009.2(3):101–11.
23. Bouadma L, Wolff M, Lucet JC. Ventilator-associated pneumonia and its prevention. *Curr Opin Infect Dis.* 2012.25(4):395–404.
24. Norbury W, Herndon DN, Tanksley J, Jeschke MG, Finnerty CC. Infection in Burns. *Surg Infect (Larchmt).* 2016.17(2):250–5.
25. Treviño González JL, Reyes Suárez LL, Hernández de León JE. Malignant otitis externa: An updated review. *Am J Otolaryngol.* 2021.42(2):102894.
26. Spornovasilis N, Psychogiou M, Poulakou G. Skin manifestations of *Pseudomonas aeruginosa* infections. *Curr Opin Infect Dis.* 2021.34(2):72–9.
27. Cystic Fibrosis Trust. UK Cystic Fibrosis Registry 2022 Annual Data Report [Internet]. London; 2023. [cited 2024 Oct 10] Available at: https://www.cysticfibrosis.org.uk/sites/default/files/2023-10/CFT_2022_Annual_Data_Report_FINAL_v8.pdf
28. Paprocka P, Durnaś B, Mańkowska A, Król G, Wollny T, Bucki R. *Pseudomonas aeruginosa* Infections in Cancer Patients. *Pathogens.* 2022.11(6).
29. Teweldemedhin M, Gebreyesus H, Atsbaha AH, Asgedom SW, Saravanan M. Bacterial profile of ocular infections: a systematic review. *BMC Ophthalmol.* 2017.17(1):212.
30. Tiew PY, Jaggi TK, Chan LLY, Chotirmall SH. The airway microbiome in COPD, bronchiectasis and bronchiectasis-COPD overlap. *Clinical Respiratory Journal.* 2021.15(2):123–33.
31. Henwood CJ, Livermore DM, James D, Warner M, *Pseudomonas* Study Group. Antimicrobial susceptibility of *Pseudomonas aeruginosa*: results of a UK survey and evaluation of the British Society for Antimicrobial Chemotherapy disc susceptibility test. *J Antimicrob Chemother.* 2001.47(6):789–99.

32. Wu W, Huang J, Xu Z. Antibiotic influx and efflux in *Pseudomonas aeruginosa*: Regulation and therapeutic implications. Vol. 17, *Microbial Biotechnology*. John Wiley and Sons Ltd; 2024.
33. Yoshimura F, Nikaido H. Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. *J Bacteriol*. 1982.152(2):636–42.
34. Livermore DM. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? *Clin Infect Dis*. 2002.34(5):634–40.
35. Botelho J, Grosso F, Peixe L. Antibiotic resistance in *Pseudomonas aeruginosa* - Mechanisms, epidemiology and evolution. *Drug Resist Updat*. 2019.44:100640.
36. Geneva: World Health Organization. WHO Bacterial Priority Pathogens List, 2024: bacterial pathogens of public health importance to guide research, development and Strategies to prevent and control antimicrobial resistance. [Internet]. 2024. [cited 2024 Sep 1]. Available at: <https://www.who.int/publications/i/item/9789240093461>
37. Micek ST, Wunderink RG, Kollef MH, Chen C, Rello J, Chastre J, et al. An international multicenter retrospective study of *Pseudomonas aeruginosa* nosocomial pneumonia: impact of multidrug resistance. *Crit Care*. 2015.19(1):219.
38. Kadri SS, Adjemian J, Lai YL, Spaulding AB, Ricotta E, Prevots DR, et al. Difficult-to-Treat Resistance in Gram-negative Bacteremia at 173 US Hospitals: Retrospective Cohort Analysis of Prevalence, Predictors, and Outcome of Resistance to All First-line Agents. *Clin Infect Dis*. 2018.67(12):1803–14.
39. Giannella M, Bussini L, Pascale R, Bartoletti M, Malagrino M, Pancaldi L, et al. Prognostic Utility of the New Definition of Difficult-to-Treat Resistance Among Patients With Gram-Negative Bloodstream Infections. *Open Forum Infect Dis*. 2019.6(12):ofz505.
40. Tamma PD, Aitken SL, Bonomo RA, Mathers AJ, van Duin D, Clancy CJ. Infectious Diseases Society of America Guidance on the Treatment of Extended-Spectrum β -lactamase Producing Enterobacterales (ESBL-E), Carbapenem-Resistant Enterobacterales (CRE), and *Pseudomonas aeruginosa* with Difficult-to-Treat Resistance (DTR-P. *aeruginosa*). *Clin Infect Dis*. 2021.72(7):e169–83.
41. Lima LM, Silva BNM da, Barbosa G, Barreiro EJ. β -lactam antibiotics: An overview from a medicinal chemistry perspective. *Eur J Med Chem*. 2020.208:112829.
42. Bush K, Bradford PA. β -Lactams and β -Lactamase Inhibitors: An Overview. *Cold Spring Harb Perspect Med*. 2016.6(8).
43. Hawkey PM, Warren RE, Livermore DM, McNulty CAM, Enoch DA, Otter JA, et al. Treatment of infections caused by multidrug-resistant Gram-negative bacteria: report of the British Society for Antimicrobial Chemotherapy/Healthcare Infection Society/British Infection Association Joint Working Party. *J Antimicrob Chemother*. 2018.73(suppl_3):iii2–78.
44. Ibrahim D, Jabbour JF, Kanj SS. Current choices of antibiotic treatment for *Pseudomonas aeruginosa* infections. *Curr Opin Infect Dis*. 2020.33(6):464–73.

45. NICE. Ceftazidime with avibactam for treating severe drug-resistant gram-negative bacterial infections Health technology evaluation [Internet]. 2022. [cited 2024 Oct 22]. Available at: www.nice.org.uk/guidance/hte1
46. NICE. Antimicrobial prescribing: ceftolozane with tazobactam for treating hospital-acquired pneumonia, including ventilator-associated pneumonia Evidence summary Key messages [Internet]. 2019. [cited 2024 Oct 22]. Available at: www.nice.org.uk/guidance/es22
47. NICE. Cefiderocol for treating severe drug-resistant gram-negative bacterial infections Health technology evaluation [Internet]. 2022. [cited 2024 Oct 22]. Available at: www.nice.org.uk/guidance/hte2
48. Tenover FC, Nicolau DP, Gill CM. Carbapenemase-producing *Pseudomonas aeruginosa* – an emerging challenge. *Emerg Microbes Infect.* 2022.11(1):811.
49. Karakonstantis S, Rousaki M, Vassilopoulou L, Kritsotakis EI. Global prevalence of cefiderocol non-susceptibility in Enterobacterales, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Stenotrophomonas maltophilia*: a systematic review and meta-analysis. *Clin Microbiol Infect.* 2024.30(2):178–88.
50. McCreary EK, Heil EL, Tamma PD. New Perspectives on Antimicrobial Agents: Cefiderocol. *Antimicrob Agents Chemother.* 2021.65(8):e0217120.
51. Krause KM, Serio AW, Kane TR, Connolly LE. Aminoglycosides: An Overview. *Cold Spring Harb Perspect Med.* 2016.6(6).
52. Taber HW, Mueller JP, Miller PF, Arrow AS. Bacterial Uptake of Aminoglycoside Antibiotics. *Microbiol Rev.* 1987.51(4):439–57.
53. Joint Formulary Committee. The British National Formulary. 2024 [cited 2024 Dec 31]. Aminoglycosides. Available from: <https://bnf.nice.org.uk/treatment-summaries/aminoglycosides/>
54. European Society of Clinical Microbiology and Infectious Diseases (EUCAST). Guidance Document on the Implementation and Use of the Revised Aminoglycoside Breakpoints. 2020.
55. Vidal L, Gafter-Gvili A, Borok S, Fraser A, Leibovici L, Paul M. Efficacy and safety of aminoglycoside monotherapy: systematic review and meta-analysis of randomized controlled trials. *J Antimicrob Chemother.* 2007.60(2):247–57.
56. Ratjen F, Brockhaus F, Angyalosi G. Aminoglycoside therapy against *Pseudomonas aeruginosa* in cystic fibrosis: a review. *J Cyst Fibros.* 2009.8(6):361–9.
57. Lister PD, Wolter DJ, Hanson ND. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin Microbiol Rev.* 2009.22(4):582–610.
58. Macfarlane ELA, Kwasnicka A, Hancock REW. Role of *Pseudomonas aeruginosa* PhoP-PhoQ in resistance to antimicrobial cationic peptides and aminoglycosides. Vol. 146, *Microbiology.* 2000.

59. Van den Bossche S, De Broe E, Coenye T, Van Braeckel E, Crabbé A. The cystic fibrosis lung microenvironment alters antibiotic activity: causes and effects. *Eur Respir Rev*. 2021.30(161).
60. Crabbé A, Ostyn L, Staelens S, Rigauts C, Risseuw M, Dhaenens M, et al. Host metabolites stimulate the bacterial proton motive force to enhance the activity of aminoglycoside antibiotics. *PLoS Pathog*. 2019.15(4).
61. Prayle A, Watson A, Fortnum H, Smyth A. Side effects of aminoglycosides on the kidney, ear and balance in cystic fibrosis. *Thorax*. 2010.65(7):654–8.
62. Fàbrega A, Madurga S, Giralt E, Vila J. Mechanism of action of and resistance to quinolones. *Microb Biotechnol*. 2009.2(1):40–61.
63. Wong A, Kassen R. Parallel evolution and local differentiation in quinolone resistance in *Pseudomonas aeruginosa*. *Microbiology (Reading)*. 2011.157(Pt 4):937–44.
64. Bove C, Baldock RA, Champigneulle O, Martin L, Bennett CL. Fluoroquinolones: old drugs, putative new toxicities. *Expert Opin Drug Saf*. 2022.21(11):1365–78.
65. Fluoroquinolone antibiotics: must now only be prescribed when other commonly recommended antibiotics are inappropriate - GOV.UK [Internet]. [cited 2024 Oct 14]. Available from: <https://www.gov.uk/drug-safety-update/fluoroquinolone-antibiotics-must-now-only-be-prescribed-when-other-commonly-recommended-antibiotics-are-inappropriate>
66. Mohapatra SS, Dwibedy SK, Padhy I. Polymyxins, the last-resort antibiotics: Mode of action, resistance emergence, and potential solutions. *J Biosci*. 2021.46(3):85.
67. Velkov T, Thompson PE, Nation RL, Li J. Structure-activity relationships of polymyxin antibiotics. Vol. 53, *Journal of Medicinal Chemistry*. 2010. p. 1898–916.
68. Falagas ME, Kasiakou SK. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin Infect Dis*. 2005.40(9):1333–41.
69. National Guideline Alliance (UK). Cystic Fibrosis: Diagnosis and management. London: National Institute for Health and Care Excellence (NICE). 2017.
70. ANDERSEN DH. CYSTIC FIBROSIS OF THE PANCREAS AND ITS RELATION TO CELIAC DISEASE. *American Journal of Diseases of Children*. 1938.56(2):344.
71. WILMERS MJ, MACKAY HMM, ANDERSON IM. Five cases of cystic fibrosis of the pancreas. *Proc R Soc Med*. 1950.43(11):829–32.
72. Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, et al. Identification of the cystic fibrosis gene: genetic analysis. *Science*. 1989.245(4922):1073–80.
73. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, et al. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science*. 1989.245(4922):1066–73.

74. Rommens JM, Iannuzzi MC, Kerem B, Drumm ML, Melmer G, Dean M, et al. Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science*. 1989.245(4922):1059–65.
75. Shteinberg M, Haq IJ, Polineni D, Davies JC. Cystic fibrosis. *Lancet*. 2021.397(10290):2195–211.
76. Cystic Fibrosis Mutation Database [Internet]. [cited 2024 Aug 11]. Available from: <http://www.genet.sickkids.on.ca/Home.html>
77. Welcome to CFTR2 | CFTR2 [Internet]. [cited 2024 Aug 11]. Available from: <http://www.cftr2.org/>
78. Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Rossier BC, et al. CFTR as a cAMP-dependent regulator of sodium channels. *Science*. 1995.269(5225):847–50.
79. Mikami Y, Grubb BR, Rogers TD, Dang H, Asakura T, Kota P, et al. Chronic airway epithelial hypoxia exacerbates injury in muco-obstructive lung disease through mucus hyperconcentration. *Sci Transl Med*. 2023.15(699):eabo7728.
80. Veit G, Avramescu RG, Chiang AN, Houck SA, Cai Z, Peters KW, et al. From CFTR biology toward combinatorial pharmacotherapy: expanded classification of cystic fibrosis mutations. *Mol Biol Cell*. 2016.27(3):424–33.
81. Zielenski J, Tsui LC. Cystic fibrosis: genotypic and phenotypic variations. *Annu Rev Genet*. 1995.29:777–807.
82. De Boeck K, Amaral MD. Rapid Review Progress in therapies for cystic fi brosis. *The Lancet Respiratory*. 2016.4:662–74.
83. Cutting GR. Modifier genes in Mendelian disorders: the example of cystic fibrosis. *Ann N Y Acad Sci*. 2010.1214:57–69.
84. Scotet V, L’Hostis C, Férec C. The Changing Epidemiology of Cystic Fibrosis: Incidence, Survival and Impact of the CFTR Gene Discovery. *Genes (Basel)*. 2020.11(6).
85. Turcios NL. Cystic Fibrosis Lung Disease: An Overview. *Respir Care*. 2020.65(2):233–51.
86. Stick SM, Brennan S, Murray C, Douglas T, von Ungern-Sternberg BS, Garratt LW, et al. Bronchiectasis in infants and preschool children diagnosed with cystic fibrosis after newborn screening. *J Pediatr*. 2009.155(5):623-8.e1.
87. Sly PD, Brennan S, Gangell C, de Klerk N, Murray C, Mott L, et al. Lung disease at diagnosis in infants with cystic fibrosis detected by newborn screening. *Am J Respir Crit Care Med*. 2009.180(2):146–52.
88. Quinton PM. Cystic fibrosis: impaired bicarbonate secretion and mucoviscidosis. *Lancet*. 2008.372(9636):415–7.
89. Walsh D, Bevan J, Harrison F. How Does Airway Surface Liquid Composition Vary in Different Pulmonary Diseases, and How Can We Use This Knowledge to Model Microbial Infections? *Microorganisms*. 2024.12(4).

