THE ROLE OF TWO-COMPONENT AND SMALL RNA REGULATORY SYSTEMS IN *PSEUDOMONAS AERUGINOSA* BIOFILMS

by

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in

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ABSTRACT

Biofilms are a crucial adaptation for bacterial survival against stresses from external environments. Biofilms are adherent colonies of sessile bacteria embedded within a self-produced matrix. Bacterial control over formation, maintenance, and response to external stresses are strictly regulated. However, complexities of intracellular signaling for biofilm regulation are still not fully understood. In this thesis, I report on two distinct regulatory systems important for biofilm formation in the opportunistic pathogen *Pseudomonas aeruginosa*. The first regulatory system I report on is the two-component system TctD-TctE. This system is involved in regulating the uptake of tricarboxylic acids such as citric acid and is involved in biofilm-specific susceptibility to aminoglycoside antibiotics. Here I describe work I performed characterizing the involvement of TctD-TctE in biofilm development when citric acid is present as a carbon source in nutrient media. In further characterizing a previously observed aminoglycoside susceptibility, I found that a strain with a deletion of TctD-TctE (Δ*tctED*) has a heightened accumulation of tobramycin in its biofilms when grown in the presence of citric acid. In Δ*tctED,* I determined that there was an inhibition of overall cell growth when citric acid was present in nutrient media. Additionally, in the presence of citric acid, Δ*tctED* displayed high levels of biofilm formation. This contrasted with normal biofilm development observed in the PA14 wild type strain where biofilm mass was reduced in the presence citric acid. The second project of this thesis reports on a novel regulatory small RNA, the Small RNA Regulator of Biofilms (SrbA). SrbA was found to be unique to *P. aeruginosa* and displayed no homology with any other sequenced bacterial species. I found that loss of SrbA resulted in a significant reduction in biofilm mass. Subsequently, loss of SrbA also leads to attenuation of *P. aeruginosa* pathogenicity in *Caenorhabditis elegans* nematodes. Bacterial biofilms possess specific regulatory programs that are still just being appreciated for their complexity. This thesis work adds to our understanding of biofilm regulation by studying roles of the two-component system TctD-TctE and the small RNA SrbA in *P. aeruginosa*.

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Lastly, it is of critical importance I acknowledge that while I was doing my PhD at the University of Ottawa, all the work presented in this thesis was done on the traditional, unceded territories of Algonquin, Anishinaabeg, and Haudenosaunee peoples.

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LIST OF ABBREVIATIONS

5', 3' UTR	5' or 3' Untranslated region
ANOVA	Analysis of variance
Ap	Ampicillin
bp	Base-pairs
c-di-GMP	Cyclic diguanylate
CA	Catalytic and ATP-binding domain
Cb	Carbenicillin
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
cfu/ml	Colony forming units per millilitre
DHp	Histidine-phosphotransfer domain
Dnr	Dissimilatory nitrate response regulator
eDNA	Extracellular DNA
EPS	Extracellular polymeric substance
Gm	Gentamicin
GFP	Green fluorescence protein
HK	Histidine kinase
HGT	Horizontal gene transfer
HSL	Homoserine lactone
IM	Inner membrane
Km	Kanamycin
LB	Lysogeny broth
MCS	Multiple cloning site
MIC	Minimal inhibitory concentration
MBC-B	Minimal bactericidal concentration in biofilms
MBC-P	Minimal bactericidal concentration in planktonic cultures
OM	Outer membrane
PAO1	*Pseudomonas aeruginosa* wild type strain UW-PAO1[1]
PA14	*Pseudomonas aeruginosa* wild type strain UCBPP-PA14[2]
PAPI-1, -2	*Pseudomonas aeruginosa* pathogenicity island 1, 2
Pel	Pellicle polysaccharides
PI	Propidium iodide
PLoS	Public Library of Science
(p)ppGpp	Guanosine tetra-/pentaphosphate
PQS	*Pseudomonas* quinolone signal
PS	Periplasmic sensor domain
Psl	Exopolysaccharides
qPCR	Semi-quantitative PCR
RBS	Ribosomal binding site
RR	Response regulator
SrbA	Small RNA regulator of biofilms A
sRNA	Small RNA
T2SS	Type II secretion system
T3SS	Type III secretion system
T6SS	Type VI secretion system
Tb	Tobramycin
Tc	Tetracycline
TCS	Two-component system
Tct	Tricarboxylic transporter

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1 INTRODUCTION

Pseudomonas aeruginosa is a versatile bacterium living in many diverse environments around the world and it is an opportunistic pathogen. It is capable of causing severe infections in plants and animals, including humans. Whether in natural environments or infecting a host, *P. aeruginosa* readily colonizes and develops biofilms on a variety of biotic and abiotic surfaces. Here I will introduce *P. aeruginosa* and our current understanding of this member of the phylum Gammaproteobacteria. I will particularly highlight what we know of *P. aeruginosa* ecology, physiology, and genetics. I will introduce these aspects of *P. aeruginosa* framed around a focus on its regulation of biofilm formation. The major aim of my thesis work was to further expand our knowledge of how *P. aeruginosa* uses two-component systems (TCSs) and non-coding, small RNAs (sRNAs) as a means of internal signalling for the regulation of biofilm formation.

1.1 *P. aeruginosa*, a Ubiquitous Environmental Bacterium and Opportunistic Pathogen

1.1.1 *P. aeruginosa* in Natural Environments

P. aeruginosa is found across the globe in soil[3–5], fresh water[6], and even marine environments[7,8]. *P. aeruginosa* is a bacillus roughly 5 μm long and 1 μm in diameter. It is capable of multiple forms of motility through use of monotrichous flagella and production of type IV pili. *P. aeruginosa* is a generalist with an extensive metabolic repertoire enabling it to utilize a diverse range of nutrient sources and is also a facultative anaerobe[9,10]. While it can grow in a range of pH, salinity, nutrient availability, and temperatures; optimal growth for *P. aeruginosa* is in temperate and damp environments[3-8]. It is a hardy bacterium capable of thriving in harsh environments in nature or in hosts when causing infections.

In addition to its ability to utilize a diverse range of nutrient sources, *P. aeruginosa* produces a wide array of secondary metabolites. These aid *P. aeruginosa* in acquiring resources, out-

competing other bacteria, and defending itself against the host immune system[11–14]. Many of these secondary metabolites therefore also have roles in *P. aeruginosa* virulence. Most prominent among these secreted products of *P. aeruginosa* are siderophores for sequestering ferric ions (Fe^{3+}) as well as quorum sensing molecules and secondary messengers (Figure 1).

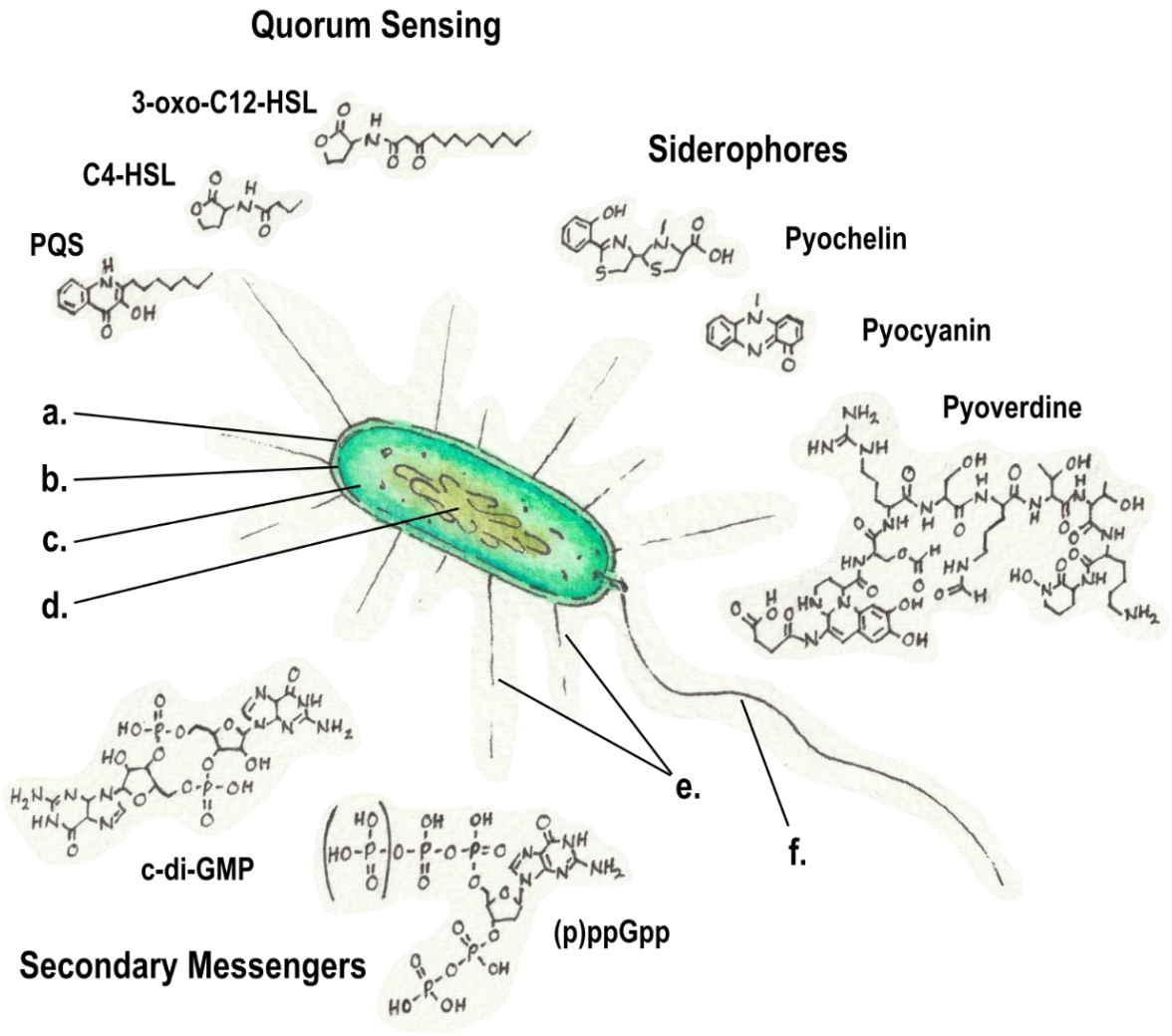

Figure 1. The *P. aeruginosa* cell and common secreted products. Major features of *P. aeruginosa* consist of the outer (**a.**) and inner (**b.**) membranes, cytoplasm (**c.**) nucleoid (**d.**), type IV pili (**e.**), and flagellum (**f.**). Major secondary metabolites that are produced by *P. aeruginosa* include compounds important

for activities such as acquiring nutrients as well as cellular communication. The homoserine lactones (HSLs) C4- and 3-oxo-C12-HSLs and the *Pseudomonas* Quinolone Signal (PQS) are major quorum sensing molecules. The siderophores pyochelin, pyocyanin, and pyoverdine sequester iron ions and have roles in virulence. Secondary messengers cyclic diguanylate (c-di-GMP) and guanosine tetra-/pentaphosphate (p)ppGpp signal for biofilm maintenance.

There are three predominant siderophores of *P. aeruginosa*: pyoverdine, pyocyanin, and pyochelin[11,13–15]. All are secreted in high levels and give *P. aeruginosa* cultures their characteristic bluish-green hue as well as fluorescence under ultraviolet light[11,14]. These siderophores are highly effective at chelating iron from the environment and are capable of out-competing similar siderophores of other organisms[11,13,14]. They also have severe cytotoxic effects on host cells[16,17]. These siderophores are therefore characterized as having important roles in *P. aeruginosa* virulence. They are highly expressed by *P. aeruginosa* when infecting hosts but also when *Pseudomonas* species are competing for iron with other microorganisms when growing in soil[16–18].

Another major system which *P. aeruginosa* uses for cell to cell communication is quorum sensing. It has three major quorum sensing molecules; the *Pseudomonas* quinolone signal (PQS) as well as two homoserine lactones (HSLs), C_4-HSL and 3-oxo-C_{12}-HSL[19,20]. These quorum sensing molecules are important for coordinating activities of biofilm populations, swarming motility, and expression of virulence factors [21]. However, PQS in particular has a role in regulating the production of pyocyanin and pyoverdine and is therefore involved in iron homeostasis[13,22]. In addition to these quorum sensing molecules, *P. aeruginosa* also uses the secondary messenger compounds cyclic diguanylate (c-di-GMP) and guanosine tetra-/pentaphosphate as major

signaling mechanisms for biofilm formation[23–27] and are gone over in greater detail below. *P. aeruginosa* uses quorum sensing in activities such as development of biofilms, communicating amongst cells in the soil microbiota, and even establish successful infections[21,28–30].

1.1.2 *P. aeruginosa* as an Opportunistic Pathogen

P. aeruginosa is most commonly encountered harmlessly in soil and tap water but it can pose as a serious threat to individuals who are immuno-compromised. *P. aeruginosa* is an opportunistic pathogen capable of colonizing and causing severe clinical infections in people with compromised natural barriers such as those with severe burn wounds[31,32]. Individuals with supressed or diminished immune systems such as transplant recipients[33–35], those afflicted with AIDS[36–38], and the elderly[39,40] are also susceptible to infections. However, the greatest incidence of *P. aeruginosa* infections occurs in individuals with the genetic condition of cystic fibrosis (CF)[41–45]. In North America, there are roughly 30,000 individuals who have CF according to the Cystic Fibrosis Foundation and Cystic Fibrosis Canada.

CF is an autosomal dominant inheritance and currently there is no cure for this condition. It arises from having inactive forms of the chloride ion-channel, cystic fibrosis transmembrane conductance regulator (CFTR)[46,47]. This chloride channel is crucial in regulating the osmosis of water across cell membrane. In CF, dysfunctional CFTR cannot transport chloride therefore affecting the ability of cells to regulate movement of water into the lumen[47]. In individuals with CF, this affects mucosal epithelia of the lungs most severely. Ultimately, this results in the CF lung having thicker mucosal linings which, is more difficult to clear.

P. aeruginosa colonizes mucosal linings of the CF lung and due to poor clearance it will persist and develop chronic infections[43,48,49]. Bacterial colonization does not require a specific receptor on the host. Rather, *P. aeruginosa* is able to develop as a biofilm within the mucosal lining of the

lungs. *P. aeruginosa* colonizes the CF lung early in life and will cause acute infections as there are series of clearances and re-infections[48,50]. By early adulthood those *P. aeruginosa* will establish chronic infections and often becomes the predominate member of the CF microbiota[43]. Isolates of *P. aeruginosa* from chronically infected CF lungs often display adaptations such as mucoidy to better survive in the host environment. Often this transition to a mucoid state is due to genetic mutation. However, *P. aeruginosa* is capable of switching between non-mucoid and mucoid states through expression of different exopolysaccharides that make up part of the biofilm matrix[12,43,49]. Pel and Psl polysaccharides (non-mucoid) and alginate (mucoid). Mucoid strains also down-regulate many virulence factors and immunogenic markers such as flagella, pili and type III secretion systems while up-regulating alginate for biofilm formation[43]. The source of *P. aeruginosa* that colonizes and leads to infection of the lung is most regularly acquired from environmental reservoirs, but it is possible for person-to-person transmission to occur[43,50,51]. Chronic *P. aeruginosa* infections have a severely negative effect on quality of life and individuals with CF ultimately succumb to organ failure due to sustained inflammation causing irredeemable tissue damage requiring lung transplantation[43,49,52,53].

P. aeruginosa is able to invade into the lungs and maintain lasting infections due to multiple factors. Many of the same factors that it uses to thrive in natural environments are also utilized when causing infections. It has been found that the ability of *P. aeruginosa* to thrive in harsh environments and achieve high cell densities is a major contributor to its success as a pathogen[54]. It shares many virulence factors and antibiotic resistance mechanisms with other bacterial pathogens, however, *P. aeruginosa* stands out for it having such a large number of mechanisms in one species. Its high tolerance to environmental stresses, diverse metabolic repertoire, and its

ability to develop resilient biofilms are all significant contributors in the success of *P. aeruginosa* in all its environments.

1.1.3 Antibiotic Resistance in *P. aeruginosa*

Beyond its ability to grow in harsh environments, the success of *P. aeruginosa* as a pathogen is owed to its high levels of antibiotic resistance. Antibiotic resistance mechanisms of *P. aeruginosa* fall into three broad categories: intrinsic, acquired and adaptive resistance mechanisms (Figure 2). Intrinsic resistance is characterized as an innate ability of bacteria to be unaffected by the bactericidal or bacteriostatic effects of an antimicrobial compound[55]. Such intrinsic attributes of *P. aeruginosa* includes low membrane permeability, expression of efflux pumps, and secretion of β-lactamases to give it native resistance to many antibiotics used clinically to treat many bacterial infections[55–61]. While these mechanisms provide intrinsic resistance, *P. aeruginosa* also has characteristics that provide it tolerance to antibiotics. Defining the boundary between tolerance and resistance is a difficult thing to place in biology as these terms can refer to nearly any stress or impact of something from an environment on a molecular mechanism, organism, community, or ecosystem. Here I opt to strictly apply the terms of tolerance and resistance to the perspective of antibiotics with clinical application against bacteria. With this in mind, I am defining resistance to antimicrobials to be the result of heritable characteristics that have been selected for though use of antimicrobial treatment[62]. I am defining tolerance to antimicrobials to be an innate characteristic bacterial biofilms whereby the structure and lifestyle of cells within provide an ability to survive without adaptation in concentrations of antimicrobial compounds that would otherwise be bactericidal to cells in any other mode of growth[62–64]. The secreted extracellular polymeric substance (EPS) matrix that *P. aeruginosa* produces for its biofilms quench the effects of antibiotics[63,65]. Additionally, multiple genes have roles in biofilm-specific antibiotic resistance and

tolerance in *P. aeruginosa*[66–68]. These characteristics of *P. aeruginosa* protect it from the effects of many antibiotics used to treat bacterial infections.

