BIOFILM RESPONSE TO ANTIBIOTICS REQUIRES OprF AND SigX

THE *PSEUDOMONAS AERUGINOSA* BIOFILM INDUCTION RESPONSE TO SUBINHIBITORY ANTIBIOTICS REQUIRES *oprF* AND *sigX*

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree

Master of Science

McMaster University MASTER OF SCIENCE (2018)
Hamilton, Ontario (Biochemistry and Biomedical Sciences)

TITLE: The *Pseudomonas aeruginosa* biofilm induction response to subinhibitory antibiotics requires *oprF* and *sigX*

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NUMBER OF PAGES: 84

LAY ABSTRACT

Pseudomonas aeruginosa is a bacterium that causes illness in patients with compromised immune systems, like patients with cystic fibrosis. This bacterium forms biofilms, which are dense groups that stick to surfaces within a protective slime that contains proteins, sugars, and DNA. Biofilms make the bacteria harder to treat with antibiotics. If the bacteria are treated with low levels of antibiotics, they respond by forming more biofilm but how this happens is unknown. We showed that adding DNA does not increase biofilm formation, while adding dead cell debris only causes a small increase. By testing a library of mutant bacteria, we found that they need two genes, *oprF* and *sigX*, to form more biofilm when they are treated with low levels of antibiotic. By studying how bacteria respond to low levels of antibiotics, we can find ways to identify new antibiotics and to make our current antibiotics work better.

ABSTRACT

Pseudomonas aeruginosa is a Gram-negative pathogen that forms biofilms, which increase tolerance to antibiotics. Biofilms are dense, surface-associated communities of bacteria that grow in a self-produced matrix of polysaccharides, proteins, and extracellular DNA (eDNA). Sub-minimal inhibitory concentration (sub-MIC) levels of antibiotics induce the formation of biofilms, indicating a potential role in response to antibiotic stress. However, the mechanisms behind sub-MIC antibiotic-induced biofilm formation are unknown. We show that treatment with sub-MIC levels of cefixime (cephalosporin), carbenicillin (β-lactam), tobramycin (aminoglycoside), chloramphenicol (chloramphenicol), thiostrepton (thiopeptide), novobiocin (aminocoumarin), ciprofloxacin (fluoroquinolone), or trimethoprim (antifolate) induces biofilm formation, with maximal induction at ~ ¼ to ½ MIC. We demonstrate that addition of exogenous eDNA or cell lysate does not stimulate biofilm formation to the same extent as antibiotics, suggesting that the release of common goods by antibiotic action does not solely drive the biofilm response. We show that increased biofilm formation upon antibiotic exposure requires the outer membrane porin OprF and the extracytoplasmic function sigma factor SigX. Through transposon mutant screening and deletion studies, we found that OprF is important for biofilm induction, as mutants lacking this protein did not form increased biofilm when exposed to sub-MIC antibiotics. OprF expression is

controlled by SigX, and its loss increases SigX activity. Loss of SigX also prevents biofilm induction by sub-MIC antibiotics. Together, these results show that antibiotic-induced biofilm formation may constitute a type of stress response. This response may be useful to screen for new antibiotics due to its ability to reveal antibiotic activity at concentrations below the MIC. Further study of this response may also provide targets for adjuvant therapies that reduce biofilm formation in *P. aeruginosa* infections and increase the efficacy of current antibiotics.

ACKNOWLEDGEMENTS

I would like to thank Dr. Lori Burrows for her constant support and utmost commitment to her graduate students and the lab as a whole, as well as my committee members Dr. Gerry Wright and Dr. Michael Surette. My committee has provided me with invaluable insight and guidance throughout this project that has helped me to focus my work and to grow as a scientist. I would especially like to thank Dr. Surette for the use of the automated colony picker, which helped considerably with the creation of the transposon mutant library. I would like Dr. Wright for valuable conversations about antibiotics and the stress that they cause within bacteria. Working with Dr. Burrows has been an absolute pleasure and I would like to thank her for valuable conversations and brainstorming when times were difficult.

My labmates that I have had the pleasure to work with over the past 2 years have been some of the most hard working and friendly people I have ever known. They have constantly inspired me to put my best foot forward and for that, I thank them all. I would like to thank Hanjeong Harvey for her invaluable knowledge of cloning and microbiological protocols as well as her friendship. I am grateful to have learned from and worked with her. I would like to thank my co-Masters student and friend Tori Marko. I appreciate all the help, conversations, and fun times that we have had throughout these past couple years. I would also like to thank all of my Biofilm Buddies – especially Sawyer

Karabelas-Pittman and Madeleine Rudolph – who started this journey with me. You have all been such amazing help and even better friends.

Finally, none of this would have been possible without the support of friends and family. I would like to thank all of my friends – Dan, Brandon, Mike, Nick, Erik, and more – for their friendship and support. I would like to thank my Mom, Dad, and sister for their love, support, and phone conversations. I would also like to thank Arielle Spilak for her love and constant support, especially during the tough times. This would have never been possible without you.

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LIST OF ABBREVIATIONS

c-di-GMP	Cyclic-di-guanosine monophosphate
DGC	Diguanylate cyclase
eATP	Extracellular Adenosine Triphosphate
ECF	Extracytoplasmic function
eDNA	Extracellular DNA
EPS	Extracellular polymeric substances
IM	Inner Membrane
MIC	Minimum inhibitory concentration
OM	Outer Membrane
PARA	*Pseudomonas aeruginosa* Response to Antagonism
PDE	Phosphodiesterase
PG	Peptidoglycan
T3SS	Type 3 secretion system
T6SS	Type 6 secretion system

DECLARATION OF ACADEMIC ACHIEVEMENT

Hanjeong Harvey helped with the creation of the PAO1 KP transposon mutant library by helping to optimize the mating protocol and by helping to hand-pick colonies for plates BBTn15-BBTn27.

Madeleine Rudolph and Sawyer Karabelas-Pittman helped to create the PAO1 KP transposon mutant library, as well as helping to optimize the biofilm screening assays. They also helped with screening the library and performed the touchdown PCR to identify the *oprF*::Himar1 mutant. They both created the pEX18Gm-Δ*oprF* plasmid and the Δ*oprF* mutant. Both performed the biofilm assays for PAO1 KP treated with carbenicillin, chloramphenicol, ciprofloxacin, novobiocin, polymyxin B, and trimethoprim.

Erin Steckley and Jessica Chee helped with screening the PAO1 KP transposon mutant library, as well as optimizing the biofilm screening assay.

Michael Ranieri performed the remaining work for this thesis and helped with all work unless otherwise noted. He also performed work on characterizing the activity and mode of entry for thiostrepton, which is in manuscript preparation. He also co-authored a review on mechanisms of biofilm stimulation by subinhibitory antibiotics.

CHAPTER 1: INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative opportunist that can cause both acute and chronic infections in humans. *P. aeruginosa* is metabolically versatile and ubiquitous in the environment. It is one of the most common causes of nosocomial infections in North American hospitals[1,2] and is particularly common in lung infections of cystic fibrosis (CF) patients[3,4] Infections with *P. aeruginosa* can be severe due to its resistance to many antibiotics[1,2], a myriad of virulence factors[5], and its ability to form biofilms, which allow for the bacteria to become antibiotic tolerant and more difficult to treat. *P. aeruginosa* can form biofilms on a variety of surfaces, including medical devices, resulting in chronic infections that are nearly impossible to eradicate. The identification of new therapies to combat *P. aeruginosa* infections is paramount to our continued treatment of these infections. It is also important to improve our understanding of *P. aeruginosa* biofilm biology to improve treatment plans and outcomes.

1.1 *P. aeruginosa* Biofilm Formation and Regulation

Biofilms are surface-associated microbial communities that grow as physical aggregates within a matrix of self-produced extracellular polymeric substances (EPS). The EPS consists of cellular components including polysaccharides, lipids, proteins, and extracellular DNA (eDNA). The

polysaccharide components of *P. aeruginosa* biofilms include Pel, Psl, and alginate[6]. The Psl and Pel polysaccharides play structural roles in mature biofilms[7] and roles in early biofilm formation such as attachment to a surface[8,9,10]. Alginate is an anionic polysaccharide that has variable expression in biofilms (strain and condition-dependent), and is dispensible for biofilm formation in certain strains and conditions[11]. Alginate production is induced in the host by exposure to reactive oxygen species produced by immune cells[12]. Alginate production contributes to a mucoid phenotype that is associated with increased antibiotic tolerance[13] and increased immune evasion[14,15].

Processes contributing to formation of biofilms by *P. aeruginosa* – such as polysaccharide production – are regulated in part by the secondary messenger cyclic di- GMP (c-di-GMP). c-di-GMP is formed from 2 GTP molecules by enzymes called diguanylate cyclases[16] (DGC). This messenger can also be broken down into GMP or pGpG by phosphodiesterases (PDE)[16]. High levels of intracellular c-di-GMP are associated with a sessile, biofilm-associated lifestyle while low levels are associated with a planktonic lifestyle. High levels of c-di-GMP stimulate the transcription of Pel[17,18] and Psl[17] biosynthetic genes, as well as the Psl-associated adhesin CdrA[19]. *P. aeruginosa* will switch from a planktonic lifestyle to a biofilm and vice versa by using DGCs and PDEs to regulate c-di-GMP levels in order to coordinate the required phenotypic changes associated with the two lifestyles.

Pseudomonas biofilm formation typically begins upon association with a surface, termed "reversible attachment". Reversible attachment is mediated by flagella at the cell pole, causing cells to associate with the surface in a perpendicular manner[16]. When forming a biofilm, swarming motility is downregulated (by increased levels of c-di-GMP) and the cell attaches to the surface via its longitudinal axis, making an irreversible attachment. This is speculated to occur via Psl or Pel polysaccharides acting as adhesins[10,16]. Growth on the surface occurs along with the production of EPS to form microcolonies that merge to create a mature biofilm. The mature biofilm and microcolonies consist of highly ordered structures that contain water channels[20,21] and interactions between eDNA and Pel polysaccharide provide structural integrity[7].

Life within a biofilm offers many benefits, including the sharing of resources[22], environmental protection, antibiotic tolerance[23], and antibiotic resistance[24]. Nutrient, oxygen, and pH gradients form because of the intense utilization of resources within the dense biofilm, resulting in phenotypic heterogeneity of the cells within the biofilm. Cells located towards the deeper regions have a more dormant phenotype compared to those that are actively growing at the periphery[23,25]. These dormant cells are more tolerant of many types of antibiotics and can persist through the course of antibiotic treatment, leading to relapse of infection. The proximity and reduced motility of cells allows

for increased horizontal gene transfer events up to 600x more than planktonic cells[24], which may result in the spread of resistance genes at higher rates. The hydrated matrix prevents desiccation and creates a barrier that can slow or inhibit the passage of certain toxic species, including some antibiotics. For example, the penetration of certain aminoglycosides into the biofilm is impeded by the cationic Pel polysaccharide[26]. All of these factors contribute to the ability of *Pseudomonas* biofilms to persist in hospital environments and tolerate antibiotic regimens. Therefore, it is important to understand how and when biofilms develop in order to prevent their formation. This work focused on how antibiotics affect biofilm formation, with a goal of understanding how this process can be leveraged to prevent biofilm formation during treatment, as well as how this behaviour can be used to identify new antibiotics.

1.2 Antibiotics as Stimulators of Bacterial Biofilm Formation

The ability of antibiotics to act as signaling molecules has been increasingly recognized as an important function[27,28,29]. Antibiotics are not typically produced in the natural environment at the high levels we use during treatment. As a result, these molecules may have other functions at levels below their minimum inhibitory concentrations (sub-MIC) that shape single-cell and community behaviours. Global gene expression profiles of multiple organisms are altered in the presence of sub-MIC antibiotics, including genes not directly

related to the mechanism of action or stress/damage pathways[27,28,30,31,88]. Examples include tetracycline-dependent induction of cytotoxicity in *P. aeruginosa* via the type III secretion system (T3SS)[27], or azithromycin-dependent downregulation of multiple quorum sensing genes in *P. aeruginosa*[32]. Sub-MIC antibiotics also have effects on biofilm formation. Sub-MIC levels of tobramycin stimulate the formation of biofilm in multiple isolates of *P. aeruginosa*[33]. Other drugs such as ciprofloxacin and tetracycline – with separate modes of action and targets – have similar effects[27]. Increases in alginate production in biofilms have also been linked to treatment with imipenem, norfloxacin, ofloxacin, and ceftazidime[30,34]. Antibiotic-induced biofilm stimulation has also been reported for species such as *Escherichia coli*, *Bacillus subtilis,* and *Staphylococcus aureus*[33,35,36]*.* Biofilm formation may be a response to antibiotic-induced stress[37] or to microbial competition[38] and it has been linked to general stress response systems such as RpoS[39]. Due to the presence of this biofilm response across a number of diverse organisms, it is possible that the stimulation of biofilm formation constitutes a general response to antibiotics that may be coordinated either through existing stress response pathways or via a novel response. A full understanding of the mechanism for the induction of biofilm formation by sub-MIC antibiotics, however, remains incomplete.

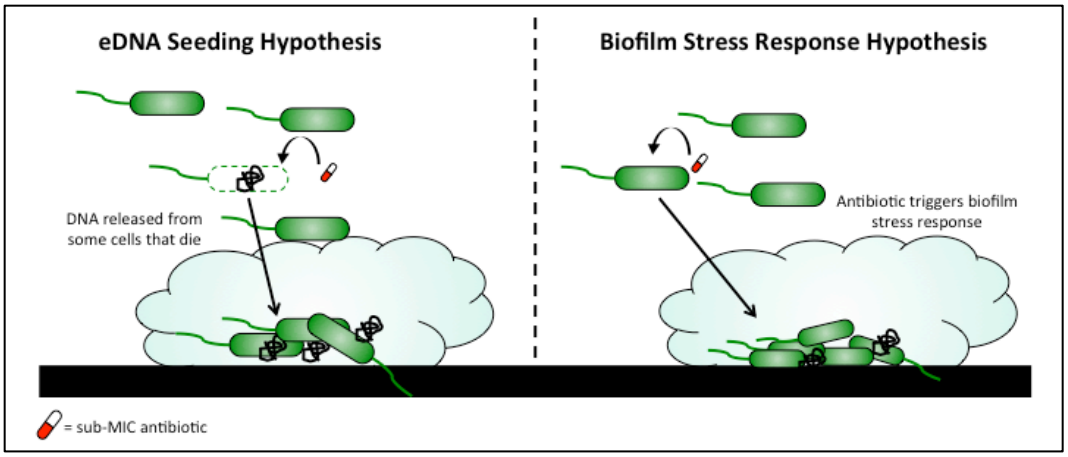


Figure 1: *Potential mechanisms governing the induction of biofilm formation by sub-MIC antibiotics*. The eDNA seeding hypothesis (left) suggests that low levels of antibiotics may kill a subpopulation of cells and induce lysis, resulting in the release of eDNA and other common goods. eDNA is an important structural component of biofilms. A biofilm stress response hypothesis suggests that bacteria may be able to sense sub-MIC antibiotics and this may cause induction of biofilm formation as a form of generalized tolerance.