90. Henderson AG, Ehre C, Button B, Abdullah LH, Cai LH, Leigh MW, et al. Cystic fibrosis airway secretions exhibit mucin hyperconcentration and increased osmotic pressure. *J Clin Invest*. 2014.124.
91. Rohmann K, Tschernig T, Pabst R, Goldmann T, Drömann D. Innate immunity in the human lung: pathogen recognition and lung disease. *Cell Tissue Res*. 2011.343(1):167–74.
92. Yonker LM, Cigana C, Hurley BP, Bragonzi A. Host-pathogen interplay in the respiratory environment of cystic fibrosis. *J Cyst Fibros*. 2015.14(4):431–9.
93. Wang G, Nauseef WM. Neutrophil dysfunction in the pathogenesis of cystic fibrosis. *Blood*. 2022.139(17):2622–31.
94. Dwyer M, Shan Q, D’Ortona S, Maurer R, Mitchell R, Olesen H, et al. Cystic Fibrosis Sputum DNA Has NETosis Characteristics and Neutrophil Extracellular Trap Release Is Regulated by Macrophage Migration-Inhibitory Factor. *J Innate Immun*. 2014.6(6):765–79.
95. Cantin AM, Hartl D, Konstan MW, Chmiel JF. Inflammation in cystic fibrosis lung disease: Pathogenesis and therapy. *Journal of Cystic Fibrosis*. 2015.14:419–30.
96. Sanders DB, Bittner RCL, Rosenfeld M, Redding GJ, Goss CH. Pulmonary exacerbations are associated with subsequent FEV1 decline in both adults and children with cystic fibrosis. *Pediatr Pulmonol*. 2011.46(4):393–400.
97. Waters V, Stanojevic S, Atenafu EG, Lu A, Yau Y, Tullis E, et al. Effect of pulmonary exacerbations on long-term lung function decline in cystic fibrosis. *Eur Respir J*. 2012.40(1):61–6.
98. Bhatt JM. Treatment of pulmonary exacerbations in cystic fibrosis. Vol. 22, *European Respiratory Review*. 2013. p. 205–16.
99. Hurley MN, Prayle AP, Flume P. Intravenous antibiotics for pulmonary exacerbations in people with cystic fibrosis. *Cochrane Database of Systematic Reviews*. 2015.2017(6).
100. Seery Donald and Hill L. Spirometry in Cystic Fibrosis. In: Lewis MD FD, editor. *Cystic Fibrosis in Primary Care : An Essential Guide to a Complex, Multi-System Disease* . Cham: Springer International Publishing; 2020. p. 27–39.
101. Beaufils F, Enaud R, Gallode F, Boucher G, Macey J, Berger P, et al. Adherence, reliability, and variability of home spirometry telemonitoring in cystic fibrosis. *Front Pediatr*. 2023.11:1111088.
102. Landini N, Ciet P, Janssens HM, Bertolo S, Ros M, Mattone M, et al. Management of respiratory tract exacerbations in people with cystic fibrosis: Focus on imaging. *Front Pediatr*. 2022.10:1084313.
103. Perrem L, Rayment JH, Ratjen F. The lung clearance index as a monitoring tool in cystic fibrosis: ready for the clinic? *Curr Opin Pulm Med*. 2018.24(6):579–85.
104. CF START - A Cystic Fibrosis Randomised Registry Trial [Internet]. [cited 2024 Oct 15]. Available from: <https://www.cfstart.org.uk/>

105. Rosenfeld M, Rayner O, Smyth AR. Prophylactic anti-staphylococcal antibiotics for cystic fibrosis. *Cochrane Database Syst Rev*. 2020.2020(9).
106. Hurley MN, Fogarty A, McKeever TM, Goss CH, Rosenfeld M, Smyth AR. Early respiratory bacterial detection and antistaphylococcal antibiotic prophylaxis in young children with cystic fibrosis. *Ann Am Thorac Soc*. 2018.15(1):42–8.
107. Zemanick ET, Hoffman LR. Cystic Fibrosis: Microbiology and Host Response. Vol. 63, *Pediatric Clinics of North America*. W.B. Saunders; 2016. p. 617–36.
108. Lund-Palau H, Turnbull AR, Bush A, Bardin E, Cameron L, Soren O, et al. *Pseudomonas aeruginosa* infection in cystic fibrosis: pathophysiological mechanisms and therapeutic approaches. *Expert Rev Respir Med*. 2016.10(6):685–97.
109. Cystic Fibrosis Foundation patient registry. 2023 Annual Data Report [internet]. Bethesda, Maryland; 2024 [Cited on 2024 Oct 13]. Available at <https://www.cff.org/medical-professionals/patient-registry>
110. Emerson J, Rosenfeld M, McNamara S, Ramsey B, Gibson RL. *Pseudomonas aeruginosa* and other predictors of mortality and morbidity in young children with cystic fibrosis. *Pediatr Pulmonol*. 2002.34(2):91–100.
111. Nixon GM, Armstrong DS, Carzino R, Carlin JB, Olinsky A, Robertson CF, et al. Clinical outcome after early *Pseudomonas aeruginosa* infection in cystic fibrosis. *J Pediatr*. 2001.138(5):699–704.
112. Konstan MW, Morgan WJ, Butler SM, Pasta DJ, Craib ML, Silva SJ, et al. Risk factors for rate of decline in forced expiratory volume in one second in children and adolescents with cystic fibrosis. *J Pediatr*. 2007.151(2):134–9, 139.e1.
113. Douglas TA, Brennan S, Gard S, Berry L, Gangell C, Stick SM, et al. Acquisition and eradication of *P. aeruginosa* in young children with cystic fibrosis. *Eur Respir J*. 2009.33(2):305–11.
114. Kidd TJ, Ramsay KA, Vidmar S, Carlin JB, Bell SC, Wainwright CE, et al. *Pseudomonas aeruginosa* genotypes acquired by children with cystic fibrosis by age 5-years. *J Cyst Fibros*. 2015.14(3):361–9.
115. Matsui H, Wagner VE, Hill DB, Schwab UE, Rogers TD, Button B, et al. A physical linkage between cystic fibrosis airway surface dehydration and *Pseudomonas aeruginosa* biofilms. *Proc Natl Acad Sci U S A*. 2006.103(48):18131–6.
116. Baltimore RS, Christie CD, Smith GJ. Immunohistopathologic localization of *Pseudomonas aeruginosa* in lungs from patients with cystic fibrosis. Implications for the pathogenesis of progressive lung deterioration. *Am Rev Respir Dis*. 1989.140(6):1650–61.
117. Pezzulo AA, Tang XX, Hoegger MJ, Abou Alaiwa MH, Ramachandran S, Moninger TO, et al. Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. Vol. 487, *Nature*. 2012. p. 109–13.
118. Stoltz DA, Meyerholz DK, Pezzulo AA, Ramachandran S, Rogan MP, Davis GJ, et al. Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. *Sci Transl Med*. 2010.2(29):29ra31.

119. Rossi E, La Rosa R, Bartell JA, Marvig RL, Haagensen JAJ, Sommer LM, et al. *Pseudomonas aeruginosa* adaptation and evolution in patients with cystic fibrosis. *Nat Rev Microbiol.* 2021.19(5):331–42.
120. Marvig RL, Sommer LM, Molin S, Johansen HK. Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. *Nat Genet.* 2015.47(1):57–64.
121. Williams D, Evans B, Haldenby S, Walshaw MJ, Brockhurst MA, Winstanley C, et al. Divergent, coexisting *Pseudomonas aeruginosa* lineages in chronic cystic fibrosis lung infections. *Am J Respir Crit Care Med.* 2015.191(7):775–85.
122. Hall KM, Pursell ZF, Morici LA. The role of the *Pseudomonas aeruginosa* hypermutator phenotype on the shift from acute to chronic virulence during respiratory infection. Vol. 12, *Frontiers in Cellular and Infection Microbiology*. Frontiers Media S.A.; 2022.
123. Oliver A, Baquero F, Blázquez J. The mismatch repair system (*mutS*, *mutL* and *uvrD* genes) in *Pseudomonas aeruginosa*: molecular characterization of naturally occurring mutants. *Mol Microbiol.* 2002.43(6):1641–50.
124. Oliver A, Cantón R, Campo P, Baquero F, Blázquez J. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science.* 2000.288(5469):1251–4.
125. Marvig RL, Johansen HK, Molin S, Jelsbak L. Genome analysis of a transmissible lineage of *pseudomonas aeruginosa* reveals pathoadaptive mutations and distinct evolutionary paths of hypermutators. *PLoS Genet.* 2013.9(9):e1003741.
126. Waine DJ, Honeybourne D, Smith EG, Whitehouse JL, Dowson CG. Association between hypermutator phenotype, clinical variables, mucoid phenotype, and antimicrobial resistance in *Pseudomonas aeruginosa*. *J Clin Microbiol.* 2008.46(10):3491–3.
127. Klockgether J, Cramer N, Fischer S, Wiehlmann L, Tümmler B. Long-Term Microevolution of *Pseudomonas aeruginosa* Differs between Mildly and Severely Affected Cystic Fibrosis Lungs. *Am J Respir Cell Mol Biol.* 2018.59(2):246–56.
128. Marvig RL, Sommer LM, Molin S, Johansen HK. Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. *Nat Genet.* 2015.47(1):57–64.
129. Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D’Argenio DA, et al. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci U S A.* 2006.103(22):8487–92.
130. Simanek KA, Paczkowski JE. Resistance Is Not Futile: The Role of Quorum Sensing Plasticity in *Pseudomonas aeruginosa* Infections and Its Link to Intrinsic Mechanisms of Antibiotic Resistance. *Microorganisms.* 2022.10(6).
131. D’Argenio DA, Wu M, Hoffman LR, Kulasekara HD, Déziel E, Smith EE, et al. Growth phenotypes of *Pseudomonas aeruginosa* *lasR* mutants adapted to the airways of cystic fibrosis patients. *Mol Microbiol.* 2007.64(2):512–33.
132. Malhotra S, Hayes D, Wozniak DJ. Cystic Fibrosis and *Pseudomonas aeruginosa*: the Host-Microbe Interface. *Clin Microbiol Rev.* 2019.32(3).

133. Martin DW, Schurr MJ, Yu H, Deretic V. Analysis of Promoters Controlled by the Putative Sigma Factor AlgU Regulating Conversion to Mucoidy in *Pseudomonas aeruginosa*: Relationship to O.E and Stress Response. *J Bacteriol.* 1994.176(21):6688–96.
134. Wood LF, Ohman DE. Use of cell wall stress to characterize σ_{22} (AlgT/U) activation by regulated proteolysis and its regulon in *Pseudomonas aeruginosa*. *Mol Microbiol.* 2009.72(1):183–201.
135. Mathee K, Mcpherson CJ, Ohman DE. Posttranslational Control of the algT (algU)-Encoded 22 for Expression of the Alginate Regulon in *Pseudomonas aeruginosa* and Localization of Its Antagonist Proteins MucA and MucB (AlgN). *J Bacteriol.* 1997.179(11):3711–20.
136. Boucher JC, Martinez-Salazar J, Schurr MJ, Mudd MH, Yu H, Deretic V. Two Distinct Loci Affecting Conversion to Mucoidy in *Pseudomonas aeruginosa* in Cystic Fibrosis Encode Homologs of the Serine Protease HtrA. *J Bacteriol.* 1996.178(2):511–23.
137. Boucher JC, Yu H, Mudd MH, Deretic V. Mucoid *Pseudomonas aeruginosa* in Cystic Fibrosis: Characterization of muc Mutations in Clinical Isolates and Analysis of Clearance in a Mouse Model of Respiratory Infection. *Infect Immun.* 1997.65(9):3838–46.
138. Okkotsu Y, Little AS, Schurr MJ. The *pseudomonas aeruginosa* AlgZR two-component system coordinates multiple phenotypes. Vol. 4, *Frontiers in Cellular and Infection Microbiology*. Frontiers Media S.A.; 2014.
139. Tart AH, Blanks MJ, Wozniak DJ. The AlgT-dependent transcriptional regulator AmrZ (AlgZ) inhibits flagellum biosynthesis in mucoid, nonmotile *Pseudomonas aeruginosa* cystic fibrosis isolates. *J Bacteriol.* 2006.188(18):6483–9.
140. Li Z, Kosorok MR, Farrell PM, Laxova A, West SEH, Green CG, et al. Longitudinal development of mucoid *Pseudomonas aeruginosa* infection and lung disease progression in children with cystic fibrosis. *JAMA.* 2005.293(5):581–8.
141. Parad RB, Gerard CJ, Zurakowski D, Nichols DP, Pier GB. Pulmonary outcome in cystic fibrosis is influenced primarily by mucoid *Pseudomonas aeruginosa* infection and immune status and only modestly by genotype. *Infect Immun.* 1999.67(9):4744–50.
142. Farrell PM, Collins J, Broderick LS, Rock MJ, Li Z, Kosorok MR, et al. Association between mucoid *Pseudomonas* infection and bronchiectasis in children with cystic fibrosis. *Radiology.* 2009.252(2):534–43.
143. Boles BR, Singh PK. Endogenous oxidative stress produces diversity and adaptability in biofilm communities. *Proc Natl Acad Sci U S A.* 2008.105(34):12503–8.
144. Mathee K, Ciofu O, Sternberg C, Lindum PW, Campbell JIA, Jensen P, et al. Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. *Microbiology (Reading).* 1999.145 (Pt 6):1349–57.
145. Limoli DH, Jones CJ, Wozniak DJ. Bacterial Extracellular Polysaccharides in Biofilm Formation and Function. *Microbiol Spectr.* 2015.3(3).

146. Pier GB, Coleman F, Grout M, Franklin M, Ohman DE. Role of alginate O acetylation in resistance of mucoid *Pseudomonas aeruginosa* to opsonic phagocytosis. *Infect Immun*. 2001.69(3):1895–901.
147. Leid JG, Willson CJ, Shirtliff ME, Hassett DJ, Parsek MR, Jeffers AK. The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN-gamma-mediated macrophage killing. *J Immunol*. 2005.175(11):7512–8.
148. Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S, Givskov M, et al. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *J Bacteriol*. 2001.183(18):5395–401.
149. Goltermann L, Tolker-Nielsen T. Importance of the Exopolysaccharide Matrix in Antimicrobial Tolerance of *Pseudomonas aeruginosa* Aggregates. 2017.
150. Starkey M, Hickman JH, Ma L, Zhang N, De Long S, Hinz A, et al. *Pseudomonas aeruginosa* rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. *J Bacteriol*. 2009.191(11):3492–503.
151. Kirisits MJ, Prost L, Starkey M, Parsek MR. Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol*. 2005.71(8):4809–21.
152. Malone JG. Role of small colony variants in persistence of *Pseudomonas aeruginosa* infections in cystic fibrosis lungs. *Infect Drug Resist*. 2015.8:237–47.
153. Pestrak MJ, Chaney SB, Eggleston HC, Dellos-Nolan S, Dixit S, Mathew-Steiner SS, et al. *Pseudomonas aeruginosa* rugose small-colony variants evade host clearance, are hyper-inflammatory, and persist in multiple host environments. *PLoS Pathog*. 2018.14(2):e1006842.
154. Valentini M, Gonzalez D, Mavridou DA, Filloux A. Lifestyle transitions and adaptive pathogenesis of *Pseudomonas aeruginosa*. *Curr Opin Microbiol*. 2018.41:15–20.
155. Bjarnsholt T, Jensen PØ, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, et al. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. *Pediatr Pulmonol*. 2009.44(6):547–58.
156. Høiby N, Ciofu O, Bjarnsholt T. *Pseudomonas aeruginosa* biofilms in cystic fibrosis. *Future Microbiol*. 2010.5(11):1663–74.
157. Schick A, Kassen R. Rapid diversification of *Pseudomonas aeruginosa* in cystic fibrosis lung-like conditions. *Proc Natl Acad Sci U S A*. 2018.115(42):10714–9.
158. Rossi E, Falcone M, Molin S, Johansen HK. High-resolution in situ transcriptomics of *Pseudomonas aeruginosa* unveils genotype independent patho-phenotypes in cystic fibrosis lungs. *Nat Commun*. 2018.9(1):3459.
159. Bartell JA, Sommer LM, Haagenen JAJ, Loch A, Espinosa R, Molin S, et al. Evolutionary highways to persistent bacterial infection. *Nat Commun*. 2019.10(1):629.
160. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. *Science (1979)*. 2002.295(5559):1487.