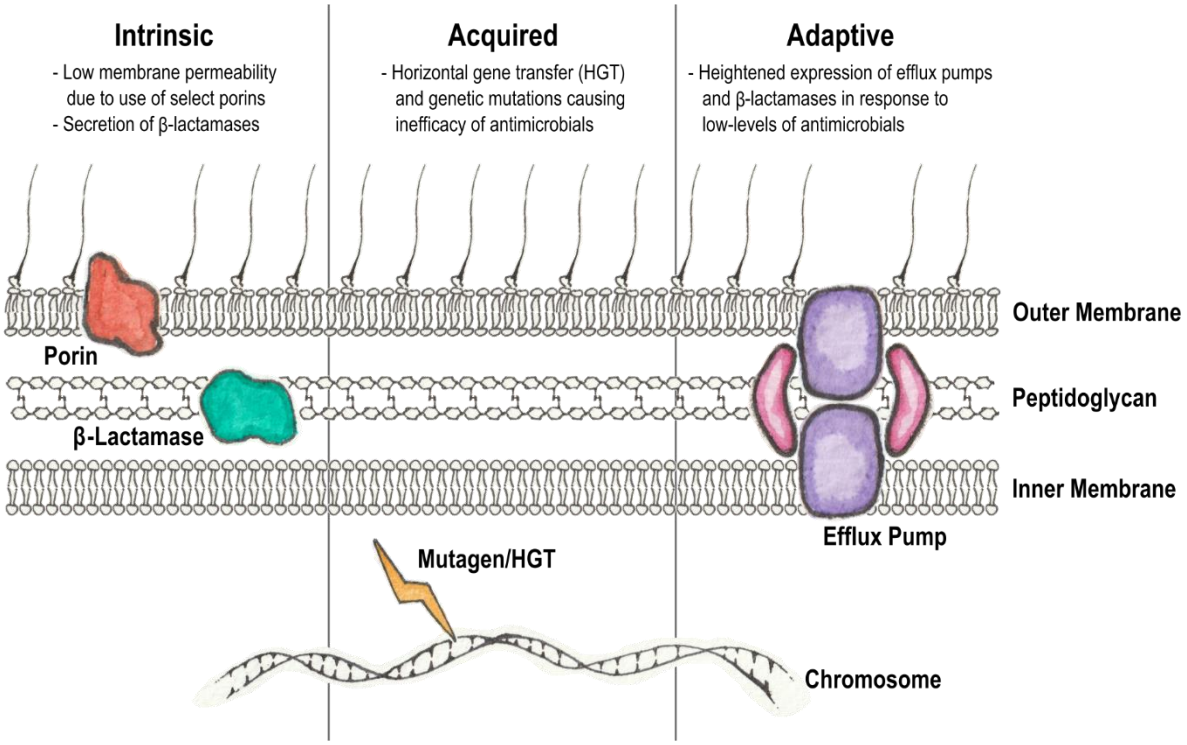

Figure 2. Mechanisms of Antibiotic Resistance in *P. aeruginosa*. Major forms of antibiotic resistance in can be grouped into categories of intrinsic, acquired, or adaptive mechanisms.

The other two categories of resistance in *P. aeruginosa* are acquired and adaptive mechanisms. Acquired resistance results from mutation which most frequently occurs due to selective pressures, environmental stresses leading to chromosomal damage, or from horizontal gene transfer between microbes[69–73]. Ultimately, a heritable genetic change occurs that leads to alteration of a target binding site for an antibiotic, constitutive expression of efflux pumps, or high secretion of β-lactamases rendering antibiotics ineffective. In *P. aeruginosa*, it has been shown that mutations in DNA repair mechanisms results in their dysregulation and cascades of unchecked mutation

throughout the genome leading to the emergence of hyper-mutator strains[74,75]. It has been demonstrated that stressful environment of the host environment and use of biocide cleaning agents in hospitals can contribute to increased levels of hyper-mutator strains and development of multi-drug resistance[76,77]. The last major category of resistance in *P. aeruginosa* is that of adaptive mechanisms. There is some overlap with mechanisms of intrinsic resistance. However, the key distinction of adaptive resistance is that it arises in response to the presence of low-levels of antibiotic[56,78,79]. Such responses include the ability to reduce membrane permeability[80–83] and heightened expression of efflux pumps[84–86]. The development of adaptive resistance need not encounter a specific antibiotic compound to provide protection against that one compound. Generic environmental stresses can elicit adaptive responses to antibiotics. Extremes of pH, abundance of additional antimicrobials, and the presence of free radicals can lead to adaptive resistance[78,87–89].

P. aeruginosa has a high-level of resistance to many antibiotics used to treat many of the most common bacterial infections. It draws on a diversity of mechanisms shared with many other bacteria making it a model system for continued study of antibiotic resistance.

1.2 The *P. aeruginosa* Genome

The ability of *P. aeruginosa* to survive in diverse natural and host environments is a result of its relatively large genome. *P. aeruginosa* cells each contain a single copy of its genome. It was one of the earliest bacterial genomes to be sequenced and in under twenty years there are now hundreds of completed *P. aeruginosa* genomes available[5,90–93]. Variation exists between *P. aeruginosa* strains in what makes up the pan-genome. However, all strains have a conserved set of genes that are considered the core genome of *P. aeruginosa*[5,90,94–98]. The *P. aeruginosa* genome is slightly greater than 6 Mb in size across all strains sequenced, but it is not larger than 6.6

Mb[5,90,92]. Within this large genome is a wide array of virulence factors, antibiotic resistance mechanisms, and metabolic pathways. The core genome encodes a large number of the genes for these systems. However, particular strains, such as PA14 used here, are adapted to more narrow niches of pathogenicity or lifestyle have additional virulence factors in its pan-genome.

For my thesis work I used the established PA14 wild type strain of *P. aeruginosa* (UCBPP-PA14) that has had its genome sequenced, annotated, and is publicly available at http://Pseudomonas.com[93]. This PA14 wildtype has one of the largest genomes of *P. aeruginosa* strains at 6.5 Mb and 5977 annotated genes. A major contribution to its larger genetic compliment that is part of its pan-genome is that it contains two large and unique insertions, the *Pseudomonas aeruginosa* Pathogenicity Islands 1 and 2 (PAPI-1 and -2)[99–101]. It is these two genetic islands, PAPI-1 and -2 that makes PA14 particularly virulent as a pathogen in humans and plants also adept at killing other bacteria. Within these exists numerous virulence factors and secretion systems not present in other strains. Of notable mention is the ExoU exotoxin and a type VI secretion system (T6SS)[99,100,102].

While the genome of *P. aeruginosa* has been extensively studied, there are still significant unknowns with regard to functional roles of encoded genes. It has been nearly twenty years since the first *P. aeruginosa* genome was sequenced, yet 45% of genes have unknown function[93]. Furthermore, with new high-throughput transcriptome sequencing methods, putative genes for non-coding RNAs have been found in intergenic regions. Novel regulatory sRNAs are of interest for the roles they may have in pathogenicity, biofilm formation and antibiotic resistance in *P. aeruginosa*.

1.3 *P. aeruginosa* Biofilms

Bacterial biofilms are an ancient adaptation conserved by all bacteria and are the natural state of bacterial colonies in the wild[62,103]. The organization of biofilm structures and coordination of bacterial colonies requires a great deal of regulatory activity. Biofilm contamination in clinical and industrial environments occurs frequently and are difficult to eliminate leading to them causing biofouling and severe infections. In clinical settings, bacterial biofilm infections are wide-spread and have a much greater level of antibiotic resistance than what has been observed through laboratory results alone[48,104]. Additionally, bacterial contamination, termed bio-fouling, in the form of biofilm growth is a constant issue in industries involved in agriculture, food production, aquaculture, and water treatment[105–107]. The complexities of the regulatory mechanisms behind biofilm formation are still not fully characterized. While important in of themselves to study for the sake of understanding our natural world, regulatory mechanisms of biofilms are worthy of studying for the impact they have on human lives.

The natural state of bacteria is to exist as a biofilm. A characteristic conserved by all bacteria is that when let to their own in their environment they will develop as aggregative sessile colonies[108,109]. Other domains of life such as Fungi[110–112] and Archaea[113–115] also form biofilms, however, bacteria do have their own unique characteristics. As mentioned above, bacterial biofilms are sessile, adherent colonies. They consist of populations of cells aggregated together in a secreted matrix of extracellular polymeric substance (EPS). In *P. aeruginosa* biofilms the EPS matrix consists of extracellular DNA (eDNA), proteins, and any combination of pellicle polysaccharides (Pel), exopolysaccharides (Psl), and/or alginate[116–119]. The formation of a mature biofilm in *P. aeruginosa* occurs through distinct stages of development starting with the attachment of a cell to an abiotic or biotic surface followed by stages of growth and EPS secretion to form a mature

biofilm (Figure 3). A mature biofilm will eventually undergo a stage of dispersal where cells become motile and planktonic, leaving the biofilm to establish new colonies. This process occurs by means that can be either passive or actively carried through signaling by cells within the biofilm itself[25]. Fluid shearing forces of the environment can remove cells from the biofilm, but there are also specific signaling molecules regulated by cells in the biofilm that trigger biofilm dispersal and are introduced below.

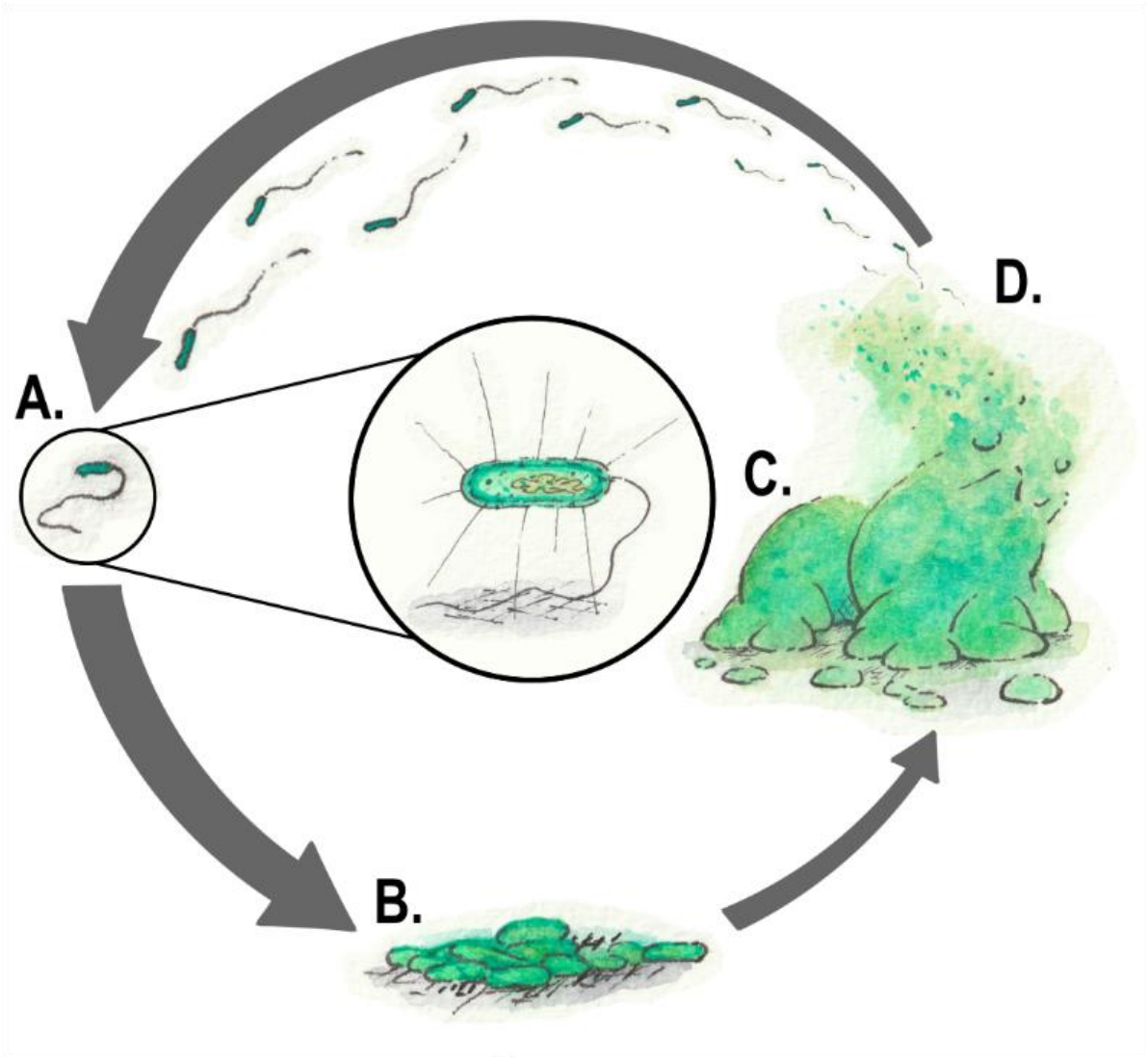

Figure 3. Biofilm formation in *P. aeruginosa*. The initial stage of biofilm formation begins with a planktonic cell transitioning to a sessile state through irreversible surface attachment (**A.**). This is followed by repeated cell growth and division as well as secretion of the extracellular polymeric substance (EPS) matrix to form microcolonies (**B.**). Continued growth, production of EPS matrix, and limited motility leads to complex biofilm morphologies in the form of macrocolonies (**C.**). Mature biofilm macrocolonies will undergo dispersion of cells through controlled signaling mechanisms and shearing forces from the environment (**D.**). Dispersed cells re-establish motility and spread to form new biofilms.

Bacterial biofilms have been referred to as being multicellular on a fairly regular basis in the literature[103,108,120–122], however, I find framing biofilm colonies that way is misleading and not representative of the unique complexities and adaptations of bacteria. A fundamentally characteristic of biofilms is that any bacterial capable of living as a single cell and is able to establish a new biofilm colony[123]. Definitions of multicellularity state that inter-dependency between cells result in them requiring to be part of the whole organism[124-126]. There are many analogous features in bacterial biofilms that occur in multicellularity but biofilms have their own form of complex organization and adaptations[123]. Here I base the definitions of multicellularity agreed upon by many authoritative sources on evolutionary biology and physiology[124–126]. I will refrain from referring to bacterial biofilms as being multicellular and rather use more accurate terms such as biofilm colonies, aggregations, or simply biofilms.

Here I will introduce the major steps of biofilm formation in *P. aeruginosa* with detailed focus on the genes, mechanisms and systems responsible for structuring biofilms as well as regulating stages of attachment, maturation, and dispersion.

1.3.1 Surface Attachment and Early Biofilm Formation

The process of biofilm development begins with a single cell adhering to a surface (Figure 3). *P. aeruginosa* is capable of adhering to any number of surfaces. During an infection it will readily form biofilms within mucosal linings and adhere to host epithelia[127]. However, it is also capable of adhering to a multitude of inert, abiotic surfaces such as stainless steel used in many clinical and industrial settings[128,129]. *P. aeruginosa* can undergo reversible attachment when encountering a surface using its single polar flagellum. However, biofilm formation truly begins when *P. aeruginosa* irreversibly adheres to a surface using both its flagellum and type IV pili[130–133]. It has been demonstrated that *P. aeruginosa* is capable of tactile sensation when attaching to a surface which results in intracellular signalling that begins its change to a biofilm mode of growth[134–136]. This transition is referred to as the motile-sessile switch as *P. aeruginosa* and is the earliest step in developing a biofilm colony[135].

There are four essential regulatory systems that are responsible for triggering the change from motile, free-swimming cell to adherent biofilms in *P. aeruginosa*. Three of these regulatory systems are two-component systems; BfiSR, BfmSR, and MifSR[134,137]. These three systems are essential for biofilms to progress from the earliest stages of adherence to development of biofilm microcolonies. The best studied of these systems is BfiSR. The transcriptional regulator BfiR has broad effects on the operons for flagellar synthesis, type IV pili, and secretion of Pel and Psl polysaccharides[116,138–140]. Regulation of these products are essential for the initial steps of formation but also are required throughout biofilm maturation. There is an added level of

complexity with the involvement of another two-component sensor, SagS which acts to regulate expression of BfiS[135]. SagS is not considered to be a major regulator of the motile-sessile switch itself but rather is part of regulation through the BfiSR system for biofilm formation. This inter-connectedness highlights the fact that regulation of biofilm formation is a highly complex process that involves multiple pathways with cross-talk between them. The fourth essential regulator for the motile-sessile switch is SadB[141]. *P. aeruginosa* can continually adhere to a surface then become motile again in a process of reversible attachment. However, to become permanently affixed on a surface it was found that SadB is required for irreversible attachment. Details of exact regulatory interactions are still not fully known but it SadB appears to require the sigma factor RpoN and the response regulator FleR for its role in irreversible attachment[141]. Coordination of these major these pathways regulate the initial steps for *P. aeruginosa* cells to undergo the motile-sessile switch and begin expressional changes transitioning from being a planktonic cells to founding the development of a biofilm colony.

1.3.2 Microcolony Development, Maturation, and Production of the EPS matrix

Biofilms begin as single cells adhered to a surface, which rapidly begin undergoing rounds of growth and cell division. The start of biofilm formation is genetically encoded and any bacterial cell is capable of establishing a new biofilm colony. With continued growth, biofilm colonies will begin to structure themselves in response to physical forces acting on them and due to requirements for efficiently accessing nutrients. This is a result of multiple factors of cells sensing their environmental conditions as well as each other within the colony. There are specific mechanisms of regulatory pathways, quorum sensing, chemical messengers, and surface receptors that work in concert to dictate the development of the biofilm. Population densities, composition of metabolites available, and presence of other species or strains have an effect on how a biofilm will form and

mature. In this section, I will outline some of the major determinants that impact colony development into mature biofilms. Cells that have adhered to a surface will undergo growth and division leading to the development of a biofilm microcolony (Figure 3). At this stage of biofilm formation, cells are secreting exopolysaccharides, extracellular DNA, and proteins that comprise the extracellular polymeric substance (EPS) matrix of the biofilm[109,138,142].

As microcolonies develop, they will merge with neighbouring colonies to form mats. It is at this point that biofilms become large enough to be observable by the unaided eye. With continued growth and secretion of the EPS matrix, microcolonies will continue to develop increasing levels of complexity. There is strong evidence to suggest that the structure of the microcolony is highly responsive and dependent upon factors such as nutrient sources available in the surrounding environment. For example, in the presence of carbon sources such as succinate and citrate, *P. aeruginosa* will maintain uniformly flat biofilm mats[108]. With other nutrient sources such as glucose, biofilms will develop into macrocolonies with a complex "mushroom cap" or domed morphology. The development of these structures is dependent on the activity of flagella and type IV pili for macrocolony formation[108,109].

The EPS matrix is expressed at every stage of biofilm development and will accumulate throughout the entire maturation of a biofilm colony. In mature biofilms, the EPS matrix makes up the largest contribution to biomass present in a biofilm[62]. This secreted mesh of polymers are crucial for cells within the biofilm to maintain a controlled environment that is protected from external chemical and physical stresses. As mentioned above, the EPS matrix is primarily comprised of protein, polysaccharides, and eDNA[65,109,138,143,144]. The proteinaceous component of the EPS matrix is largely comprised of flagella and type IV pili adhesins which help shape biofilms through some limited motility of cells within the biofilm colony[108,109,143]. Type IV pili also add to

integrity of the EPS matrix via cross-linking other polymer components such as eDNA and polysaccharides[65,109,145].

P. aeruginosa uses three separate polysaccharides for structural integrity of its biofilms: Pel, Psl, and alginate[116–119]. For all three of these polysaccharides there appears to be some functional redundancy. It has been argued that alginate does not play a predominant role in *P. aeruginosa* biofilm formation[146]. However, it is still evident that alginate can play a crucial role in protecting *P. aeruginosa* cells within a biofilm[65,117,147]. At the very least, Pel and Psl make up the largest proportion of secreted polysaccharides in the EPS matrix of non-mucoid strains. These two appear to also have redundant roles for one another[65,116]. For formation of the EPS matrix, Pel and Psl are likely performing as the primary exopolysaccharide structural components with at least some functional redundancy while alginate is a minor contributor cross-linking with adhesins to add structural support to the biofilm. While there may be functional overlap, there is evidence to suggest that *P. aeruginosa* maintains each of these specific polysaccharides for when growing biofilms in particular environments[65,139].