While mechanisms behind the antibiotic-induced increase in biofilm formation of *P. aeruginosa* and other bacteria are currently unknown, two compatible hypotheses have emerged (Figure 1). One proposes that increased biofilm is a result of lysis of a subpopulation due to the actions of the antibiotic, "seeding" biofilm formation by the release of extracellular DNA[40,41]. eDNA is a critical component of the biofilm EPS and is important for the development of biofilms[42]. *P. aeruginosa* coordinates the explosive cell lysis of a subpopulation of cells to release eDNA into the environment and initiate biofilm formation[43], a process that may be accelerated by antibiotic exposure. The second hypothesis proposes that the increase in biofilm is part of a coordinated response to the

presence of sub-MIC antibiotics[27,35]. This involves the detection of antibiotics, directly or as a result of the cellular stress that results from their activity, and a response that involves transcription of adhesins, polysaccharide biosynthesis genes, eDNA release genes, and other factors. Increases in cell-surface interactions occur as a result of sub-MIC antibiotic treatment in both *P. aeruginosa* and *S. aureus*, including up-regulation of adhesion-related proteins and changes in cell surface hydrophobicity[44,45]. In another example, tobramycin induces biofilm formation in a subset of *P. aeruginosa* strains in an *arr*-dependent manner[33]. Some groups have provided evidence for mechanisms that combine elements of both hypotheses. For example, sub-MIC genotoxic antibiotics like ciprofloxacin induce transcription of holin and endolysin genes in a *recA*-dependent manner, causing increased explosive cell lysis and release of eDNA in a subpopulation of *P. aeruginosa* cells[43].

1.3 The roles of the general outer membrane porin OprF in *P. aeruginosa*

A large number of proteins spanning numerous cellular functions have been implicated in contributing to, or regulating biofilm formation in *P. aeruginosa*, resulting in a complex regulatory hierarchy that controls this behaviour. For a number of these proteins, their specific contribution to their associated phenotypes remains unclear, which indicates that there may be unknown regulatory pathways that can control biofilm formation in response to

certain stimuli. One of these proteins is the general outer membrane porin OprF. Porins are proteins that form hydrophilic channels in the outer membrane and permit the passive diffusion of small, hydrophilic species such as ions and sugars that are necessary for bacterial growth. The general outer membrane porin OprF in *P. aeruginosa* is one of the most abundant and is associated with the outer membrane via an N-terminal beta barrel as well as with the peptidoglycan (PG) via a C-terminal globular domain containing a PG-binding motif[46,47]. OprF allows for passage of compounds up to ~1,500 Da[48]. *oprF* expression is controlled by the extracytoplasmic function (ECF) sigma factors SigX and AlgU[49,50,51].

OprF plays multiple roles in the cell beyond the diffusion of ions and sugars. OprF plays a role in sensing the immune system, specifically binding interferon-γ and increasing the expression of the adhesin LecA in a quorum sensing-dependent manner[52]. Loss of OprF results in sensitivity to low-osmolarity media, leakage of periplasmic proteins, reduction in cell length, and activation of membrane stress-responsive sigma factors AlgU and SigX[53,54], which indicates its importance to maintenance of cell shape and outer membrane stability[54]. Loss of OprF also leads to a reduction in virulence[55]. The mutant had impaired adhesion to eukaryotic cells, impaired secretion of ExoS and ExoT type III effectors, altered pyocyanin production, and altered production of quorum sensing molecules[55]. OprF loss also elevates levels of c-di-GMP, increasing biofilm formation[53]. With

multiple established roles in membrane homeostasis, virulence, and immune system detection, OprF may play a direct or indirect role in sensing and detection of other stressors.

1.4 Roles of the ECF Sigma Factor SigX in *P. aeruginosa*

It has been proposed that the effects on biofilm formation observed in an *oprF* mutant may be linked to the action of the ECF sigma factor SigX. Specifically, loss of OprF results in increased activity of SigX[53], which would have downstream consequences due to altered regulation of genes in the SigX regulon (Figure 2). ECF sigma factors are common in bacteria and are responsible for coupling environmental stimuli to transcriptional responses. Many ECF sigma factors are coupled with cognate anti-sigma factors that maintain them in an inactive state[56]. When a stimulus is sensed by the anti-sigma factor, the sigma factor is released to modulate transcription accordingly[56]. Other ECF sigma factors, including SigX, lack anti-sigma factors and rely on a transcriptional method of control[56]. The environmental stimuli that trigger SigX activity are not well defined, however its activity has been linked to levels of metabolites like sucrose and to membrane stress[49,57]. The SigX regulon is estimated to contain upwards of 250 genes, including proteins involved in stress responses as well as c-di-GMP metabolic enzymes[58,59] (Figure 2). Due to its potential role in responding to stress, control of c-di-GMP metabolic enzymes, and its activation

in an Δ*oprF* mutant, SigX may play an important role in the response to antibiotic-related stress.

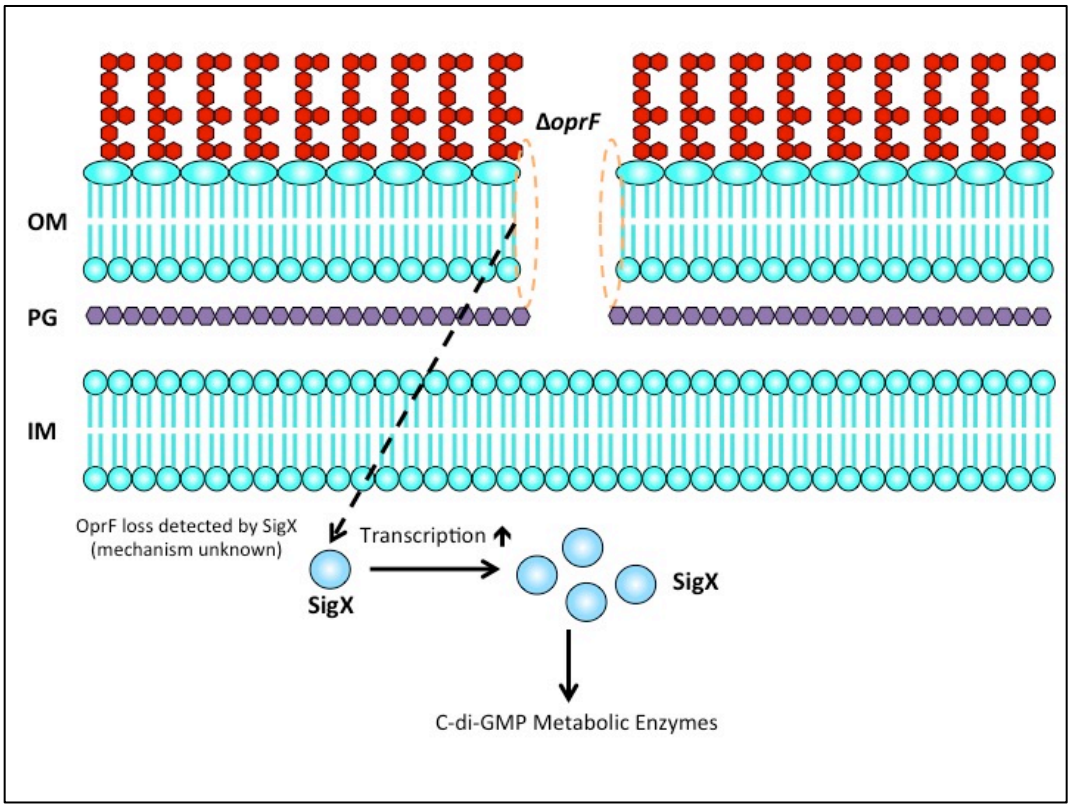


Figure 2: *Loss of OprF increases sigX activity in* P. aeruginosa*.* Loss of the general outer membrane porin OprF results in increased activity of SigX. Unlike other ECF sigma factors, SigX is not known to have a cognate anti-sigma factor. The SigX regulon contains over 250 genes, including multiple c-di-GMP metabolic enzymes that may contribute to the regulation of biofilm formation.

1.5 Hypothesis & Aims

Numerous reports have linked sub-MIC concentrations of antibiotics to shifts in the transcriptional landscape of the cell and to changes in behaviour, including biofilm formation. With this in mind, I hypothesized that increased biofilm

formation in response to sub-MIC antibiotic exposure is a part of a conserved stress response in bacteria. In order to test this hypothesis, my work has been split into three aims:

1. Testing the biofilm stimulating effects of eDNA and cell lysates to see if antibiotic-induced cell lysis can seed biofilm formation.
2. Searching for genes that are required for a biofilm response to antibiotics by screening a transposon mutant library for mutants deficient in this response.
3. Characterizing genes involved in the biofilm response to antibiotics and the roles that they play, as well as any cues to which they respond.

To address whether biofilm formation can be stimulated by eDNA or cell lysate, we prepared purified genomic DNA or cell lysates from *P. aeruginosa*. These were used in biofilm dose-response assays using an adapted crystal violet staining method[60,61], showing that neither stimulated biofilm formation to the same extent as sub-MIC antibiotics. Using transposon mutagenesis, we created a mutant library in *P. aeruginosa* PAO1 KP and screened for mutants that failed to show increases in biofilm formation after treatment with sub-MIC antibiotics. This work, along with deletion and complementation studies, identified the OprF porin as important for the ability to mount the biofilm stimulation response. The

ECF sigma factor SigX was also identified as important for this response through deletion and complementation studies. From these results, we conclude that sub-MIC antibiotics may stimulate biofilm formation in *P. aeruginosa* by activating a programmed stress response. Further, the ability to mount this response requires the presence of OprF and SigX.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions

All cells were grown at 37˚C, 200 rpm for liquid cultures and at 37˚C for solid media. Ninety-six well plates were incubated in humidified containers to prevent evaporation in peripheral wells. Lysogeny Broth (LB) media (Bioshop) contained 10g/L tryptone, 5g/L yeast extract, and 5g/L sodium chloride. The 10% LB-PBS media was LB diluted 1:10 in 1x phosphate buffered saline (PBS). With the exception of polymyxin B and ciprofloxacin, which were solubilized in sterile milliQ water, all drugs were solubilized in DMSO and diluted in growth media such that the final concentration of DMSO never exceeded 1.33% (v/v). Complementation experiments were performed with 0.05% L-arabinose as an inducer, except for *oprF*::Himar1 complementation.

2.2 Antibiotic-induced biofilm formation assays

Antibiotic-induced biofilm formation assays were performed as previously described with modifications[60,61]. Bacteria were cultivated overnight at 37˚C, 200 rpm in 10% LB-PBS media (unless otherwise noted). Overnight cultures were diluted 1:25 in 10% LB-PBS media and subcultured to OD_{600} = 0.1 under the same growth conditions. Subcultures were then diluted 1:500 in fresh 10% LB-PBS. Assays were prepared in 96-well plates with 96-peg lids (Nunc). Wells contained 150µL of total culture, with 148µL of the diluted subculture

added to each well (sterility control wells contained 148µL of media in place of the subculture) and 2µL of either an antibiotic or DMSO. Antibiotic-treated wells contained 2µL of antibiotic suspended in DMSO, while the vehicle and sterility control wells contained 2µL DMSO. Assays were incubated at 37˚C, 200rpm for 16h in humidified containers, except for biofilm assays using *P. aeruginosa oprF* or *sigX*-complemented strains (PAO1 KP + pBADGr, PAO1 KP + *oprF*, PAO1 KP + *sigX*, Δ*oprF* + pBADGr, Δ*oprF* + *oprF*, Δ*oprF* + *sigX*, Δ*sigX* + pBADGr, Δ*sigX* + *oprF*, and Δ*sigX* + *sigX*) which were incubated for 22h under the same conditions. Peg lids were removed from the plates and the 96-well plate was scanned using a plate reader measuring optical density at 600nm to quantify planktonic growth. Peg lids were submerged in 1x PBS for 10 min to remove loosely attached cells, then transferred to 0.1% crystal violet for 15 min to stain adhered cells. Peg lids were removed from crystal violet and washed immediately by submerging in 70 ml milliQ water in a basin, then transferred to a fresh milliQ water basin for 10 min. Three additional 10-min washes with milliQ water were performed in succession to remove excess stain. After washing, peg lids were allowed to air dry for a minimum of 30 minutes. Stained biofilms were solubilized in 200µL of 33.3% acetic acid in a 96-well plate for 5 min. The absorbance of the eluted crystal violet dye was quantified at 600nm using a plate reader. Optical density (planktonic growth) and absorbance values (biofilm) were plotted as the percent of the DMSO control values (corrected for background).

2.3 Creation of a PAO1 KP Himar1 Mariner transposon library

Transposon mutagenesis was performed as previously described[62], with modifications. *E. coli* SM10 λ pir cells were transformed with pBT20 (carrying the Himar1 Mariner transposon) to create *E. coli* SM10 λ pir /pBT20. Successful transformants were selected with ampicillin on solid media. *P. aeruginosa* PAO1 KP was grown on an LB agar plate overnight and *E. coli* SM10 λ pir /pBT20 was grown on an LB agar + 15µg/mL gentamicin plate overnight at 37˚C. A full inoculating loop of cells was scraped from each plate and resuspended in either 1mL of LB media (for PAO1 KP) or 1mL of LB + 15µg/mL gentamicin (for *E. coli* SM10 λ pir /pBT20). Five hundred microlitres of the *E. coli* SM10 λ pir /pBT20 and 500 µL of PAO1 KP were mixed together in a new tube and centrifuged to pellet the cells. Eight hundred microliters of supernatant were removed and the mixed cell pellet was resuspended in the remaining supernatant. A mating spot was created by placing 100µL of the resuspended mixed cell pellet in a single spot in the middle of an LB agar plate. The mating spot was dried at room temperature for 20 min and then incubated at 37˚C overnight. The mating spot was collected using a sterile loop and resuspended in 1mL of LB media. *P. aeruginosa* PAO1 KP transposon mutants were selected by plating 100µL of the mating spot cell suspension on *Pseudomonas* Isolation Agar (PIA) (BD Difco) + 100µg/mL gentamicin. PIA contains 25µg/mL irgasan, which selects against *E. coli*. The

100µg/mL gentamicin selects against *P. aeruginosa* PAO1 KP that did not receive the transposon cassette containing a gentamicin resistance marker. Single colonies were picked either manually using sterile toothpicks or using an automated colony picker (Micro10x, Hudson Robotics) and arrayed into 96-well plates containing 100µL of LB + 30µg/mL gentamicin. Six wells containing LB media per 96-well plate were inoculated with the parental strain PAO1 KP (Wells H1-6) and another 6 wells containing LB media were left blank as sterility controls (H7-12). The 96-well plates were incubated overnight at 37˚C, 200rpm in humidified containers. After incubation, 100µL of LB + 30% glycerol was added to each well of the 96-well plates and the plates were stored at -80˚C. We collected a total of 165 96-well plates, totaling 13,776 mutants.

2.4 Screening PAO1 KP transposon mutants for antibiotic-induced biofilm formation

The screening protocol was developed based on a previously described crystal violet biofilm assay[60,61]. *P. aeruginosa* PAO1 KP transposon mutants were inoculated from 15% glycerol freezer stocks into a 96-well plate containing 10% LB-PBS media (150µL/well). Wells H1-H6 contained wild type PAO1 KP, wells H7-H12 were sterility controls, and all other wells contained individual mutants. The plates were incubated overnight at 37˚C, 200rpm in a humidified container. Using this overnight culture, a 96-well subculture plate containing 10% LB-PBS media (150µL/well) was inoculated with a 96-pin tool that transferred ~0.2µL per

well. The resulting subculture was incubated at 37˚C, 200rpm for 2h. Using the subculture plate and the 96-pin tool, we inoculated four assay (96 peg lid) plates containing 148µL of 10% LB-PBS and of one of the following: 2µL of cefixime (5µM final), tobramycin (0.2µM final), thiostrepton (0.63µM final), or DMSO (1.33% v/v final). Sterility and vehicle control wells in row H contained 1.33% (v/v) DMSO instead of antibiotic for antibiotic-treated plates. A polystyrene 96-peg lid (Nunc) was used for all assay plates to support biofilm growth. The plates were incubated overnight at 37˚C, 200rpm in humidified containers. The planktonic growth and biofilm for all plates were quantified as per the protocol in "Antibiotic-induced biofilm formation assays" (see above).