161. Turnbull L, Toyofuku M, Hynen AL, Kurosawa M, Pessi G, Petty NK, et al. Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat Commun.* 2016.7.
162. Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, et al. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol Microbiol.* 2006.59(4):1114–28.
163. Chiang WC, Nilsson M, Jensen PØ, Høiby N, Nielsen TE, Givskov M, et al. Extracellular DNA shields against aminoglycosides in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother.* 2013.57(5):2352–61.
164. Wilton M, Charron-Mazenod L, Moore R, Lewenza S. Extracellular DNA acidifies biofilms and induces aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* 2016.60(1):544–53.
165. Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T. Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. *Mol Microbiol.* 2008.68(1):223–40.
166. Ciofu O, Tolker-Nielsen T. Tolerance and Resistance of *Pseudomonas aeruginosa* Biofilms to Antimicrobial Agents-How *P. aeruginosa* Can Escape Antibiotics. *Front Microbiol.* 2019.10(MAY):913.
167. Huszczyński SM, Lam JS, Khursigara CM. The Role of *Pseudomonas aeruginosa* Lipopolysaccharide in Bacterial Pathogenesis and Physiology. *Pathogens.* 2019.9(1).
168. Weiss J, Barker J. Diverse pro-inflammatory endotoxin recognition systems of mammalian innate immunity. *F1000Res.* 2018.7:516.
169. Venkataranganayaka Abhilasha K, Kedihithlu Marathe G. Bacterial lipoproteins in sepsis. *Immunobiology.* 2021.226(5):152128.
170. Zeth K, Sancho-Vaello E. The Human Antimicrobial Peptides Dermcidin and LL-37 Show Novel Distinct Pathways in Membrane Interactions. *Front Chem.* 2017.5:86.
171. Ernst RK, Yi EC, Guo L, Lim KB, Burns JL, Hackett M, et al. Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. *Science.* 1999.286(5444):1561–5.
172. Ernst RK, Moskowitz SM, Emerson JC, Kraig GM, Adams KN, Harvey MD, et al. Unique lipid modifications in *Pseudomonas aeruginosa* isolated from the airways of patients with cystic fibrosis. *J Infect Dis.* 2007.196(7):1088–92.
173. Lo Sciuto A, Cervoni M, Stefanelli R, Mancone C, Imperi F. Effect of lipid A aminoarabinylation on *Pseudomonas aeruginosa* colistin resistance and fitness. *Int J Antimicrob Agents.* 2020.55(5).
174. Hajjar AM, Tsai JH, Wilson CB, Miller SI. Human Toll-like receptor 4 recognizes host-specific LPS modifications. *Nat Immunol.* 2002.3(4):354–9.

175. Thaipisuttikul I, Hittle LE, Chandra R, Zangari D, Dixon CL, Garrett TA, et al. A divergent *Pseudomonas aeruginosa* palmitoyltransferase essential for cystic fibrosis-specific lipid A. *Mol Microbiol*. 2014.91(1):158–74.
176. Bell SC, Mall MA, Gutierrez H, Macek M, Madge S, Davies JC, et al. The future of cystic fibrosis care: a global perspective. *Lancet Respir Med*. 2020.8(1):65–124.
177. Yang C, Montgomery M. Dornase alfa for cystic fibrosis. *Cochrane Database Syst Rev*. 2021.3(3):CD001127.
178. Nevitt SJ, Thornton J, Murray CS, Dwyer T. Inhaled mannitol for cystic fibrosis. *Cochrane Database Syst Rev*. 2020.5(5):CD008649.
179. Wark P, McDonald VM, Smith S. Nebulised hypertonic saline for cystic fibrosis. *Cochrane Database of Systematic Reviews*. 2023.2023(6).
180. Boyle MP, De Boeck K. A new era in the treatment of cystic fibrosis: correction of the underlying CFTR defect. *Lancet Respir Med*. 2013.1(2):158–63.
181. Van Goor F, Hadida S, Grootenhuis PDJ, Burton B, Cao D, Neuberger T, et al. Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proc Natl Acad Sci U S A*. 2009.106(44):18825–30.
182. Taylor-Cousar JL, Robinson PD, Shteinberg M, Downey DG. CFTR modulator therapy: transforming the landscape of clinical care in cystic fibrosis. Vol. 402, *The Lancet*. Elsevier B.V.; 2023. p. 1171–84.
183. Van Goor F, Hadida S, Grootenhuis PDJ, Burton B, Stack JH, Straley KS, et al. Correction of the F508del-CFTR protein processing defect in vitro by the investigational drug VX-809. *Proc Natl Acad Sci U S A*. 2011.108(46):18843–8.
184. Taylor-Cousar JL, Munck A, McKone EF, van der Ent CK, Moeller A, Simard C, et al. Tezacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del. *N Engl J Med*. 2017.377(21):2013–23.
185. Heijerman HGM, McKone EF, Downey DG, Van Braeckel E, Rowe SM, Tullis E, et al. Efficacy and safety of the elexacaftor plus tezacaftor plus ivacaftor combination regimen in people with cystic fibrosis homozygous for the F508del mutation: a double-blind, randomised, phase 3 trial. *The Lancet*. 2019.394(10212):1940–8.
186. Keating D, Marigowda G, Burr L, Daines C, Mall MA, McKone EF, et al. VX-445–Tezacaftor–Ivacaftor in Patients with Cystic Fibrosis and One or Two Phe508del Alleles. *New England Journal of Medicine*. 2018.379(17):1612–20.
187. Nichols DP, Morgan SJ, Skalland M, Vo AT, Van Dalfsen JM, Singh SB, et al. Pharmacologic improvement of CFTR function rapidly decreases sputum pathogen density, but lung infections generally persist. *J Clin Invest*. 2023.133(10).
188. Hisert KB, Heltshe SL, Pope C, Jorth P, Wu X, Edwards RM, et al. Restoring Cystic Fibrosis Transmembrane Conductance Regulator Function Reduces Airway Bacteria and Inflammation in People with Cystic Fibrosis and Chronic Lung Infections. *Am J Respir Crit Care Med*. 2017.195(12):1617–28.

189. Southern KW, Solis-Moya A, Kurz D, Smith S. Macrolide antibiotics (including azithromycin) for cystic fibrosis. *Cochrane Database of Systematic Reviews*. 2024.2024(2).
190. Somayaji R, Parkins MD, Shah A, Martiniano SL, Tunney MM, Kahle JS, et al. Antimicrobial susceptibility testing (AST) and associated clinical outcomes in individuals with cystic fibrosis: A systematic review. *Journal of Cystic Fibrosis*. 2019.18(2):236–43.
191. Jones AP, Pgdi H, Williamson PR, Langton Hower S, Langton Hower SC, Smyth AR, et al. Intravenous versus oral antibiotics for eradication of *Pseudomonas aeruginosa* in cystic fibrosis (TORPEDO-CF): a randomised controlled trial. *The Lancet Respiratory*. 2020.8:975–86.
192. Holland P, Jahnke N. Single versus combination intravenous anti-pseudomonal antibiotic therapy for people with cystic fibrosis. *Cochrane Database Syst Rev*. 2021.6(6):CD002007.
193. Smith S, Rowbotham NJ. Inhaled anti-pseudomonal antibiotics for long-term therapy in cystic fibrosis. *Cochrane Database Syst Rev*. 2022.11(11):CD001021.
194. Jensen T, Pedersen SS, Garne S, Heilmann C, Høiby N, Koch C. Colistin inhalation therapy in cystic fibrosis patients with chronic *Pseudomonas aeruginosa* lung infection. *J Antimicrob Chemother*. 1987.19(6):831–8.
195. Ramsey BW, Dorkin HL, Eisenberg JD, Gibson RL, Harwood IR, Kravitz RM, et al. Efficacy of aerosolized tobramycin in patients with cystic fibrosis. *N Engl J Med*. 1993.328(24):1740–6.
196. Lenoir G, Antypkin YG, Miano A, Moretti P, Zanda M, Varoli G, et al. Efficacy, safety, and local pharmacokinetics of highly concentrated nebulized tobramycin in patients with cystic fibrosis colonized with *Pseudomonas aeruginosa*. *Paediatr Drugs*. 2007.9 Suppl 1:11–20.
197. Konstan MW, Geller DE, Minić P, Brockhaus F, Zhang J, Angyalosi G. Tobramycin inhalation powder for *P. aeruginosa* infection in cystic fibrosis: the EVOLVE trial. *Pediatr Pulmonol*. 2011.46(3):230–8.
198. Galeva I, Konstan MW, Higgins M, Angyalosi G, Brockhaus F, Piggott S, et al. Tobramycin inhalation powder manufactured by improved process in cystic fibrosis: the randomized EDIT trial. *Curr Med Res Opin*. 2013.29(8):947–56.
199. Schuster A, Haliburn C, Döring G, Goldman MH, Freedom Study Group. Safety, efficacy and convenience of colistimethate sodium dry powder for inhalation (Colobreathe DPI) in patients with cystic fibrosis: a randomised study. *Thorax*. 2013.68(4):344–50.
200. Hodson ME, Gallagher CG, Govan JRW. A randomised clinical trial of nebulised tobramycin or colistin in cystic fibrosis. *Eur Respir J*. 2002.20(3):658–64.
201. Bonyadi P, Saleh NT, Dehghani M, Yamini M, Amini K. Prevalence of antibiotic resistance of *Pseudomonas aeruginosa* in cystic fibrosis infection: A systematic review and meta-analysis. *Microb Pathog*. 2022.165:105461.
202. Bergen PJ, Li J, Nation RL, Turnidge JD, Coulthard K, Milne RW. Comparison of once-, twice- and thrice-daily dosing of colistin on antibacterial effect and emergence of

- resistance: studies with *Pseudomonas aeruginosa* in an in vitro pharmacodynamic model. *J Antimicrob Chemother.* 2008.61(3):636–42.
203. Koyama Y, Kurosasa A, Tsuchiya A, Takakuta K. A new antibiotic 'colistin' produced by spore-forming soil bacteria. *J Antibiot.* 1950.3:457–8.
 204. Binsker U, Käsbohrer A, Hammerl JA. Global colistin use: a review of the emergence of resistant Enterobacterales and the impact on their genetic basis. *FEMS Microbiol Rev.* 2022.46(1):1–37.
 205. Umair M, Hassan B, Farzana R, Ali Q, Sands K, Mathias J, et al. International manufacturing and trade in colistin, its implications in colistin resistance and One Health global policies: a microbiological, economic, and anthropological study. *Lancet Microbe.* 2023.4(4):e264–76.
 206. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular biological study. *Lancet Infect Dis.* 2016.16(2):161–8.
 207. Zhu Y, Monsel A, Roberts JA, Pontikis K, Mimoz O, Rello J, et al. Nebulized Colistin in Ventilator-Associated Pneumonia and Tracheobronchitis: Historical Background, Pharmacokinetics and Perspectives. *Microorganisms.* 2021.9(6):219–49.
 208. Yapa SWS, Li J, Patel K, Wilson JW, Dooley MJ, George J, et al. Pulmonary and systemic pharmacokinetics of inhaled and intravenous colistin methanesulfonate in cystic fibrosis patients: Targeting advantage of inhalational administration. *Antimicrob Agents Chemother.* 2014.58(5):2570–9.
 209. Magréault S, Mankikian J, Marchand S, Diot P, Couet W, Flament T, et al. Pharmacokinetics of colistin after nebulization or intravenous administration of colistin methanesulphonate (Colimycin®) to cystic fibrosis patients. *Journal of Cystic Fibrosis.* 2020.19(3):421–6.
 210. Ratjen F, Rietschel E, Kasel D, Schwiertz R, Starke K, Beier H, et al. Pharmacokinetics of inhaled colistin in patients with cystic fibrosis. *Journal of Antimicrobial Chemotherapy.* 2006.57(2):306–11.
 211. Lu Q, Girardi C, Zhang M, Bouhemad B, Louchahi K, Petitjean O, et al. Nebulized and intravenous colistin in experimental pneumonia caused by *Pseudomonas aeruginosa*. *Intensive Care Med.* 2010.36(7):1147–55.
 212. Li J. Reviving Polymyxins: Achievements, Lessons and the Road Ahead. In: Li J, Nation RL, Kaye KS, editors. *Polymyxin Antibiotics: From Laboratory Bench to Bedside.* Springer Cham; 2019. p. 1–8.
 213. El-Sayed Ahmed MAEG, Zhong LL, Shen C, Yang Y, Doi Y, Tian GB. Colistin and its role in the Era of antibiotic resistance: an extended review (2000-2019). *Emerg Microbes Infect.* 2020.9(1):868–85.
 214. Thomas CJ, Surolia N, Surolia A. Surface plasmon resonance studies resolve the enigmatic endotoxin neutralizing activity of polymyxin B. *J Biol Chem.* 1999.274(42):29624–7.