The last major component of the EPS matrix that provides a structural role in biofilms is eDNA. It acts as a lattice that is cross-linked by other components such as Pel and type IV pili[145,148]. The majority of eDNA present in the biofilm matrix results from release of cellular contents upon death but it has not been ruled out that a tightly regulated mechanism, such as type IV secretion, might be used to secrete eDNA[65,138,144]. Additionally, it has not been ruled out that DNA contribution from the host such as with the release of DNA nets from neutrophils may be incorporated into *P. aeruginosa* biofilms[65,144]. In addition to the role eDNA has in providing structural support to the biofilm, it also provides a protective measure against reactive and bactericidal compounds.

Multiple studies have shown that eDNA is capable of chelating cations and providing tolerance to aminoglycoside antibiotics as well as antimicrobial peptides[149–151].

Adhesins, polysaccharides, and eDNA components make up the EPS matrix and cells within the biofilm continuously produce these factors throughout the growth of biofilms. The EPS matrix is a necessary part of biofilms. Its role is not limited to providing the structural support that cells of the biofilm cling to. The EPS matrix provides protection and shelter from both physical shearing forces from the environment but also the bactericidal activity of antimicrobial compounds.

1.3.3 Mature Biofilm Macrocolonies and Dispersal

Inevitably, cells within a biofilm disperse. There are many factors that can lead to this. Physical shearing forces of the fluid environment a biofilm is in or chemical/enzymatic degradation of the EPS matrix may liberate cells back into a planktonic environment. However, there are also controlled internal mechanisms that *P. aeruginosa* uses to break down its biofilms.

As biofilm colonies continue to grow, they eventually develop macrocolonies (Figure 3). These large biofilm structures consist of a milieu of heterogeneous populations of cells within a thick EPS matrix. This heterogeneity arises due to the layers or depth into the biofilm that is created as it grows and matures. Macrocolonies have an expanded volume and this puts limitations on nutrient availability for subpopulations of cells. Biofilms are quite permeable and there is little limitation to compounds and molecules diffusing through the EPS matrix[62,88]. However, cells near to the outer edges of biofilm macrocolonies are in closer contact with the external environment. These cells take up and use nutrients more rapidly than the compounds can effectively diffuse into the biofilm. This creates a gradient where moving further into the interior of the macrocolony, the environment is increasingly anoxic, nutrient poor, and there is a higher accumulation of waste products. Therefore, cells deeper within biofilm macrocolonies exhibit lower metabolic activities.

Among subpopulations in the deeper layers of biofilms are a particular group of cells referred to as persisters[152,153]. Whether these persister cells are merely responding to their nutrient poor environment or their presence within the biofilm is a controlled mechanism is not entirely understood as there is evidence to support either conclusion[62,152,154,155]. Persister cells provide an adaptive advantage as it has been well demonstrated that these subpopulations are capable of re-establishing biofilms after clearance of the rest of the biofilm[156].

Without chemical or enzymatic clearance of cells in a biofilm, there is also natural sloughing of biofilm cells as the strain of the fluid environment disrupts outer portions of the biofilm. There are not only passive mechanisms of disruption of a biofilm, however. *P. aeruginosa* does actively regulate its own dispersion from a biofilm[25,157]. *P. aeruginosa* actively expresses two secondary messengers that are critical for maturation and maintenance of biofilms. The molecules c-di-GMP and (p)ppGpp are important secondary messengers in biofilms. These two compounds both act as signals to maintain a biofilm state of growth[23–27]. In a mature biofilm, there will be a transition in signaling from maintenance of a biofilm to dispersion of cells. It is likely that cues such as nutrient availability, microbiota composition, and oxygen availability are major triggers of this[25,158,159]. Levels of c-di-GMP and (p)ppGpp will decline signaling to cells within the biofilm to switch from sessile to motile states[23–27]. Biofilm dispersion will occur as layers of cells will express flagella and swim off of the biofilm. These motile cells then disperse and will eventually adhere to another surface elsewhere and continue the cycle of biofilm development. Thus, for every stage of biofilm formation, there are specific regulatory signals cells use to interact with one another within the colony as well as respond to cues from the surrounding external environment.

While many of the major systems have been characterized, it is still not clear to what degree cross-talk is being exploited between signaling pathways and how many other signaling pathways may be involved in the complexity of biofilms.

1.4 The Role of Two-Component Systems in *P. aeruginosa* Biofilms

1.4.1 Two-Component Systems in *P. aeruginosa*

Two-component systems (TCSs) are a major mechanism for bacteria to sense and respond to their environment. The systems consist of a sensor histidine kinase (HK) in the cellular membrane and a cognate transcriptional regulator, the response regulator (RR), within the cytoplasmic space (Figure 4). The process of receiving a signal to then exerting a regulatory response begins with the HK. The HK possesses a periplasmic sensor (PS) domain that directly interacts with its activating signal. A given TCS will be responsive to a specific signal that may be chemical or physical such as small molecules, ions, osmotic pressure, or temperature. Interaction of the PS domain with its ligand causes a conformational change through the now activated HK. Within the cytoplasm, the HK has a catalytic and ATP-binding (CA) domain plus a histidine-phosphotransfer (DHp) domain. With these two domains, the HK uses a bound ATP at the CA domain to phosphorylate a histidine residue of the DHp domain in a process of autophosphorylation. The RR associates with the DHp domain in a specific recognition between a given HK and RR. However, it has been found that there are systems where the HK may interact with multiple different RRs[160,161]. The RR is activated through transfer of a phosphate from a histidine of the DHp domain to an aspartate residue on the RR. The activated RR then carries out its regulatory role. The function of an RR can range from having effects on transcriptional expression on a small number of genes to potentially wide-spread effects throughout the genome. While the general mechanism of TCSs is presented here, the molecular steps of activation, phosphorylation, and phosphotransfer can be highly complex. Many

specific exceptions and variations on this general mechanism have been characterized and are thoroughly detailed by Zschiedrich, et al (2016)[161].

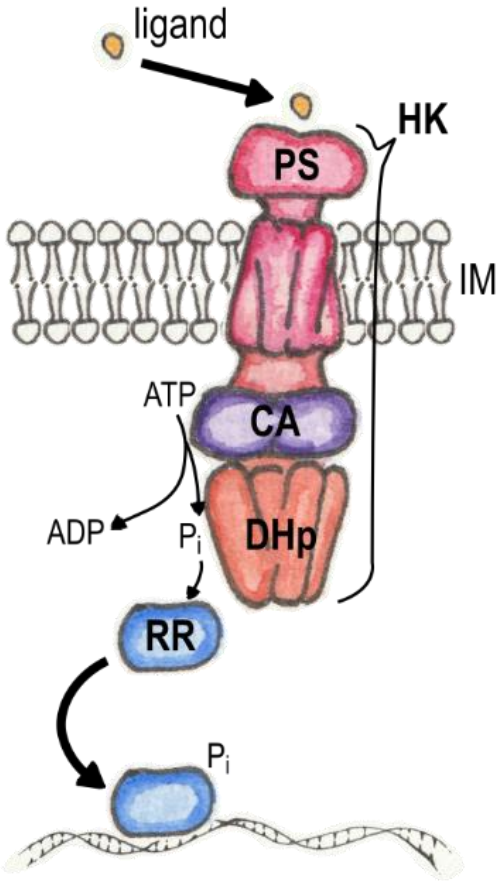

Figure 4. General mechanism of bacterial two-component systems. Embedded within the inner membrane (IM) of the cell is the histidine kinase (HK) which contains the periplasmic sensor (PS) domain, the catalytic and ATP-binding (CA) domain and the histidine-phosphotransfer (DHp) domain. The HK will activate when ligand is bound by the PS domain causing conformational changes and activation of the HK. Autophosphorylation of the active HK occurs through the CA domain using one ATP and transferring a phosphate (P_i) to the DHp domain. The HK's cognate response regulator (RR) interacts with the DHp

domain where it is activated by phosphorylation. When active, the RR dissociates into the cytoplasm to carry out its transcriptional regulation.

Gram-negative bacteria rely heavily on two-component systems (TCSs) for initiating responses to external stimuli[92,93,162–166]. *P. aeruginosa* has over 60 TCSs that are involved in regulation of effectively every aspect of its biological functions[167]. The biological roles that *P. aeruginosa* has adapted TCSs include metabolic homeostasis, social behaviours, biofilm formation, motility, virulence, and antibiotic resistance[134,161,162,165,168–173]. TCSs are not limited to a single biological role as many systems will have a significant amount of cross-talk between pathways[167,168]. This creates a level of complexity that enables *P. aeruginosa* to have nuanced and finely-tuned specific responses. This is particularly beneficial for biofilm formation where sensation of environmental cues and stresses are critical for the survival of sessile populations of cells. The expansive and inter-connected roles that TCSs have in activities such as biofilm formation are still poorly understood and of great importance for further study.

1.4.2 TctD-TctE Regulation of OpdH for Tricarboxylic Acid Uptake in *P. aeruginosa*

In this thesis, I characterize a novel role of the TctD-TctE (**T**ri**c**arboxylic **t**ransporter) TCS in *P. aeruginosa* biofilm regulation. The Tct system was originally characterized in *Salmonella typhimurium* where it was found to be involved in metabolism of tricarboxylic acids (citric acid, isocitric acid, acontic acid, and trimesic acid)[174]. Subsequent work determined that the *tct* system is responsible for uptake of tricarboxylic acids[175–177]. The *tct* operon in *Salmonella typhimurium* consists of three genes (*tctCBA*) that encode structural proteins for the porin responsible for uptake and a fourth gene (*tctD*) that is a transcriptional regulator[175–177]. *P. aeruginosa* does not have conserved *tctCBA* genes but it does encode a gene homologous with *tctD*. In *P. aeruginosa*, this

homolog is the transcriptional regulator member of a TCS. Separate work investigating tricarboxylic uptake in *P. aeruginosa* determined that it uses the porin OpdH for transport of citrate metabolites[178]. Furthermore, this work found that the TctD homolog and its cognate sensor kinase (TctE) regulate the expression of *opdH*.

Work carried out in the Mah lab[68] found that deletion of the TctD-TctE system in *P. aeruginosa* results in a biofilm-specific susceptibility to aminoglycoside antibiotics. This finding exposed a possible connection of citrate metabolism having an effect on tolerance and resistance to antibiotics. Specific metabolic activity affecting susceptibility to antibiotics is not an entirely unheard of concept. Previous studies have found that metabolic activity of bacterial cells can result in significant changes in the efficacy of antibiotic treatment[179,180]. Since TCSs in *P. aeruginosa* have expansive biological roles that affect biofilms, it was of interest to further investigate TctD-TctE for their roles in biofilm formation.

1.5 The Role of small RNA regulators in *P. aeruginosa* Biofilms

1.5.1 Categories of sRNAs and Mechanisms of Action

To date, there have been over 500 non-coding RNAs discovered in *P. aeruginosa*[181]. Mechanisms that use non-coding RNAs for activity are diverse and regulate many biological activities. In bacteria, regulatory RNA systems such as riboswitches, CRISPR, as well as *cis*- and *trans*-sRNAs have specific roles and mechanisms of action. sRNA mechanisms of action for post-transcriptional regulation can be grouped into *cis*- and *trans*- acting sRNAs (Figure 5). Generally speaking, sRNAs that act to modulate levels of translation. *Cis*-sRNAs do so in a relatively limited capacity. A *cis*-sRNA is termed as such due to its activity on the polycistronic transcript that it is expressed with[182] and it is often expressed at the 3' untranslated region (UTR) of a transcript or encoded on the opposite strand of a target gene. A *cis*-sRNA will regulate translation either through

folding of the whole RNA transcript or by post-transcriptional cleavage of the *cis*-sRNA[182,183]. The mechanism of action of a *cis*-sRNA relies on a high-degree of antisense complementarity with its target. A *cis*-sRNA is typically in the range of 50 nucleotides and will form double-stranded RNA with its target. This interaction limits levels of translation for a target or signals the mRNA for degradation by endonucleases[183]. The other major class of sRNAs, and the main focus of this thesis work, are *trans*-sRNAs. These have been characterized as having significantly more expansive regulatory roles in *P. aeruginosa* than *cis*-sRNAs[182,184,185]. As *cis*-sRNAs are termed for being encoded and acting in close proximity with their regulatory targets, *trans*-sRNAs are termed for their independent expression from their regulatory targets. For the remainder of this thesis, I will be discussing only *trans*-sRNAs and will simply use "sRNA" in reference to these specific non-coding RNAs. Only relatively recently has the variety and number of sRNAs present in bacterial genomes been appreciated. These are encoded as separate genes and are rarely part of any operon. This has led many sRNAs going unnoticed in genomic sequencing until recent methods of high-throughput transcriptomic sequencing illuminated the large numbers of sRNAs present[181,186,187]. This thesis work focused specifically on the post-transcriptional regulatory role that *trans*-sRNAs have in biofilm formation.

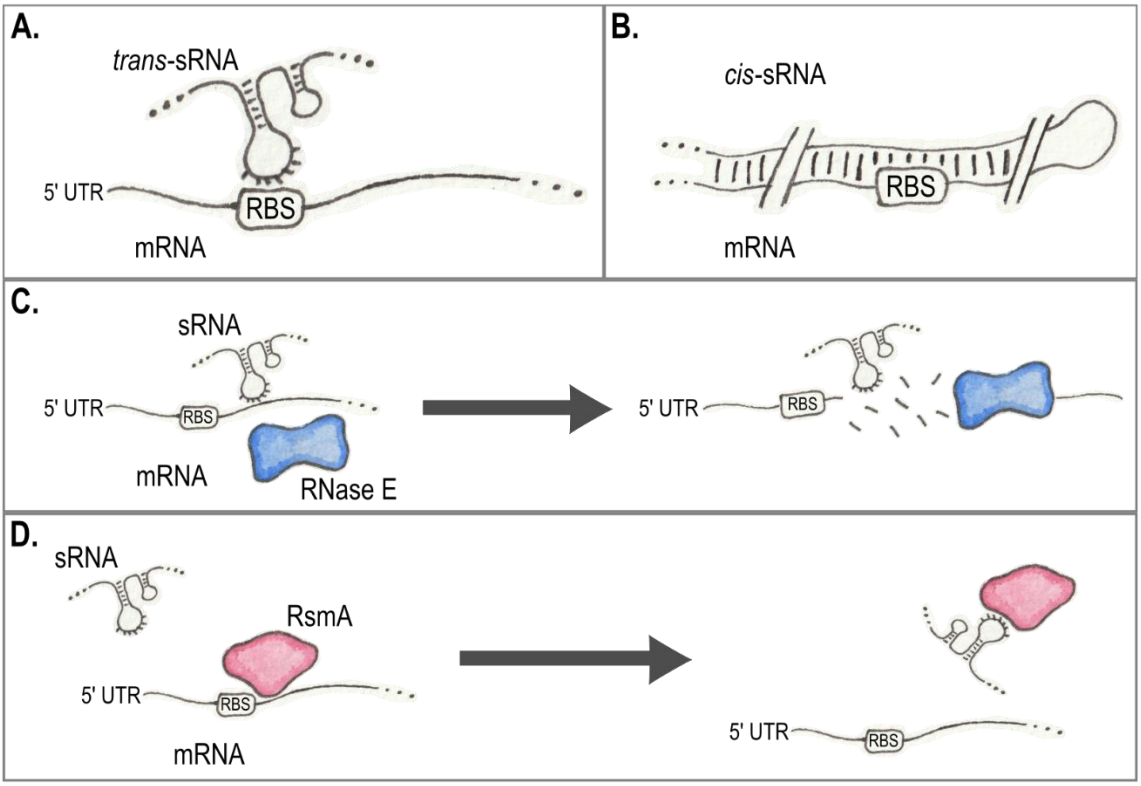

Figure 5. Mechanisms of action for small RNAs. Non-coding small RNA (sRNA) transcripts in bacteria have post-transcriptional regulatory effects on mRNA through mechanisms of either as separate *trans*-sRNA transcripts targeting mRNAs through short regions of antisense homology to regulate access to the ribosomal binding sites (RBS) in the 5' untranslated region (5' UTR) (**A.**), with high homology antisense base pairing to affect RBS access in *cis*-sRNAs (**B.**), recruitment of endonuclease RNase E for degradation of mRNA (**C.**), or sequestering RNA-binding proteins (RsmA) thereby releasing mRNA transcripts for translation (**D.**).

There is greater variety in the mechanisms of action used by sRNAs compared to other regulatory RNAs such as *cis*-sRNAs. This is due to the fact that they are encoded as separate genes from their targets. Due to this independence, sRNAs have numerous interaction targets and will act widely throughout the transcriptome. sRNAs have very low sequence complementarity with their mRNA targets. While sRNAs range from ~50-400 nucleotides in length, they typically only use ~7 nucleotide base-pairing "seeder" regions for recognition of their targets[182,183,188–190]. Therefore, sRNAs possess multiple potential "seeder" regions in the length of their coding sequence. While there is currently little direct evidence to support the idea, it is likely that RNA folding and tertiary structures make a significant contribution for recognition of targets in sRNAs.

The most extensively studied sRNAs are RsmY and RsmZ. This system is highly conserved across many Gammaproteobacteria species[188,191,192]. RsmY and Z have a high degree of redundancy and act specifically with only the RNA-binding protein RsmA. It has been found that RsmY and Z are involved in a multitude of roles within the cell such as development of antibiotic resistance, response to environmental stress, and formation of biofilms. RsmA has affinity for single stranded RNA and acts as a negative regulator of its mRNA targets. RsmY and Z therefore tend to have positive regulatory effects as they sequester this RNA-binding protein (Figure 5). By binding RsmA these sRNAs reduce the effects of this negative regulator leaving mRNAs free to be bound by ribosomes and translated.

While Rsm sRNAs are conserved across multiple Gammaproteobacteria and have been adapted to regulate multiple pathways, their strict interaction with RsmA is not the predominant mechanism of action for sRNAs. Typically, the only RNA-binding protein most sRNAs interact with is the RNA stabilizing protein Hfq[183,192]. The most common mechanism of action in sRNAs is to alter RNA-folding of its mRNA target. This action can have downstream effects of 1) acting

as a negative regulator through the binding of and occlusion of the ribosomal binding site (RBS), 2) being a positive regulator through binding elsewhere in the mRNA transcript alleviating secondary structures of an mRNA thereby increasing access for the ribosome, 3) promoting mRNA stability for long-term expression while associated with Hfq by binding and creating double-stranded RNA, or 4) leading to recruiting endonucleases by creating double-stranded RNA[183]. The broad mechanisms of action and regulatory potential of sRNAs make them a particularly interesting area of study with regard to complex adaptations in *P. aeruginosa* such as biofilm formation.

1.5.2 sRNAs in *P. aeruginosa*

Many sRNAs have been studied for their roles in complex adaptations and behaviours in *P. aeruginosa.* Among the best studied, other than the Rsm sRNAs introduced above, are the PrrF/H sRNAs, PhrS, and CrcZ. Each of these systems were found for their critical role in their respective regulatory pathways which have impacts on larger aspects such as virulence and biofilm formation in *P. aeruginosa*.