2.5 Identifying the transposon insertion site

The transposon insertion site was identified using a touchdown PCR method[61] based off of a semi-random, two-step (ST)-PCR method[63]. For the first round of PCR, a hybrid consensus-degenerate primer Rnd1-ARB1 (5' GGCCACGCGTCGACTAGTACNNNNNNNNNNAGAG 3') and a transposon specific primer Rnd1-TnM20 (5' TATAATGTGTGGAATTGTGAGCGG 3') were used to amplify the unknown chromosomal region adjacent to the transposon insertion site. Cells from a single colony were used as template for the PCR. Random annealing by the Rnd1-ARB1 primer was facilitated by starting with an annealing temperature at 49˚C and reducing the temperature by 1˚C every cycle for 15

cycles. Then, the annealing temperature was kept constant at 60˚C for a further 20 cycles of PCR. Then, 2µL of the product from this first round of PCR was removed and used as template DNA for a second round of PCR with the Rnd2-ARB primer (5' GGCCACGCGTCGACTAGTAC 3') and the Rnd2-TnM20 primer (5' ACAGGAAACAGGACTCTAGAGG 3'). This second round of PCR used an annealing temperature of 60˚C, a 2-minute extension time, and repeated for 20 cycles. The PCR product was run on a 1% agarose gel with ethidium bromide as a visualizing agent at 120V for 30 minutes. The bands were excised from the gel and purified using a GeneJET gel extraction kit (Thermo) followed by Sanger sequencing of the resulting gel-purified PCR fragments using the sequencing primer TnM20Seq (5' CACCCAGCTTTCTTGTACAC 3'). The resulting sequences were mapped to the *P. aeruginosa* PAO1 (reference) genome found at www.pseudomonas.com[64] using the BLAST[65] search function on www.pseudomonas.com[64].

2.6 eDNA and cell lysate biofilm formation assays

Chromosomal DNA was isolated from *P. aeruginosa* PAO1 KP cells using a Promega Wizard Genomic DNA Purification Kit. Purified genomic DNA (gDNA) was resuspended to 10ng/µL in nuclease-free water. Cell lysates were prepared from *P. aeruginosa* PAO1 KP cells grown to OD_{600} = 1.8 in LB media using freeze-thaw cycling. Seven hundred microlitres of 1.8 OD_{600} culture was incubated at -80˚C for 30 min and thawed at room temperature for 30 min. This freeze-thaw

cycle was repeated at least five times for a total of at least six cycles. Biofilm formation assays were set up as described in "Antibiotic-induced biofilm formation assays", except 15µL of gDNA or cell lysate (at the indicated concentrations) were added to each treated well, with 135µL of bacterial subculture (prepared as described in "antibiotic-induced biofilm formation assays"). For vehicle and sterility control wells, water was used in place of eDNA or LB was used in place of cell lysate. The assays were performed in 10% LB-PBS media at 37˚C, 200rpm for 16h. Biofilms were stained and analyzed as described above in "Antibiotic-induced biofilm formation assays". Planktonic growth measurements were taken using a plate reader measuring optical density at 600nm. The results for planktonic growth and biofilm were graphed as the percentage of a vehicle control.

2.7 Construction of Δ*sigX* and Δ*oprF* mutants

The Δ*sigX* and Δ*oprF* clean deletion mutants were constructed using a gene replacement method and pEX18Gm vector as previously reported[66,67]. Approximately 700bp of upstream and downstream sequences flanking *sigX* or *oprF* (+50bp at the 5' and 3' ends of *sigX* or *oprF*) were cloned into the pEX18Gm plasmid to create the pEX18Gm-Δ*oprF* and pEX18Gm-Δ*sigX* plasmids. The insert for pEX18GM-Δ*sigX* used SacI and BamHI restriction sites at the 5' and 3' ends, respectively, with an NdeI restriction site joining the upstream and downstream

sequences. The upstream flanking region for *sigX* was amplified using the forward primer SigXUP (5' GAACGAGCTCAACTGGTGAACAGCGTCGTG 3') and the reverse primer SigX5' (5' GCGCCATATGGCCGCATCAATTCTTCATAG 3'). The downstream flanking region for *sigX* was amplified using the forward primer SigX3' (5' CGCGCATATGCGAAAAGTTTTCAGATGCGAC 3') and the reverse primer SigXDWN (5' CTGCGGATCCCTTTCGAACCACCGAAGTTG 3'). The insert for pEX18Gm-Δ*oprF* used SacI and HindIII restriction sites at the 5' and 3' ends, respectively, with a BamHI restriction site joining the upstream and downstream sequences. The upstream flanking region for *oprF* was amplified using the forward primer oprf_F1 (5' NNNNGAGCTCGGCAGCCGCGAGGTCGCCGG 3') and the reverse primer oprf_R1 (5' NNNNGGATCCCAAAGGCGTTCATTGCCGAA 3'). The downstream flanking region for *oprF* was amplified using the forward primer oprf_F2 (5' NNNNGGATCCACAACGCCACCGCTGAAGGC 3') and the reverse primer oprf_R2 (5' NNNNAAGCTTGGCACCGGGACGACCCAGCC 3'). Each deletion construct was transformed into *E. coli* DH5α cells for large-scale purification of plasmid DNA. The gene replacement strategy used a *sacB*-based method previously described[67]. Plasmid DNA was isolated using a GeneJET Plasmid Miniprep Kit (Thermo) and used to transform *E. coli* SM10 cells. *E. coli* SM10 containing the deletion construct and *P. aeruginosa* PAO1 KP were grown overnight on solid media (LB + 15μg/mL gentamicin for *E. coli* SM10 with deletion constructs, LB media for PAO1 KP) at 37˚C. Sterile inoculating loops

were used to scrape cells from each plate and both were resuspended in a single 1mL aliquot of LB media. The cell mixture was pelleted and then 800µL of supernatant was removed. The cell pellet was resuspended in the remaining supernatant and then 200µL of the cell suspension was plated in a single spot at the center of an LB plate. The mating spot was allowed to dry for 20 min at room temperature and then was incubated at 37˚C overnight. The mating spot was scraped using a sterile loop and resuspended in 1mL of LB media. Various amounts of the cell suspension (50µL, 100µL, and 200µL) were plated on PIA (BD Difco) + 100µg/mL gentamicin. Colonies were restreaked on LB agar + 5% sucrose to select against merodiploids. Sucrose-resistant colonies were restreaked onto both LB agar and LB agar + 30µg/mL gentamicin to ensure the vector DNA was lost. The deletion mutants were verified using PCR with *oprF* or *sigX* internal primers (SigXMID: 5' CGTGGCTATATAGCATCACGTACAACG 3'. OprFMID: 5' CGTCGGCTTCAACTTCGGTGGTTC 3') to amplify the deleted region (compared against a PAO1 KP control), as well as Sanger sequencing using the oprf_F1 primer (5' NNNNGAGCTCGGCAGCCGCGAGGTCGCCGG 3') to sequence the Δ*sigX* mutant and using the oprf_F1 primer (5' NNNNGAGCTCGGCAGCCGCGAGGTCGCCGG 3') and the oprf_R2 primer (5' NNNNAAGCTTGGCACCGGGACGACCCAGCC 3') to sequence the Δ*oprF* mutant.

2.8 Construction of *oprF*-pUCP20, *oprF*-pBADGr, and *sigX*-pBADGr

PCR was performed using primers specific to the 5' and 3' ends of each gene, with the forward primer containing an EcoRI site and the reverse primer containing a HindIII site. Primers used to amplify the *oprF* insert for *oprF*-pUCP20 and *oprF*-pBADGr plasmids were OprFFW (5' GTACGAATTCGATGGGGATTTAACGGATG 3') and OprFRV (5' GCATAAGCTTGCTCAGCCGATTACTTG 3'). Primers used to amplify the *sigX* insert for the *sigX*-pBADGr plasmid were SigXFW (5' CTGAGAATTCGCACTCGGAGCTGTTCCAC 3') and SigXRV (5' CCGCAAGCTTCTTCCCGCATAGAGAGAC 3') The amplified DNA inserts were purified by gel electrophoresis and extracted using the GeneJET gel extraction kit (Thermo). The purified DNA inserts and either pUCP20 or pBADGr were digested with both EcoRI and HindIII for 30 min. Reactions were placed at -20˚C for 30 min to stop the restriction digest. The cut DNA insert and cut plasmid were purified using a GeneJET PCR purification kit (Thermo). The purified DNA insert and plasmid were then ligated together with T4 DNA ligase at 4˚C overnight. Five microliters of ligation product was used to transform *E. coli* DH5α competent cells. Transformants were selected on LB + 100µg/mL ampicillin (for *oprF*-pUCP20 transformants) or LB + 15µg/mL gentamicin (for *oprF*-pBADGr and *sigX*-pBADGr transformants) plates with 70µL of X-Gal (20mg/mL in DMF) spread on the surface of the plate to allow for blue-white colony screening. All plates were incubated overnight at 37˚C. Colonies with white colony morphology were

selected and used to inoculate 5mL of LB media containing either 100µg/mL ampicillin (for *oprF*-pUCP20 transformants) or 30µg/mL gentamicin (for *oprF*-pBADGr or *sigX*-pBADGr transformants). These cultures were incubated overnight at 37˚C, 200rpm. Plasmids were purified from the resulting cultures using a GeneJET Plasmid Miniprep Kit (Thermo) and digested using EcoRI and HindIII. DNA gel electrophoresis was used to analyze the restriction digests of the plasmids to identify the plasmids with the correct sized insert. Sanger sequencing was used to confirm the identities of the *oprF*-pBADGr and *sigX*-pBADGr plasmids using pBADGr forward (5' GCACGGCGTCACACTTTGCTATGCCA 3') and reverse (5' CGGCATGGGGTCAGGTGGGA 3') primers.

2.9 Data analysis and graphs

All experiments were performed a minimum of 3 times, unless otherwise stated. Data from plate reader scans was analyzed using Excel and Prism 7. All graphs were created using Prism 7. One-way ANOVAs were used to determine if there was significant variation between any of the mean biofilm levels from a particular experiment. Dunnett's post-test was performed for all one-way ANOVAs to further identify whether each specific concentration-vehicle control comparison pairs had a variation that was significant. One-way ANOVAs with Dunnett's post-test were performed using Prism 7. Sequencing data was analyzed using FinchTV and Geneious 6.0.6.

2.10 Strains used in this study

Table 1: *Strains used in this study.*

Strain Name	Genotype/Characteristics	Reference
Pseudomonas aeruginosa		
PAO1 KP	PAO1 wild type strain. Graciously donated by Keith Poole (Queen's University, Kingston, Canada)	[68]
PA14 Tn	UCBPP-PA14 strain. Used in creation of PA14 nonredundant transposon mutant library. Graciously donated by Michael Surette (McMaster University, Hamilton, Canada)	[69, 70]
PAO1 KP *oprF*::Himar1	*oprF*::Himar1. Himar1 transposon insertional mutant in *oprF.* Gentamicin resistant.	This Study
PAO1 KP *oprF*::Himar1 + pUCP20	*oprF*::Himar1. Himar1 transposon insertional mutant in *oprF* with pUCP20. Ampicillin/carbenicillin resistant. Gentamicin resistant. Vector control.	This Study
PAO1 KP *oprF*::Himar1 + pUCP20-oprF	*oprF*::Himar1. Himar1 transposon insertional mutant in *oprF* expressing WT *oprF* on a pUCP20 plasmid. Ampicillin/Carbenicillin resistant. Gentamicin resistant.	This Study
PAO1 KP Δ*oprF*	Δ*oprF*. An *oprF* clean deletion mutant	This Study
PAO1 KP Δ*oprF* + pBADGr	Δ*oprF*. An *oprF* clean deletion mutant with a pBADGr plasmid. Gentamicin resistant. Vector Control.	This Study
PAO1 KP Δ*oprF* + pBADGr-oprF	Δ*oprF*. An *oprF* clean deletion mutant expressing WT *oprF* on a pBADGr plasmid. Gentamicin resistant.	This Study
PAO1 KP Δ*oprF* + pBADGr-sigX	Δ*oprF*. An *oprF* clean deletion mutant expressing WT *sigX* on a pBADGr plasmid. Gentamicin resistant.	This Study
PAO1 KP Δ*sigX*	Δ*sigX*. A *sigX* clean deletion mutant	This Study
PAO1 KP Δ*sigX* + pBADGr	Δ*sigX*. A *sigX* clean deletion mutant with a pBADGr plasmid. Gentamicin resistant. Vector Control.	This Study
PAO1 KP Δ*sigX* + pBADGr-sigX	Δ*sigX*. A *sigX* clean deletion mutant expressing WT *sigX* on a pBADGr plasmid. Gentamicin resistant.	This Study
PAO1 KP Δ*sigX* + pBADGr-oprF	Δ*sigX*. A *sigX* clean deletion mutant expressing WT *oprF* on a pBADGr plasmid. Gentamicin resistant.	This Study
Escherichia coli		
DH5α	F– *endA1 gln*V44 *thi*-1 *recA1 relA1 gyrA96 deoR nupG purB20 φ80dlacZΔM 15 Δ(lacZYA-argF)U169*, *hsdR17(rK–mK+)*, λ–. Used for amplifying plasmid DNA, high transformation efficiency. Allows for blue-white colony screening.	Invitrogen
DH5α + pUCP20	DH5α expressing pUCP20. Ampicillin resistant.	This Study
DH5α + pUCP20-oprF	DH5α expressing pUCP20-oprF. Ampicillin resistant.	This Study
DH5α + pBADGr	DH5α expressing pBADGr. Gentamicin resistant.	This Study
DH5α + pBADGr-oprF	DH5α expressing pBADGr-oprF. Gentamicin resistant.	This Study
DH5α + pBADGr-sigX	DH5α expressing pBADGr-sigX. Gentamicin resistant.	This Study
DH5α + pEX18Gm-Δ*oprF*	DH5α expressing pEX18Gm-Δ*oprF*. Gentamicin resistant.	This Study
DH5α + pEX18Gm-Δ*sigX*	DH5α expressing pEX18Gm-Δ*sigX*. Gentamicin resistant.	This Study
SM10	Used for efficient conjugative transfer of plasmid DNA to *P. aeruginosa*.	[71]
SM10 + pEX18Gm-Δ*oprF*	SM10 expressing pEX18Gm-Δ*oprF*. Gentamicin resistant. Used for conjugative transfer of plasmid to *P. aeruginosa*.	This Study
SM10 + pEX18Gm-Δ*sigX*	SM10 expressing pEX18Gm-Δ*sigX*. Gentamicin resistant. Used for conjugative transfer of plasmid to *P. aeruginosa*.	This Study
SM10-λ*pir*	SM10 containing λ*pir* which allows for replication of plasmids with oriR6K origins.	[71, 72]
SM10-λ*pir* + pBT20	SM10 containing λ*pir* which allows for replication of plasmids with *oriR6K* origins. Used to transfer pBT20 to *P. aeruginosa* PAO1 KP.	This Study

2.11 Plasmids used in this study

Table 2: *Plasmids used in this study.*

Plasmid Name	Relevant Characteristics	Reference
pBT20	Carries Himar1 transposon and the Himar1 Mariner C9 transposase. Contains *oriT* and *oriR6K.* *bla* selectable marker.	[73]
pUCP20	Escherichia-Pseudomonas shuttle vector with *lac* promoter and *lacZ* α-subunit. *bla* selectable marker. Derived from pUC18/19.	[74]
pUCP20-oprF	*oprF* gene cloned into pUCP20	This Study
pBADGr	Arabinose-inducible promoter, gentamicin resistance.	[75]
pBADGr-oprF	Arabinose-inducible expression of *oprF*	This Study
pBADGr-sigX	Arabinose-inducible expression of *sigX*	This Study
pEX18Gm	Gene replacement vector, gentamicin resistance, *sacB*+, *oriT*+. Contains pUC18 MCS.	[66]
pEX18Gm-oprF	Contains fusion of ~700bp upstream and downstream *oprF* flanking regions.	This Study
pEX18Gm-sigX	Contains fusion of ~700bp upstream and downstream *sigX* flanking regions.	This Study

CHAPTER 3: RESULTS

3.1 Sub-MIC levels of cefixime, tobramycin, and thiostrepton induce biofilm formation in *P. aeruginosa* PAO1 KP

We first chose an appropriate strain of *P. aeruginosa* to study the biofilm response to sub-MIC antibiotics, optimized growth conditions, and selected multiple antibiotics to stimulate biofilm formation. We used the common laboratory strain PAO1 KP because it forms robust biofilms and has a well-annotated genome, and sub-MIC cefixime, tobramycin, and thiostrepton. Cefixime is a cephalosporin that targets peptidoglycan synthesis, tobramycin is an aminoglycoside that causes translational errors by binding the ribosomal A site, and thiostrepton is a thiopeptide antibiotic that inhibits translation by inhibiting GTPase activity of the ribosome. These antibiotics are structurally and functionally diverse, which allowed us to address whether the biofilm response occurs only for certain antibiotics. Cefixime and tobramycin are also relevant antibiotics used to treat *P. aeruginosa* infections. Thiostrepton is not used in humans, however it was selected based on previous work in the lab that identified it as a stimulator of biofilm formation (Uyen T. Nguyen and Michael Ranieri, manuscript in preparation). In PAO1 KP, sub-MIC cefixime, tobramycin, and thiostrepton all stimulated biofilm formation to varying degrees when compared to a DMSO control (Figure 3). Maximal stimulation was observed with 5µM cefixime, 0.2µM tobramycin, and at 10µM thiostrepton. For subsequent experiments, concentration ranges for each antibiotic were selected to flank these maximal stimulatory concentrations. In the cases of cefixime and tobramycin, the maximal stimulatory concentrations were approximately ¼ to ½ of the MIC. Thiostrepton is not known to be active against Gram-negative species; the observed maximal stimulatory concentration appeared at the

highest concentration tested, which was constrained by solubility. We also tested strain PA14 with sub-MIC levels of the same antibiotics and found that results were far less consistent across multiple biological replicates compared to PAO1 KP (data not shown). Therefore, PAO1 KP was selected to study the biofilm response to sub-MIC antibiotics.