215. Thomas CJ, Gangadhar BP, Surolia N, Surolia A. Kinetics and mechanism of the recognition of endotoxin by polymyxin B. *J Am Chem Soc.* 1998.120(48):12428–34.
216. Pristovsek P, Kidric J. Solution structure of polymyxins B and E and effect of binding to lipopolysaccharide: an NMR and molecular modeling study. *J Med Chem.* 1999.42(22):4604–13.
217. Moore RA, Bates NC, Hancock RE. Interaction of polycationic antibiotics with *Pseudomonas aeruginosa* lipopolysaccharide and lipid A studied by using dansyl-polymyxin. *Antimicrob Agents Chemother.* 1986.29(3):496–500.
218. Simpson BW, Trent MS. Pushing the envelope: LPS modifications and their consequences. *Nature Reviews Microbiology* 2019 17:7. 2019.17(7):403–16.
219. Whitfield C, Trent MS. Biosynthesis and export of bacterial lipopolysaccharides. *Annu Rev Biochem.* 2014.83:99–128.
220. Guest RL, Rutherford ST, Silhavy TJ. Border Control: Regulating LPS Biogenesis. *Trends Microbiol.* 2021.29(4):334–45.
221. Khadka NK, Aryal CM, Pan J. Lipopolysaccharide-Dependent Membrane Permeation and Lipid Clustering Caused by Cyclic Lipopeptide Colistin. *ACS Omega.* 2018.3(12):17828–34.
222. Schindler M, Osborn MJ. Interaction of divalent cations and polymyxin B with lipopolysaccharide. *Biochemistry.* 1979.18(20):4425–30.
223. Buchholz KR, Reichelt M, Johnson MC, Robinson SJ, Smith PA, Rutherford ST, et al. Potent activity of polymyxin B is associated with long-lived super-stoichiometric accumulation mediated by weak-affinity binding to lipid A. *Nat Commun.* 2024.15(1).
224. Tsubery H, Ofek I, Cohen S, Fridkin M. Structure - Function studies of Polymyxin B nonapeptide: Implications to sensitization of Gram-negative bacteria. *J Med Chem.* 2000.43(16):3085–92.
225. Dixon RA, Chopra I. Polymyxin B and polymyxin B nonapeptide alter cytoplasmic membrane permeability in *Escherichia coli*. *J Antimicrob Chemother.* 1986.18(5):557–63.
226. Duwe AK, Rupar CA, Horsman GB, Vas SI. In vitro cytotoxicity and antibiotic activity of polymyxin B nonapeptide. *Antimicrob Agents Chemother.* 1986.30(2):340–1.
227. Sabnis A, Hagart KL, Klöckner A, Becce M, Evans LE, Furniss RCD, et al. Colistin kills bacteria by targeting lipopolysaccharide in the cytoplasmic membrane. *Elife.* 2021.10.
228. Humphrey M, Larrouy-Maumus GJ, Furniss RCD, Mavridou DAI, Sabnis A, Edwards AM. Colistin resistance in *Escherichia coli* confers protection of the cytoplasmic but not outer membrane from the polymyxin antibiotic. *Microbiology (Reading).* 2021.167(11).
229. Cajal Y, Rogers J, Berg OG, Jain MK. Intermembrane molecular contacts by polymyxin B mediate exchange of phospholipids. *Biochemistry.* 1996.35(1):299–308.
230. Clausell A, Rabanal F, Garcia-Subirats M, Asunción Alsina M, Cajal Y. Membrane association and contact formation by a synthetic analogue of polymyxin B and its fluorescent derivatives. *J Phys Chem B.* 2006.110(9):4465–71.

231. Sampson TR, Liu X, Schroeder MR, Kraft CS, Burd EM, Weiss DS. Rapid killing of *Acinetobacter baumannii* by polymyxins is mediated by a hydroxyl radical death pathway. *Antimicrob Agents Chemother*. 2012.56(11):5642–9.
232. Brochmann RP, Toft A, Ciofu O, Briales A, Kolpen M, Hempel C, et al. Bactericidal effect of colistin on planktonic *Pseudomonas aeruginosa* is independent of hydroxyl radical formation. *Int J Antimicrob Agents*. 2014.43(2):140–7.
233. Deris ZZ, Akter J, Sivanesan S, Roberts KD, Thompson PE, Nation RL, et al. A secondary mode of action of polymyxins against Gram-negative bacteria involves the inhibition of NADH-quinone oxidoreductase activity. *Journal of Antibiotics*. 2014.67(2):147–51.
234. Lenhard JR, Nation RL, Tsuji BT. Synergistic combinations of polymyxins. *Int J Antimicrob Agents*. 2016.48(6):607–13.
235. Brennan-Krohn T, Pironti A, Kirby JE. Synergistic Activity of Colistin-Containing Combinations against Colistin-Resistant Enterobacteriaceae. 2018.
236. Armengol E, Kragh KN, Tolker-Nielsen T, Sierra JM, Higazy D, Ciofu O, et al. Colistin Enhances Rifampicin's Antimicrobial Action in Colistin-Resistant *Pseudomonas aeruginosa* Biofilms. *Antimicrob Agents Chemother*. 2023.
237. Armengol E, Asunción T, Viñas M, Sierra JM. When Combined with Colistin, an Otherwise Ineffective Rifampicin-Linezolid Combination Becomes Active in *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. *Microorganisms*. 2020.8(1).
238. Matzneller P, Strommer S, Drucker C, Petroczi K, Schörgenhofer C, Lackner E, et al. Colistin Reduces LPS-Triggered Inflammation in a Human Sepsis Model In Vivo: A Randomized Controlled Trial. *Clin Pharmacol Ther*. 2017.101(6):773–81.
239. Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol*. 2014.5(NOV):643.
240. Lin QY, Tsai YL, Liu MC, Lin WC, Hsueh PR, Liaw SJ. *Serratia marcescens* arn, a PhoP-regulated locus necessary for polymyxin B resistance. *Antimicrob Agents Chemother*. 2014.58(9):5181–90.
241. Cox AD, Wilkinson SG. Ionizing groups in lipopolysaccharides of *Pseudomonas cepacia* in relation to antibiotic resistance. *Mol Microbiol*. 1991.5(3):641–6.
242. Sidorchuk Z, Zähringer U, Rietschel ET. Chemical structure of the lipid A component of the lipopolysaccharide from a *Proteus mirabilis* Re-mutant. *Eur J Biochem*. 1983.137(1–2):15–22.
243. Cervoni M, Sposato D, Lo Sciuto A, Imperi F. Regulatory Landscape of the *Pseudomonas aeruginosa* Phosphoethanolamine Transferase Gene *eptA* in the Context of Colistin Resistance. *Antibiotics (Basel)*. 2023.12(2).
244. McPhee JB, Lewenza S, Hancock REW. Cationic antimicrobial peptides activate a two-component regulatory system, PmrA-PmrB, that regulates resistance to polymyxin B and cationic antimicrobial peptides in *Pseudomonas aeruginosa*. *Mol Microbiol*. 2003.50(1):205–17.

245. Moskowitz SM, Ernst RK, Miller SI. PmrAB, a Two-Component Regulatory System of *Pseudomonas aeruginosa* that Modulates Resistance to Cationic Antimicrobial Peptides and Addition of Aminoarabinose to Lipid A. *J Bacteriol.* 2004.186(2):575–9.
246. Macfarlane EL, Kwasnicka A, Ochs MM, Hancock RE. PhoP-PhoQ homologues in *Pseudomonas aeruginosa* regulate expression of the outer-membrane protein OprH and polymyxin B resistance. *Mol Microbiol.* 1999.34(2):305–16.
247. Fernández L, Jenssen H, Bains M, Wiegand I, Gooderham WJ, Hancock REW. The two-component system CprRS senses cationic peptides and triggers adaptive resistance in *Pseudomonas aeruginosa* independently of ParRS. *Antimicrob Agents Chemother.* 2012.56(12):6212–22.
248. Gutu AD, Sgambati N, Strasbourger P, Brannon MK, Jacobs MA, Haugen E, et al. Polymyxin resistance of *Pseudomonas aeruginosa* phoQ mutants is dependent on additional two-component regulatory systems. *Antimicrob Agents Chemother.* 2013.57(5):2204–15.
249. Nowicki EM, O'Brien JP, Brodbelt JS, Trent MS. Extracellular zinc induces phosphoethanolamine addition to *Pseudomonas aeruginosa* lipid A via the ColRS two-component system. *Mol Microbiol.* 2015.97(1):166–78.
250. Cervoni M, Lo Sciuto A, Bianchini C, Mancone C, Imperi F. Exogenous and Endogenous Phosphoethanolamine Transferases Differently Affect Colistin Resistance and Fitness in *Pseudomonas aeruginosa*. *Front Microbiol.* 2021.12.
251. Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, et al. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother.* 2012.56(2):1019–30.
252. Abraham N, Kwon DH. A single amino acid substitution in PmrB is associated with polymyxin B resistance in clinical isolate of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett.* 2009.298(2):249–54.
253. Schurek KN, Sampaio JLM, Kiffer CR V, Sinto S, Mendes CMF, Hancock REW. Involvement of pmrAB and phoPQ in Polymyxin B Adaptation and Inducible Resistance in Non-Cystic Fibrosis Clinical Isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* 2009.53(10):4345–51.
254. Miller AK, Brannon MK, Stevens L, Johansen HK, Selgrade SE, Miller SI, et al. PhoQ mutations promote lipid A modification and polymyxin resistance of *Pseudomonas aeruginosa* found in colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother.* 2011.55(12):5761–9.
255. Carroll LM, Gaballa A, Guldemann C, Sullivan G, Henderson LO, Wiedmann M. Identification of Novel Mobilized Colistin Resistance Gene mcr-9 in a Multidrug-Resistant, Colistin-Susceptible *Salmonella enterica* Serotype Typhimurium Isolate. *mBio.* 2019.10(3).
256. Khuntayaporn P, Thirapanmethee K, Chomnawang MT. An Update of Mobile Colistin Resistance in Non-Fermentative Gram-Negative Bacilli. *Front Cell Infect Microbiol.* 2022.12:882236.

257. Moffatt JH, Harper M, Adler B, Nation RL, Li J, Boyce JD. Insertion sequence ISAb11 is involved in colistin resistance and loss of lipopolysaccharide in *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. 2011.55(6):3022–4.
258. Lee JY, Park YK, Chung ES, Na IY, Ko KS. Evolved resistance to colistin and its loss due to genetic reversion in *Pseudomonas aeruginosa*. *Sci Rep*. 2016.6(1):25543.
259. Döbelmann B, Willmann M, Steglich M, Bunk B, Nübel U, Peter S, et al. Rapid and Consistent Evolution of Colistin Resistance in Extensively Drug-Resistant *Pseudomonas aeruginosa* during Morbidostat Culture. *Antimicrob Agents Chemother*. 2017.61(9).
260. Puja H, Bolard A, Noguès A, Plésiat P, Jeannot K. The Efflux Pump MexXY/OprM Contributes to the Tolerance and Acquired Resistance of *Pseudomonas aeruginosa* to Colistin. *Antimicrob Agents Chemother*. 2020.64(4).
261. Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2000.44(12):3322–7.
262. Young ML, Bains M, Bell A, Hancock RE. Role of *Pseudomonas aeruginosa* outer membrane protein OprH in polymyxin and gentamicin resistance: isolation of an OprH-deficient mutant by gene replacement techniques. *Antimicrob Agents Chemother*. 1992.36(11):2566–8.
263. Bell A, Bains M, Hancock RE. *Pseudomonas aeruginosa* outer membrane protein OprH: expression from the cloned gene and function in EDTA and gentamicin resistance. *J Bacteriol*. 1991.173(21):6657–64.
264. Michael AJ. Polyamines in Eukaryotes, Bacteria, and Archaea. *J Biol Chem*. 2016.291(29):14896–903.
265. Johnson L, Mulcahy H, Kanevets U, Shi Y, Lewenza S. Surface-localized spermidine protects the *Pseudomonas aeruginosa*: Outer membrane from antibiotic treatment and oxidative stress. *J Bacteriol*. 2012.194(4):813–26.
266. Conrad RS, Wulf RG, Clay DL. Effects of carbon sources on antibiotic resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 1979.15(1):59–66.
267. Sauvage S, Gaviard C, Tahrioui A, Coquet L, Le H, Alexandre S, et al. Impact of Carbon Source Supplementations on *Pseudomonas aeruginosa* Physiology. *J Proteome Res*. 2022.21(6):1392–407.
268. Hill D, Rose B, Pajkos A, Robinson M, Bye P, Bell S, et al. Antibiotic Susceptibilities of *Pseudomonas aeruginosa* Isolates Derived from Patients with Cystic Fibrosis under Aerobic, Anaerobic, and Biofilm Conditions. *J Clin Microbiol*. 2005.43(10):5085–90.
269. Kolpen M, Appeldorff CF, Brandt S, Mousavi N, Kragh KN, Aydogan S, et al. Increased bactericidal activity of colistin on *Pseudomonas aeruginosa* biofilms in anaerobic conditions. *Pathog Dis*. 2018.74(1).
270. Zemke AC, Shiva S, Burns JL, Moskowitz SM, Pilewski JM, Gladwin MT, et al. Nitrite modulates bacterial antibiotic susceptibility and biofilm formation in association with airway epithelial cells. *Free Radic Biol Med*. 2014.77:307–16.

271. Pinezich MR, Tamargo MA, Fleischer S, Reimer JA, Hudock MR, Hozain AE, et al. Pathological remodeling of distal lung matrix in end-stage cystic fibrosis patients. *J Cyst Fibros*. 2022.21(6):1027–35.
272. Coates AL, Allen PD, MacNeish CF, Ho SL, Lands LC. Effect of size and disease on estimated deposition of drugs administered using jet nebulization in children with cystic fibrosis. *Chest*. 2001.119(4):1123–30.
273. Montgomery ST, Mall MA, Kicic A, Stick SM, AREST CF. Hypoxia and sterile inflammation in cystic fibrosis airways: mechanisms and potential therapies. *Eur Respir J*. 2017.49(1).
274. Whitsett JA. Airway Epithelial Differentiation and Mucociliary Clearance. *Ann Am Thorac Soc*. 2018.15(Suppl 3):S143–8.
275. Ruhluel D, O'Brien S, Fothergill JL, Neill DR. Development of liquid culture media mimicking the conditions of sinuses and lungs in cystic fibrosis and health. *F1000Res*. 2022.11:1007.
276. Palmer KL, Aye LM, Whiteley M. Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. *J Bacteriol*. 2007.189(22):8079–87.
277. Thomas SR, Ray A, Hodson ME, Pitt TL. Increased sputum amino acid concentrations and auxotrophy of *Pseudomonas aeruginosa* in severe cystic fibrosis lung disease. *Thorax*. 2000.55(9):795–7.
278. Sriramulu DD, Lünsdorf H, Lam JS, Römling U. Microcolony formation: A novel biofilm model of *Pseudomonas aeruginosa* for the cystic fibrosis lung. *J Med Microbiol*. 2005.54(7):667–76.
279. Shrout JD, Chopp DL, Just CL, Hentzer M, Givskov M, Parsek MR. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. *Mol Microbiol*. 2006.62(5):1264–77.
280. Santos AS, Sampaio APW, Vasquez GS, Santa Anna LM, Pereira N, Freire DMG. Evaluation of different carbon and nitrogen sources in production of rhamnolipids by a strain of *Pseudomonas aeruginosa*. *Appl Biochem Biotechnol*. 2002.98–100:1025–35.
281. Rojo F. Carbon catabolite repression in *Pseudomonas* : optimizing metabolic versatility and interactions with the environment. *FEMS Microbiol Rev*. 2010.34(5):658–84.
282. Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev*. 2006.86(1):245–78.
283. Henke MO, John G, Germann M, Lindemann H, Rubin BK. MUC5AC and MUC5B mucins increase in cystic fibrosis airway secretions during pulmonary exacerbation. *Am J Respir Crit Care Med*. 2007.175(8):816–21.
284. Wheeler KM, Cárcamo-Oyarce G, Turner BS, Dellos-Nolan S, Co JY, Lehoux S, et al. Mucin glycans attenuate the virulence of *Pseudomonas aeruginosa* in infection. *Nat Microbiol*. 2019.4(12):2146–54.
285. Wang BX, Wheeler KM, Cady KC, Lehoux S, Cummings RD, Laub MT, et al. Mucin Glycans Signal through the Sensor Kinase RetS to Inhibit Virulence-Associated Traits in *Pseudomonas aeruginosa*. *Curr Biol*. 2021.31(1):90-102.e7.