The PrrF/H sRNAs were found originally as a tandem pair of genes with a high-degree of sequence homology. The sequence identity of these two sRNAs is so close that they are in fact considered to be alleles, PrrF1 and PrrF2[193]. Later it was found that a third sRNA is encoded in the region which occurs through reading both PrrF genes as one transcript and expressed as PrrH. This third sRNA was found to be distinct and displayed unique regulatory activity from the PrrF sRNAs[194]. All three of the PrrF/H sRNAs have roles in regulating iron homeostasis within *P. aeruginosa*. Biologically available iron is recognized as one of the most essential yet scarcest ion required[195]. The PrrF/H sRNAs were found to be responsive to iron levels and have a critical role in regulating the secretion of siderophores in *P. aeruginosa*[193,194]. These sRNAs are positively

regulated by Fur (Ferric uptake regulator), a major regulator of iron homeostasis conserved in Gammaproteobacteria. They have been found to regulate translation of mRNA transcripts involved in iron metabolism throughout the genome in *P. aeruginosa*[194,195]. Their activity provided mechanistic explanations for how Fur appeared to have regulatory effects on genes that did not possess a recognizable DNA binding motif. Therefore, the PrrF/H sRNAs potentially have a role important for biological activities such as virulence and formation of biofilm infection through regulation of iron homeostasis but has not been demonstrated in *P. aeruginosa*[192,194,196].

The sRNA PhrS was also found to have an indirect role in virulence and biofilm formation. PhrS was characterized as being a critical regulator of the translation of key biosynthetic enzymes for the production of the quorum sensing molecule PQS[197]. A major quorum sensing system of *P. aeruginosa*, PQS signaling also has effects on production of siderophores and communication in biofilms. PhrS was found to interact with the mRNA transcript of PqsR, the major transcriptional regulator of the *pqs* operon. PhrS binding alleviated secondary structures of the mRNA exposing an alternative ribosomal binding site[197]. While PhrS appears to carry out the majority of its regulatory activity through *pqsR*, it is in fact a *trans*-sRNA existing as an independent gene. It is through regulation over PQS production that it has a large downstream effect throughout the *P. aeruginosa* genome.

Lastly, the sRNA CrcZ interacts specifically with Crc in *P. aeruginosa*. Its mechanism of action is similar to that of the Rsm system described above, CrcZ sequesters the RNA-binding protein Crc and inhibits it from interacting with mRNA targets[198]. Crc is a major regulator of metabolic activity in *P. aeruginosa*. The regulation of this system has a significant role in cell growth and therefore also has a significant impact on biofilm formation[185,198,199].

These sRNA regulate specific pathways which have larger impacts on the cellular activities and behaviours of *P. aeruginosa*. With the identification of over 500 sRNAs in *P. aeruginosa,* it is of great interest to determine their functional roles as there are potentially many more sRNAs that are important for biofilm formation, virulence, and the development of antibiotic resistance.

1.6 Overall Hypothesis and Rationale

The overall hypothesis of this thesis is that there are uncharacterized regulatory two-component systems and sRNAs that have critical roles in biofilm formation of *P. aeruginosa*. Furthermore, these roles in biofilm formation also have effects on *P. aeruginosa* pathogenicity in hosts and the development of antibiotic resistance. The rationale for this arises from the fact that biofilm formation is a complex adaptation requiring a high-degree of regulation across the entire transcriptome. Additionally, recent work has begun to appreciate that regulation by TCSs and sRNAs are intricately involved in biofilm formation and maintenance in *P. aeruginosa*. There is still a large number of TCSs and sRNAs found in *P. aeruginosa* with an incomplete understanding of their biological roles. Specifically, my main hypotheses in this thesis work is that 1) the antibiotic resistance phenotype previously observed in a deletion strain of TctD-TctE in *P. aeruginosa* is due to this TCSs role in biofilm formation and 2) the putative sRNA SrbA has a role in biofilm maturation which affects pathogenicity of *P. aeruginosa*. For my first project focusing on TctD-TctE, my main objectives were to 1) further characterize the tobramycin susceptibility phenotype in biofilms, and 2) understand if there is any pleiotropic effects or contribution of the role TctD-TctE has in citric acid uptake on tobramycin susceptibility and biofilm formation in *P. aeruginosa*. For my second project on SrbA my main objectives were to 1) characterize any effects loss of expression would have on biofilm formation in *P. aeruginosa* and 2) whether there was a greater effect on pathogenicity.

2 MATERIALS AND METHODS

2.1 Strains and Growth Conditions

All strains used for my thesis work (Table 1) were stocked for long-term storage at 80°C in lysogeny broth/Luria-Bertani (LB) media with a final concentration of 15% (volume glycerol/volume LB) sterile glycerol. Before experimental work was performed, strains were streaked out on LB agar plates and grown overnight at 37°C. From these plates single colonies were picked, inoculated in LB, and grown overnight at 37°C on a roller or shaker (with continuous agitation) to maintain monoclonal isolate use for each experimental run. Each colony picked was considered to be distinct and a single biological replicate for experiments.

Table 1. Strains and Plasmids.

	Description[a]	**Source**
Strains		
PA14	*P. aeruginosa* UCBPP-PA14 wild type strain	[2]
Δ*tctED*	PA14 containing a chromosomal deletion of *tctED*	[68]
PA14 VC	PA14 complemented with pJB866, Tc^R	[68]
tctED$^+$	Δ*tctED* background complemented with pJB866::*tctED*	[68]
PA14^{efflux}	PA14 complemented with pJB866::PA1875-1877	[200]
Δ*tctED*^{efflux}	Δ*tctED* complemented with pJB866::PA1875-1877	[200]
Δ*srbA*	UCBPP-PA14 containing a chromosomal deletion of *srbA*	[201]
PA14 pUCP18	UCBPP-PA14 background complemented with pUCP18, Cb^R	[201]
srbA$^+$	Δ*srbA* background complemented with pUC*srbA*, Cb^R	[201]
PA14 *srbA*$^+$	UCBPP-PA14 background complemented with pUC*srbA*, Cb^R	[201]
DH5α	*Escherichia coli* λ^-, φ80*lacZ*ΔM15, F^-, Δ(*lacZYA-argF*)*U169*, *endA1*, *gyr*A96, *hsdR17*(r_k^-, m_k^+), *phoA*, *recA1*, *relA1*, *supE44*, *thi-1*	[202]
S17-1	*Escherichia coli* λ*pir*, RP4-Tc::Mu Km::Tn7, *hsdR*$^-$, *hsdM*$^+$, *pro*, *recA*, *thi*, Sm^R, Tp^R	[203]
OP50	*E. coli* uracil auxotroph, Sm^R	[204]
C. elegans	N2 Bristol wild type strain	[201]

Plasmids		
pJB866	RK2 expression vector for broad-host range, *Pm m*-toluic acid inducible promoter, *oriT*$^{+}$, Tc^{R}	[205]
pJB866::*tctED*	pJB866 carrying insertion of *tctED* open reading frames without native promoter in MCS, Tc^{R}	[68]
pJB866::PA1875-1877	pJB866 carrying PA1875-1877 efflux system genes, Tc^{R}	[67]
pEX18Gm	Gene replacement vector, *oriT*$^{+}$, *sacB*$^{+}$, MCS from pUC18, Gm^{R}	[206]
pEXΔ*srbA*	pEX18Gm carrying a 2kb insertion in the MCS consisting of flanking regions but lacking *srbA*, Gm^{R}	[201]
pUCP18	Cloning and expression vector for use in *E. coli* and *P. aeruginosa*, MCS from pUC18, c*olE1*$^{+}$, *ori1600*$^{+}$, Ap^{R} (*E. coli*)/Cb^{R} (*P. aeruginosa*)	[207]
pBBR1MCS-5	Broad-host-range cloning and expression vector, *lacZα*$^{+}$, Gm^{R}	[208,209]
pMQ72	Expression vector for broad-host range in bacteria and *Saccharomyces cerevisiae*, *URA3*$^{+}$, *oriT*$^{+}$, P_{oriV}-*rep*$^{+}$, *colE1*$^{+}$, P_{BAD}, Km^{R}	[210]
pUC*srbA*	pUCP18 with insertion of *srbA* in the MCS, Cb^{R}	[201]
pBBR1MCS-5::*srbA*	pBBR1MCS-5 with insertion of *srbA* in the MCS, Gm^{R}	This thesis
pMQ*srbA*	pMQ72 with insertion of *srbA* in the MCS, Km^{R}	This thesis

a. Abbreviated antibiotics with "^{R}" denotes resistance and was used as positive selection marker.

With the exception of LB and 1% (weight/volume) tryptone, other media used for experiments had the same basic composition of M63 phosphate buffer (22 mM KH_2PO_4, 40 mM K_2HPO_4, 15 mM $(NH_4)_2SO_4$) with the addition of 10 μM $FeSO_4$, 1 mM $MgSO_4$, and a carbon source. Experimental carbon sources added to media included: 23 mM arginine; 2, 5, 10, and 20 mM citric acid; as well as 2.2 mM glucose. Under particular experimental conditions certain media contained both arginine and a concentration of citric acid as carbon sources. It is stated in the results which experiments use media with both arginine and citric. The purpose for this is due to the fact that, as demonstrated in the results, Δ*tctED* was incapable of growing when citric acid was provided as a sole carbon source. For strains that carry a plasmid, cultures were grown in the presence of

antibiotics to maintain plasmids: 100 μg/ml Ap, 100 μg/ml Cb, 15 μg/ml Gm, 15 μg/ml Km, 10 μg/ml Tc.

2.2 Construction of Deletion and Complemented Expression Strains

For Δ*srbA*, a chromosomal deletion was generated in the *srbA* gene locus by allelic exchange[211]. One kilobase-pair fragments flanking the *srbA* gene were amplified using PCR primers "*srbA* deletion 1" through "4" (Table 2). The two 1 kb PCR amplifications were digested with BamHI and ligated together generating a 2 kb construct of the chromosomal locus lacking the *srbA* gene. The 2 kb construct and pEX18Gm were digested using EcoRI and SalI. The digested 2 kb construct and linearized pEX18Gm were then ligated together to generate the pEXΔ*srbA* deletion construct. pEXΔ*srbA* was first introduced into chemically competent *Escherichia coli* strains DH5α by a heat-shock method used in our lab (mix DH5α and pEXΔ*srbA*; place for 30 min on ice; 2 min at 42°C, 5 min on ice, add 1 ml LB and incubate at 37°C for 1 h, plate on selective LB agar and incubate overnight at 37°C). Transformation of DH5α was used for purposes of amplifying pEXΔ*srbA* and for long-term storage of the construct. pEXΔ*srbA* was isolated again and introduced into the conjugative transfer *E. coli* strain S17-1. Finally, PA14 was conjugated with S17-1 to transfer pEXΔ*srbA*. PA14 carrying pEXΔ*srbA* were isolated by growth on LB agar plates containing 15 μg/ml gentamicin. These LB agar plates also contained 30 μg/ml nalidixic acid to negatively select against S17-1 cells. Healthy *P. aeruginosa* colonies were then streaked out on LB agar plates containing 10% (w/v) sucrose to negatively select isolates still containing pEXΔ*srbA* and thereby expressing SacB of the suicide vector. Healthy PA14 isolates believed to contain a chromosomal deletion of *srbA* were confirmed by PCR as well as by sequencing at the StemCore facility of the Ottawa Hospital Research Institute.

Table 2. Primers used for this thesis.

Primer	Sequence (5’ to 3’)
srbA (RT-qPCR, forward)	ACTCGCTGTCGAGTCTTTCG
srbA (RT-qPCR, reverse)	TGCGACTGGAACGAAGTTGT
rpoD (RT-qPCR, forward)	TCCTGGCCGACTACAATCGC
rpoD (RT-qPCR, reverse)	TTGACCGGCTCCACCTCTTC
aceA (RT-qPCR, forward)	TACGGCATTGATCTTGGCGA
aceA (RT-qPCR, reverse)	GTGTCCGACGAGAAGCAGTG
tag (RT-qPCR, forward)	GTTCCTCTGGTCCTTCGTCG
tag (RT-qPCR, reverse)	CTCGGCTTCCGGGGTAATC
pcaK (RT-qPCR, forward)	CAAGGTGATCGGCCTGTTCTA
pcaK (RT-qPCR, reverse)	CAGGGTTGCCAGTAGGGTG
PA14_03560 (RT-qPCR, forward)	CCTGTCCCTGCAATACCTGG
PA14_03560 (RT-qPCR, reverse)	TCCTGGCCGAGATAGTCCC
nirJ (RT-qPCR, forward)	GACATCTGCAACGGCAACAC
nirJ (RT-qPCR, reverse)	TCCTGATCGCTGAGGTAGCA
PA14_08310 (RT-qPCR, forward)	GTTACCGGTACTGGCACACA
PA14_08310 (RT-qPCR, reverse)	TCGTACCAACAACCATCGGG
secE (RT-qPCR, forward)	TCGCGTTCTCGGTATTCTCG
secE (RT-qPCR, reverse)	TAAAGAAGGCCTGCCCCTTG
hpaA (RT-qPCR, forward)	CCGAAGATTTCCGTGCCTCT
hpaA (RT-qPCR, reverse)	GTAGATCTCACGGTCGTCGC
PA14_11790 (RT-qPCR, forward)	CGAGGAAACCAAGAACCCCA
PA14_11790 (RT-qPCR, reverse)	ACCGACGACCAGAATCAGTG
PA14_12740 (RT-qPCR, forward)	TCAGTTTGCCGATCCCGAG
PA14_12740 (RT-qPCR, reverse)	GCTATCGCTGAAATCGCCATC
PA14_12910 (RT-qPCR, forward)	ACAGTGGACTGCTGGCG
PA14_12910 (RT-qPCR, reverse)	CAGCAGACGGGCTTCCAG
PA14_12920 (RT-qPCR, forward)	TCGCTACCCCGTTCGTTTC
PA14_12920 (RT-qPCR, reverse)	TGACGATCTTCACCTTGGACG
recJ (RT-qPCR, forward)	GAGCATCCAGGAGTTCCACC
recJ (RT-qPCR, reverse)	GAACATCGGCTCGGGAAAGT
lasB (RT-qPCR, forward)	ACGCTTGACCTGTTGTTCGT
lasB (RT-qPCR, reverse)	GGGAGTTTGGACACGTCGAT
PA14_16870 (RT-qPCR, forward)	TCGATCCGAACCAGAAGCAC
PA14_16870 (RT-qPCR, reverse)	ATGGGTGGAGATGATCACGATG
PA14_17650 (RT-qPCR, forward)	AGGTGGAAGGCAAGCGAC
PA14_17650 (RT-qPCR, reverse)	GTAGGGACTCTTGCGGAACAT
fruI (RT-qPCR, forward)	GCCGATTTCCCACGAAGAGA
fruI (RT-qPCR, reverse)	CAACTGGGTCTCGAGGATCTG
mexQ (RT-qPCR, forward)	ACCGATCATGGCGATTACCTC
mexQ (RT-qPCR, reverse)	ATAGAACTCGCCCTGCAGAC
PA14_20290 (RT-qPCR, forward)	ACTCCTACCTACTCCAGCCG
PA14_20290 (RT-qPCR, reverse)	ACTTCTGCGATCTGCTCACG
PA14_21750 (RT-qPCR, forward)	GATCGTCCCGGAATTCACCA
PA14_21750 (RT-qPCR, reverse)	TCCAGATTCACATGCCGGAC

PA14_23090 (RT-qPCR, forward)	GCCGCTTCAAGCTGTTTCC
PA14_23090 (RT-qPCR, reverse)	GATATCGGGGAATGGTCCCG
serC (RT-qPCR, forward)	ACGACTACTTCGCCATTCCG
serC (RT-qPCR, reverse)	GTCTCGTTGGAGGCGTAGTG
PA14_25020 (RT-qPCR, forward)	GAGCAGACGGTGATCGACAA
PA14_25020 (RT-qPCR, reverse)	GCCGAGATAGCTGAGCACAT
PA14_25400 (RT-qPCR, forward)	CAGGGGCTTCACGTCTCG
PA14_25400 (RT-qPCR, reverse)	AAGTCTGTGATCAGGCTGTCC
PA14_26810 (RT-qPCR, forward)	GGCCTGTCGATCAGCTACAA
PA14_26810 (RT-qPCR, reverse)	GCTGATGAGGAAGCGGGTT
PA14_28300 (RT-qPCR, forward)	TCTCGACGATCTACCTGGTCT
PA14_28300 (RT-qPCR, reverse)	GAAGTCGATGAACAGCCGGT
PA14_29230 (RT-qPCR, forward)	AGATCAAGGTTGGACAGCTCG
PA14_29230 (RT-qPCR, reverse)	CGATCCACTTGATGGCCTCT
PA14_29260 (RT-qPCR, forward)	AACTGGTTTTCGATGGCGGA
PA14_29260 (RT-qPCR, reverse)	TCCGGTGCTGGCACTTTATT
PA14_31030 (RT-qPCR, forward)	GCAATACGAATGTGGGCGAC
PA14_31030 (RT-qPCR, reverse)	CACGCAGGTCGATCCATACA
PA14_32750 (RT-qPCR, forward)	CTGGAGTTCCACCACAACCT
PA14_32750 (RT-qPCR, reverse)	AGTCGAGGAACCAGAACATCG
PA14_33190 (RT-qPCR, forward)	TGCAACGCTATCGGACGTT
PA14_33190 (RT-qPCR, reverse)	ATGTCCGCCCTCCTCTTCT
pslL (RT-qPCR, forward)	GGGCATCCTGATCATCGTCTT
pslL (RT-qPCR, reverse)	GAACGGAGAGAGCAACGGAT
cynS (RT-qPCR, forward)	ACGAGATGCTGCAAGTCTACG
cynS (RT-qPCR, reverse)	GAAGTTGATCGCGCTGATGATG
PA14_38090 (RT-qPCR, forward)	TGCTGTTCTCGACCAATCCC
PA14_38090 (RT-qPCR, reverse)	TCGCTTCGTAGTACTTGTCGATG
PA14_38290 (RT-qPCR, forward)	GCTGGAGGAACTGGACATCG
PA14_38290 (RT-qPCR, reverse)	CCCAGTCGCGGATGACATAG
PA14_40260 (RT-qPCR, forward)	ATACCGGTACCACGCAACTG
PA14_40260 (RT-qPCR, reverse)	ACGACCTGTACCGTGTTGTC
modA (RT-qPCR, forward)	GGCCTACCAGTTCGTTTCCA
modA (RT-qPCR, reverse)	GGTTGCGACTTTACCATCCTTG
pscU (RT-qPCR, forward)	TGAAGCGCGAGTACAAGGAG
pscU (RT-qPCR, reverse)	TGCTCGATTGCAGTTCCTGA
PA14_43710 (RT-qPCR, forward)	GAGGTCTATCACAGCGCCTT
PA14_43710 (RT-qPCR, reverse)	ACAGCCAAGCCAGTACCAC
fliK (RT-qPCR, forward)	GACGCCTGATGTAAAGCCCA
fliK (RT-qPCR, reverse)	CGGAGAAGCTGGAAGTCTTGT
PA14_46530 (RT-qPCR, forward)	AGCCCAGGATGCACAATCTT
PA14_46530 (RT-qPCR, reverse)	CAAGCGCAGGCATGATGTAG
ilvA2 (RT-qPCR, forward)	GGCGATCAAGGACATCTACGA
ilvA2 (RT-qPCR, reverse)	ACTTCTTGATCCCCGCTACG
cobT (RT-qPCR, forward)	CTGACCAACCTGCTGCTCT