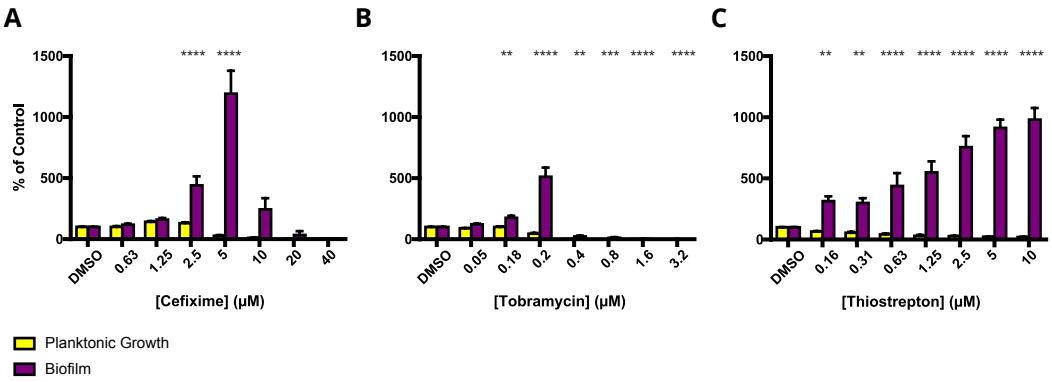


Figure 3: *Effects of sub-MIC antibiotics on biofilm formation in* Pseudomonas aeruginosa *PAO1 KP.* Structurally and functionally diverse antibiotics (A) cefixime, (B) tobramycin, and (C) thiostrepton cause dose-dependent increases in biofilm formation approaching the minimal inhibitory concentration. A one-way ANOVA with Dunnett's test was performed between DMSO and each drug treatment. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. N = 3, with 3 technical replicates per biological replicate. Planktonic growth (OD_{600}, yellow) and amount of biofilm (A_{600}, purple) both reported as percentage of a DMSO control.

3.2 Increased cell lysis is not the primary driver of the biofilm response to sub-MIC antibiotics

3.2.1 Addition of eDNA does not induce biofilm formation in P. aeruginosa PAO1 KP

Multiple hypotheses have been advanced to explain how sub-MIC antibiotics might induce biofilm formation in bacteria. The "eDNA seeding" hypothesis states that sub-MIC antibiotics kill a vulnerable sub-population of cells and release extracellular (e)DNA, which then seeds biofilm formation. eDNA is an important structural component of biofilms, and its release is linked to the ability to initiate formation of biofilms in *P. aeruginosa* and other species[36,42]. To test whether the addition of eDNA to *P. aeruginosa* PAO1 KP cultures could stimulate biofilm formation, we purified PAO1 KP genomic DNA and performed dose-response biofilm assays. The eDNA concentration range tested was guided by previous work on eDNA and *P. aeruginosa* biofilms[76,77] and based on the amounts of DNA per cell and estimates of the number of cells undergoing lysis. Specifically, genomic DNA concentrations were estimated based on the mass of a single *P. aeruginosa* genome weighing ~6.799×10^{-6} ng (assuming 6.3 million bp genome and an average 650 g/mol per base pair). Under assay conditions, there are approximately 1.5×10^{7} CFU/mL. Assuming a maximal cell lysis of 50%, we calculated that the total eDNA released would weigh ~51 ng, corresponding to 0.34 ng/µL. Therefore, we selected a concentration range of genomic DNA that would encompass this estimate. The addition of eDNA alone did not significantly increase biofilm formation in PAO1 KP above 200% of control (Figure 4). Biofilm formation was also not significantly reduced upon addition of eDNA.

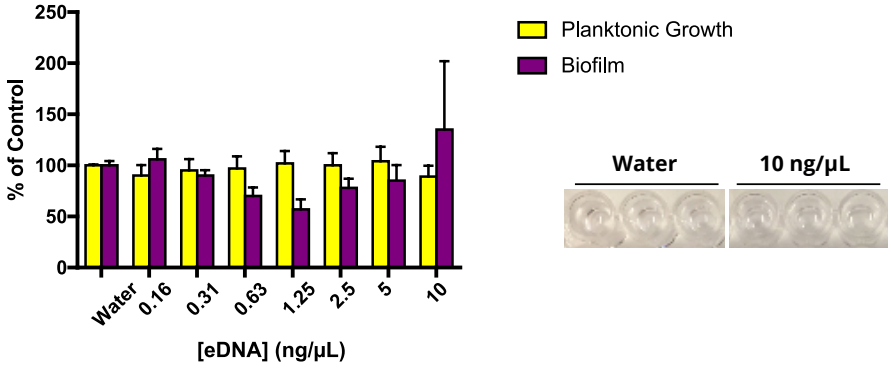


Figure 4: *Addition of eDNA fails to increase biofilm formation in PAO1 KP*. Addition of purified genomic DNA from *P. aeruginosa* PAO1 KP did not increase biofilm formation at any of the concentrations tested. Images on the left show 3 technical replicates of a water control and of a 10ng/µL eDNA treated biofilm. A one-way ANOVA with Dunnett's test was performed between DMSO and each drug treatment. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. N = 3. Planktonic growth (OD_{600}, yellow) and biofilm (A_{600}, purple) reported as percentage of a water-treated control.

3.2.2 Addition of P. aeruginosa PAO1 KP cell lysate induces a small increase in biofilm formation

The lack of biofilm stimulation seen after the addition of purified genomic DNA might be due to the lack of other factors released upon cell lysis by sub-MIC antibiotics, or a lack of DNA-binding proteins associated with the DNA. A previous report noted that an unknown factor released upon *P. aeruginosa* lysis acts as a warning signal for kin cells, resulting in activation of the Gac/Rsm pathway that is involved in up-regulating type 6 secretion and biofilm formation[78]. Another report found that extracellular ATP (eATP) can influence twitching motility-mediated biofilm expansion in *P. aeruginosa*[79]. Therefore, we performed dose-response biofilm assays using PAO1 KP whole cell lysate. Concentrations used were based on cell densities at time of inoculation (OD_{600} = 0.1 culture diluted 1:500 in growth media, or $1.1x10^4$ cfu/mL) and densities at

the assay endpoint (OD_{600} = ~0.35, or ~$1.9x10^7$ cfu/mL), aiming to include concentrations of cell lysate that could represent 50-75% of cells in the assay undergoing lysis. We lysed cells using a freeze-thaw method that resulted in >99% cell lysis, verified by plating lysate pre- and post-lysis on LB agar. A small, significant increase in biofilm formation by PAO1 KP was observed (<200% of control) at the highest concentrations of cell lysate, along with a corresponding decrease in planktonic growth (Figure 5). The magnitude of this stimulation however is far lower than what is seen with many sub-MIC antibiotics, including cefixime or thiostrepton (Figure 3). These results indicated that the lysis of cells is not likely the main driver of the biofilm response to sub-MIC antibiotics. Altogether, increases in the concentration of eDNA or in cell lysis events likely do not drive the biofilm response to antibiotics. However, the importance of eDNA to biofilm development and previous literature that shows cell lysis and eDNA are important for biofilm formation suggests that these factors may still contribute to the response in some capacity.

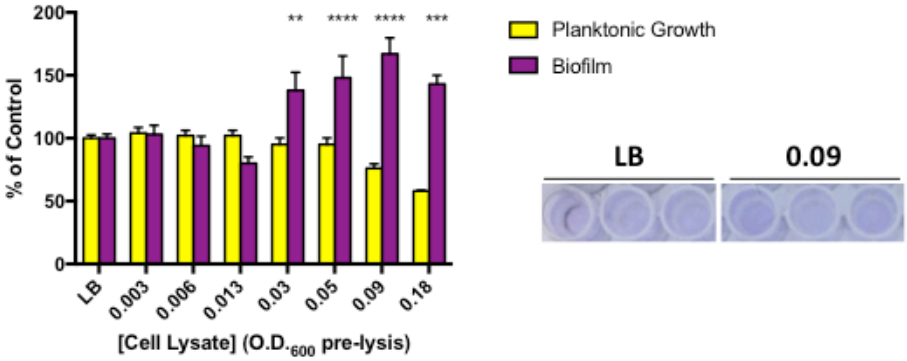


Figure 5: *Addition of cell lysate induces a small increase in biofilm formation by PAO1 KP*. Addition of cell lysate does not induce biofilm formation above 200% at any of the concentrations tested, however significant biofilm induction occurs at 0.03 and above. Images on the right show 3 technical replicates of an LB control biofilm and of a 0.09 OD_{600} pre-lysis lysate-treated biofilm. A one-way ANOVA with Dunnett's test was performed between DMSO and each drug treatment. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. N = 3. Planktonic growth (OD_{600}) and

biofilm (A_{600}) reported as percentage of a LB-treated control. Cell lysate concentrations are provided as optical density of the culture pre-lysis.

3.3 An *oprF* mutant is deficient in the biofilm response to sub-MIC antibiotics

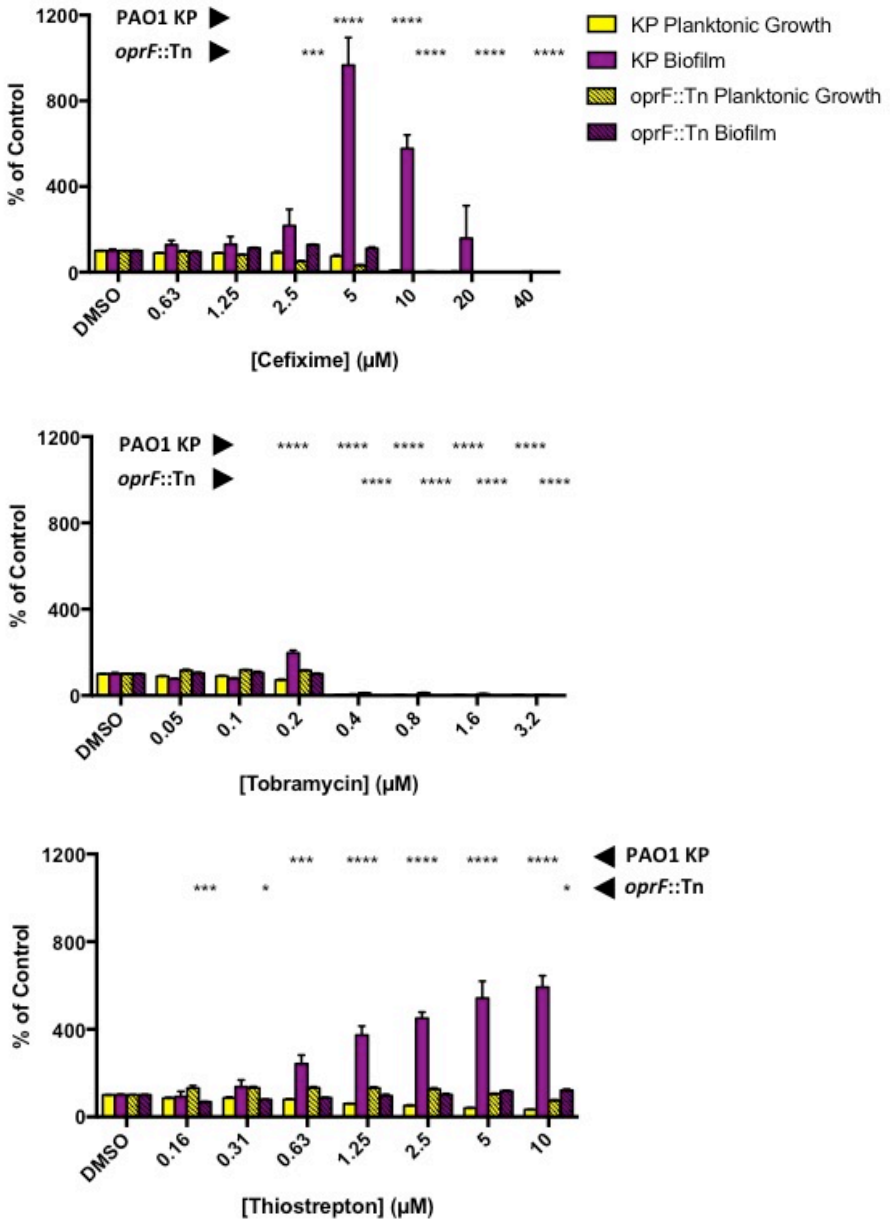
Because addition of eDNA or cell lysate did not recapitulate the biofilm stimulation phenotype we saw with antibiotics, we sought to test an alternate hypothesis, that sub-MIC antibiotics provoke stress responses that result in increased biofilm formation. To test this hypothesis in an unbiased manner, we elected to screen for mutants unable to respond to sub-inhibitory concentrations of antibiotics with increased biofilm formation. We generated a transposon mutant library in *P. aeruginosa* PAO1 KP using the Himar1 Mariner transposon and screened for mutants deficient in the biofilm response to sub-MIC concentrations of cefixime, tobramycin, and thiostrepton. We generated a random transposon mutant library containing approximately 13,800 single transposon mutants. To screen the library, we used 5µM cefixime, 0.2µM tobramycin, and 0.63µM thiostrepton. The concentrations for cefixime and tobramycin were selected because they were the maximal biofilm stimulatory concentrations previously determined (Figure 3). The screening concentration for thiostrepton was chosen because no defined maximal stimulatory concentration was found under the screening conditions (Figure 3) and because 0.63µM is ½ MIC for thiostrepton when *P. aeruginosa* is grown under iron-limited conditions where thiostrepton is most active against *P. aeruginosa* (Uyen T. Nguyen & Michael Ranieri, manuscript in preparation). We set an arbitrary cutoff of 200% of a DMSO control for significant biofilm stimulation to account for the inherent noise in the biofilm assay where growth of individual mutants was not standardized.