286. Venkatakrishnan V, Packer NH, Thaysen-Andersen M. Host mucin glycosylation plays a role in bacterial adhesion in lungs of individuals with cystic fibrosis. *Expert Rev Respir Med*. 2013.7(5):553–76.
287. Huang JX, Blaskovich MAT, Pelingon R, Ramu S, Kavanagh A, Elliott AG, et al. Mucin binding reduces colistin antimicrobial activity. *Antimicrob Agents Chemother*. 2015.59(10):5925–31.
288. Müller L, Murgia X, Siebenbürger L, Börger C, Schwarzkopf K, Sewald K, et al. Human airway mucus alters susceptibility of *Pseudomonas aeruginosa* biofilms to tobramycin, but not colistin. *Journal of Antimicrobial Chemotherapy*. 2018.73(10):2762–9.
289. Hunt BE, Weber A, Berger A, Ramsey B, Smith AL. Macromolecular mechanisms of sputum inhibition of tobramycin activity. *Antimicrob Agents Chemother*. 1995.39(1):34–9.
290. Butnarusu C, Barbero N, Pacheco D, Petrini P, Visentin S. Mucin binding to therapeutic molecules: The case of antimicrobial agents used in cystic fibrosis. *Int J Pharm*. 2019.564:136–44.
291. Sun E, Gill EE, Falsafi R, Yeung A, Liu S, Hancock REW. Broad-Spectrum Adaptive Antibiotic Resistance Associated with *Pseudomonas aeruginosa* Mucin-Dependent Surfing Motility. *Antimicrob Agents Chemother*. 2018.62(9).
292. Brandt T, Breitenstein S, von der Hardt H, Tummler B. DNA concentration and length in sputum of patients with cystic fibrosis during inhalation with recombinant human DNase. *Thorax*. 1995.50(8):880–2.
293. Piva TC, Luft C, Antunes KH, Marostica PJC, Pinto LA, Donadio MVF. Extracellular DNA in sputum is associated with pulmonary function and hospitalization in patients with cystic fibrosis. *Respir Med*. 2020.172.
294. Chance DL, Mawhinney TP. Observations of, and Insights into, Cystic Fibrosis Mucus Heterogeneity in the Pre-Modulator Era: Sputum Characteristics, DNA and Glycoprotein Content, and Solubilization Time. *Journal of Respiration*. 2020.1(1):8–29.
295. Smith AL, Redding G, Doershuk C, Goldmann D, Gore E, Hilman B, et al. Sputum changes associated with therapy for endobronchial exacerbation in cystic fibrosis. *J Pediatr*. 1988.112(4):547–54.
296. Alhede M, Alhede M, Qvortrup K, Kragh KN, Jensen PØ, Stewart PS, et al. The origin of extracellular DNA in bacterial biofilm infections in vivo. *Pathog Dis*. 2020.78(2).
297. Lethem MI, James SL, Marriott C, Burke JF. The origin of DNA associated with mucus glycoproteins in cystic fibrosis sputum. *Eur Respir J*. 1990.3(1):19–23.
298. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. *Nature*. 2000.408(6813):740–5.
299. Buzzo JR, Devaraj A, Gloag ES, Jurcisek JA, Robledo-Avila F, Kesler T, et al. Z-form extracellular DNA is a structural component of the bacterial biofilm matrix. *Cell*. 2021.184(23):5740-5758.e17.

300. Mulcahy H, Charron-Mazenod L, Lewenza S. Extracellular DNA Chelates Cations and Induces Antibiotic Resistance in *Pseudomonas aeruginosa* Biofilms. Gilmore MS, editor. PLoS Pathog. 2008.4(11):e1000213.
301. Mulcahy H, Charron-Mazenod L, Lewenza S. *Pseudomonas aeruginosa* produces an extracellular deoxyribonuclease that is required for utilization of DNA as a nutrient source. Environ Microbiol. 2010.12(6):1621–9.
302. Hull J, South M, Phelan P, Grimwood K. Surfactant composition in infants and young children with cystic fibrosis. Am J Respir Crit Care Med. 1997.156(1):161–5.
303. Griesse M, Birrer P, Demirsoy A. Pulmonary surfactant in cystic fibrosis. Eur Respir J. 1997.10(9):1983–8.
304. Meyer KC, Sharma A, Brown R, Weatherly M, Moya FR, Lewandoski J, et al. Function and composition of pulmonary surfactant and surfactant-derived fatty acid profiles are altered in young adults with cystic fibrosis. Chest. 2000.118(1):164–74.
305. Deschamps E, Schaumann A, Schmitz-Afonso I, Afonso C, Dé E, Loutelier-Bourhis C, et al. Membrane phospholipid composition of *Pseudomonas aeruginosa* grown in a cystic fibrosis mucus-mimicking medium. Biochim Biophys Acta Biomembr. 2021.1863(1).
306. Baker LY, Hobby CR, Siv AW, Bible WC, Glennon MS, Anderson DM, et al. *Pseudomonas aeruginosa* responds to exogenous polyunsaturated fatty acids (PUFAs) by modifying phospholipid composition, membrane permeability, and phenotypes associated with virulence. BMC Microbiol. 2018.18(1):117.
307. Son MS, Matthews WJ, Kang Y, Nguyen DT, Hoang TT. In vivo evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of cystic fibrosis patients. Infect Immun. 2007.75(11):5313–24.
308. Kang Y, Zarzycki-Siek J, Walton CB, Norris MH, Hoang TT. Multiple FadD Acyl-CoA Synthetases Contribute to Differential Fatty Acid Degradation and Virulence in *Pseudomonas aeruginosa*. PLoS One. 2010.5(10):13557.
309. Grasemann H, Shehnaz D, Enomoto M, Leadley M, Belik J. L-Ornithine Derived Polyamines in Cystic Fibrosis Airways. PLoS One. 2012.7(10):46618.
310. Twomey KB, Alston M, An SQ, O’Connell OJ, McCarthy Y, Swarbreck D, et al. Microbiota and metabolite profiling reveal specific alterations in bacterial community structure and environment in the cystic fibrosis airway during exacerbation. PLoS One. 2013.8(12):e82432.
311. Kwon DH, Lu CD. Polyamines induce resistance to cationic peptide, aminoglycoside, and quinolone antibiotics in *Pseudomonas aeruginosa* PAO1. Antimicrob Agents Chemother. 2006.50(5):1615–22.
312. Hasan CM, Pottenger S, Green AE, Cox AA, White JS, Jones T, et al. *Pseudomonas aeruginosa* utilizes the host-derived polyamine spermidine to facilitate antimicrobial tolerance. JCI Insight. 2022.7(22).
313. Tata F, Stanier P, Wicking C, Halford S, Kruyer H, Lench NJ, et al. Cloning the mouse homolog of the human cystic fibrosis transmembrane conductance regulator gene. Genomics. 1991.10(2):301–7.

314. Snouwaert JN, Brigman KK, Latour AM, Malouf NN, Boucher RC, Smithies O, et al. An Animal Model for Cystic Fibrosis Made by Gene Targeting. *Science* (1979). 1992.257(5073):1083–8.
315. Dorin JR, Dickinson P, Alton EW, Smith SN, Geddes DM, Stevenson BJ, et al. Cystic fibrosis in the mouse by targeted insertional mutagenesis. *Nature*. 1992.359(6392):211–5.
316. Colledge WH, Abella BS, Southern KW, Ratcliff R, Jiang C, Cheng SH, et al. Generation and characterization of a delta F508 cystic fibrosis mouse model. *Nat Genet*. 1995.10(4):445–52.
317. Delaney SJ, Alton EW, Smith SN, Lunn DP, Farley R, Lovelock PK, et al. Cystic fibrosis mice carrying the missense mutation G551D replicate human genotype-phenotype correlations. *EMBO J*. 1996.15(5):955–63.
318. Davidson DJ, Dorin JR. The CF mouse: an important tool for studying cystic fibrosis. *Expert Rev Mol Med*. 2001.2001:1–27.
319. Semaniakou A, Croll RP, Chappe V. Animal Models in the Pathophysiology of Cystic Fibrosis. *Front Pharmacol*. 2018.9:1475.
320. Mccarron A, Parsons D, Donnelley M. Animal and Cell Culture Models for Cystic Fibrosis Which Model Is Right for Your Application? *Am J Pathol*. 2021.191:228–42.
321. Sathe M, Houwen R. Meconium ileus in Cystic Fibrosis. *J Cyst Fibros*. 2017.16 Suppl 2:S32–9.
322. Collawn JF, Lazrak A, Bebok Z, Matalon S. The CFTR and ENaC debate: how important is ENaC in CF lung disease? *Am J Physiol Lung Cell Mol Physiol*. 2012.302(11):L1141–6.
323. Van Heeckeren A, Walenga R, Konstan MW, Bonfield T, Davis PB, Ferkol T. Excessive Inflammatory Response of Cystic Fibrosis Mice to Bronchopulmonary Infection with *Pseudomonas aeruginosa*. *J Clin Invest*. 1997.100(11):2810–5.
324. Kent G, Iles R, Bear CE, Huan LJ, Griesenbach U, McKerlie C, et al. Lung disease in mice with cystic fibrosis. *J Clin Invest*. 1997.100(12):3060–9.
325. Durie PR, Kent G, Phillips MJ, Ackerley CA. Characteristic multiorgan pathology of cystic fibrosis in a long-living cystic fibrosis transmembrane regulator knockout murine model. *Am J Pathol*. 2004.164(4):1481–93.
326. Guilbault C, Martin P, Houle D, Boghdady ML, Guiot MC, Marion D, et al. Cystic fibrosis lung disease following infection with *Pseudomonas aeruginosa* in *Cftr* knockout mice using novel non-invasive direct pulmonary infection technique. *Lab Anim*. 2005.39(3):336–52.
327. Mall M, Grubb BR, Harkema JR, O’Neal WK, Boucher RC. Increased airway epithelial Na⁺ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med*. 2004.10(5):487–93.
328. Zhou Z, Duerr J, Johannesson B, Schubert SC, Treis D, Harm M, et al. The ENaC-overexpressing mouse as a model of cystic fibrosis lung disease. *J Cyst Fibros*. 2011.10 Suppl 2(2):S172–82.

329. Mall MA, Harkema JR, Trojanek JB, Treis D, Livraghi A, Schubert S, et al. Development of chronic bronchitis and emphysema in beta-epithelial Na⁺ channel-overexpressing mice. *Am J Respir Crit Care Med*. 2008.177(7):730–42.
330. Van Goor F, Hadida S, Grootenhuis PDJ, Burton B, Cao D, Neuberger T, et al. Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *Proc Natl Acad Sci U S A*. 2009.106(44):18825–30.
331. Bose SJ, Bijvelds MJC, Wang Y, Liu J, Cai Z, Bot AGM, et al. Differential thermostability and response to cystic fibrosis transmembrane conductance regulator potentiators of human and mouse F508del-CFTR. *Am J Physiol Lung Cell Mol Physiol*. 2019.317(1):L71–86.
332. Gawenis LR, Hodges CA, McHugh DR, Valerio DM, Miron A, Cotton CU, et al. A BAC Transgene Expressing Human CFTR under Control of Its Regulatory Elements Rescues Cfr Knockout Mice. *Sci Rep*. 2019.9(1):11828.
333. Trezise AE, Szpirer C, Buchwald M. Localization of the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) in the rat to chromosome 4 and implications for the evolution of mammalian chromosomes. *Genomics*. 1992.14(4):869–74.
334. Tuggle KL, Birket SE, Cui X, Hong J, Warren J. Characterization of Defects in Ion Transport and Tissue Development in Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)-Knockout Rats. *PLoS One*. 2014.9(3):91253.
335. McCarron A, Cmielewski P, Reyne N, McIntyre C, Finnie J, Craig F, et al. Phenotypic Characterization and Comparison of Cystic Fibrosis Rat Models Generated Using CRISPR/Cas9 Gene Editing. *Am J Pathol*. 2020.190(5):977–93.
336. Dreano E, Bacchetta M, Simonin J, Galmiche L, Usal C, Slimani L, et al. Characterization of two rat models of cystic fibrosis-KO and F508del CFTR-Generated by Crispr-Cas9. *Animal Model Exp Med*. 2019.2(4):297–311.
337. Birket SE, Davis JM, Fernandez CM, Tuggle KL, Oden AM, Chu KK, et al. Development of an airway mucus defect in the cystic fibrosis rat. *JCI Insight*. 2018.3(1).
338. Reyne N, Cmielewski P, McCarron A, Smith R, Schneider-Futschik E, Eikelis N, et al. Effect of elexacaftor-tezacaftor-ivacaftor on nasal potential difference and lung function in Phe508del rats. *Front Pharmacol*. 2024.15:1362325.
339. Fisher JT, Zhang Y, Engelhardt JF. Comparative biology of cystic fibrosis animal models. *Methods Mol Biol*. 2011.742:311–34.
340. Sun X, Yan Z, Yi Y, Li Z, Lei D, Rogers CS, et al. Adeno-associated virus-targeted disruption of the CFTR gene in cloned ferrets. *J Clin Invest*. 2008.118(4):1578–83.
341. Sun X, Sui H, Fisher JT, Yan Z, Liu X, Cho HJ, et al. Disease phenotype of a ferret CFTR-knockout model of cystic fibrosis. *J Clin Invest*. 2010.120(9):3149–60.
342. Sun X, Olivier AK, Liang B, Yi Y, Sui H, Evans TIA, et al. Lung phenotype of juvenile and adult cystic fibrosis transmembrane conductance regulator-knockout ferrets. *Am J Respir Cell Mol Biol*. 2014.50(3):502–12.