cobT (RT-qPCR, reverse)	AGCTCGCCGAGACATTCC
PA14_48010 (RT-qPCR, forward)	CAACATGGTCTACGGCAACG
PA14_48010 (RT-qPCR, reverse)	CCTTGAGCGAACCGGTGAA
flgJ (RT-qPCR, forward)	CGGTGAGCCTGTCGAAGAAT
flgJ (RT-qPCR, reverse)	CCTGGAGCCTTGCTTCATCT
phnB (RT-qPCR, forward)	CGATGCCGATGGTGAGATCAT
phnB (RT-qPCR, reverse)	GTGAGAATCGACTCGGGATGG
acsA (RT-qPCR, forward)	GCCCTGACCAACCCTGAAA
acsA (RT-qPCR, reverse)	TGGTTCCACTTGATCTCCGC
PA14_53780 (RT-qPCR, forward)	CTACTTCCCGGTGATGGTCTG
PA14_53780 (RT-qPCR, reverse)	CCCATCCCCAACGGTGATT
PA14_54040 (RT-qPCR, forward)	TCGCTGCAGATGGTGATGTT
PA14_54040 (RT-qPCR, reverse)	GGAATGGTCTTGGTCGGGTT
hxcR (RT-qPCR, forward)	CAGCGCCTACTCCGATACC
hxcR (RT-qPCR, reverse)	GGATATCGTCCATCAGGCGG
nemO (RT-qPCR, forward)	GCTCGAACTCGACGAGAACT
nemO (RT-qPCR, reverse)	ACGAAGCTCTTCCAGCCTTG
recC (RT-qPCR, forward)	GTGAATGCGTTCTTCCAGCAG
recC (RT-qPCR, reverse)	TTCGAAAGGCTCTTCGTCGT
ampG (RT-qPCR, forward)	CCATCACCCTCGACAACTTCA
ampG (RT-qPCR, reverse)	CGGAGAACTTCAGGTTGGTCA
PA14_59010 (RT-qPCR, forward)	CCTTGCCGAACGACTGTTTG
PA14_59010 (RT-qPCR, reverse)	CTCTCGATGCGCTTTTCGTG
PA14_59030 (RT-qPCR, forward)	TTCGCTCAAGCAGAAGGCT
PA14_59030 (RT-qPCR, reverse)	AGAAATGAAGATCGAGGCGCA
dtd (RT-qPCR, forward)	ATAGAAATACCGGCCGTGAGC
dtd (RT-qPCR, reverse)	ACCGTGGTATGGCTCAGATG
PA14_61990 (RT-qPCR, forward)	TTCGGCAAGTACAGCATGGA
PA14_61990 (RT-qPCR, reverse)	GCAGCATCAGTTCGAGGAGAA
PA14_64280 (RT-qPCR, forward)	GTGATCCGCCATCTCTACGG
PA14_64280 (RT-qPCR, reverse)	ACCAGTTGGATCAGCACCAG
PA14_64530 (RT-qPCR, forward)	CCACCCTGACCGCCAAC
PA14_64530 (RT-qPCR, reverse)	GCAGTTCGACGCTGGACA
PA14_66380 (RT-qPCR, forward)	GCAAAGGTCTCAACGAGCG
PA14_66380 (RT-qPCR, reverse)	GGTCACGGTGAGGAATACCG
PA14_66510 (RT-qPCR, forward)	CTGGCATTACTACGGCTGGA
PA14_66510 (RT-qPCR, reverse)	CAGGTGCAGCGAGACCAATA
PA14_68670 (RT-qPCR, forward)	AACCCTACTACCGCCTGGA
PA14_68670 (RT-qPCR, reverse)	CAATCGGTGAACGAGCCCA
adhA (RT-qPCR, forward)	AATTCTGCCGAAAAACGTCGAA
adhA (RT-qPCR, reverse)	GTCTGCTTGAGCCCCTTGT
srbA deletion 1	GGTGGTGAATTCAAGGAGTTGTACAGGTCGCC
srbA deletion 2	GGTGGTGGATCCTTTGAGTGGGTCAGTCGGTG
srbA deletion 3	GGTGGTGGATCCCCGTCGTCAAACGTTTTGTAGT
srbA deletion 4	GGTGGTGTCGACGTGATCCCGCTCAACTACCG

srbA complementation (forward)	GGTGGTGTCGACATCAGGGGCTCTGAAACGAC
srbA complementation (reverse)	GGTGGTGAATTCCACCGACTGACCCACTCAAA
srbA chromosomal flank (forward)	ACCATCCAACCGGACAGGTA
srbA chromosomal flank (reverse)	GGTGTCAGCGAACAGAACCA

Electroporation of PA14 was used to create a complementation strain that restored expression of *srbA* (*srbA*$^{+}$) and strains expressing the PA1875-1877 efflux system (PA14^{efflux} and Δ*tctED*^{efflux}). Firstly, complementation plasmids were constructed. For *srbA*$^{+}$, the entire 239 bp *srbA* coding region was amplified by PCR using *srbA* complementation primers (Table 2) followed by digestion with EcoRI and SalI restriction enzymes. The digested product was used for ligations into the MCS of pUCP18, pBBR1MCS-5, and pMQ72. The digested product was ligated into linearized plasmids orienting *srbA* to produce native PA14 SrbA transcript. Complementation constructs were introduced into chemically competent *E. coli* DH5α by the heat-shock method described above. After isolation and purification using the PureLink™ plasmid isolation kit (Catalog number: K210011) as per the manufacturer's (Thermo Fisher) instructions, PA14 was transformed with the complementation constructs by electroporation based on a previously established protocol[212]: 5 ms pulses at 2.5 kV in 0.2 cm electroporation cuvettes. PA14 cells successfully transformed were selected for on LB agar containing 100 μg/ml carbenicillin for pUC*srbA*, 15 μg/ml gentamicin for pBBR1MCS-5::*srbA*, or 15 μg/ml kanamycin for pMQ*srbA*. This method of electroporation was also applied to PA14 and to Δ*tctED* for transformation with pJB866::PA1875-1877 having isolated the plasmid construct from a DH5α strain previously stocked[67]. PA14 and Δ*tctED* transformed with pJB866::PA1875-1877 were selected for on 50 μg/ml tetracycline as carried out in previous work. The relatively high concentration of tetracycline used in *P. aeruginosa* is due its tolerance to this bacteriostatic antibiotic. All transformation

isolates were confirmed to be carrying respective plasmids by restriction enzyme digestion and subsequent visualization on agarose gels with ethidium staining.

2.3 Minimal Inhibitory Concentration (MIC), Minimal Bactericidal Concentration of Planktonic cultures (MBC-P), and Minimal Bactericidal Concentration of Biofilms (MBC-B) Assays

MICs, MBC-Ps, and MBC-Bs were performed in 96-well microtitre plates using both LB and M63 media. MICs were performed based on the assay described by Wiegand et al[213]. Briefly, a final volume of 100 µl was dispensed to each well. Antibiotic concentrations consisted of 2-fold serial dilutions with wells for no drug present. Plates were then inoculated by the addition of 1 µl of overnight culture to each well. Plates were incubated at 37°C for 24 h before measuring the MIC which was taken as the well with the lowest concentration of antibiotic without visible culture growth.

MBC-Ps were assayed by taking 5 µl of culture from each well of MIC plates and inoculating onto LB agar plates containing no antibiotics. LB agar plates were then incubated overnight at 37°C. The MBC-P was taken as the spot on the LB agar containing no antibiotic from a sampled well with the lowest concentration of antibiotic with no colony growth. Any spots that had growth on the LB agar was considered to have still had viable cells present after treatment with antibiotic and therefore the concentration was not sufficient to be entirely bactericidal of the culture.

MBC-Bs were prepared as previously described[214]. Biofilms were grown first before the addition of antibiotics. Plates were inoculated with overnight cultures in a 1/100 dilution and grown at 37°C for 24 h without antibiotics present. After 24 h media was removed and wells were washed with buffer. Washed plates then had 100 µl of fresh media added and antibiotics were added in 2-fold serial dilutions. Plates were again incubated for 24 h at 37°C. After 48 h total time

plates were removed from incubation and media with antibiotics were removed and 100 µl of fresh media without antibiotics were added to wells. Plates were further incubated for 24 h at 37°C. At 72 h total time plates were removed from incubation and cultures from wells were spotted onto LB agar plates using a multi-pronged stamp. Inoculated LB agar plates were then grown overnight at 37°C before reading the MBC-B. The MBC-B was taken as the well with the lowest concentration of antibiotic with no colony growth.

2.4 Accumulation Assays

Accumulation of tobramycin in biofilms was performed as previously described[67]. Static biofilms of PA14 and Δ*tctED* which grow predominantly at the air-liquid interface were cultured in 12-well plates for 24 h. M63-arginine media containing either 10 mM citric acid or without the addition of citric acid were used for this assay. After 24 h a final concentration 200 µg/ml tobramycin was added to the cultures. These cultures were then incubated for 8 h at 37°C after which they were washed with M63 buffer. M63 was removed and replaced with 0.5 ml sterile 0.1 M glycine (pH 3). The 12-well plates were incubated at 37°C for 16 h to fully lyse biofilms. This would then create a biofilm lysate that contained any antibiotic that was retained within the biofilm but without any live cells. Culture wells containing biofilm-cell lysate in glycine buffer were dried fully before re-suspending in sterile water. This solution was then absorbed into sterile paper diffusion disks. The disks were then placed on LB agar plates spread with lawns of *E. coli* DH5α the same day. The plates were then incubated overnight at 37°C. The zone of clearance around the diffusion disk, measured by its diameter, was used as the indication of relative amount of tobramycin in the biofilm lysate.

2.5 Crystal Violet Staining of Biofilms

Biofilms first grown at the air-liquid interface were grown in either 96-well microtitre plates

or 12-well culture plates angled at ~30-45°. Overnight planktonic cultures were diluted 1/100 into fresh media as specified in the experimental results below. Biofilm cultures were grown for 24 h at 37°C after which wells were washed with deionized water and 100 µl (for 96-well) or 1 ml (for 12-well) of 0.1% (w/v) crystal violet was added to each well. Plates were incubated at room temperature for at least 20 minutes before removing crystal violet and washing away excess with deionized water. Biofilm formation was quantified by solubilizing crystal violet in 110 µl per well (96-well) or 1.1 ml (12-well) of 70% (volume of absolute ethanol/total volume) ethanol. Absorbance was read at 595 nm with a Synergy H1 hybrid multi-mode plate reader by BioTek, Inc. to measure relative differences in biofilm.

2.6 Microscopy

A Leica DMI6000 B was used to perform phase-contrast and fluorescence microscopy. For fluorescence microscopy, Texas Red and GFP filters from Leica were used. All image processing of overlaying channels for composite fluorescent images was done with the Leica Application Suite software provided (version 1.5.1 build 869). No other image manipulation was performed. Each image is representative of 30 fields of view each for 3 biological replicates.

2.7 Growth Assays

To assess strains under planktonic conditions, growth studies were performed by sampling cultures at multiple time points. Overnight cultures were inoculated 1 in 100 into 100 µl fresh media in 96-well microtitre plates. For growth assays both LB, 1% Tryptone, and M63 media were used. In conditions that used M63, carbon sources of 2, 5, 10, 20mM citric acid, 2.2 mM glucose, and 23 mM arginine were used alone or in combination. Conditions that utilized multiple carbon sources are specified in the results. Additionally, M63-arginine media with adjusted pHs of 7.0, 6.8, 6.2, and 5.1 were used to test the effects of pH that mimic the addition of citric acid. Plates

were incubated at 37°C for 18 h in a Synergy H1 hybrid multi-mode plate reader by BioTek, Inc. with continuous orbital shaking. To determine growth, optical densities (OD_{600}) of cultures were taken every 30 min. To convert OD_{600} measurements into cfu/ml, colony counts were performed by plating samples collected at a specific OD_{600} for multiple samplings. OD_{600} readings were taken from liquid LB cultures with constant shaking. This experiment was repeated 6 times using both PA14 and Δ*tctED*. No significant difference was found between the strains for these experiments and experiments from both strains were used to calculate cfu/ml from OD_{600} readings. By this method, it was determined that an OD_{600} of 1.0 equals a culture population of $6x10^{10}$ cfu/ml. All figures in this work that provide cfu/ml were converted from OD_{600} data based on this experiment.

2.8 Phenotype Microarrays Testing Various Carbon Sources

Assessing growth on a variety of carbon sources was done using MicroPlates™ phenotypic microarrays manufactured by Biolog, Inc. To prepare for these assays, overnight cultures were diluted to a McFarland standard of 0.25 in buffer provided by Biolog, Inc. An additional dye also provided by Biolog, Inc. was then added. Plates PM1 and PM2A were utilized to assess growth for various carbon sources. Plate organization and layouts are provided at the following URL: https://biolog.com/products-portfolio-overview/phenotype-microarrays-for-microbial-cells/. Assays were carried out with a GEN III OMNILOG® ID system located in Dr. Franco Pagotto's Lab at the Microbial Hazard Lab with Health Canada where plates were incubated for 24 h and readings taken every 15 min. Three biological replicates were performed for each of PA14 and Δ*tctED* with each plate of PM1 and PM2A.

2.9 Gradient Agar Plates

Agar plates with gradual transitions of one nutrient medium to another were prepared as previously described[215]. Square 12 cm plates were set with one edge raised at an angle of ~7° as

one nutrient medium was poured and allowed to set. Once solidified plates were laid flat and a second nutrient medium was poured over the first. Allowing the plates to dry and cool after this created a one dimensional gradient of one medium over the other at the surface of the agar. Plates were inoculated using a loop of overnight culture that was dragged along this gradient.

2.10 RNA Isolation

Whole cell RNA was isolated by lysing cells from planktonic (exponential, early stationary, and late stationary growth phases) as well as biofilm cultures. Planktonic cultures were grown by placing an aliquot of overnight cultures into fresh media at a dilution of 1/100. Sub-cultures were incubated at 37°C with constant shaking until reaching an OD_{600} approaching 0.5 (exponential phase cells), 0.5-1.0 (early stationary phase), or >1.0 (late stationary phase) before pelleting by centrifugation followed by lysis. Colony biofilms were cultured by spotting forty-eight 5 µl aliquots of overnight culture onto M63-arginine agar plates (1% w/v). Plates were first grown for 24 h at 37°C, followed by an additional 24 h at room temperature as done previously for work from the lab[68]. After this incubation period, colony biofilms were collected using a sterile glass spreader. Cultures were pelleted by centrifugation, the media was removed and the bacterial pellet was re-suspended in at least 1 ml TRIzol®. Repeated pipetting was used to break up biofilms. The aqueous fraction of the TRIzol® lysate suspension was isolated by chloroform separation. RNA was purified from lysate using the PureLink® RNA Minikit (catalog number: 12183018A) according to instructions from Thermo Fisher Scientific, Inc. RNA samples were cleared of DNA through use of DNase digestion and samples were checked for DNA contamination by PCR.

2.11 DNA Microarrays

DNA Microarrays for analysis of global changes in gene expression were performed using Affymetrix® GeneChip® for the *P. aeruginosa* PAO1 annotated genome. RNA samples were

isolated as described above and sent to the Genome Québec and Innovation Centre at McGill University, Montréal, QC, Canada to perform the workflow. Raw data in the form of .cel files was received and analyzed in-house. For analysis, the expression console software, build 1.3.1.187, and the annotated *P. aeruginosa* PAO1 library (Pae_G1a) were acquired from the Affymetrix® website. Work here investigated gene expression in PA14 strains but due to the close genetic homology of the genomes between *P. aeruginosa* strains, it was sufficient to use PAO1 DNA microarray chips and the Pae_G1a library. The Robust Multi-array Average method was used for data normalization with the housekeeping gene *rpoD* selected for expression normalization. Changes in gene expression are represented as mean fold-changes in Δ*tctED* relative to PA14 for planktonic or biofilm conditions from 2 biological replicates for each condition. Raw microarray data are available from the Gene Expression Omnibus, GEO accession: GSE114431[200].

2.12 Semi-Quantitative PCR (qPCR)

For qPCR, cDNA was generated using the iScript™ kit from Bio-Rad Laboratories, Inc. (catalog number: 170-8891) according to the manufacturer's directions. Every sample used for analysis in the work for this thesis was normalized with 0.7 μg of RNA being used as template for synthesis of cDNA. qPCR was performed using the MyIQ™ system from Bio-Rad Laboratories, Inc. with expression fold-changes being calculated using the ΔΔCt method from raw Ct values using *rpoD* as a reference gene. All primers used for analysis of gene expression by qPCR in this thesis are listed in Table 2.

2.13 Swarming, Swimming, and Colony Biofilm Agar Plates

Agar plate based assays for motility and colony biofilms were performed according to previously established techniques. Swarming and colony biofilms were grown on M63-glucose and -arginine agar plates, respectively, while swimming assays were performed on LB agar plates.

Swarm plates were prepared based on previous methods[216]. Overnight cultures were diluted 1 in 100 into fresh M63-glucose media and grown to mid-log phase (OD_{600} 0.5 or ~3 h) at 37°C with shaking. From mid-log phase cultures, 2 µl was inoculated onto swarming agar plates. Swarm Plates were made using M63 buffer lacking $(NH_4)_2SO_4$ and contained 2.2 mM glucose, 0.1% (w/v) casamino acids, and 0.5% (w/v) agar. A consistent volume of 20 ml of swarm agar was poured into each plate and allowed to solidify with lids partially off. Inoculated swarm plates were then incubated for 18 h at 37°C before photographing swarming. Swim plates were made as described previously[217], plates consisted of LB with 0.3% (w/v) agar. Plates were inoculated on the agar surface with 2 µl of overnight cultures. Inoculated swim plates were incubated for 18 h at 37°C after which the diameter of the swimming colonies were measured.

2.14 Flow Cell Chamber Biofilm Growth Assays

The flow cell system used for work in this thesis was set up as described by Tolker-Nielsen and Sternberg[218]. A Watson Marlow 205U peristaltic pump and IBI 3-channel flow cells with in-house manufactured bubble traps were used to assemble the flow cell system. To prepare the flow cell system a solution of 0.5% bleach was loaded and then run through at 0.5 rpm. The system was then flushed with sterile water and loaded with sterile M63 media containing 2.2 mM glucose, 1 mM $MgSO_4$, and 10 µM $FeSO_4$. Sterile M63 media was then run through the system for 2 h at 2.0 rpm. The system was inoculated by injection of a 1/1000 dilution of overnight cultures up-stream from the IBI 3-channel flow cells (the tubing between bubble trap and flow cells). Cultures were left with no flow for an hour to allow adherence of cells before pumps were started and run at 0.5 rpm. Flow cell biofilm cultures were grown for 72 h before pumps were stopped and cultures were stained with Syto 9 for live cells and propidium iodide (PI) for dead/permeable cells to be imaged by microscopy.

2.15 Slowing Killing Model in *Caenorhabditis elegans*

Assessment of *P. aeruginosa* pathogenicity and ability to establish biofilm infections was done through use of a *C. elegans* animal model as described previously[68,204,219,220]. *C. elegans* worms were maintained on agar plates with nematode growth medium (0.25% (w/v) peptone, 50 mM NaCl, 25 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 0.2% (w/v) agar adjusted to pH 6). *P. aeruginosa* strains or *E. coli* OP50 were spread on the agar plates using 100 µl of overnight cultures for the worms to ingest. OP50 is an accepted food source for *C. elegans* and was used as a negative control for mortality in this work[68,204,219,220]. Plates were incubated overnight at 37°C to grow bacterial lawns fully before introducing worms. Two technical replicates were carried out for each of three biological runs of the experiment. One technical replicate had a total of 30 synchronized L4 stage worms seeded on a plate. Once worms were seeded onto plates they were incubated in a humid environment at room temperature for 72 h. Counts of dead worms were taken every 24 h. Worms were considered dead if unresponsive to touch.