We screened 4,327 transposon mutants, with further screening in progress, and identified two mutants that failed to form increased biofilm in the presence of sub-MIC antibiotics (Figure 6a,b). One of these mutants was the transposon mutant BBTn1_G5. The PAO1 KP wild-type biofilms increased to 200-1000% of the DMSO control when stimulated with sub-MIC cefixime, tobramycin, or thiostrepton, while BBTn1_G5 remained below 200% of control. Of note, this mutant formed more biofilm in the presence of DMSO only (2-3x), (Figure 6b) compared to the wild type and grew to a lower terminal density than the wild type (~30-50% of the wild type terminal OD_{600}). Using touchdown PCR and sequencing, we identified the gene disrupted in BBTn1_G5 as *oprF*. OprF is a highly abundant outer membrane porin, homologous to *E. coli* OmpA[54], and allows for the passive diffusion of small molecules such as sugars and ions. OprF has important roles in *P. aeruginosa* virulence[55], sensing the immune system[52], and a structural role in the outer membrane[47]. Loss of OprF is associated with membrane stress and leakage[54], as well as elevated levels of the secondary messenger c-di-GMP[53]. Complementation of the *oprF*::Himar1 mutant with *oprF* in trans resulted in restoration of both the biofilm response to sub-MIC cefixime and near-wild type planktonic growth (Figure 6c), confirming that this mutation was likely responsible for loss of the biofilm stimulation response and for the growth defect. The second mutant had a disruption in the *dsbA* gene. DsbA is a periplasmic thiol oxidoreductase that is responsible for helping the OprF protein fold into a conformation that allows for it to bind peptidoglycan[80]. The role of DsbA in proper OprF folding suggested that the phenotype observed was potentially linked to OprF, therefore we focused our study on the *oprF* mutant.

We constructed an Δ*oprF* deletion mutant to validate the above results. Similar results were obtained for Δ*oprF*, with sub-MIC cefixime, tobramycin, and thiostrepton failing to induce biofilm formation (Figure 7). Biofilm formation in

the Δ*oprF* mutant did not increase above 150% of control, while PAO1 KP biofilm formation increased to 200-1200% of control when these strains were treated with sub-MIC cefixime, tobramycin, and thiostrepton. Δ*oprF* also produced higher baseline levels of biofilm and grew to a lower density compared to the wild type, similar to the *oprF*::Himar1 mutant.

A


PAO1 KP
oprF::Tn
KP Planktonic Growth
KP Biofilm
oprF::Tn Planktonic Growth
oprF::Tn Biofilm
% of Control
1200
800
400
0
DMSO
0.63
1.25
2.5
5
10
20
40
[Cefixime] (μM)
PAO1 KP
oprF::Tn
% of Control
DMSO
0.05
0.1
0.2
0.4
0.8
1.6
3.2
[Tobramycin] (μM)
PAO1 KP
oprF::Tn
% of Control
DMSO
0.16
0.31
0.63
1.25
2.5
5
10
[Thiostrepton] (μM)

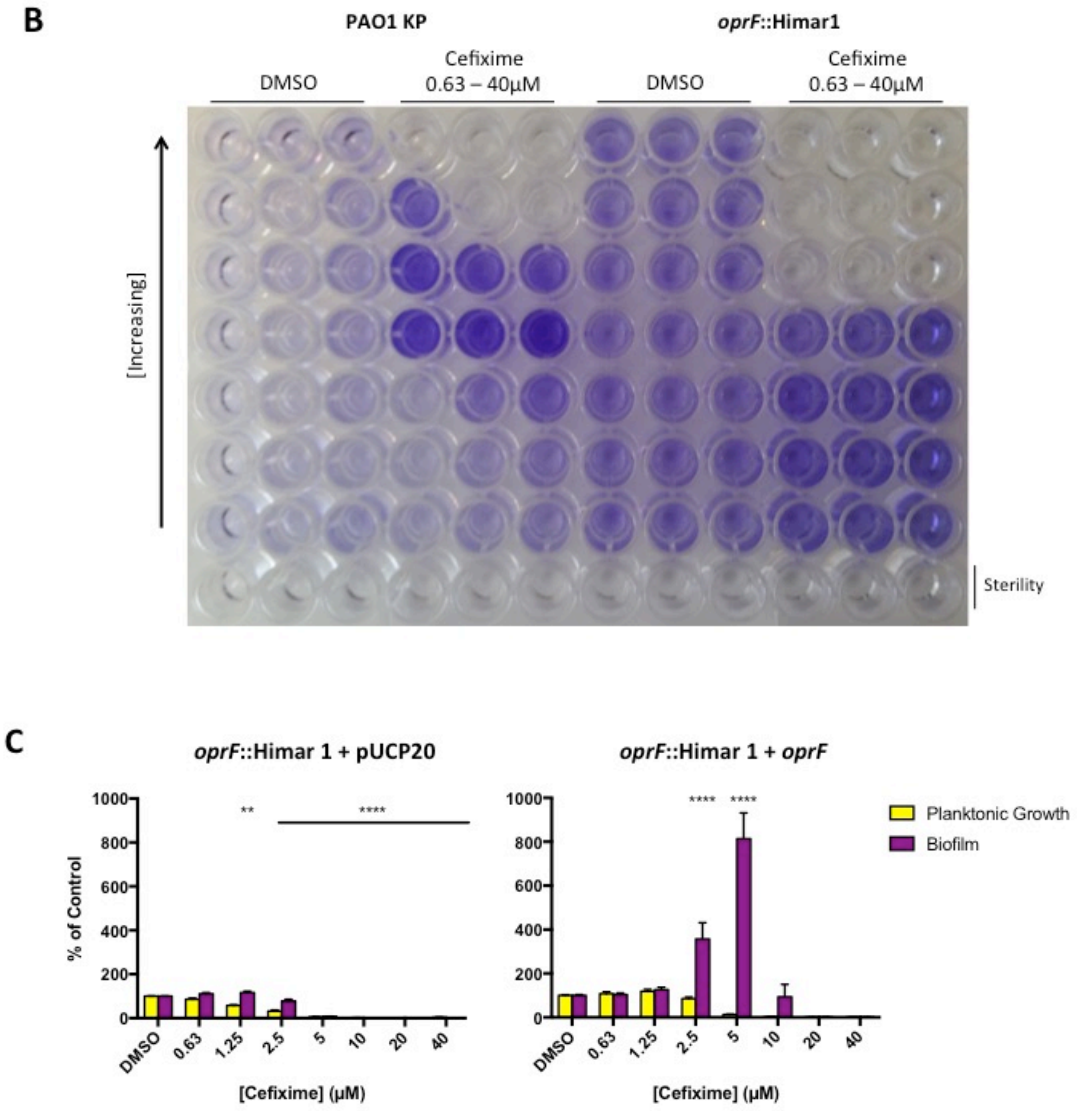
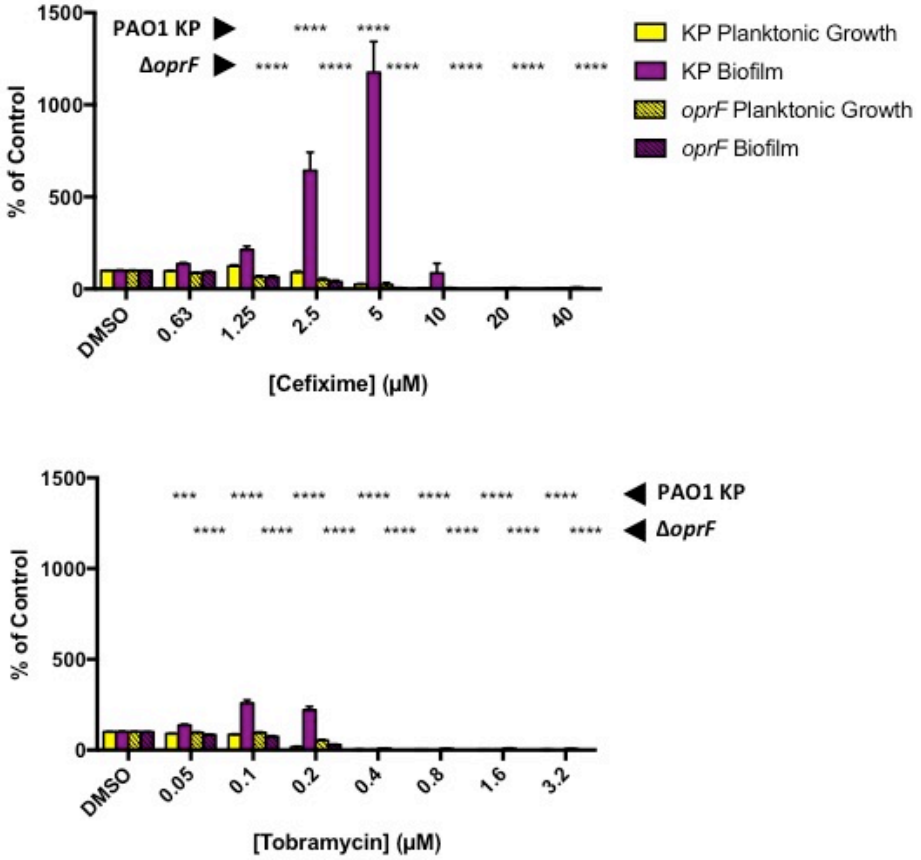


Figure 6: *Sub-MIC antibiotics fail to induce biofilm formation of an* oprF*::Himar1 transposon mutant*. (A) Sub-MIC antibiotics fail to induce induce biofilm formation above 200% of a DMSO control in an *oprF*::Himar1 (denoted *oprF*::Tn, patterned bars) mutant. Sub-MIC antibiotics induced biofilm formation between 200-1000% in the parent strain PAO1 KP (denoted KP, solid colour bars). N=1. Planktonic growth (OD_{600}) and biofilm (A_{600}) reported as percentage of a DMSO-treated control. (B) Biofilm staining plate image of sub-MIC cefixime results from panel A, left graph. Intensity of crystal violet correlates with amount of biofilm formed. (C) Sub-MIC cefixime does not induce biofilm formation in a non-complemented *oprF*::Himar1 strain (left). Complementation with *oprF* on a pUCP20 plasmid restores the biofilm response to sub-MIC cefixime. N=3. Planktonic growth (OD_{600}) and biofilm (A_{600}) reported as percentage of a DMSO-

treated control. A one-way ANOVA with Dunnett's test was performed for both (A) and (C) between DMSO and each drug treatment. For (A), the upper row of * refers to KP biofilm and the lower refers to *oprF*::Tn biofilm. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

A



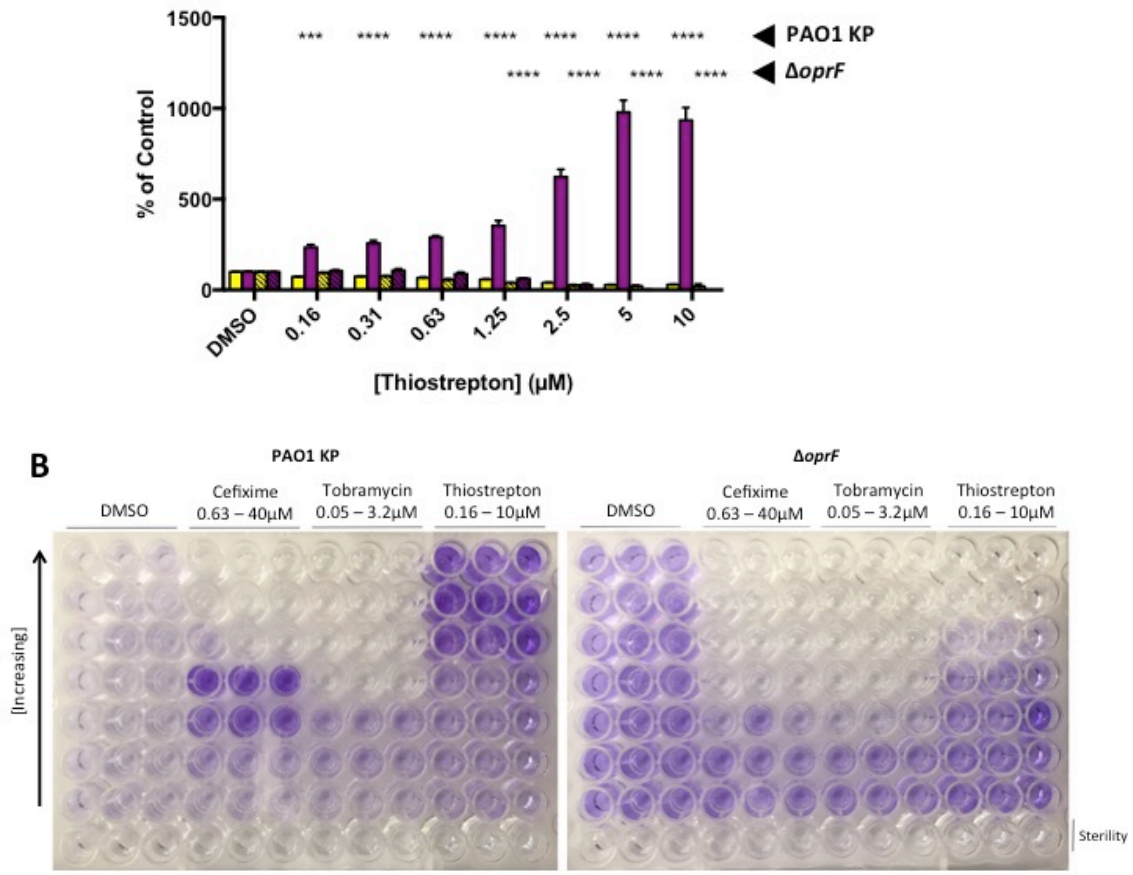


Figure 7: *Sub-MIC antibiotics fails to induce biofilm formation in an* ΔoprF *mutant*. (A) None of the antibiotics used, including cefixime, tobramycin, and thiostrepton, were able to induce biofilm formation in an Δ*oprF* mutant above 200% of a DMSO control. (B) Biofilm staining plate images of the results in panel A. A one-way ANOVA with Dunnett's test was performed between DMSO and each drug treatment. The upper row of * refers to KP biofilm and the lower refers to Δ*oprF* biofilm. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. N=3. Planktonic growth (OD_{600}) and biofilm (A_{600}) reported as percentage of a DMSO-treated control.

3.4 Loss of OprF inhibits the biofilm stimulation response to many antibiotics

Cefixime, tobramycin, and thiostrepton induce biofilm formation in the wild type and fail to induce biofilm formation in an Δ*oprF* mutant. We next tested a broader group of antibiotics, selecting 6 that were bacteriostatic or

bactericidal and with various mechanisms of action. This group included carbenicillin, chloramphenicol, ciprofloxacin, novobiocin, polymyxin B, and trimethoprim (Table 3). We treated PAO1 KP and Δ*oprF* with a range of concentrations of each antibiotic and found that all antibiotics stimulated biofilm formation of PAO1 KP to varying degrees, but that the maximal stimulation generally occurred at ¼ - ½ MIC (Figure 8a-f). Polymyxin B initially failed to stimulate biofilm formation of PAO1 KP, however low levels of stimulation (slightly below 200% of control) were detected when the concentration range was expanded (Figure 9). For Δ*oprF*, none of the 6 antibiotics tested stimulated biofilm formation above 200% of control (Figure 8a-f). MICs for the antibiotics against Δ*oprF* either remained similar to the wild type or were decreased. Interestingly, Δ*oprF* had highly increased susceptibility to novobiocin and trimethoprim, with MICs for both drugs decreased by ~8 fold (Figure 8d, f). PAO1 KP and Δ*oprF* had similar MIC values for all other antibiotics.

Table 3: *List of antibiotics used for biofilm stimulation assay*. MIC values reflect the results in Figures 5, 6, and 7. MIC values reflect those performed in 10% LB-PBS media.