343. Keiser NW, Birket SE, Evans IA, Tyler SR, Crooke AK, Sun X, et al. Defective innate immunity and hyperinflammation in newborn cystic fibrosis transmembrane conductance regulator-knockout ferret lungs. *Am J Respir Cell Mol Biol*. 2015.52(6):683–94.
344. Sun X, Yi Y, Yan Z, Rosen BH, Liang B, Winter MC, et al. In utero and postnatal VX-770 administration rescues multiorgan disease in a ferret model of cystic fibrosis. *Sci Transl Med*. 2019.11(485).
345. Ostedgaard LS, Rogers CS, Dong Q, Randak CO, Vermeer DW, Rokhlina T, et al. Processing and function of CFTR-DeltaF508 are species-dependent. *Proc Natl Acad Sci U S A*. 2007.104(39):15370–5.
346. Pabst R, Binns RM. The immune system of the respiratory tract in pigs. *Vet Immunol Immunopathol*. 1994.43(1–3):151–6.
347. Maina JN, van Gils P. Morphometric characterization of the airway and vascular systems of the lung of the domestic pig, *Sus scrofa*: comparison of the airway, arterial and venous systems. *Comp Biochem Physiol A Mol Integr Physiol*. 2001.130(4):781–98.
348. Rogers CS, Hao Y, Rokhlina T, Samuel M, Stoltz DA, Li Y, et al. Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. *J Clin Invest*. 2008.118(4):1571–7.
349. Welsh MJ, Rogers CS, Stoltz DA, Meyerholz DK, Prather RS. Development of a porcine model of cystic fibrosis. *Trans Am Clin Climatol Assoc*. 2009.120:149–62.
350. Stoltz DA, Meyerholz DK, Pezzulo AA, Ramachandran S, Rogan MP, Davis GJ, et al. Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. *Sci Transl Med*. 2010.2(29):29ra31.
351. Meyerholz DK, Stoltz DA, Namati E, Ramachandran S, Pezzulo AA, Smith AR, et al. Loss of cystic fibrosis transmembrane conductance regulator function produces abnormalities in tracheal development in neonatal pigs and young children. *Am J Respir Crit Care Med*. 2010.182(10):1251–61.
352. Ehrhardt C, Collnot EM, Baldes C, Becker U, Laue M, Kim KJ, et al. Towards an in vitro model of cystic fibrosis small airway epithelium: characterisation of the human bronchial epithelial cell line CFBE41o-. *Cell Tissue Res*. 2006.323(3):405–15.
353. Castellani S, Di Gioia S, di Toma L, Conese M. Human Cellular Models for the Investigation of Lung Inflammation and Mucus Production in Cystic Fibrosis. *Anal Cell Pathol (Amst)*. 2018.2018(1):3839803.
354. Grassi L, Crabbé A. Recreating chronic respiratory infections in vitro using physiologically relevant models. *Eur Respir Rev*. 2024.33(173):107832.
355. Brodlie M, McKean MC, Johnson GE, Perry JD, Nicholson A, Verdon B, et al. Primary bronchial epithelial cell culture from explanted cystic fibrosis lungs. *Exp Lung Res*. 2010.36(2):101–10.
356. Dvorak A, Tilley AE, Shaykhiev R, Wang R, Crystal RG. Do airway epithelium air-liquid cultures represent the in vivo airway epithelium transcriptome? *Am J Respir Cell Mol Biol*. 2011.44(4):465–73.

357. Martinovich KM, Iosifidis T, Buckley AG, Looi K, Ling KM, Sutanto EN, et al. Conditionally reprogrammed primary airway epithelial cells maintain morphology, lineage and disease specific functional characteristics. *Sci Rep.* 2017.7(1):17971.
358. Yonker LM, Mou H, Chu KK, Pazos MA, Leung H, Cui D, et al. Development of a Primary Human Co-Culture Model of Inflamed Airway Mucosa. *Sci Rep.* 2017.7(1):8182.
359. Moreau-Marquis S, Bomberger JM, Anderson GG, Swiatecka-Urban A, Ye S, O'Toole GA, et al. The DeltaF508-CFTR mutation results in increased biofilm formation by *Pseudomonas aeruginosa* by increasing iron availability. *Am J Physiol Lung Cell Mol Physiol.* 2008.295(1):L25-37.
360. Zemke AC, Gladwin MT, Bomberger JM. Sodium nitrite blocks the activity of aminoglycosides against *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother.* 2015.59(6):3329–34.
361. Lashua LP, Melvin JA, Deslouches B, Pilewski JM, Montelaro RC, Bomberger JM. Engineered cationic antimicrobial peptide (eCAP) prevents *Pseudomonas aeruginosa* biofilm growth on airway epithelial cells. *J Antimicrob Chemother.* 2016.71(8):2200–7.
362. Murgia X, Yasar H, Carvalho-Wodarz C, Loretz B, Gordon S, Schwarzkopf K, et al. Modelling the bronchial barrier in pulmonary drug delivery: A human bronchial epithelial cell line supplemented with human tracheal mucus. *Eur J Pharm Biopharm.* 2017.118:79–88.
363. Lewin GR, Kapur A, Cornforth DM, Duncan RP, Diggle FL, Moustafa DA, et al. Application of a quantitative framework to improve the accuracy of a bacterial infection model. *Proc Natl Acad Sci U S A.* 2023.120(19):e2221542120.
364. Barrila J, Radtke AL, Crabbé A, Sarker SF, Herbst-Kralovetz MM, Ott CM, et al. Organotypic 3D cell culture models: using the rotating wall vessel to study host-pathogen interactions. *Nat Rev Microbiol.* 2010.8(11):791–801.
365. Crabbé A, Liu Y, Matthijs N, Rigole P, De La Fuente-Núñez C, Davis R, et al. Antimicrobial efficacy against *Pseudomonas aeruginosa* biofilm formation in a three-dimensional lung epithelial model and the influence of fetal bovine serum. *Sci Rep.* 2017.7:43321.
366. Grassi L, Batoni G, Ostyn L, Rigole P, Van den Bossche S, Rinaldi AC, et al. The Antimicrobial Peptide lin-SB056-1 and Its Dendrimeric Derivative Prevent *Pseudomonas aeruginosa* Biofilm Formation in Physiologically Relevant Models of Chronic Infections. *Front Microbiol.* 2019.10:198.
367. Van den Bossche S, Ostyn L, Vandendriessche V, Rigauts C, De Keersmaecker H, Nickerson CA, et al. The development and characterization of in vivo-like three-dimensional models of bronchial epithelial cell lines. *European Journal of Pharmaceutical Sciences.* 2023.190.
368. Ingber DE. Human organs-on-chips for disease modelling, drug development and personalized medicine. *Nat Rev Genet.* 2022.23(8):467–91.
369. Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE. Reconstituting organ-level lung functions on a chip. *Science.* 2010.328(5986):1662–8.

370. Plebani R, Potla R, Soong M, Bai H, Izadifar Z, Jiang A, et al. Modeling pulmonary cystic fibrosis in a human lung airway-on-a-chip. *J Cyst Fibros*. 2022.21(4):606–15.
371. Vazquez-Armendariz AI, Tata PR. Recent advances in lung organoid development and applications in disease modeling. *J Clin Invest*. 2023.133(22).
372. Paolicelli G, Luca A De, Jose SS, Antonini M, Teloni I, Fric J, et al. Using Lung Organoids to Investigate Epithelial Barrier Complexity and IL-17 Signaling During Respiratory Infection. *Front Immunol*. 2019.10:323.
373. Leoni Swart A, Laventie BJ, Sütterlin R, Junne T, Lauer L, Manfredi P, et al. *Pseudomonas aeruginosa* breaches respiratory epithelia through goblet cell invasion in a microtissue model. *Nat Microbiol*. 2024.9(7):1725–37.
374. Berical A, Lee RE, Lu J, Beermann M Lou, Le Suer JA, Mithal A, et al. A multimodal iPSC platform for cystic fibrosis drug testing. *Nat Commun*. 2022.13(1):4270.
375. Pollard BS, Pollard HB. Induced pluripotent stem cells for treating cystic fibrosis: State of the science. *Pediatr Pulmonol*. 2018.53(S3):S12–29.
376. Cidem A, Bradbury P, Traini D, Ong HX. Modifying and Integrating in vitro and ex vivo Respiratory Models for Inhalation Drug Screening. *Front Bioeng Biotechnol*. 2020.8:581995.
377. Harrison F, Muruli A, Higgins S, Diggle SP. Development of an ex vivo porcine lung model for studying growth, virulence, and signaling of *Pseudomonas aeruginosa*. *Infect Immun*. 2014.82(8):3312–23.
378. Harrison F, Diggle SP. An ex vivo lung model to study bronchioles infected with *Pseudomonas aeruginosa* biofilms. *Microbiology (Reading)*. 2016.162(10):1755–60.
379. Hassan MM, Harrington NE, Sweeney E, Harrison F. Predicting Antibiotic-Associated Virulence of *Pseudomonas aeruginosa* Using an ex vivo Lung Biofilm Model. *Front Microbiol*. 2020.11:568510.
380. Cornforth DM, Dees JL, Ibberson CB, Huse HK, Mathiesen IH, Kirketerp-Møller K, et al. *Pseudomonas aeruginosa* transcriptome during human infection. *Proc Natl Acad Sci U S A*. 2018.115(22):E5125–34.
381. Harrington NE, Littler JL, Harrison F. Transcriptome Analysis of *Pseudomonas aeruginosa* Biofilm Infection in an Ex Vivo Pig Model of the Cystic Fibrosis Lung. *Appl Environ Microbiol*. 2022.88(3):e0178921.
382. Ghani M, Soothill JS. Ceftazidime, gentamicin, and rifampicin, in combination, kill biofilms of mucoid *Pseudomonas aeruginosa*. *Can J Microbiol*. 1997.43(11):999–1004.
383. Fung C, Naughton S, Turnbull L, Tingpej P, Rose B, Arthur J, et al. Gene expression of *Pseudomonas aeruginosa* in a mucin-containing synthetic growth medium mimicking cystic fibrosis lung sputum. *J Med Microbiol*. 2010.59(9):1089–100.
384. Hare NJ, Cho †, Soe Z, Rose B, Harbour C, Codd R, et al. Proteomics of *Pseudomonas aeruginosa* Australian Epidemic Strain 1 (AES-1) Cultured under Conditions Mimicking the Cystic Fibrosis Lung Reveals Increased Iron Acquisition via the Siderophore Pyochelin. *J Proteome Res*. 2012.11:776–95.

385. Quinn RA, Whiteson K, Lim YW, Salamon P, Bailey B, Mienardi S, et al. A Winogradsky-based culture system shows an association between microbial fermentation and cystic fibrosis exacerbation. *ISME Journal*. 2015.9:1024–38.
386. Kirchner S, Fothergill JL, Wright EA, James CE, Mowat E, Winstanley C. Use of artificial sputum medium to test antibiotic efficacy against *Pseudomonas aeruginosa* in conditions more relevant to the cystic fibrosis lung. *Journal of Visualized Experiments*. 2012.(64):1–8.
387. Aiyer A, Manos J. The Use of Artificial Sputum Media to Enhance Investigation and Subsequent Treatment of Cystic Fibrosis Bacterial Infections. *Microorganisms*. 2022.10(7).
388. Turner KH, Wessel AK, Palmer GC, Murray JL, Whiteley M. Essential genome of *Pseudomonas aeruginosa* in cystic fibrosis sputum. *Proc Natl Acad Sci U S A*. 2015.112(13):4110–5.
389. Cornforth DM, Diggle FL, Melvin JA, Bomberger JM, Whiteley M. Quantitative Framework for Model Evaluation in Microbiology Research Using *Pseudomonas aeruginosa* and Cystic Fibrosis Infection as a Test Case. *mBio*. 2020.11(1).
390. Hoang TT, Kutchma AJ, Becher A, Schweizer HP. Integration-proficient plasmids for *Pseudomonas aeruginosa*: Site- specific integration and use for engineering of reporter and expression strains. *Plasmid*. 2000.43(1):59–72.
391. Figurski DH, Helinski DR. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci U S A*. 1979.76(4):1648–52.
392. Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, Ausubel FM. Common Virulence Factors for Bacterial Pathogenicity in Plants and Animals. *Science* (1979). 1995.268(5219):1899–902.
393. Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, et al. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc Natl Acad Sci U S A*. 2006.103(8):2833–8.
394. Hachani A, Allsopp LP, Oduko Y, Filloux A. The VgrG proteins are “à la carte” delivery systems for bacterial type VI effectors. *Journal of Biological Chemistry*. 2014.289(25):17872–84.
395. Herrero M, de Lorenzo V, Timmis KN. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J Bacteriol*. 1990.172(11):6557–67.
396. European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clinical Microbiology and Infection*. 2003.9(8):ix–xv.
397. National Committee for Clinical Laboratory Standards, Barry AL. Methods for determining bactericidal activity of antimicrobial agents : approved guideline. Vol. 19. Wayne, PA: National Committee for Clinical Laboratory Standards; 1999.

398. Pader V, Hakim S, Painter KL, Wigneshweraraj S, Clarke TB, Edwards AM. *Staphylococcus aureus* inactivates daptomycin by releasing membrane phospholipids. *Nat Microbiol.* 2016.2:16194.
399. Matuschek E, Brown DFJ, Kahlmeter G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clinical Microbiology and Infection.* 2014.20(4).
400. Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, et al. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J Natl Cancer Inst.* 1973.51(5):1417–23.
401. Qamar W, Khan MR, Arafah A. Optimization of conditions to extract high quality DNA for PCR analysis from whole blood using SDS-proteinase K method. *Saudi J Biol Sci.* 2017.24(7):1465–9.
402. Helander IM, Mattila-Sandholm T. Fluorometric assessment of gram-negative bacterial permeabilization. *J Appl Microbiol.* 2000.88(2):213–9.
403. Jeannot K, Hagart K, Dortet L, Kostrzewa M, Filloux A, Plesiat P, et al. Detection of Colistin Resistance in *Pseudomonas aeruginosa* Using the MALDIxin Test on the Routine MALDI Biotyper Sirius Mass Spectrometer. *Front Microbiol.* 2021.12.
404. Barth AL, Pitt TL. The high amino-acid content of sputum from cystic fibrosis patients promotes growth of auxotrophic *Pseudomonas aeruginosa*. *J Med Microbiol.* 1996.45(2):110–9.
405. Hoffman LR, Richardson AR, Houston LS, Kulasekara HD, Martens-Habbena W, Klausen M, et al. Nutrient availability as a mechanism for selection of antibiotic tolerant *Pseudomonas aeruginosa* within the CF airway. *PLoS Pathog.* 2010.6(1):e1000712.
406. Baker EH, Clark N, Brennan AL, Fisher DA, Gyi KM, Hodson ME, et al. Hyperglycemia and cystic fibrosis alter respiratory fluid glucose concentrations estimated by breath condensate analysis. *J Appl Physiol.* 2007.102(5):1969–75.
407. Garnett JP, Nguyen TT, Moffatt JD, Pelham ER, Kalsi KK, Baker EH, et al. Proinflammatory mediators disrupt glucose homeostasis in airway surface liquid. *J Immunol.* 2012.189(1):373–80.
408. Henke MO, John G, Germann M, Lindemann H, Rubin BK. MUC5AC and MUC5B Mucins Increase in Cystic Fibrosis Airway Secretions during Pulmonary Exacerbation. *Am J Respir Crit Care Med.* 2007.175(8):816–21.
409. Hoegger MJ, Fischer AJ, McMenimen JD, Ostedgaard LS, Tucker AJ, Awadalla MA, et al. Impaired mucus detachment disrupts mucociliary transport in a piglet model of cystic fibrosis. *Science.* 2014.345(6198):818–22.
410. Schneider-Futschik EK, Paulin OKA, Hoyer D, Roberts KD, Ziogas J, Baker MA, et al. Sputum Active Polymyxin Lipopeptides: Activity against Cystic Fibrosis *Pseudomonas aeruginosa* Isolates and Their Interactions with Sputum Biomolecules. *ACS Infect Dis.* 2018.4(5):646–55.