2.16 Bioinformatic Analyses

Both BLASTn and TargetRNA2 were used to determine sequence complementarity of SrbA with other regions of the genome as potential regulatory targets[221–223]. The full coding sequence of SrbA was used as a query to search the UCBPP-PA14 reference genome for homology. All homologies returned were manually curated to check that there could be theoretical complementary base-pairing between sRNA and mRNA transcripts. Multiple sequence alignments to the SrbA transcript were done using Clustal Omega[224]. Potential motifs and database searches for comparisons were performed using PRODORIC servers[225]. SrbA transcript was also checked for whether it could potentially encode any protein coding sequence using ExPASy software[226]. Predicted folding and structures of SrbA were created using the RNAfold servers working with

the ViennaRNA package Version 2.4.8 (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi)[227]. Default parameters were used to predict RNA structures and no imposed constraints were entered.

3 ROLES OF THE TWO-COMPONENT SYSTEM TctD-TctE IN RESISTANCE TO TOBRAMYCIN SUSCEPTIBILITY AND BIOFILM FORMATION

As a disclaimer, the work presented in this chapter has been published in the journal mSphere[200]. For results presented here, I performed the experiments and analyzed the data. Results for MBC-B and MBC-P data which I refer to in the publication were performed by Li Zhang. Dr. Thien-Fah Mah and Li Zhang were both involved in designing the experiments in this chapter. They were also both involved in editing the manuscript that was published from this work.

In this thesis, I present data demonstrating that TctD-TctE are important for regulation of biofilm formation in *P. aeruginosa* in response to citric acid. Previous research in our lab demonstrated that a strain of *P. aeruginosa* with a genetic deletion of both *tctD* and *tctE* (here presented as Δ*tctED* for the order of the genes starting from the promoter and previously presented as ΔPA0756-0757) resulted in a mutant with an increased susceptibility to the aminoglycoside antibiotics tobramycin and gentamicin[68]. Furthermore, this phenotype was only observable in Δ*tctED* biofilm cultures. Planktonic cultures displayed no difference in resistance to antibiotics compared to the parental wild type. Tobramycin is an antibiotic that *P. aeruginosa* is susceptible to and it is therefore relied upon for the treatment of *P. aeruginosa* infections[68,84,104] Since TctD-TctE is involved in sensing tricarboxylic acids, it raised the question of whether the presence of citrate metabolites affects *P. aeruginosa* resistance and tolerance to antibiotics. There is evidence that metabolism has a role in the development of antibiotic resistance as recently it has been demonstrated that the metabolic activity of *E. coli* affects susceptibility to clinically relevant antibiotics[179,180]. Therefore, I tested whether the presence of citric acid affects *P. aeruginosa* biofilms and their resistance to tobramycin through the contribution of TctD-TctE.

3.1 Accumulation of Tobramycin in Δ*tctED* Biofilms

In previously published data from our lab it was found that the Δ*tctED* strain had a 4-fold increase in its susceptibility to the aminoglycoside antibiotics tobramycin and gentamicin relative to PA14[68]. However, this susceptibility phenotype was only observed in biofilms; planktonic cultures of Δ*tctED* displayed no difference in its minimum bactericidal concentration (MBC) compared to PA14. I was curious as to what effect the inclusion of citric acid would have on the Δ*tctED* strain. I hypothesized that the addition of citric acid to the culture medium when challenging Δ*tctED* with tobramycin would further exacerbate the susceptibility phenotype previously observed[68]. MBC-Bs performed by Li Zhang replicated previous observations of a 4-fold increase in susceptibility to tobramycin in Δ*tctED* biofilms. However, when citric acid was added to nutrient media there was no further change to susceptibility and a 4-fold change was also observed in Δ*tctED*[200].

Following these observations, I was further interested as to what features of Δ*tctED* biofilms may be contributing to this tobramycin susceptibility phenotype. Biofilms are passively absorbent and can readily accumulate both resources as well as toxic compounds present[62,228,229]. I thereby questioned whether there was any difference in the accumulation of tobramycin occurring in Δ*tctED*. I performed accumulation assays by first growing biofilms in media with or without 10 mM citric acid. Mature biofilms were then treated with 200 μg/ml tobramycin[66,67]. Treated biofilm cultures were lysed and this lysate was then absorbed onto diffusion disks. These diffusion disks were placed on pre-grown *E. coli* lawns and relative accumulation of tobramycin was determined by measuring the diameter of clearance on the *E. coli* lawns.

Upon determining clearance zones, I found that biofilm lysates from cultures grown without any citric acid present displayed no difference between PA14 and Δ*tctED* and thus no difference

in tobramycin accumulation (Figure 6). However, lysates of Δ*tctED* cultures grown in the presence of citric acid showed a statistically significant ($p<0.05$) increase in zones of clearance compared to PA14, indicating greater accumulation of tobramycin in Δ*tctED* biofilms (Figure 6). In conditions where strains were grown in M63-arginine with 10 mM citric acid there was a mean difference of 3 mm in the zone of clearance between PA14 and Δ*tctED*. For these assays, differences in diameters of ≥3 mm for zones of clearance is considered definitive for determining a clinically relevant change in susceptibility[230].

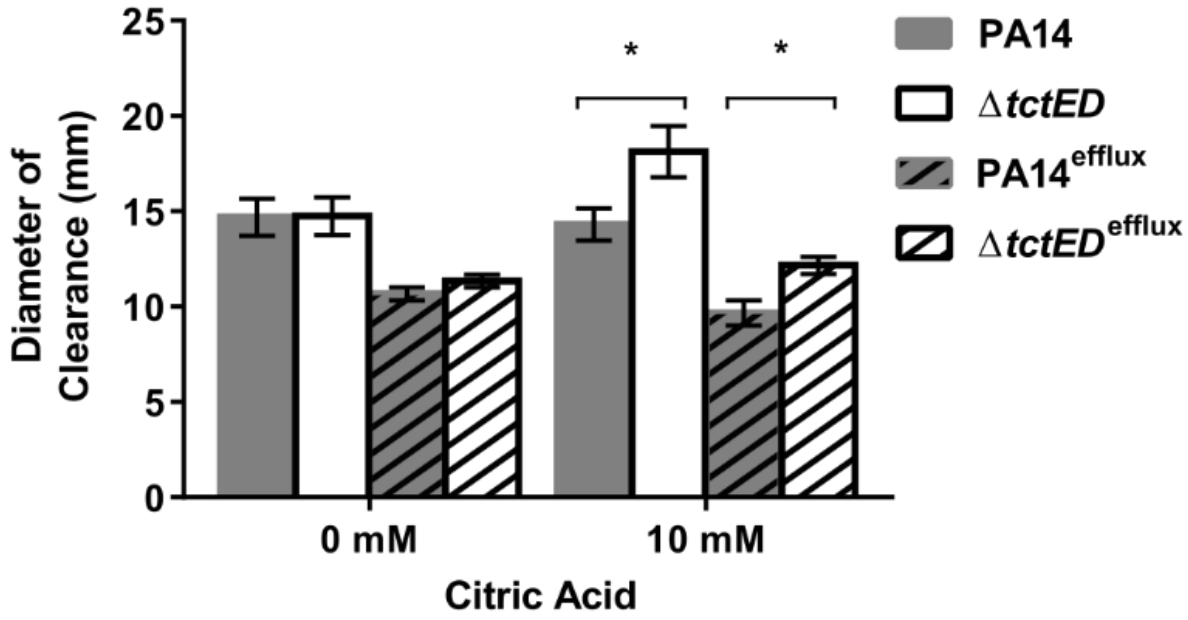

Figure 6. Tobramycin accumulation in Δ*tctED*. Through use of a disk diffusion assay, lysates were assessed for variations in levels of tobramycin taken up by biofilm cells. Disks with absorbed lysates were placed onto LB agar plates that had been spread with lawns of DH5α *E. coli* where incubated at 37°C overnight to allow *E. coli* to grow while lysate diffused from the disks into LB agar. Error bars are the SEM and columns are the mean of 3 biological repeats.

A Bonferroni-Dunn test was used to determine whether differences were significant and * indicates $p < 0.05$.

In *P. aeruginosa*, efflux is a major resistance mechanism against aminoglycosides. Because of this, I was curious whether lower levels of efflux in Δ*tctED* was the reason for the increased accumulation observed when citric acid was present in media. To test this possibility, I created strain of PA14 and Δ*tctED* expressing the PA1875-1877 efflux system that was characterized in our lab previously[67]. I have designated these new strains expressing PA1875-1877 as PA14^{efflux} and Δ*tctED*^{efflux}, respectively. I reasoned that if efflux was disrupted in Δ*tctED* then constitutive expression of PA1875-1877 in Δ*tctED*^{efflux} should restore wild-type levels of tobramycin accumulation. PA14^{efflux} served as a control for over-expression of PA1875-1877. I found that there was overall a moderate reduction in zones of clearance for both PA14^{efflux} and Δ*tctED*^{efflux} strains under conditions with and without citric acid but the of trend of greater accumulation was still observed in Δ*tctED*^{efflux} relative to PA14^{efflux} in the presence of citric acid (Figure 6). Therefore, accumulation of tobramycin in Δ*tctED* biofilms is likely not due to increased retention within cells. It is possible that the accumuled tobramycin is being absorbed by the biofilm matrix. Previous work has characterized cationic compounds such as aminoglycosides like tobramycin as being readily absorbed and chelated within the biofilm matrix[65]. This has been demonstrated to reduce their efficacy in treatment of infections[104].

3.2 Biofilms of Δ*tctED* have a Divergent Biofilm Phenotype from PA14 in the Presence of Citric Acid

Since I observed that Δ*tctED* biofilms accumulate greater amounts of tobramycin in the presence of citric acid compared to PA14, and that this phenotype is not due to issues of efflux, I hypothesized that Δ*tctED* may be dysregulated in its ability to develop biofilms and mature biofilm

architecture. To assess biofilm formation, I used a crystal violet-staining assay as well as microscopy

For these experiments, I used M63 with 23 mM arginine as a basal medium and added 0, 2, 10, or 20 mM citric acid. In the absence of citric acid, PA14 and Δ*tctED* displayed the same levels of biofilm formation as determined by crystal violet staining (Figure 7). For these experiments I opted to use arginine as a basal carbon source for the sake of recreating nutrient conditions used in the original study that characterized the Δ*tctED* tobramycin susceptibility phenotype[68]. Crystal violet is a generic stain of organic polymers and peptides that is easily solubilized in ethanol. Stained biofilm cultures can be washed with water, solubilized and levels of staining can be measured by absorbance or optical density at 595 nm wavelength. With the addition of citric acid, I observed that Δ*tctED* biofilms displayed consistent levels of staining for all concentrations of citric acid tested (Figure 7). In stark contrast to this, with increasing concentrations of citric acid, I observed that there was less staining of PA14 biofilms. This was most apparent at the highest concentrations of citric acid (10 and 20 mM) (Figure 7). From these experiments, it appears that there is some dysregulation affecting biofilm formation occurring in Δ*tctED*. I was curious whether the inclusion of tobramycin would cause any further effects on Δ*tctED* biofilms. To test this, I repeated these experiments with sub-inhibitory levels of tobramycin but there was no further effect with the presence of tobramycin in addition to citric acid in media (Figure 7).

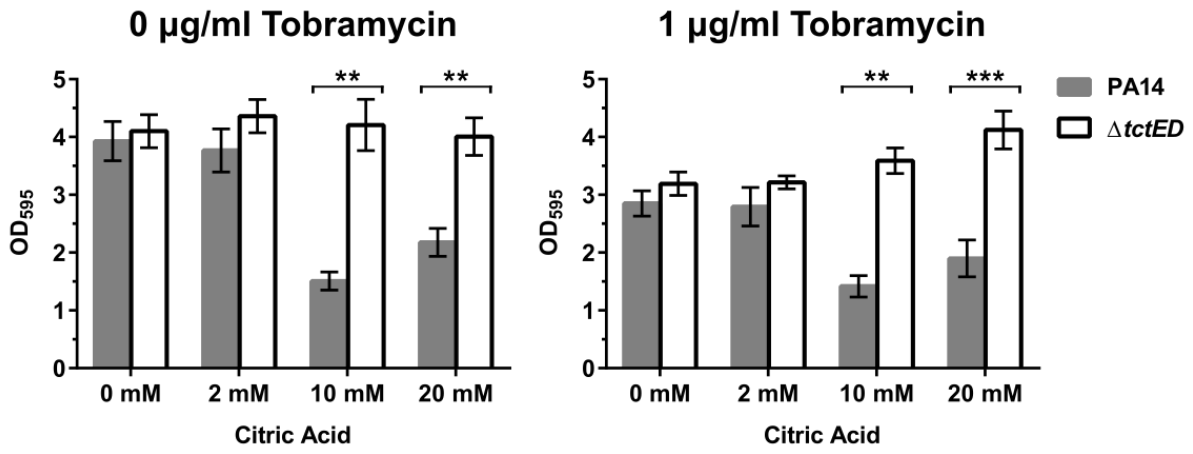

Figure 7. Crystal violet staining of Δ*tctED* biofilms in the presence of citric acid. Biofilm cultures were grown with varying concentrations of citric acid while unchallenged or challenged with a sub-inhibitory concentration of tobramycin. Columns represent the mean of 3 biological replicates and error bars represent the SEM. To assess significance a Bonferroni-Dunn method was used where ** = $p < 0.01$ and *** = $p < 0.001$.

To determine if structural components of *P. aeruginosa* biofilms were affected in Δ*tctED*, I tested different forms of motility. Both flagella and type IV pili are parts of biofilm architecture and important for early stages of biofilm formation in *P. aeruginosa*[109,143]. Flagella are essential for swimming motility in *P. aeruginosa*, while swarming motility uses both flagella and type IV pili. Swimming motility is uncoordinated in *P. aeruginosa* and cells freely moves individually propelled by their polar flagellum. Swarming motility is a social behaviour where cells in the colony have coordinated activity through grouping via type IV pili and movement through unified use of flagella. Both these forms of motility were assayed in PA14 and Δ*tctED* to assess if flagella and type IV pili formation or functionality was dysregulated. I observed no deficiencies or hyper-motility in either swimming or swarming motilities (Figure 8). This suggests that dysregulation in

biofilms of Δ*tctED* occurs at some later stage or regulatory step of biofilm formation.

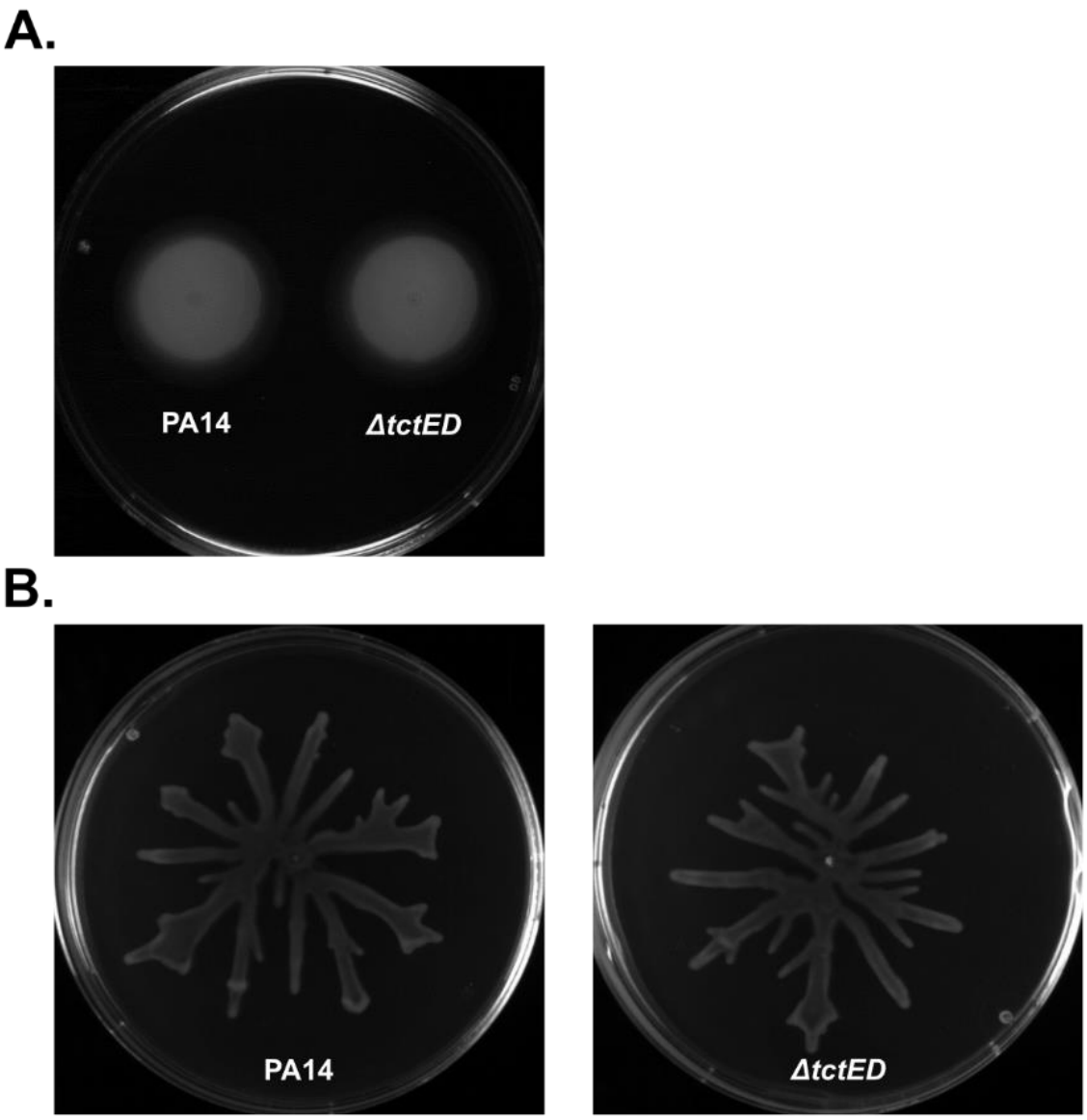

Figure 8. Assessment of motility in Δ*tctED*. Flagella and pili function were tested through use of swimming (A.) and swarming (B.) motilities. A volume of 2 µl of overnight cultures were inoculated for both swarm and swim plates. Plates were incubated for 18 h at 37°C. Each photo is a representative biological replicate of at least 3 performed for each strain. For swarm plates PA14 and Δ*tctED* shown are from the same biological replicate.