Antibiotic	Mechanism of Action	Bacteriostatic vs. Bactericidal	MIC for PAO1 KP	MIC for Δ*oprF*
Cefixime	Inhibits peptidoglycan synthesis	Bactericidal	4.5-9.1 µg/mL	4.5 µg/mL
Tobramycin	Binds ribosomal A site, causes mistranslation	Bactericidal	0.19 µg/mL	0.19 µg/mL
Thiostrepton	Binds ribosomal GTPase-associated centre	Bacteriostatic	>16.6 µg/mL	16.6 µg/mL
Carbenicillin	Inhibits peptidoglycan synthesis	Bactericidal	>200 µg/mL	200µg/mL
Chloramphenicol	Inhibits protein chain elongation	Bacteriostatic	16 µg/mL	2-4 µg/mL
Ciprofloxacin	Inhibits DNA gyrase, topoisomerase IV	Bactericidal at ≥2x MIC. Bacteriostatic at 1x MIC [81]	0.016 µg/mL	0.008 µg/mL
Novobiocin	Inhibits DNA gyrase	Bacteriostatic	1200-2400 µg/mL	300 µg/mL
Polymyxin B	Disrupts outer and inner membranes	Bactericidal	0.7 µg/mL	0.5-1 µg/mL
Trimethoprim	Inhibits dihydrofolate reductase	Bactericidal	32 µg/mL	<4 µg/mL

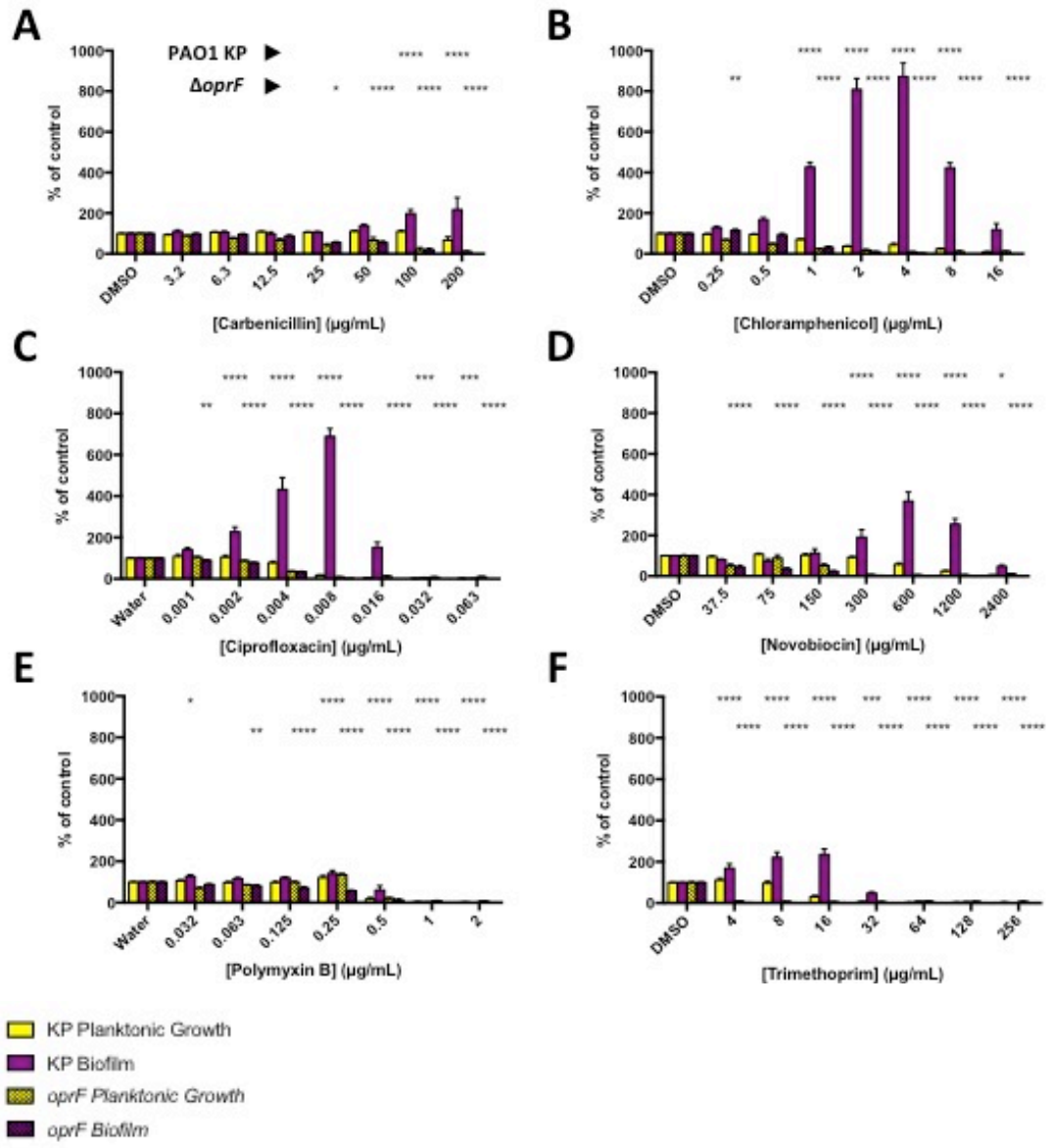


Figure 8: *Multiple classes of sub-MIC antibiotics fail to induce biofilm formation in an Δ*oprF *mutant*. (A-F) None of the 6 antibiotics tested induced biofilm formation in an Δ*oprF* mutant (patterned bars), while nearly all induced biofilm formation in PAO1 KP (solid bars). Polymyxin B (E) did not induce biofilm formation above 200% of a water control at the concentrations tested in PAO1 KP. Trimethoprim (F) sensitivity increased dramatically in an Δ*oprF* mutant. A one-way ANOVA with Dunnett's post-test was performed for A-F between DMSO and each drug treatment. The upper row of * refers to PAO1 KP biofilm and the lower refers to Δ*oprF* biofilm. *

= p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. N=3. Planktonic growth (OD_{600}) and biofilm (A_{600}) reported as percentage of a DMSO-treated control.

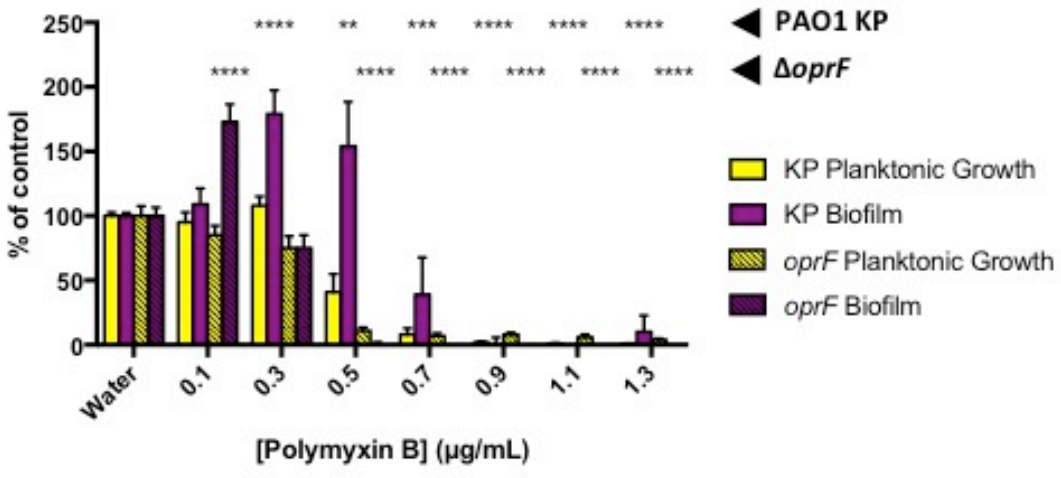
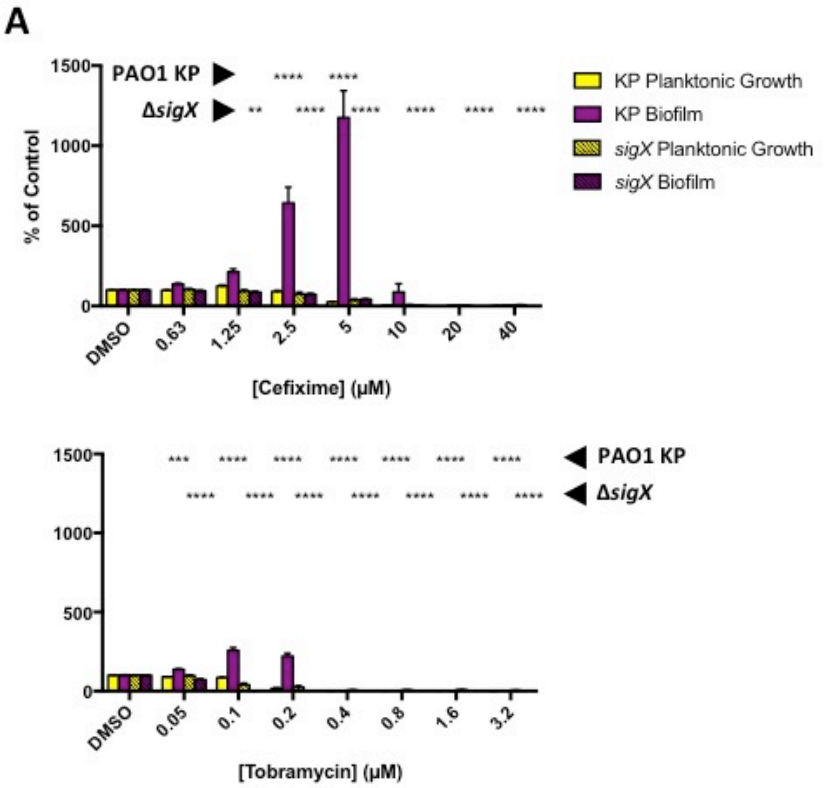


Figure 9: *Polymyxin B weakly induces biofilm formation in PAO1 KP*. Polymyxin B induced more biofilm formation within an expanded concentration range, but the amount of biofilm formed was slightly less than 200% of a water control. A one-way ANOVA with Dunnett's test was performed between DMSO and each drug treatment. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. N=3. Planktonic growth (OD_{600}) and biofilm (A_{600}) reported as percentage of a DMSO-treated control.

3.5 A Δ*sigX* mutant is deficient in the biofilm response to antibiotics

To further investigate the role of OprF in a biofilm response to antibiotics, we searched for other proteins connected with OprF. Prior studies linked *oprF* transcription to the ECF sigma factor SigX. When *oprF* is lost, SigX activity increases. SigX controls the transcription of more than 250 genes[58,59], including c-di-GMP metabolic enzymes[53,58], and the *sigX* gene is located next to *oprF* on the chromosome. ECF sigma factors commonly play roles in responses to environmental stimuli. However, the specific stimuli to which SigX responds have

not been well defined in *P. aeruginosa*, although it has been linked to outer membrane stress and osmotic shock responses[49,57]. Due to its predicted roles in responses to environmental stimuli and stress, and link to OprF expression, we hypothesized that *sigX* may be important for the biofilm response to sub-MIC antibiotics. A Δ*sigX* mutant was created in a similar manner to the Δ*oprF* mutant and verified by PCR and Sanger sequencing. When Δ*sigX* was tested for biofilm stimulation by sub-MIC cefixime, tobramycin, and thiostrepton, the results were similar to Δ*oprF* (Figure 10). The Δ*sigX* mutant displayed elevated baseline levels of biofilm formation that were 1.5-2x higher than Δ*oprF*, but these levels did not increase as antibiotic was added. Like Δ*oprF*, Δ*sigX* MIC values were consistent with wild type MIC values (Figure 10) and the Δ*sigX* mutant grew more slowly than PAO1 KP.



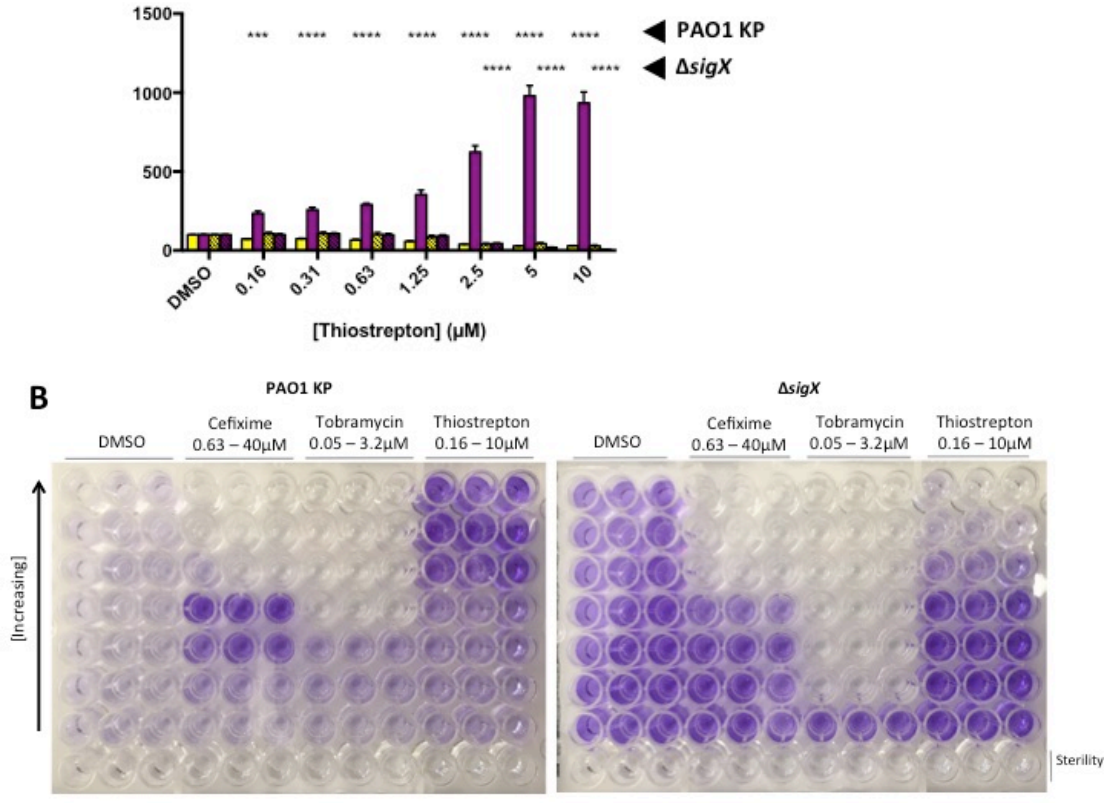


Figure 10: *Sub-MIC antibiotics fail to further increase biofilm formation in a Δ*sigX *mutant*. (A) Sub-MIC cefixime, tobramycin, and thiostrepton do not further increase biofilm formation in a Δ*sigX* mutant. (B) Biofilm staining plate images of the results in panel A. Loss of *sigX* greatly increases baseline biofilm formation. A one-way ANOVA with Dunnett's test was performed between DMSO and each drug treatment. The upper row of * refers to KP biofilm and the lower refers to Δ*sigX* biofilm. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001. N=3. Planktonic growth (OD_{600}) and biofilm (A_{600}) reported as percentage of a DMSO-treated control.