411. Hubeau C, Lorenzato M, Couetil JP, Hubert D, Dusser D, Puchelle E, et al. Quantitative analysis of inflammatory cells infiltrating the cystic fibrosis airway mucosa. *Clin Exp Immunol.* 2001.124(1):69–76.
412. Caceres SM, Malcolm KC, Taylor-Cousar JL, Nichols DP, Saavedra MT, Bratton DL, et al. Enhanced in vitro formation and antibiotic resistance of nonattached *Pseudomonas aeruginosa* aggregates through incorporation of neutrophil products. *Antimicrob Agents Chemother.* 2014.58(11):6851–60.
413. Rosenecker J, Naundorf S, Rudolph C. Airway surface liquid contains endogenous DNase activity which can be activated by exogenous magnesium. *Eur J Med Res.* 2009.14(7):304–8.
414. McShane D, Davies JC, Davies MG, Bush A, Geddes DM, Alton EFW. Airway surface pH in subjects with cystic fibrosis. *Eur Respir J.* 2003.21(1):37–42.
415. Schultz A, Puvvadi R, Borisov SM, Shaw NC, Klimant I, Berry LJ, et al. Airway surface liquid pH is not acidic in children with cystic fibrosis. *Nat Commun.* 2017.8(1):1409.
416. Mozaheb N, Rasouli P, Kaur M, Van Der Smissen P, Larrouy-Maumus G, Mingeot-Leclercq MP. A Mildly Acidic Environment Alters *Pseudomonas aeruginosa* Virulence and Causes Remodeling of the Bacterial Surface. *Gilk SD, editor. Microbiol Spectr.* 2023.11(4):e0483222.
417. Cramer N, Klockgether J, Wrasman K, Schmidt M, Davenport CF, Tümmler B. Microevolution of the major common *Pseudomonas aeruginosa* clones C and PA14 in cystic fibrosis lungs. *Environ Microbiol.* 2011.13(7):1690–704.
418. Marvig RL, Johansen HK, Molin S, Jelsbak L. Genome analysis of a transmissible lineage of *pseudomonas aeruginosa* reveals pathoadaptive mutations and distinct evolutionary paths of hypermutators. *PLoS Genet.* 2013.9(9):e1003741.
419. Cornforth DM, Dees JL, Ibberson CB, Huse HK, Mathiesen IH, Kirketerp-Møller K, et al. *Pseudomonas aeruginosa* transcriptome during human infection. *Proc Natl Acad Sci U S A.* 2018.115(22):E5125–34.
420. Rossi E, Falcone M, Molin S, Johansen HK. High-resolution in situ transcriptomics of *Pseudomonas aeruginosa* unveils genotype independent patho-phenotypes in cystic fibrosis lungs. *Nat Commun.* 2018.9(1):3459.
421. Govan JR, Deretic V. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev.* 1996.60(3):539–74.
422. D'Argenio DA, Wu M, Hoffman LR, Kulasekara HD, Déziel E, Smith EE, et al. Growth phenotypes of *Pseudomonas aeruginosa* lasR mutants adapted to the airways of cystic fibrosis patients. *Mol Microbiol.* 2007.64(2):512–33.
423. De Vos D, De Chial M, Cochez C, Jansen S, Tümmler B, Meyer JM, et al. Study of pyoverdine type and production by *Pseudomonas aeruginosa* isolated from cystic fibrosis patients: prevalence of type II pyoverdine isolates and accumulation of pyoverdine-negative mutations. *Arch Microbiol.* 2001.175(5):384–8.

424. Ernst RK, Adams KN, Moskowitz SM, Kraig GM, Kawasaki K, Stead CM, et al. The *Pseudomonas aeruginosa* lipid A deacylase: Selection for expression and loss within the cystic fibrosis airway. *J Bacteriol.* 2006.188(1):191–201.
425. Lo Sciuto A, Imperi F. Aminoarabinylation of Lipid A Is Critical for the Development of Colistin Resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* 2018.62(3).
426. Neve RL, Carrillo BD, Phelan V V. Impact of Artificial Sputum Medium Formulation on *Pseudomonas aeruginosa* Secondary Metabolite Production. O'Toole G, editor. *J Bacteriol.* 2021.203(21):e0025021.
427. Bucki R, Byfield FJ, Janmey PA. Release of the antimicrobial peptide LL-37 from DNA/F-actin bundles in cystic fibrosis sputum. *European Respiratory Journal.* 2007.29(4):624–32.
428. Ma L, Conover M, Lu H, Parsek MR, Bayles K. Assembly and Development of the *Pseudomonas aeruginosa* Biofilm Matrix. *PLoS Pathog.* 2009.5(3):1000354.
429. Kumar SS, Penesyan A, Elbourne LDH, Gillings MR, Paulsen IT. Catabolism of Nucleic Acids by a Cystic Fibrosis *Pseudomonas aeruginosa* Isolate: An Adaptive Pathway to Cystic Fibrosis Sputum Environment. *Front Microbiol.* 2019.10:1199.
430. Halverson TWR, Wilton M, Poon KKH, Petri B, Lewenza S. DNA is an antimicrobial component of neutrophil extracellular traps. *PLoS Pathog.* 2015.11(1):e1004593.
431. Hoffman CL, Lalsiamthara J, Aballay A. Host Mucin Is Exploited by *Pseudomonas aeruginosa* To Provide Monosaccharides Required for a Successful Infection. *mBio.* 2020.11(2).
432. Flynn JM, Niccum D, Dunitz JM, Hunter RC. Evidence and Role for Bacterial Mucin Degradation in Cystic Fibrosis Airway Disease. *PLoS Pathog.* 2016.12(8).
433. Sweeney E, Sabnis A, Edwards AM, Harrison F. Effect of host-mimicking medium and biofilm growth on the ability of colistin to kill *Pseudomonas aeruginosa*. *Microbiology (N Y).* 2020.166(12):1171–80.
434. Bucki R, Namiot DB, Namiot Z, Savage PB, Janmey PA. Salivary mucins inhibit antibacterial activity of the cathelicidin-derived LL-37 peptide but not the cationic steroid CSA-13. *Journal of Antimicrobial Chemotherapy.* 2008.62(2):329–35.
435. Meldrum OW, Yakubov GE, Bonilla MR, Deshmukh O, McGuckin MA, Gidley MJ. Mucin gel assembly is controlled by a collective action of non-mucin proteins, disulfide bridges, Ca²⁺-mediated links, and hydrogen bonding. *Sci Rep.* 2018.8(1):5802.
436. Turnidge John and Sei K and MJ. Polymyxin Susceptibility Testing and Breakpoint Setting. In: Li Jian and Nation RL and KKS, editor. *Polymyxin Antibiotics: From Laboratory Bench to Bedside.* Cham: Springer International Publishing; 2019. p. 117–32.
437. Wheeler KM, Cárcamo-Oyarce G, Turner BS, Dellos-Nolan S, Co JY, Lehoux S, et al. Mucin glycans attenuate the virulence of *Pseudomonas aeruginosa* in infection. *Nat Microbiol.* 2019.4:2146.

438. Magréault S, Mankikian J, Marchand S, Diot P, Couet W, Flament T, et al. Pharmacokinetics of colistin after nebulization or intravenous administration of colistin methanesulphonate (Colimycin®) to cystic fibrosis patients. *Journal of Cystic Fibrosis*. 2020.19(3):421–6.
439. Johnson L, Mulcahy H, Kanevets U, Shi Y, Lewenza S. Surface-localized spermidine protects the *Pseudomonas aeruginosa*: Outer membrane from antibiotic treatment and oxidative stress. *J Bacteriol*. 2012.194(4):813–26.
440. Lewenza S, Johnson L, Charron-Mazenod L, Hong M, Mulcahy-O'grady H. Extracellular DNA controls expression of *Pseudomonas aeruginosa* genes involved in nutrient utilization, metal homeostasis, acid pH tolerance and virulence. *J Med Microbiol*. 2020.69:895–905.
441. Jochumsen N, Marvig RL, Damkiær S, Jensen RL, Paulander W, Molin S, et al. The evolution of antimicrobial peptide resistance in *Pseudomonas aeruginosa* is shaped by strong epistatic interactions. *Nat Commun*. 2016.7(1):13002.
442. Lee JY, Ko KS. Mutations and expression of PmrAB and PhoPQ related with colistin resistance in *Pseudomonas aeruginosa* clinical isolates. *Diagn Microbiol Infect Dis*. 2014.78(3):271–6.
443. Ekkelenkamp MB, Cantón R, Díez-Aguilar M, Tunney MM, Gilpin DF, Bernardini F, et al. Susceptibility of *Pseudomonas aeruginosa* Recovered from Cystic Fibrosis Patients to Murepavadin and 13 Comparator Antibiotics. *Antimicrob Agents Chemother*. 2020.64(2).
444. Wang BX, Cady KC, Oyarce GC, Ribbeck K, Lauba MT. Two-Component Signaling Systems Regulate Diverse Virulence- Associated Traits in *Pseudomonas Aeruginosa*. *Appl Environ Microbiol*. 2021.87(11):1–18.
445. Capra EJ, Laub MT. Evolution of two-component signal transduction systems. *Annu Rev Microbiol*. 2012.66:325–47.
446. Huang J, Li C, Song J, Velkov T, Wang L, Zhu Y, et al. Regulating polymyxin resistance in Gram-negative bacteria: roles of two-component systems PhoPQ and PmrAB. *Future Microbiol*. 2020.(6):445–59.
447. Fernández L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock REW. Adaptive resistance to the “last hope” antibiotics polymyxin B and colistin in *Pseudomonas aeruginosa* is mediated by the novel two-component regulatory system ParR-ParS. *Antimicrob Agents Chemother*. 2010.54(8):3372–82.
448. Ainsaar K, Mumm K, Ilves H, Hōrak R. The ColRS signal transduction system responds to the excess of external zinc, iron, manganese, and cadmium. *BMC Microbiol*. 2014.14:162.
449. Wiesmann CL, Zhang Y, Alford M, Hamilton CD, Dosanjh M, Thoms D, et al. The ColR/S two-component system is a conserved determinant of host association across *Pseudomonas* species. *ISME J*. 2023.17(2):286–96.
450. Barrow K, Kwon DH. Alterations in two-component regulatory systems of phoPQ and pmrAB are associated with polymyxin B resistance in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2009.53(12):5150–4.

451. McPhee JB, Bains M, Winsor G, Lewenza S, Kwasnicka A, Brazas MD, et al. Contribution of the PhoP-PhoQ and PmrA-PmrB two-component regulatory systems to Mg²⁺-induced gene regulation in *Pseudomonas aeruginosa*. *J Bacteriol*. 2006.188(11):3995–4006.
452. Lethem MI, James SL, Marriott C, Burke JF. The origin of DNA associated with mucus glycoproteins in cystic fibrosis sputum. *Eur Respir J*. 1990.3(1):19–23.
453. Helander IM, Mattila-Sandholm T. Fluorometric assessment of gram-negative bacterial permeabilization. *J Appl Microbiol*. 2000.88(2):213–9.
454. Brennan-Krohn T, Pironti A, Kirby JE. Synergistic Activity of Colistin-Containing Combinations against Colistin-Resistant Enterobacteriaceae. *Antimicrob Agents Chemother*. 2018.62(10).
455. Nicas TI, Hancock RE. Outer membrane protein H1 of *Pseudomonas aeruginosa*: involvement in adaptive and mutational resistance to ethylenediaminetetraacetate, polymyxin B, and gentamicin. *J Bacteriol*. 1980.143(2):872–8.
456. Hachani A, Allsopp LP, Oduko Y, Filloux A. The VgrG proteins are “à la carte” delivery systems for bacterial type VI effectors. *J Biol Chem*. 2014.289(25):17872–84.
457. Hittle LE, Powell DA, Jones JW, Tofigh M, Goodlett DR, Moskowitz SM, et al. Site-specific activity of the acyltransferases HtrB1 and HtrB2 in *Pseudomonas aeruginosa* lipid A biosynthesis. *FEMS Pathogens and Disease*. 2015.73:53.
458. Nowicki EM, O’Brien JP, Brodbelt JS, Trent MS. Characterization of *Pseudomonas aeruginosa* LpxT reveals dual positional lipid A kinase activity and co-ordinated control of outer membrane modification. *Mol Microbiol*. 2014.94(3):728–41.
459. Bakthavatchalam YD, Pragasam AK, Biswas I, Veeraraghavan B. Polymyxin susceptibility testing, interpretative breakpoints and resistance mechanisms: An update. *J Glob Antimicrob Resist*. 2018.12:124–36.
460. Guo Z, Wang Y, Yang A, Yang G. The effect of pH on charge inversion and condensation of DNA. *Soft Matter*. 2016.12(31):6669–74.
461. Gao T, Zhang W, Wang Y, Yang G. DNA Compaction and Charge Neutralization Regulated by Divalent Ions in very Low pH Solution. *Polymers (Basel)*. 2019.11(2).
462. Owczarzy R, Moreira BG, You Y, Behlke MA, Wälder JA. Predicting stability of DNA duplexes in solutions containing magnesium and monovalent cations. *Biochemistry*. 2008.47(19):5336–53.
463. Song Y, Zhang S, Zhao N, Nong C, He Y, Bao R. *Pseudomonas aeruginosa* two-component system CprRS regulates HigBA expression and bacterial cytotoxicity in response to LL-37 stress. *PLoS Pathog*. 2024.20(1):e1011946.
464. Chamnongpol S, Dodson W, Cromie MJ, Harris ZL, Groisman EA. Fe(III)-mediated cellular toxicity. *Mol Microbiol*. 2002.45(3):711–9.
465. Wösten MM, Kox LF, Chamnongpol S, Soncini FC, Groisman EA. A signal transduction system that responds to extracellular iron. *Cell*. 2000.103(1):113–25.