Crystal violet staining provided insight into changes in biofilm mass that was being produced or remaining adhered to culture wells. To learn more about biofilm formation in Δ*tctED*, I visualized biofilms with phase-contrast microscopy. I used the same media and culture conditions from crystal violet staining assays for cultures to be visualized by microscopy. For conditions lacking citric acid, both PA14 and Δ*tctED* biofilms were thick, macrocolonies were readily seen, and biofilm growth covered the entire air-liquid interface of the culture well (Figure 9). However, at higher concentrations of citric acid, PA14 biofilms were increasingly flatter and more sparsely spread over the culture well surface (Figure 9A). In agreement with crystal violet staining assays, I found Δ*tctED* biofilms maintained thick growth at every concentration of citric acid tested (Figure 9A). As further confirmation, I also stained these cultures used for microscopy with crystal violet. This staining made the decrease of PA14 biofilms clearly apparent with increasing concentrations of citric acid. PA14 biofilms were nearly non-staining and hardly visible in 20 mM citric acid, the highest concentration tested (Figure 9B). An additional interesting observation was that Δ*tctED* actually had an overall increasing amount of biofilm formation (Figure 9B). This is also in agreement with observations presented above (Figure 8). Staining of Δ*tctED* in conditions of 10 and 20 mM citric acid shows biofilm growth spreading across the surface of the well. Biofilm cultures of Δ*tctED* with 0 and 2 mM citric acid maintained tighter bands of growth at the air-liquid interface like in PA14 cultures. This heightened biofilm formation in Δ*tctED* for conditions of 10 and 20 mM citric acid present in the media is of interest as *P. aeruginosa* typically develops the greatest amount of biofilm formation at the air-liquid interface of media. I incubated the culture plates for this assay at a 45° angle so that the biofilm that formed at the air-liquid interface would be easily visualized by microscopy. For Δ*tctED* cultures grown in 10 and 20 mM citric acid, this demonstrates that biofilm was forming consistently down into media beyond the air-liquid

interface. These contrasting responses between PA14 and Δ*tctED* biofilms in the presence of high concentrations of citric acid support that there is dysregulation occurring in Δ*tctED* that affects its ability to form biofilms.

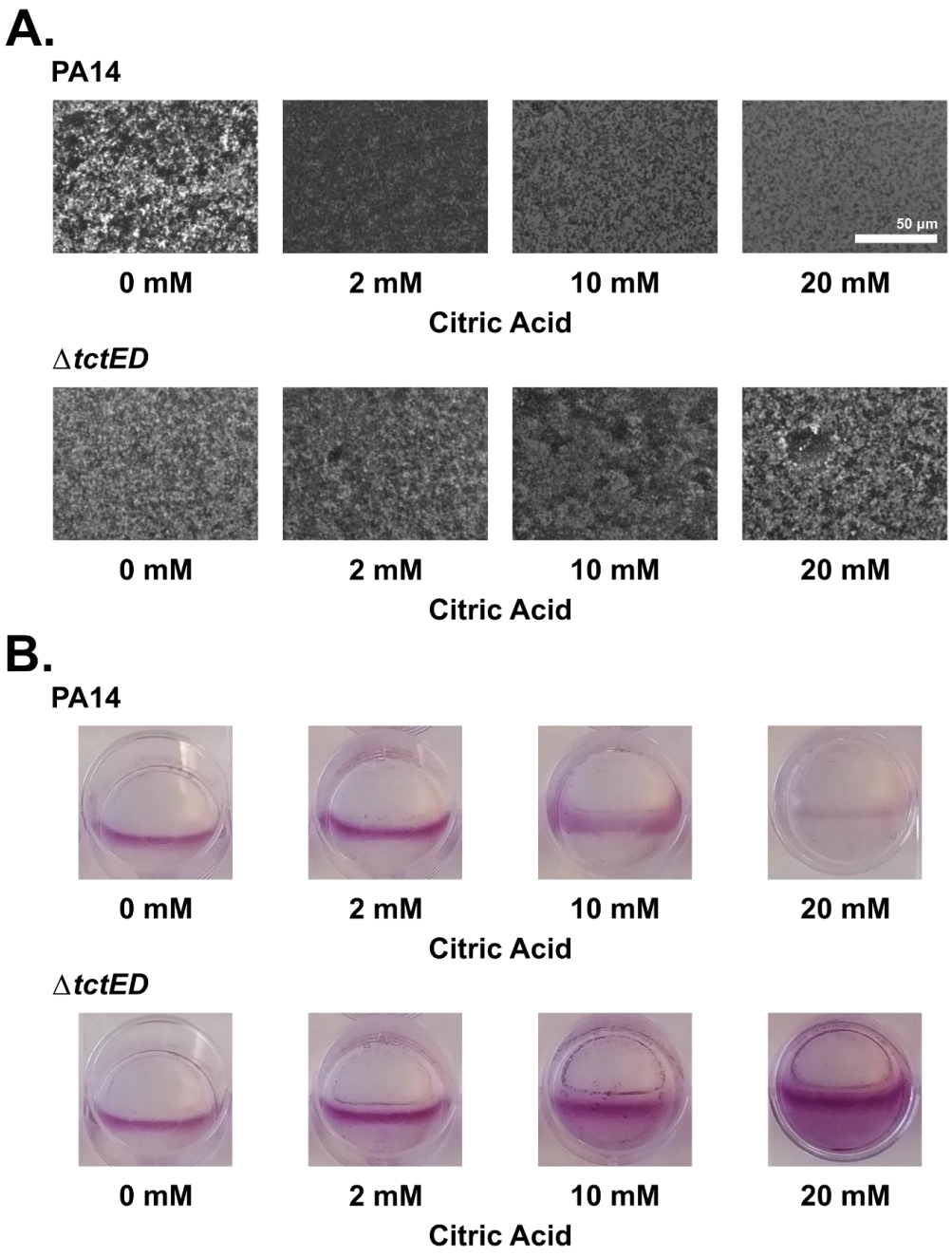

Figure 9. Visualization of PA14 and Δ*tctED* biofilms in the presence of citric acid. Biofilms from the air-liquid interface of 12-well culture plates were used for phase-contrast microscopy (A.) or whole culture staining with 0.1% crystal violet (B.). In these experiments the basal medium used was M63-arginine in which different concentrations of citric acid were then added. For microscopy, at least 30 fields of view from each of 3 biological replicates were taken. Images presented here are representative and taken from the same biological replicate for both microscopy and staining.

While the M63 basal medium used for these experiments is buffered and the pH is adjusted to 7.0 the addition of citric acid alters the pH of experimental media. I was curious as to whether pH changes that occur with the addition of citric acid could be contributing to the growth deficiency phenotype I was observing in Δ*tctED*. To address this, I tested the effect of altering pH without adding citric acid. I measured the pH of M63 media with citric acid concentrations (0, 2, 10, and 20 mM) and then prepared M63-arginine with no citric acid but adjusted pH to match media with citric acid (pH 7.0, 6.8, 6.2, and 5.1, respectively). However, I found there was no effect on Δ*tctED* growth when testing these pH levels (Figure 10).

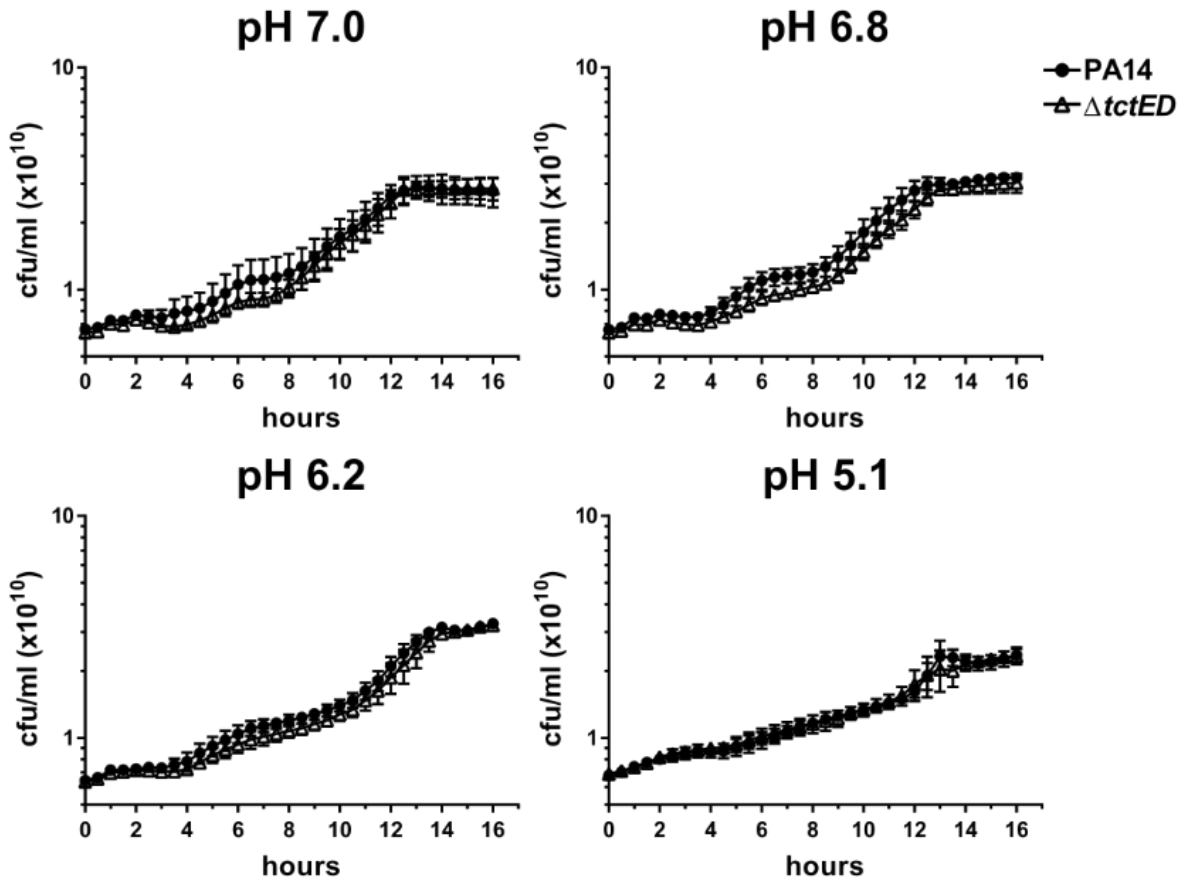

Figure 10. Growth of Δ*tctED* in varying pH. PA14 (●) and Δ*tctED* (Δ) were grown in liquid M63-arginine cultures with constant shaking for 16 h to assess growth at varying pH. Data points are the mean of 3 biological replicates and error bars are the SEM.

3.3 Δ*tctED* is Inhibited by Citric Acid Present in Nutrient Media

The divergent phenotype of Δ*tctED* biofilms compared to PA14 led me to question if there were any other differences in growth phenotypes of PA14 and Δ*tctED*. I hypothesized that there could be a growth deficiency in Δ*tctED* using citric acid (and related citrate metabolites) as a carbon source for growth. I based this hypothesis on the fact that previously it has been found that high ratios of citrate metabolites in media can have inhibitive effects on growth and metabolism

in *P. aeruginosa*[231,232]. It is a core substrate of the TCA cycle, however, on its own is not a preferred carbon source for *P. aeruginosa* to use[231,232]. With loss of TctD-TctE regulation over citrate uptake in Δ*tctED*, it seemed possible that there could be a situation in which intracellular levels of citrate metabolites would be skewed and result in broader effects on regulation of cellular metabolism.

To test this hypothesis, I first performed growth assays in M63 media with varying concentrations of citric acid as the sole carbon source provided. I found that regardless of the concentration of citric acid provided Δ*tctED* displayed no growth (Figure 11). However, PA14 grew at each concentration of citric acid tested. It is worth noting that I found using M63 with 2.2 mM glucose, there was no observable difference between PA14 and Δ*tctED* growth curves. For this experiment glucose was used to provide a preferred carbon source over arginine or citric acid as the conditions were entirely to assess growth and not recreate nutrient conditions from the study that originally characterized the Δ*tctED* tobramycin susceptibility phenotype. It is also worth noting that when grown in 2.2 mM glucose both PA14 and Δ*tctED* cultures looked characteristically like *P. aeruginosa* cultures. Both PA14 and Δ*tctED* cultures were consistently homogenously opaque and bright blue-green at the end of the experiment once entering stationary phase. This indicates that Δ*tctED* is a stable strain as unwanted mutations and genetic instability in *P. aeruginosa* leads to variability in mucoidy and the production of phenazine pigments[233,234].

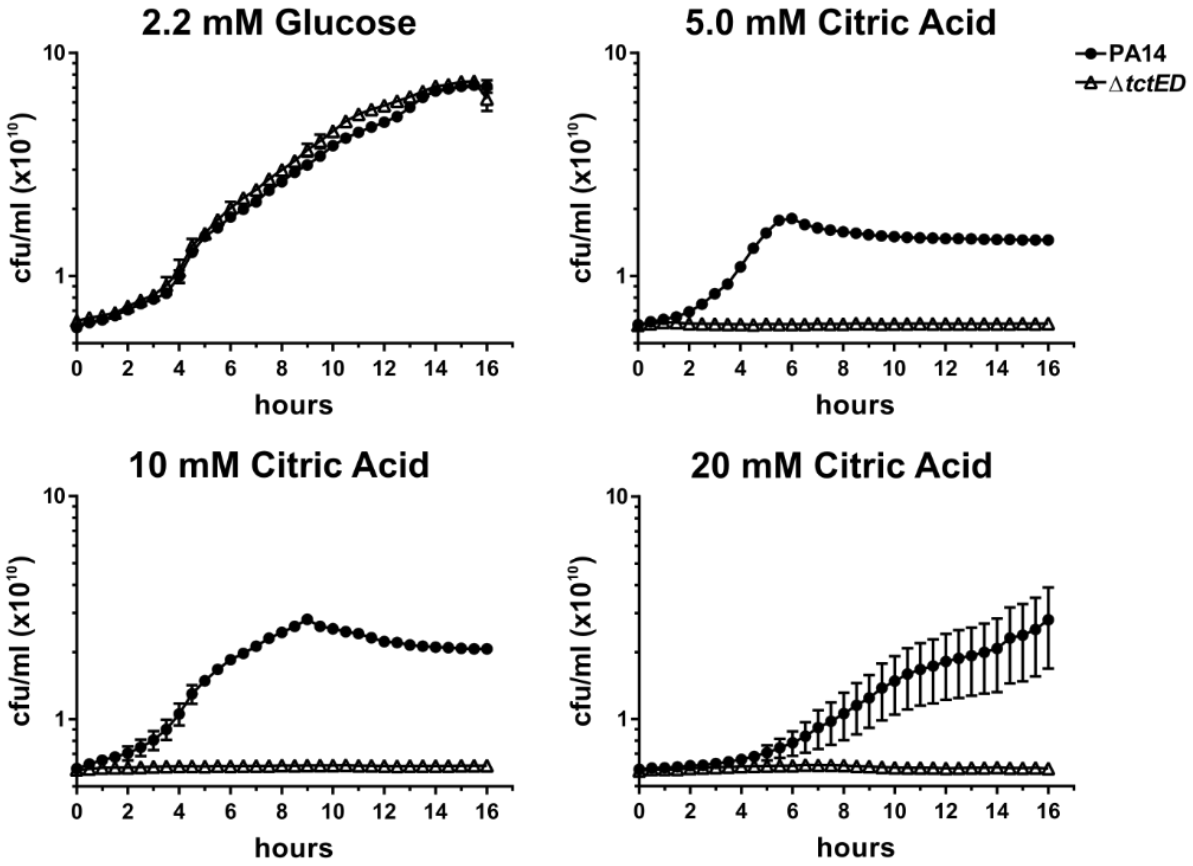

Figure 11. Growth of Δ*tctED* with citric acid as sole carbon source. Cultures of PA14 (●) or Δ*tctED* (Δ) were grown for 16 h with shaking and sampling every 30 min. Each growth curve presented represents cultures grown in media containing the carbon source labelled as the sole carbon source. For these experiments, OD_{600} was measured and cfu/ml was extrapolated based on an independent experiment where I found an OD_{600} of 1.0 equated to $6.0x10^{10}$ cfu/ml. Data are the mean of 3 biological replicates and error bars represent the SEM.

Facing this issue of Δ*tctED* displaying no observable growth when citric acid was used as the carbon source, I questioned whether this was due to an inability for Δ*tctED* to metabolize citric

acid or if there was some regulatory inhibition of metabolism occurring due to the presence of citric acid. Because TctD-TctE is itself a regulatory system and no changes to metabolic enzymes have been directly made in Δ*tctED*, I hypothesized that the latter was occurring. I repeated the planktonic growth assays under similar media conditions used for the biofilm assays performed previously. For these assays, I was also curious if, in addition to the effect of having citric acid present, there would be any more effect by adding sub-inhibitory (1 μg/ml) and inhibitory (4 μg/ml) concentrations of tobramycin. While Δ*tctED* was able to grow when arginine was provided as an alternative carbon source, there was reduced growth with increasing concentrations of citric acid present in media (Figure 12). In low (2 mM) and moderate (10 mM) concentrations of citric acid, there was a lag in growth of the Δ*tctED* relative to PA14 (Figure 12B and C). It is notable that the decrease and delay in growth observed in Δ*tctED* was greater in 10 mM than 2 mM citric acid present. However, Δ*tctED* was able to reach similar levels of culture density as PA14 with 0 μg/ml and 1 μg/ml tobramycin (Figure 12C). In the presence of 10 mM citric acid and 4 μg/ml tobramycin, Δ*tctED* was unable to achieve the same cell density as PA14 (Figure 12C). There is the possibly that Δ*tctED* displayed reduced growth throughout this condition due to the combined stress of citric acid and tobramycin concentrations.

The greatest difference in growth between PA14 and Δ*tctED*, however, was observed in media with 20 mM citric acid present. Here Δ*tctED* strain was severely reduced in its ability to grow and was largely unable to reach the same culture density as PA14 (Figure 12D).