3.6 Complementing Δ*oprF* and Δ*sigX* with *oprF* or *sigX in trans* does not restore the biofilm response to sub-MIC antibiotics

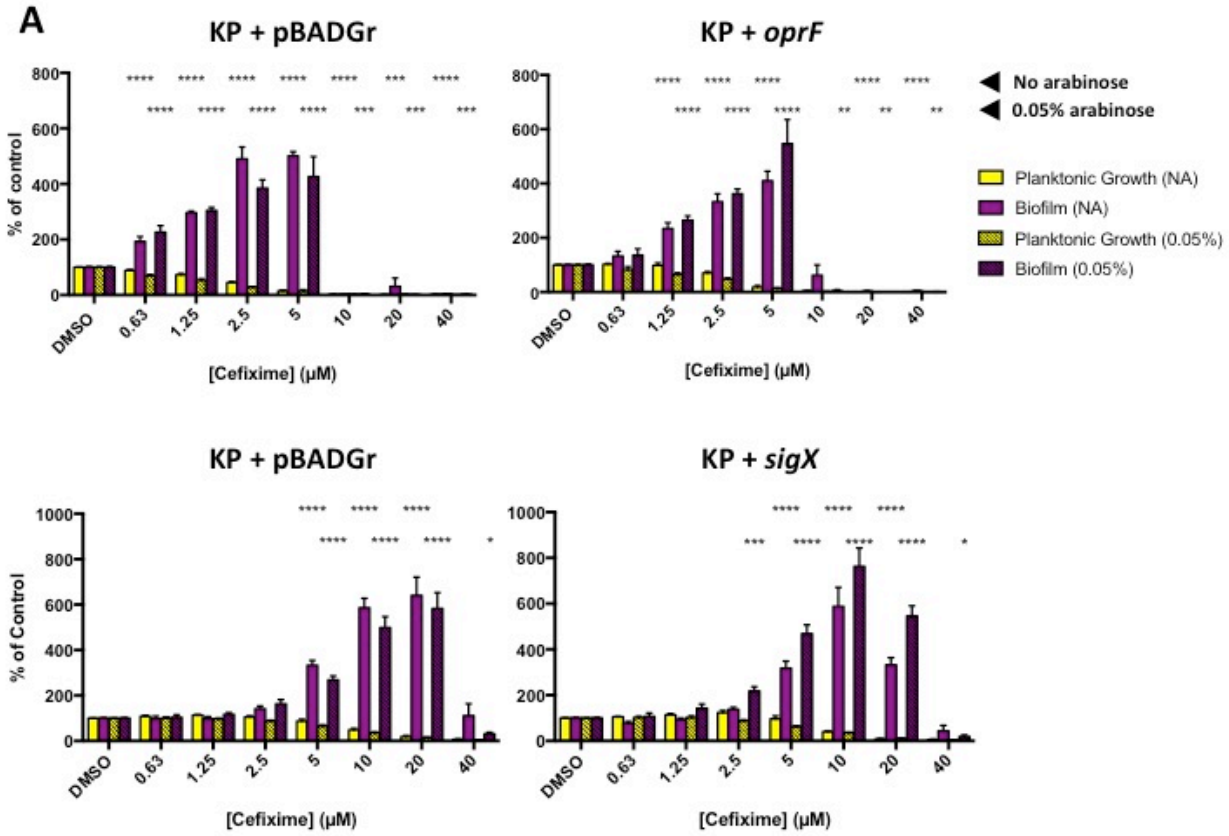
One issue with the above experiment is that transcription of *oprF* and *sigX* is closely linked[50,53]. When *oprF* is deleted, *sigX* expression increases in response[52]. When *sigX* is lost, *oprF* expression is reduced due to decreased transcriptional activation by *sigX*[50]. Therefore, it was not clear whether the *oprF*-

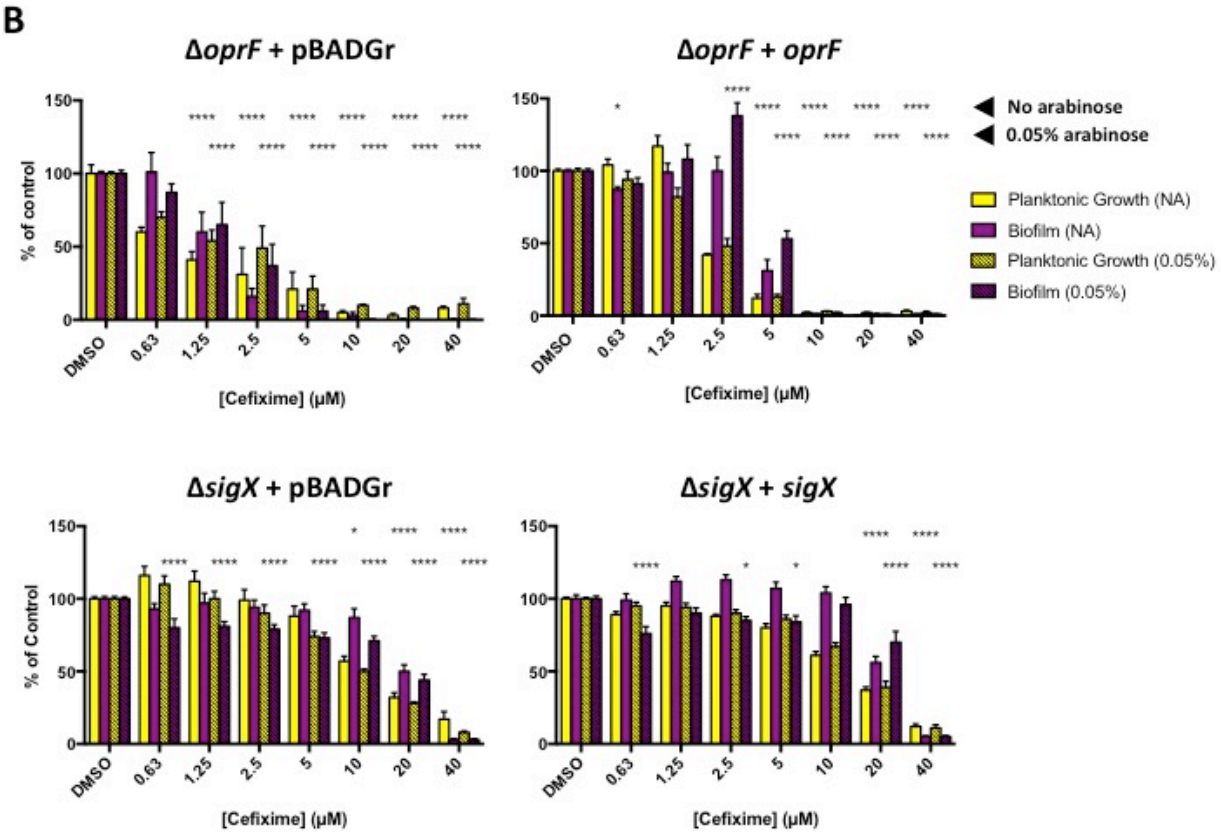
like phenotype of the *sigX* mutant was due to reduced OprF levels. Therefore, we created L-arabinose-inducible *oprF* and *sigX* complementation constructs to express the two genes *in trans* in the Δ*oprF* and Δ*sigX* backgrounds (Figure 11). The empty vector (pBADGr), without or with 0.05% arabinose, had no effect on the wild type biofilm response to sub-MIC cefixime (Figure 9A). For all *sigX* complementation experiments (Figure 11a,b,c bottom 2 graphs per section) the cefixime stock was sourced from a different bottle. This resulted in a 2-4x shift up in MIC values compared to what is normally seen for PAO1 KP (Figure 3), Δ*oprF* (Figure 7), and Δ*sigX* (Figure 10).

Interestingly, both the Δ*oprF* + *oprF* and Δ*sigX* + *sigX* complementation strains failed to return to the wild type biofilm response. They both displayed similar patterns to their non-complemented parent strains, where no increases in biofilm formation are observed in the presence of sub-MIC cefixime (Figure 9B). In the case of Δ*oprF* + *oprF*, there was an increase in the baseline biofilm levels as well as a slight increase in biofilm formation (135% of control) at 2.5µM cefixime, but this increase was not above the 200%-of-control cutoff (Figure 10). The growth defect present in the Δ*oprF* mutant however, was reverted back to near-wild type in Δ*oprF* + *oprF*, suggesting that complementation restored some of the phenotypes disrupted in the mutant.

Sub-MIC cefixime failed to induce biofilm formation of the Δ*oprF* + *sigX* and Δ*sigX* + *oprF* strains at any concentration tested (Figure 9C). Growth was similar to the control strains Δ*oprF* + pBADGr and Δ*sigX* + pBADGr, with all showing a slow growth phenotype. These results showed that the lack of biofilm response to antibiotics found in Δ*oprF* and Δ*sigX* mutants could not be complemented *in trans* through the addition of *oprF* or *sigX*. However, the growth rate of the Δ*oprF* mutant could be returned to wild type levels in an

Δ*oprF* + *oprF* strain, indicating that the lack of complementation may only pertain to the biofilm response.




B
ΔoprF + pBADGr
ΔoprF + oprF
ΔsigX + pBADGr
ΔsigX + sigX
% of control
% of Control
[Cefixime] (μM)
DMSO
0.63
1.25
2.5
5
10
20
40
No arabinose
0.05% arabinose
Planktonic Growth (NA)
Biofilm (NA)
Planktonic Growth (0.05%)
Biofilm (0.05%)

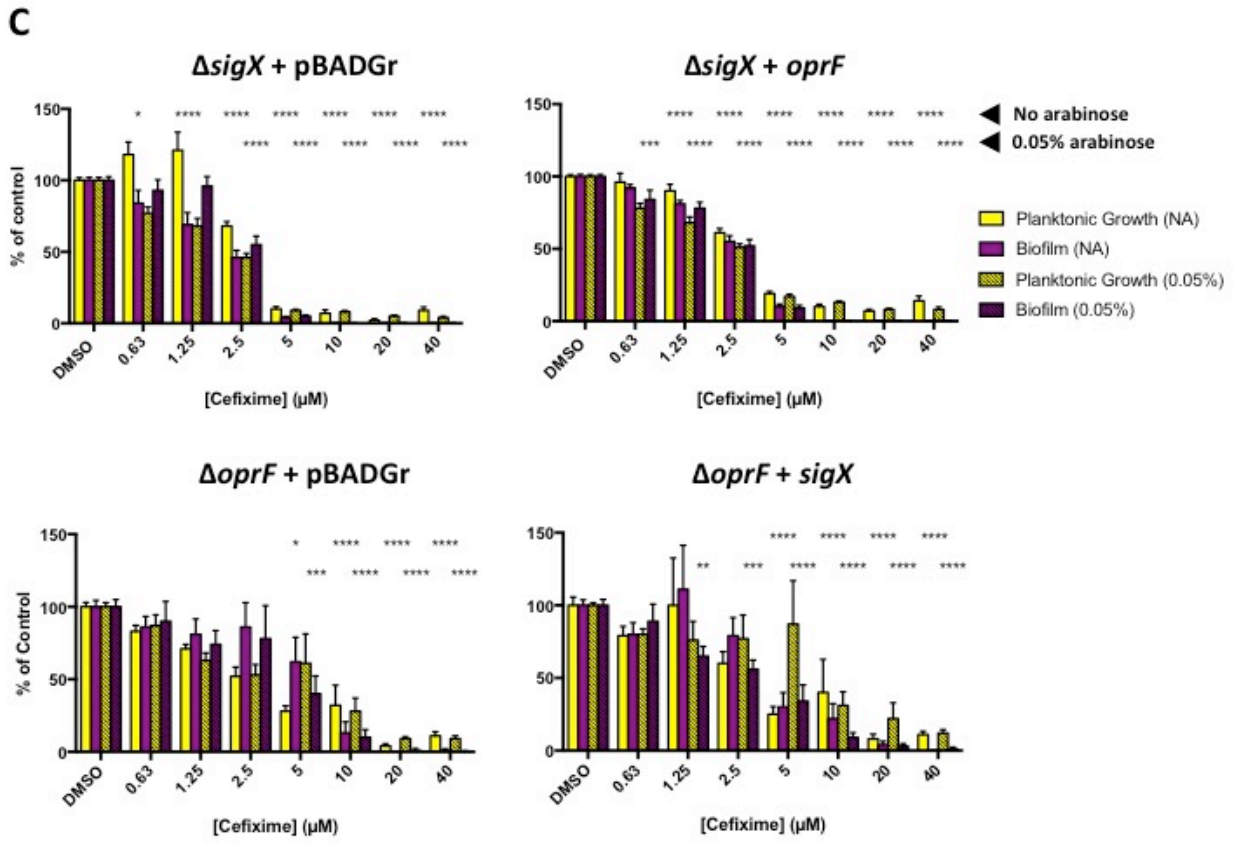


Figure 11: *Expression of* oprF *or* sig*X in trans does not complement the biofilm response to sub-MIC cefixime in an Δ*oprF *or Δ*sigX *mutant*. Bottom graphs for (A,B,C) were performed with a new stock of cefixime, producing MIC values 2-4x higher than expected. (A) *oprF* or *sigX* were expressed in trans in the wild type, without inducer (NA) and with inducer (0.05% L-arabinose). Expression of each gene in trans did not alter biofilm stimulation patterns or the MIC compared to vector controls. (B) Expression of *oprF* or *sigX* in trans within the corresponding mutant does not restore the biofilm response to sub-MIC cefixime. Complementation of the Δ*oprF* growth defect occurred in the Δ*oprF* + *oprF* strain, along with mild biofilm stimulation (135% of DMSO control). (C) Expression of *oprF* or *sigX* in trans within the partner mutant (ie. the opposite gene) did not restore the biofilm response to sub-MIC cefixime, nor did it complement the growth defect observed in both mutants. A one-way ANOVA with Dunnett's test was performed between DMSO and each drug treatment. The upper row of * refers to no L-arabinose biofilm and the lower refers to 0.05% L-arabinose biofilm. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. N=3, except for the top graphs in panel A which are N=2. Planktonic growth (OD_{600})

and biofilm (A_{600}) reported as percentage of a DMSO-treated control. No L-arabinose = solid bars, patterned bars = 0.05% L-arabinose.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

4.1 Antibiotic-induced cell lysis does not drive the biofilm response to sub-MIC antibiotics

One of the current hypotheses for how sub-MIC antibiotics induce biofilm formation suggests that these molecules kill a subpopulation of cells releasing common goods, such as eDNA, that seed biofilm formation. eDNA is an important structural component of biofilms, and its release is linked to the ability to initiate biofilm formation in *P. aeruginosa* and other species[36,42]. The release of eDNA by antibiotic action has been suggested to be the driver of this biofilm response to sub-MIC antibiotics[40,41]. These studies examined the response in other species, e.g. *Enterococcus faecalis* and *Haemophilus influenzae*, and only observed the effect with antibiotics that targeted peptidoglycan synthesis. Other classes of antibiotics, such as protein synthesis inhibitors or fluoroquinolones, did not induce biofilm formation[40,41]. In contrast, our results in *P. aeruginosa* showed that a variety of antibiotics can induce biofilm formation at sub-MIC levels regardless of their mechanism of action (Table 3). This indicates that there may be differences between the *P. aeruginosa* response to antibiotics and those observed in *E. faecalis* or *H. influenzae*. Indeed, it has been noted that sub-MIC levels of fluoroquinolones could induce a self-lysis response via activation of

prophage elements in *P. aeruginosa*[43], releasing eDNA. This response has not been noted in *E. faecalis* or *H. influenzae*. We tested whether addition of genomic DNA alone could stimulate biofilm formation in order to remove antibiotic-induced lysis as a variable. Dosing cultures of *P. aeruginosa* PAO1 KP with increasing concentrations of purified genomic DNA failed to increase biofilm formation (Figure 4), indicating that an increased release of DNA is unlikely to be the sole driver of the biofilm response to sub-MIC antibiotics in *P. aeruginosa*. It has been suggested that the lack of biofilm stimulation by exogenous genomic DNA could be due to the DNA being evenly distributed in the medium instead of concentrated at the substratum[43]. This explanation, however, would require the subpopulation of lysed cells to already be associated with the surface. A limitation of using purified genomic DNA is that numerous DNA-associated proteins would be removed during purification, which may lead to the lack of a response if these proteins are required.