466. Herrera CM, Hankins J V., Trent MS. Activation of PmrA inhibits LpxT-dependent phosphorylation of lipid A promoting resistance to antimicrobial peptides. *Mol Microbiol.* 2010.76(6):1444–60.
467. Tran AX, Whittimore JD, Wyrick PB, McGrath SC, Cotter RJ, Trent MS. The lipid A 1-phosphatase of *Helicobacter pylori* is required for resistance to the antimicrobial peptide polymyxin. *J Bacteriol.* 2006.188(12):4531–41.
468. Rice A, Wereszczynski J. Atomistic Scale Effects of Lipopolysaccharide Modifications on Bacterial Outer Membrane Defenses. *Biophysj.* 2018.114:1389–99.
469. Jiang X, Yang K, Han ML, Yuan B, Li J, Gong B, et al. Outer Membranes of Polymyxin-Resistant *Acinetobacter baumannii* with Phosphoethanolamine-Modified Lipid A and Lipopolysaccharide Loss Display Different Atomic-Scale Interactions with Polymyxins. *ACS Infect Dis.* 2020.6(10):2698–708.
470. Greipel L, Fischer S, Klockgether J, Dorda M, Mielke S, Wiehlmann L, et al. Molecular epidemiology of mutations in antimicrobial resistance loci of *Pseudomonas aeruginosa* isolates from airways of cystic fibrosis patients. *Antimicrob Agents Chemother.* 2016.60(11):6726–34.
471. Han ML, Velkov T, Zhu Y, Roberts KD, Le Brun AP, Chow SH, et al. Polymyxin-Induced Lipid A Deacylation in *Pseudomonas aeruginosa* Perturbs Polymyxin Penetration and Confers High-Level Resistance. *ACS Chem Biol.* 2018.13(1):121–30.
472. Marcos V, Zhou-Suckow Z, Önder Yildirim A, Bohla A, Hector A, Vitkov L, et al. Free DNA in cystic fibrosis airway fluids correlates with airflow obstruction. *Mediators Inflamm.* 2015.2015:408935.
473. Henke MO, Ratjen F. Mucolytics in cystic fibrosis. *Paediatr Respir Rev.* 2007.8(1):24–9.
474. Konstan MW, Ratjen F. Effect of dornase alfa on inflammation and lung function: potential role in the early treatment of cystic fibrosis. *J Cyst Fibros.* 2012.11(2):78–83.
475. Halverson TWR, Wilton M, Poon KKH, Petri B, Lewenza S. DNA is an antimicrobial component of neutrophil extracellular traps. *PLoS Pathog.* 2015.11(1):e1004593.
476. Siew R, Ou TL, Dahesh S, Akong K, Nizet V. Bicarbonate Effects on Antibacterial Immunity and Mucus Glycobiology in the Cystic Fibrosis Lung: A Review With Selected Experimental Observations. *Infectious microbes & diseases.* 2022.4(3):103–10.
477. Gorrieri G, Scudieri P, Caci E, Schiavon M, Tomati V, Sirici F, et al. Goblet Cell Hyperplasia Requires High Bicarbonate Transport To Support Mucin Release. *Sci Rep.* 2016.6:36016.
478. Garcia MAS, Yang N, Quinton PM. Normal mouse intestinal mucus release requires cystic fibrosis transmembrane regulator-dependent bicarbonate secretion. *Journal of Clinical Investigation.* 2009.119(9):2613–22.
479. Alaiwa MHA, Reznikov LR, Gansemer ND, Sheets KA, Horswill AR, Stoltz DA, et al. pH modulates the activity and synergism of the airway surface liquid antimicrobials β -defensin-3 and LL-37. *Proc Natl Acad Sci U S A.* 2014.111(52):18703–8.

480. Khan MA, Philip LM, Cheung G, Vadakepeedika S, Grasemann H, Sweezey N, et al. Regulating NETosis: Increasing pH Promotes NADPH Oxidase-Dependent NETosis. *Front Med (Lausanne)*. 2018.5(13):19.
481. Kaushik KS, Stolhandske J, Shindell O, Smyth HD, Gordon VD. Tobramycin and bicarbonate synergise to kill planktonic *Pseudomonas aeruginosa*, but antagonise to promote biofilm survival. *NPJ Biofilms Microbiomes*. 2016.2:16006.
482. Dobay O, Laub K, Stercz B, Kéri A, Balázs B, Tóthpál A, et al. Bicarbonate Inhibits Bacterial Growth and Biofilm Formation of Prevalent Cystic Fibrosis Pathogens. *Front Microbiol*. 2018.9:2245.
483. Jaikumpun P, Ruksakiet K, Stercz B, Pállinger É, Steward M, Lohinai Z, et al. Antibacterial Effects of Bicarbonate in Media Modified to Mimic Cystic Fibrosis Sputum. *Int J Mol Sci*. 2020.21(22).
484. Garcia MAS, Yang N, Quinton PM. Normal mouse intestinal mucus release requires cystic fibrosis transmembrane regulator-dependent bicarbonate secretion. *J Clin Invest*. 2009.119(9):2613–22.
485. Andolina G, Bencze LC, Zerbe K, Müller M, Steinmann J, Kocherla H, et al. A Peptidomimetic Antibiotic Interacts with the Periplasmic Domain of LptD from *Pseudomonas aeruginosa*. *ACS Chem Biol*. 2018.13(3):666–75.
486. Sperandeo P, Martorana AM, Polissi A. The lipopolysaccharide transport (Lpt) machinery: A nonconventional transporter for lipopolysaccharide assembly at the outer membrane of Gram-negative bacteria. *J Biol Chem*. 2017.292(44):17981–90.
487. Dale GE, Halabi A, Petersen-Sylla M, Wach A, Zwingelstein C. Pharmacokinetics, tolerability, and safety of murepavadin, a novel antipseudomonal antibiotic, in subjects with mild, moderate, or severe renal function impairment. *Antimicrob Agents Chemother*. 2018.62(9).
488. Li D, Schneider-Futschik EK. Current and Emerging Inhaled Antibiotics for Chronic Pulmonary *Pseudomonas aeruginosa* and *Staphylococcus aureus* Infections in Cystic Fibrosis. *Antibiotics (Basel)*. 2023.12(3).
489. Wei X, Gao J, Zhou D, Xu C, Chen P, Chen S, et al. Murepavadin promotes the killing efficacies of aminoglycoside antibiotics against *Pseudomonas aeruginosa* by enhancing membrane potential. *Antimicrob Agents Chemother*. 2024.68(4).
490. Sader HS, Flamm RK, Dale GE, Rhomberg PR, Castanheira M. Murepavadin activity tested against contemporary (2016-17) clinical isolates of XDR *Pseudomonas aeruginosa*. *J Antimicrob Chemother*. 2018.73(9):2400–4.
491. Pulmozyme P. PRODUCT MONOGRAPH [Internet]. 1993.
492. Guérout M, Picot D, Abi-Ghanem J, Hartmann B, Baaden M. How cations can assist dnase i in DNA binding and hydrolysis. *PLoS Comput Biol*. 2010.6(11).
493. Parsiegla G, Noguere C, Santell L, Lazarus RA, Bourne Y. The structure of human DNase I bound to magnesium and phosphate ions points to a catalytic mechanism common to members of the DNase I-like superfamily. *Biochemistry*. 2012.51(51):10250–8.

494. Sanders NN, Franckx H, De Boeck K, Haustraete J, De Smedt SC, Demeester J. Role of magnesium in the failure of rhDNase therapy in patients with cystic fibrosis. *Thorax*. 2006.61(11):962–8.
495. Ghassani A, Triponney P, Bour M, Plésiat P, Jeannot K, MucoMicrobes study Group. Mutations in genes *lpxL1*, *bamA*, and *pmrB* impair the susceptibility of cystic fibrosis strains of *Pseudomonas aeruginosa* to murepavadin. *Antimicrob Agents Chemother*. 2024.68(1):e0129823.
496. VANECKO S, LASKOWSKI M. Studies of the specificity of deoxyribonuclease I. III. Hydrolysis of chains carrying a monoesterified phosphate on carbon 5'. *J Biol Chem*. 1961.236(12):3312–6.
497. Díez-Aguilar M, Hernández-García M, Morosini MI, Fluit A, Tunney MM, Huertas N, et al. Murepavadin antimicrobial activity against and resistance development in cystic fibrosis *Pseudomonas aeruginosa* isolates. *J Antimicrob Chemother*. 2021.76(4):984–92.
498. Romano KP, Warriar T, Poulsen BE, Nguyen PH, Loftis AR, Saebi A, et al. Mutations in *pmrB* Confer Cross-Resistance between the LptD Inhibitor POL7080 and Colistin in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2019.63(9).
499. Camus L, Vandenesch F, Moreau K. From genotype to phenotype: adaptations of *Pseudomonas aeruginosa* to the cystic fibrosis environment. *Microb Genom*. 2021.7(3).
500. Mikacenic C, Moore R, Dmyterko V, West TE, Altemeier WA, Liles WC, et al. Neutrophil extracellular traps (NETs) are increased in the alveolar spaces of patients with ventilator-associated pneumonia. *Crit Care*. 2018.22(1).
501. Jochumsen N, Marvig RL, Damkiær S, Jensen RL, Paulander W, Molin S, et al. The evolution of antimicrobial peptide resistance in *Pseudomonas aeruginosa* is shaped by strong epistatic interactions. *Nature Communications* 2016 7:1. 2016.7(1):1–10.
502. Lee JY, Chung ES, Na IY, Kim H, Shin D, Ko KS. Development of colistin resistance in *pmrA*-, *phoP*-, *parR*- and *cprR*-inactivated mutants of *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy*. 2014.69(11):2966–71.
503. Liu YY, Chandler CE, Leung LM, McElheny CL, Mettus RT, Shanks RMQ, et al. Structural modification of lipopolysaccharide conferred by *mcr-1* in gram-negative ESKAPE pathogens. *Antimicrob Agents Chemother*. 2017.61(6).
504. Tate S, MacGregor G, Davis M, Innes JA, Greening AP. Airways in cystic fibrosis are acidified: detection by exhaled breath condensate. *Thorax*. 2002.57(11):926–9.
505. Gomez CCS, Parazzi PLF, Clinckspoor KJ, Mauch RM, Pessine FBT, Levy CE, et al. Safety, Tolerability, and Effects of Sodium Bicarbonate Inhalation in Cystic Fibrosis. *Clin Drug Investig*. 2020.40(2):105–17.
506. Coakley RJ, Taggart C, McElvaney NG, O'Neill SJ. Cytosolic pH and the inflammatory microenvironment modulate cell death in human neutrophils after phagocytosis. *Blood*. 2002.100(9):3383–91.
507. Ostedgaard LS, Meyerholz DK, Chen JH, Pezzulo AA, Karp PH, Rokhlina T, et al. The $\Delta F508$ mutation causes CFTR misprocessing and cystic fibrosis-like disease in pigs. *Sci Transl Med*. 2011.3(74):74ra24.

508. O'Toole GA, Crabbé A, Kümmerli R, LiPuma JJ, Bomberger JM, Davies JC, et al. Model Systems to Study the Chronic, Polymicrobial Infections in Cystic Fibrosis: Current Approaches and Exploring Future Directions. Vol. 12, mBio. American Society for Microbiology; 2021.
509. Pankratz D, Gomez NO, Nielsen A, Mustafayeva A, Gür M, Arce-Rodriguez F, et al. An expanded CRISPR–Cas9-assisted recombineering toolkit for engineering genetically intractable *Pseudomonas aeruginosa* isolates. Nat Protoc. 2023.18(11):3253–88.
510. Muhl D, Filloux A. Site-Directed Mutagenesis and Gene Deletion Using Reverse Genetics. In: Filloux A, Ramos JL, editors. *Pseudomonas methods and protocols*. New York, NY: Springer New York; 2014. p. 521–40.
511. Barquist L, Mayho M, Cummins C, Cain AK, Boinett CJ, Page AJ, et al. The TraDIS toolkit: Sequencing and analysis for dense transposon mutant libraries. Bioinformatics. 2016.32(7):1109–11.
512. Ersoy SC, Heithoff DM, Barnes L, Tripp GK, House JK, Marth JD, et al. Correcting a Fundamental Flaw in the Paradigm for Antimicrobial Susceptibility Testing. EBioMedicine. 2017.20:173–81.
513. Elborn JS, Blasi F, Burgel PR, Peckham D. Role of inhaled antibiotics in the era of highly effective CFTR modulators. Eur Respir Rev. 2023.32(167):220154.
514. Greenwald MA, Wolfgang MC. The changing landscape of the cystic fibrosis lung environment: From the perspective of *Pseudomonas aeruginosa*. Curr Opin Pharmacol. 2022.65:102262.
515. Ledger EL, Smith DJ, Teh JJ, Wood ME, Whibley PE, Morrison M, et al. Impact of CFTR Modulation on *Pseudomonas aeruginosa* Infection in People With Cystic Fibrosis . J Infect Dis. 2024.
516. Wainwright CE. A New Era for Cystic Fibrosis and Cystic Fibrosis Transmembrane Conductance Regulator Modulator Trials in Infants. Am J Respir Crit Care Med. 2022.206(10):1193–5.
517. Henke MO, Renner A, Huber RM, Seeds MC, Rubin BK. MUC5AC and MUC5B Mucins Are Decreased in Cystic Fibrosis Airway Secretions. Am J Respir Cell Mol Biol. 2004.31(1):86–91.
518. Hull J, South M, Phelan P, Grimwood K. Surfactant Composition in Infants and Young Children with Cystic Fibrosis. Vol. 156, Am J Respir Crit Care Med. 1997.
519. Meyer KC, Sharma A, Brown R, Weatherly M, Moya FR, Lewandoski J, et al. Function and composition of pulmonary surfactant and surfactant-derived fatty acid profiles are altered in young adults with cystic fibrosis. Chest. 2000.118(1):164–74.
520. Korgaonkar AK, Whiteley M. *Pseudomonas aeruginosa* enhances production of an antimicrobial in response to N-acetylglucosamine and peptidoglycan. J Bacteriol. 2011.193(4):909–17.
521. Haley CL, Colmer-Hamood JA, Hamood AN. Characterization of biofilm-like structures formed by *Pseudomonas aeruginosa* in a synthetic mucus medium. BMC Microbiol. 2012.12:181.

- 522. Moskowitz SM, Foster JM, Emerson J, Burns JL. Clinically Feasible Biofilm Susceptibility Assay for Isolates of *Pseudomonas aeruginosa* from Patients with Cystic Fibrosis. *J Clin Microbiol.* 2004.42(5):1915–22.
- 523. Hanssens LS, Duchateau J, Casimir GJ. CFTR Protein: Not Just a Chloride Channel? *Cells.* 2021.10(11).
- 524. Zajac M, Lepissier A, Dréano E, Chevalier B, Hatton A, Kelly-Aubert M, et al. Putting bicarbonate on the spot: pharmacological insights for CFTR correction in the airway epithelium. *Front Pharmacol.* 2023.14:1293578.