Others have reported that cell lysis can release a signal that induces expression of the type 6 secretion system in *P. aeruginosa* via activation of the Gac/Rsm regulatory system[78]. This signal is predicted to act as a warning for cells and prepares them for warfare with other bacteria by inducing type 6 secretion. However, biofilm formation is also induced via the Gac/Rsm pathway and is regulated similarly to the type 6 secretion system[82]. Therefore, it may be possible

that this signal released by cell lysis helps to drive the biofilm response to sub-MIC antibiotics. Due to the unknown nature of this signaling molecule, we treated *P. aeruginosa* PAO1 KP with whole cell lysate at various concentrations and found that biofilm formation was not induced above 200% of the LB control (Figure 5). This suggests that cell lysis via sub-MIC antibiotics alone is likely not driving this response. However, it is possible that the freeze-thaw process may denature components of the lysate that play a role in stimulating biofilm formation. Interestingly, a small increase in biofilm formation occurred at the highest cell lysate concentrations concurrent with a decrease in planktonic growth. This could be the result of *P. aeruginosa* response to antagonism (PARA) activation by lysate, which has been previously described[78]. Concentrations used by LeRoux et. al ($5x10^4$ lysed cells/µL)[78] to stimulate this response were similar to concentrations used in this study ($9x10^3$ lysed cells/µL at maximal stimulatory concentration). The magnitude of this response, however, does not match the observed magnitude of the biofilm response to sub-MIC antibiotics. Therefore, PARA may play a role in the biofilm response to sub-MIC antibiotics but it is not the sole driver. The negative effect observed on the planktonic growth could be a recruitment of cells from the planktonic state into the biofilm, potentially driven by the PARA response.

The amount of potential cell lysis that occurs in a population exposed to sub-MIC antibiotics would be influenced by the antibiotic mechanism of action and the sensitivity of the bacteria to the antibiotic. Antibiotics can be stratified into two classes: bactericidal and bacteriostatic. Bactericidal antibiotics act by killing the bacteria, while bacteriostatic antibiotics only inhibit their growth. We tested both types of antibiotics, including chloramphenicol (a bacteriostatic antibiotic) and polymyxin B (a bactericidal antibiotic). Polymyxin B acts by binding to the LPS and displacing the divalent cations present, as well as using its lipophilic tail to create transient holes in the membrane. We reasoned that this mechanism would likely cause a greater degree of cell lysis compared to chloramphenicol, which inhibits translation. If cell lysis is important for the biofilm response to antibiotics, we would expect a weaker response from chloramphenicol than polymyxin B. Instead, we observed high levels of biofilm stimulation from sub-MIC chloramphenicol and comparatively weak biofilm stimulation from polymyxin B (Figures 8,9). It is possible that bacteriostatic antibiotics may allow more time for the cell to mount the response while bactericidal antibiotics that target the outside of the cell (like polymyxin B) may kill too quickly for the bacteria to activate the required transcriptional programs. This would support the existence of a biofilm response to antibiotics and would suggest that fact-acting bactericidal antibiotics may reduce the amount of biofilm formed during treatment. It is also possible that our concentration

intervals were too wide to observe maximal biofilm stimulation by sub-MIC polymyxin B. Future work should aim to decrease the concentration interval size for polymyxin B to see if this has any effect, as well as testing the speed of polymyxin B killing compared to other antibiotics.

Increased resistance to an antibiotic would also result in decreased cell lysis upon exposure to sub-MIC antibiotics. Therefore, mutants that do not show increased biofilm formation after treatment with sub-MIC antibiotics may simply be more resistant. However, an Δ*oprF* mutant had similar MIC values or, in some cases, was more sensitive than PAO1 KP, but unlike its parent failed form more biofilm when challenged with 9 different sub-MIC antibiotics (Figures 8,9). If cell lysis was the primary driver of biofilm formation, we would have expected an Δ*oprF* mutant to have increased biofilm at similar or lower concentrations of drug than the wild type. Further experiments examining the effects of increased and decreased drug resistance as well as the effects of different growth rates on the biofilm response to antibiotics should be performed moving forward to characterize the role that antibiotic action has on the biofilm response and whether the effects can be separated.

4.2 OprF and SigX: a genetic response to sub-MIC antibiotic stress?

Apart from its roles as a non-specific porin and structural protein, OprF has previously been linked to *P. aeruginosa* virulence in multiple models[55] and

has been assigned a role in sensing/responding to the immune system by binding interferon-γ[52]. Loss of OprF increases c-di-GMP levels in the cell, resulting in enhanced baseline biofilm formation[53], as well as altered production of quorum sensing ligands like the *Pseudomonas* Quinolone Signal (PQS)[55]. Our screening of a *P. aeruginosa* PAO1 KP transposon mutant library revealed that an *oprF* mutant was deficient in the biofilm response to sub-MIC cefixime, tobramycin, and thiostrepton. This phenotype was recapitulated in an Δ*oprF* mutant and was observed across 9 different antibiotics with differing targets within the cell. Further, our identification of a *dsbA* transposon mutant with a similar biofilm phenotype to the *oprF* transposon mutant also indicates that properly folded OprF may play a role in this response, especially the peptidoglycan-binding conformation[80]. Our work suggests that loss of OprF increases baseline biofilm levels and results in the inability to increase these levels during sub-MIC antibiotic stress. This indicates that OprF may play a direct or indirect role in the regulation of a biofilm induction response to antibiotics. A direct role could involve OprF acting as a sensor for an extracellular signal, much like its role in binding interferon-γ[52], or as and indicator of changes in membrane homeostasis, which can then be detected by a sensor. An indirect role could involve altered regulation of another protein as a consequence of OprF loss. Due to the role that OprF plays in anchoring the outer membrane to the peptidoglycan, its loss could drastically alter the regulation of many genes. Loss of lipoproteins that connect

the outer membrane to the peptidoglycan like Braun's lipoprotein (Lpp) in *E. coli* or multiple membrane-bound lytic transglycosylases (mLTs) in *P. aeruginosa* has been shown to compromise the outer membrane to varying degrees[83,84]. Further, the loss of multiple mLTs in *P. aeruginosa* was shown to increase biofilm formation[83]. This suggests that membrane instability resulting from the loss of outer membrane-peptidoglycan bridging proteins like OprF may be perceived as a signal similar to damage from β-lactam antibiotics. In this way, loss of OprF could partially pre-activate a biofilm response to sub-MIC antibiotics, or may alter the regulation of the response.

We initially hypothesized that loss of OprF may result in the altered regulation of other proteins in the cell, leading to a blunted response to sub-MIC antibiotics. We looked in the literature for proteins that either interacted with OprF or regulated OprF activity. Loss of OprF had been previously associated with increased activity of the ECF sigma factor SigX[53]. ECF sigma factors play key roles in responses to environmental changes or stress. Examples include PvdS, activated in low-iron environments[85], or AlgU by envelope or oxidative stress[86, 87]. While SigX has also been linked to responses to certain types of membrane or osmotic stress, the environmental cues that activate it are not well defined. SigX controls a large regulon of over 250 genes, including multiple c-di-GMP metabolism enzymes that may contribute to biofilm regulation[58,59]. With its role

in stress responses, control of c-di-GMP metabolic enzymes, and increased activity in an Δ*oprF* mutant, we hypothesized that SigX may play a role in the biofilm induction response to antibiotics. We deleted *sigX* and found that the phenotypes of a Δ*sigX* mutant were very similar to an Δ*oprF* mutant, with higher baseline biofilm, no stimulation of biofilm formation upon treatment with sub-MIC antibiotics, and reduced planktonic growth (Figure 10). We reasoned this could be due to loss of *oprF* transcription, since SigX is the predominant sigma factor that regulates *oprF*[49]. Therefore, we performed complementation experiments with Δ*oprF* + *oprF* and Δ*sigX* + *sigX* to test whether these phenotypes would return to wild type, as well as with Δ*oprF* + *sigX* and Δ*sigX* + *oprF* to test whether expressing the "partner gene" *in trans* would return the mutants back to wild type responses. Our reasoning for including the partner gene complement strains was to see whether *oprF* complementation in a Δ*sigX* background restored biofilm formation, suggesting that *sigX* is not involved and that the observed Δ*sigX* phenotype was due to reduced *oprF* transcription. None of these combinations returned the wild type biofilm induction response to sub-MIC cefixime (Figure 11). However, we did see complementation of the Δ*oprF* growth defect in an Δ*oprF* + *oprF* strain which suggested that *oprF* was successfully being expressed from the plasmid and that the planktonic growth phenotype and biofilm phenotypes can be separated from each other. A different complementation construct (pUCP20-*oprF*) complements the original

oprF transposon mutant for all phenotypes (Figure 6), suggesting that complementation of the biofilm response should be possible and that it is not separated from the growth defect. We did not see restoration of wild type planktonic growth with any of the other complemented strains. While *oprF* complementation in the Δ*oprF* + *oprF* strain restored growth, the lack of biofilm stimulation and the increased baseline biofilm formation in this strain may be due to polar effects from the chromosomal deletion (Figure 11). The gene encoding SigX lies immediately upstream of the *oprF* gene in *P. aeruginosa*, which increases the risk that mutations may arise during deletion of either gene. However, sequencing results showed that the region contained no mutations in Δ*oprF* or Δ*sigX* mutants when compared to a PAO1 reference genome. Deletion of *oprF* may have resulted in destabilization of the *sigX* transcript, which could explain the lack of complementation in the Δ*oprF* + *oprF* strain. Lack of Δ*sigX* + *sigX* complementation may be due to incorrect levels of SigX being present. Due to the complexity of the SigX regulon and the lack of the native promoter in the complementation plasmid, we predict that SigX complement levels would not reflect those found in wild type cells and that this may have pleiotropic effects. Future work should involve expressing the entire wild type *sigX-oprF* genomic region on a plasmid in the Δ*sigX* and Δ*oprF* mutants, or inserting a wild type copy of the genomic region into a different location on the chromosome. These experiments may shed light on the relative contributions of *sigX* and *oprF* in the

biofilm response to sub-MIC antibiotics. Future work should also include RNA-seq analysis of PAO1 KP and an Δ*oprF* mutant under normal conditions and after treatment with sub-MIC antibiotic. While prior studies have analyzed the transcriptional changes after exposure to sub-MIC antibiotics[88,89], studies using RNA-seq to determine differences in transcriptional modulation after exposure to sub-MIC antibiotics between a wild type strain and a strain deficient in the biofilm response to sub-MIC antibiotics have not been performed. This work would help to identify groups of genes that may be involved in the biofilm response to antibiotics and would complement data from our transposon mutant screen.

4.3 Increased sensitivity of an *oprF* mutant to novobiocin and trimethoprim

The loss of OprF had no effect on the MIC of specific antibiotics (tobramycin, polymyxin B), while slightly sensitizing (2-4 fold reduction in MIC) the bacteria to others (ciprofloxacin, cefixime, chloramphenicol) (Figures 8,9). However, there were two notable exceptions: novobiocin and trimethoprim. Sensitivity to novobiocin has previously been reported for an *oprF* mutant[55] and may be due to alteration of the outer membrane lipid content[54]. Overexpression of SigX increases transcription of a number of fatty acid biosynthesis genes, leading to an increase in C16:0 lipids and an increase in membrane fluidity[90]. However, in *Listeria monocytogenes,* increases in C16:0 membrane lipids correlated with decreased membrane fluidity and increased cell surface

hydrophobicity[91]. These reports suggest that alterations in membrane lipid content could lead to increased permeability of the outer membrane to hydrophobic compounds like novobiocin. A small decrease in the efficiency of this barrier may not greatly affect smaller molecules like cefixime or ciprofloxacin, which can more easily gain access through porins, while making a dramatic difference for novobiocin which is larger and may not go through porins efficiently.

Increased antibiotic sensitivity in an *oprF* mutant has been described previously for multiple antibiotics, including trimethoprim[92]. Trimethoprim normally inhibits dihydrofolate reductase and folate levels, which are not directly associated with OprF or the outer membrane. This sensitivity could indicate a secondary activity for trimethoprim against the membrane or peptidoglycan. Evidence for such a secondary activity has been reported in *L. monocytogenes*, with sub-inhibitory trimethoprim concentrations leading to elongated cell morphologies (indicative of cell division defects) similar to those arising from β-lactam antibiotic action[93]. Trimethoprim also causes accumulation of UDP-Mur-NAc pentapeptide in *Enterobacter cloacae*, another characteristic of β-lactam antibiotic treatment[94]. Due to the role of OprF as a peptidoglycan binding protein, its loss may also alter peptidoglycan morphology or metabolism in a way that increases sensitivity to a potential secondary activity for trimethoprim. This

unexpected activity presents an opportunity to repurpose an old antibiotic for use against *P. aeruginosa*. Future work should aim to find whether trimethoprim activity can be potentiated by inhibiting or blocking OprF, or by destabilizing the membrane by other means such as co-administering trimethoprim with a membrane-disrupting agent. Co-administration of trimethoprim with β-lactam antibotics should be performed to see if any synergistic interactions occur.

4.4 Screening for new antibiotics using the biofilm induction response to sub-MIC antibiotics

An understanding of how low levels of antibiotics affect bacterial physiology may help us to find new antibiotics to combat the resistance crisis. Many current antibiotic screens use cell death as the readout for activity. However, unless antibiotic concentrations are sufficiently high, an active compound may be passed over in a screen. In natural product screens that search microbial culture extracts for activity, a compound present in the extract can be active but may be produced at levels that are too low to cause discernible cell death. Compounds that are highly active only under certain environmental or nutrient conditions may also be routinely missed. Understanding the physiological and behavioural changes that occur in the presence of sub-MIC antibiotics may allow for more sensitive screening for antibiotic compounds.

Our results showed that *P. aeruginosa* biofilm formation was consistently and significantly stimulated at ¼ - ½ of the MIC for 9 separate antibiotics, but for

some compounds like chloramphenicol, ciprofloxacin, or thiostrepton this effect was seen at even lower concentrations. Thiostrepton was found to have potent anti-pseudomonal activity under iron limiting conditions through previous work in our lab, and this activity was initially identified by increased biofilm formation after treatment of *P. aeruginosa* with thiostrepton in dilute LB media (Uyen T. Nguyen & Michael Ranieri, manuscript in preparation). Together, these results suggest that biofilm stimulation is a broad reporter for sub-inhibitory antibiotic activity.

Screening for physiological changes using fluorescence microscopy is already being used to identify new compounds with antibiotic activity[95]. If regulatory mechanisms for the various responses to sub-MIC antibiotics are identified, then the promoter regions associated with the genes involved can be used to create reporter-based assays for antibiotic activity. This could potentially be adapted for high throughput screening. In order to do this, future work should aim to identify other players in the biofilm response to sub-MIC antibiotics in order to better understand the response and to increase the number of candidates for these reporters. Once the regulatory mechanisms are better characterized, reporter plasmids can be used along with promoters that are activated during the biofilm response to sub-MIC antibiotics in order to identify antibiotic activity at low levels.

CHAPTER 5: CONCLUSIONS AND SIGNIFICANCE

The modulation of bacterial behaviour by subinhibitory concentrations of antibiotics is a well-noted phenomenon observed in a wide range of bacterial species. Stimulation of biofilm formation in particular occurs in multiple species, including *P. aeruginosa*. Here, we have provided evidence for a programmed biofilm response to stress induced by 9 separate subinhibitory antibiotics. We propose that increases in biofilm formation induced by sub-MIC antibiotics are not simply the result of antibiotic-induced lysis of a subpopulation of cells that releases factors that seed biofilm formation. Instead, we propose that antibiotic stress induces biofilm formation via a mechanism that depends on the presence of the OM porin OprF and the ECF sigma factor SigX. While their exact roles in this response remain elusive, we propose that the loss of OprF may alter regulation of genes involved in the response, or that its loss pre-activates such a response, diminishing the ability of an *oprF* mutant to make additional biofilm. We propose that the loss of SigX also results in dysregulation of genes involved in the response, due to its role in regulating the transcription of many genes, including DGCs and PDEs that control c-di-GMP levels. A deeper understanding of this bacterial response to sub-MIC antibiotics will allow for the design of more sensitive antibiotic screening assays that can detect sub-MIC levels of antibiotic, as well as the identification of targets for adjuvant therapies to make our current

antibiotic arsenal more effective. As antibiotic-resistant pathogens like *P. aeruginosa* become more commonplace, the increased discovery of new antibiotics and improvement of current therapies will be vital for treating these infections.

CHAPTER 6: REFERENCES

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