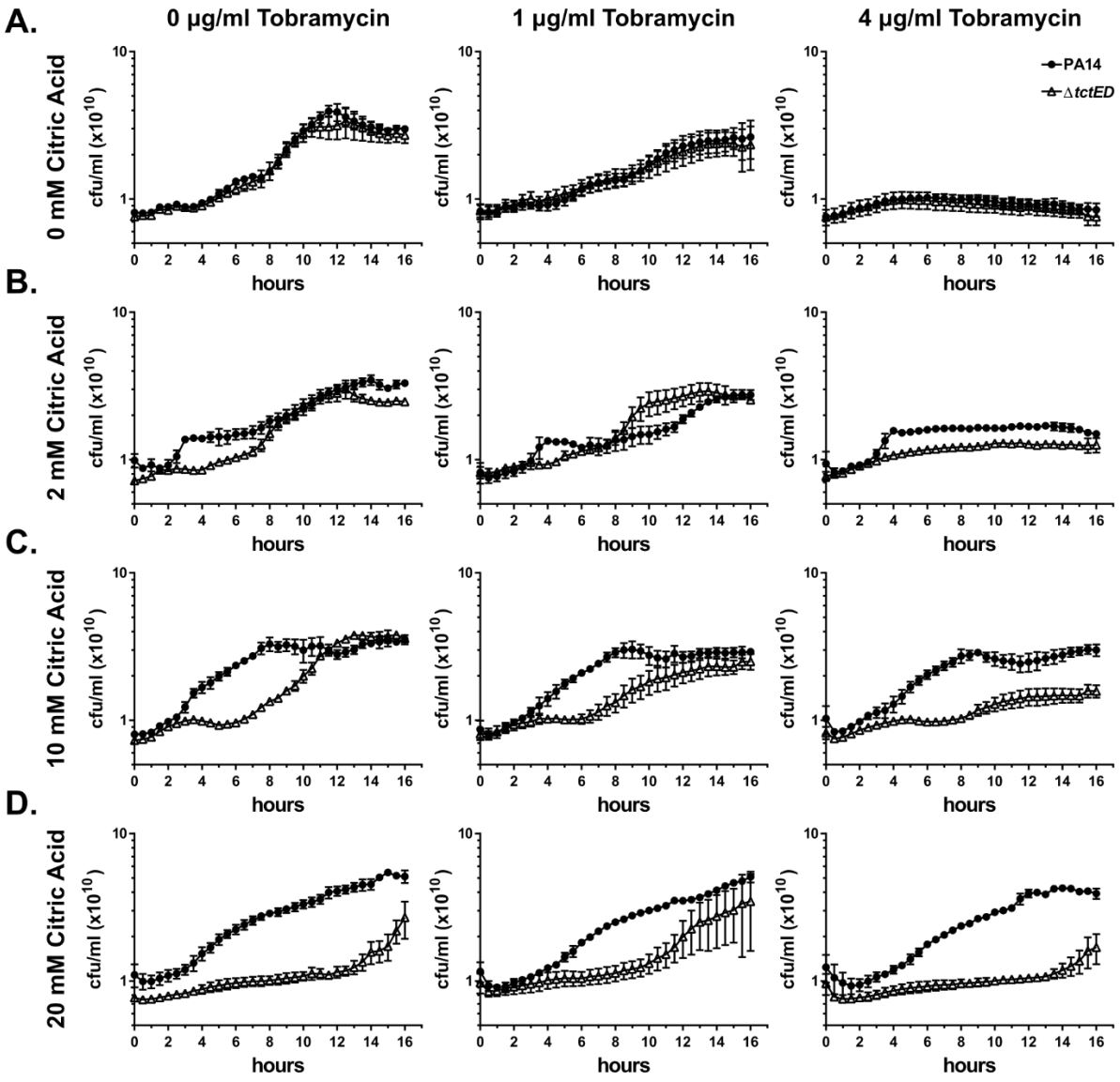

Figure 12. Growth of Δ*tctED* in media containing citric acid. Beginning with a basal medium of M63-arginine either 0 mM (A), 2 mM (B), 10 mM (C), or 20 mM citric acid (D) were added to test cultures of PA14 (●) or Δ*tctED* (Δ) while concomitantly being challenged with zero, sub-inhibitory (1 µg/ml), and inhibitory (4 µg/ml) tobramycin concentrations. Data are the mean of 3 biological replicates where error bars are the SEM. Where no error bars are visible the SEM was smaller than what could be displayed.

Δ*tctED* has a previously characterized aminoglycoside biofilm-specific susceptibility. As mentioned above, I was also interested as to whether there would be any further effect on the growth of Δ*tctED* in M63-arginine media which had citric acid as well as tobramycin added. I used sub-inhibitory (1 μg/ml) and inhibitory (4 μg/ml) concentrations of tobramycin based on findings of susceptibility reported here in this thesis as well as previously published work from our lab[68]. However, regardless of the concentration of tobramycin, there was no consistent observable added effect on Δ*tctED* having further reduced growth in the presence of both citric acid and tobramycin (Figure 12). The one condition of 10 mM citric acid and 4 μg/ml tobramycin (Figure 12C) was the only condition that may provide support for the possibility that the presence of citric acid may further exacerbate the tobramycin susceptibility observed in Δ*tctED*. Overall, the growth deficiencies of Δ*tctED* under these planktonic culture conditions appear to be largely dependent on only the presence of citric acid in the media. Furthermore, this growth discrepancy between PA14 and Δ*tctED* that appears to be dose dependent on the presence of citric acid, and not tobramycin, suggests that in fact there is an inhibition of growth occurring in Δ*tctED* due to citric acid.

While Δ*tctED* displayed a clear inhibition of growth in the presence of citric acid, I set out to be sure this phenotype was not due to polar effects. The porin gene that TctD-TctE regulates is encoded immediately upstream to the *tctED* operon. To confirm that growth deficiency was due to loss of TctD-TctE, a complementation strain (*tctED*$^{+}$) was struck out on gradient agar plates that had a continuous transition from M63 with 23 mM arginine to M63 with 10 mM citric acid. Gradient agar plates effectively consist of two layers of media which taper along a single dimension with each medium tested at full concentration at either end of the gradient plate. I found that expression of TctD-TctE in Δ*tctED* (*tctED*$^{+}$) restored the ability of this strain to grow on citric

acid to wild-type levels (Figure 13). Additionally, this growth was maintained in the presence of tobramycin.

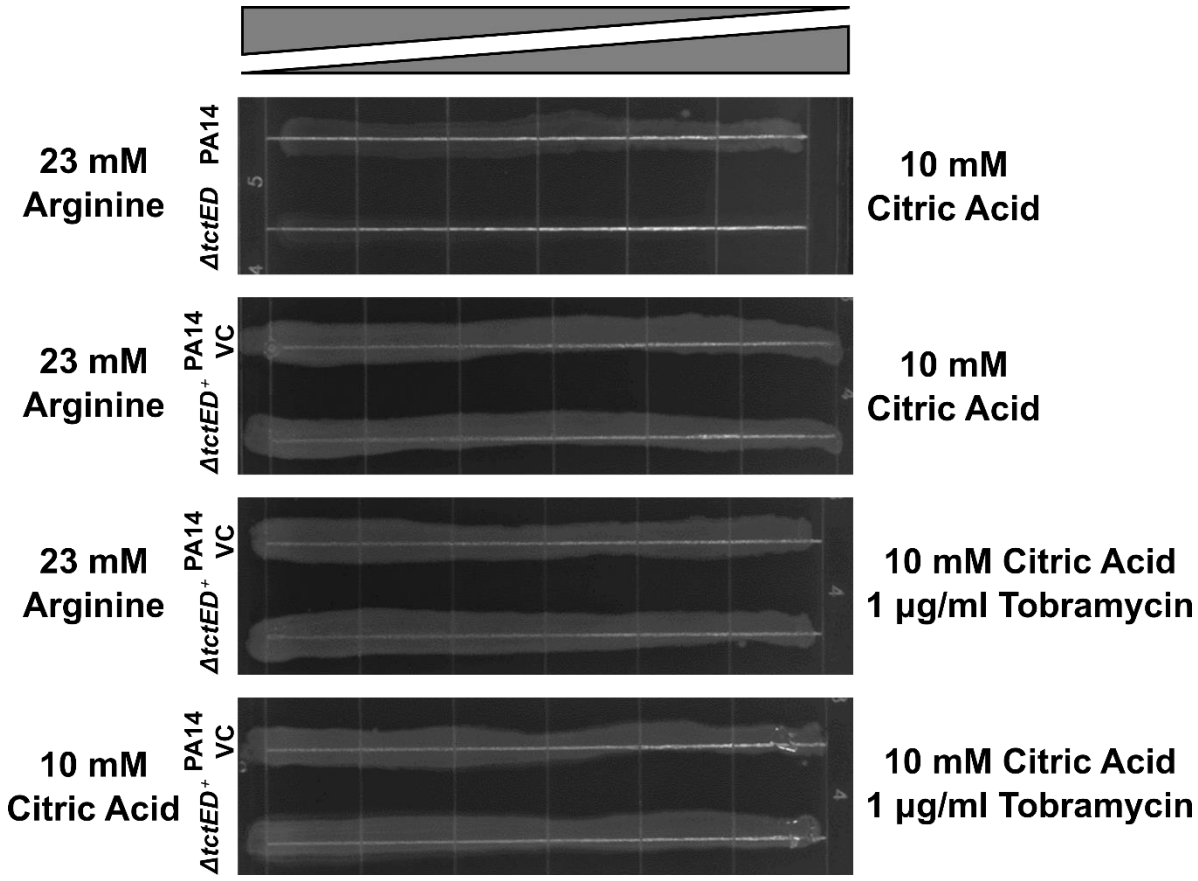

Figure 13. Complementation of Δ*tctED* (*tctED*⁺) restored growth on citric acid. PA14 was transformed with pJB866 (PA14 VC [vector control]) as control for complementation to enable side-by-side comparison of growth on selective media. Triangles at the top of the figure represent gradients at either maximum for nutrient condition listed on both the left and right of gradient plate images.

3.4 The Δ*tctED* Growth Deficiency is Unique to Citric Acid as a Carbon Source

Having characterized growth inhibition of Δ*tctED* on citric acid, I wondered whether this effect was unique to citric acid and related citrate metabolites. I performed a high-throughput phenotypic

microarray testing a diverse array of potential utilizable carbon metabolites in *P. aeruginosa*[235]. This assay uses changes in absorbance resulting from colour change in the reduction of a dye added to the plates. Metabolically active cells undergoing oxidative phosphorylation cause reduction of the dye and thereby indirectly provides a measure for cell growth (see materials and methods, chapter 2.16, for a link to the manufacturer's website). Δ*tctED* had moderately reduced growth on the carbon sources of α-hydroxy butyric acid (PM1 E7, plate and well position) and D,L-carnitine (PM2A H5) compared to PA14 (Table 3 and Figure 29). Additionally, there were moderate increases in growth of Δ*tctED* relative to PA14 on carbon sources of adenosine (PM1 E12), α-keto-butyric acid (PM1 D7), putrescine (PM2A H8), and uridine (PM1 D12) (Table 3 and Figure 29). However, the only carbon source that displayed a stark and clear difference between PA14 and Δ*tctED* was citric acid (PM1 F2) (Figure 29). In these phenotypic microarrays, I found that Δ*tctED* was unable to grow when citric acid was provided as the sole carbon source. These results also provide confirmation for the experiments I performed and described above. All of these small molecules may be indirectly linked to the metabolism through the core TCA cycle and thereby citric acid in *P. aeruginosa* but there is no direct connection apparent with regard to any pathways, metabolism or uptake between these carbon sources.

Table 3. Assessment of Δ*tctED* growth on various carbon sources.

	Δ*tctED* growth relative to PA14[a]
α-Hydroxy-butyric acid	**-**
α-Keto-butyric acid	**+**
Adenosine	**+**
Citric acid	**-**
D,L-Carnitine	**-**
Putrescine	**+**

Uridine	+

a. Experiments were performed in triplicate with a representative assay provided in Figure 29 of the appendix. Differences in growth for a given carbon source presented in this table were observed for all three experiments.

3.5 Multiple Genes Representing Different Pathways are Dysregulated in Δ*tctED*

Lastly, since TctD-TctE is a TCS, I was interested in global gene expression changes in Δ*tctED* which I investigated through use of DNA microarrays In total, I found that 94 genes in planktonic cultures and 23 genes in biofilm cultures were significantly dysregulated (>2-fold) in Δ*tctED* relative to the PA14 (Table 4). Of the 94 genes in planktonic cultures, I determined that 15 have roles in metabolism in *P. aeruginosa* by consulting the *Pseudomonas* Genome Database[93] for functional characterizations. Of the 23 genes in biofilm cultures, I found that 8 genes have a role in metabolic processes. These genes are indicated by bold font in Table 4. Interestingly, I found no overlap in dysregulated in genes that are known to be involved in metabolism between planktonic and biofilm cultures. There was one gene, PA14_02520 which is of unknown function, that displayed significant difference in fold change between planktonic and biofilm conditions. This does not rule out the possibility that among the genes with unknown roles there may be metabolic functions that have not been characterized yet. This is suggestive of there either being unique regulomes of TctD-TctE in each of these modes of growth. While metabolic genes were not consistently dysregulated between planktonic and biofilm conditions of Δ*tctED*, the transcriptional regulator genes *cheY*, *pmrA*, and *dnr*, as well as the two-component sensor gene *phoQ* were (Table 4). These data demonstrated that Δ*tctED* has wide reaching effects within the cell. Likely consisting of direct and indirect effects on these pathways it is apparent that Δ*tctED* has a complex role in effecting regulation of biofilm formation in *P. aeruginosa*.

Table 4. Genes with significant changes in expression in Δ*tctED*.

Gene Locus	Gene Name[a]	Gene Function	Fold Change Difference[b,c]	
			Planktonic	**Biofilm**
PA14_72260			ns[d]	5.54
PA14_02520			2.54	5.26
PA14_01310			4.80	ns
PA14_70010			4.73	ns
PA14_01320	***coIII***	**cytochrome c oxidase, subunit III**	**4.51**	**ns**
PA14_60190	*clpB*	ClpB protein	3.81	ns
PA14_07660			3.77	ns
PA14_49130	***dctA***	**C4-dicarboxylate transport protein**	**3.63**	**ns**
PA14_42860			ns	3.51
PA14_46900			ns	3.41
PA14_70040	***dadA***	**D-amino acid dehydrogenase, small subunit**	**3.06**	**ns**
PA14_56540			ns	2.94
PA14_55750			ns	2.92
PA14_38840			2.88	ns
PA14_17550			2.82	ns
PA14_07680			2.78	ns
PA14_68840			2.75	ns
PA14_58690			2.68	ns
PA14_61600			2.68	ns
PA14_61610			2.60	ns
PA14_44480			2.57	ns
PA14_22350			ns	2.52
PA14_45620	*cheY*	two-component response regulator CheY	2.48	ns

Gene Locus	Gene Name[a]	Gene Function	Fold Change Difference[b,c]	
PA14_17700			2.48	ns
PA14_56690			ns	2.47
PA14_44470	***hemN***	**oxygen-independent coproporphyrinogen III oxidase**	**ns**	**2.46**
PA14_60700	***ccpR***	**cytochrome c551 peroxidase precursor**	**ns**	**2.44**
PA14_10730			2.44	ns
PA14_24770			2.38	ns
PA14_68260			2.36	ns
PA14_45610	*cheZ*	chemotaxis protein CheZ	2.29	ns
PA14_16260			2.29	ns
PA14_15030	***leuA***	**2-isopropylmalate synthase**	**2.28**	**ns**
PA14_61840			2.28	ns
PA14_60520			2.26	ns
PA14_16680			2.19	ns
PA14_30830			2.17	ns
PA14_44860			2.15	ns
PA14_71720			2.14	ns
PA14_49170	*phoQ*	two-component sensor PhoQ	ns	2.11
PA14_52800	***acsA***	**acetyl-coenzyme A synthetase**	**ns**	**2.08**
PA14_19870	***ldh***	**leucine dehydrogenase**	**2.08**	**ns**
PA14_06870	***dnr***	**transcriptional regulator Dnr**	**ns**	**2.06**
PA14_05060			2.06	ns
PA14_24760			2.05	ns
PA14_26590			2.04	ns
PA14_20890	***rfaD***	**ADP-L-glycero-D-mannoheptose 6-epimerase**	**ns**	**2.02**
PA14_51850			2.01	ns

Gene Locus	Gene Name[a]	Gene Function	Fold Change Difference[b,c]	
PA14_27210	*efp*	translation elongation factor P	-2.01	ns
PA14_66140			-2.01	ns
PA14_17080	***pyrH***	**uridylate kinase**	**-2.02**	**ns**
PA14_64560			-2.02	ns
PA14_65605	*parC*	topoisomerase IV subunit A	-2.03	ns
PA14_54370	*lepA*	GTP-binding protein LepA	-2.04	ns
PA14_25490			-2.04	ns
PA14_18350			-2.04	ns
PA14_09980	***dkgB***	**2,5-diketo-D-gluconate reductase B**	**ns**	**-2.06**
PA14_61770	***prs***	**ribose-phosphate pyrophosphokinase**	**-2.06**	**ns**
PA14_61780			-2.06	ns
PA14_17060	*rpsB*	30S ribosomal protein S2	-2.09	ns
PA14_25740	***tmk***	**thymidylate kinase**	**-2.10**	**ns**
PA14_51840			-2.12	ns
PA14_62710	***pnp***	**polyribonucleotide nucleotidyltransferase**	**-2.12**	**ns**
PA14_21760	*capB*	cold acclimation protein B	-2.13	ns
PA14_47420			ns	-2.13
PA14_65160			-2.14	ns
PA14_14440	***valS***	**valyl-tRNA synthetase**	**-2.15**	ns
PA14_09030	*rpmD*	50S ribosomal protein L30	-2.15	ns
PA14_66230	***waaG***	**UDP-glucose:(heptosyl) LPS alpha 1,3-glucosyltransferase**	**-2.15**	**ns**
PA14_17675	***dgkA***	**diacylglycerol kinase**	**-2.16**	**ns**
PA14_09050	*secY*	secretion protein SecY	-2.18	ns
PA14_55590			ns	-2.19
PA14_70640	*rubA1*	Rubredoxin 1	-2.20	ns

Gene Locus	Gene Name[a]	Gene Function	Fold Change Difference[b,c]	
PA14_73420	*rnpA*	ribonuclease P protein component	-2.20	ns
PA14_25760	*holB*	DNA polymerase III, delta prime subunit	-2.21	ns
PA14_57960	***ptsN***	**nitrogen regulatory IIA protein**	**ns**	**-2.21**
PA14_00990			ns	-2.22
PA14_57580	*rpsI*	30S ribosomal protein S9	-2.22	ns
PA14_09020	*rpsE*	30S ribosomal protein S5	-2.23	ns
PA14_56980			ns	-2.23
PA14_52070			ns	-2.24
PA14_63120			-2.24	ns
PA14_25730			-2.25	ns
PA14_65170	*rpsR*	30S ribosomal protein S18	-2.25	ns
PA14_58130	*mreC*	rod shape-determining protein MreC	-2.26	ns
PA14_41250	*tig*	trigger factor	-2.27	ns
PA14_08860	*rplD*	50S ribosomal protein L4	-2.30	ns
PA14_12550			-2.33	ns
PA14_09000	*rplF*	50S ribosomal protein L6	-2.36	ns
PA14_17220	***lpxB***	**lipid A-disaccharide synthase**	**-2.40**	**ns**
PA14_25630	*rpmF*	50S ribosomal protein L32	-2.41	ns
PA14_09010	*rplR*	50S ribosomal protein L18	-2.44	ns
PA14_00580			-2.48	ns
PA14_07560	*rpsU*	30S ribosomal protein S21	-2.48	ns
PA14_63110			-2.48	ns
PA14_14610			-2.49	ns
PA14_63150	*pmrA*	two-component regulator system response regulator	-2.49	ns
PA14_62830	***tpiA***	**triosephosphate isomerase**	**-2.53**	**ns**

Gene Locus	Gene Name[a]	Gene Function	Fold Change Difference[b,c]	
PA14_61820			-2.55	ns
PA14_09040	*rplO*	50S ribosomal protein L15	-2.58	ns
PA14_65180	*rpsF*	30S ribosomal protein S6	-2.58	ns
PA14_15980	*rimM*	16S rRNA processing protein	-2.59	ns
PA14_73410			-2.62	ns
PA14_58570			-2.68	ns
PA14_10170	***fepB***	**ferrienterobactin-binding periplasmic protein precursor**	**ns**	**-2.70**
PA14_25270	*aroP1*	aromatic amino acid transport protein AroP1	-2.76	ns
PA14_44060	***sdhC***	**succinate dehydrogenase (C subunit)**	**-2.80**	**ns**
PA14_58120	*mreD*	rod shape-determining protein MreD	-2.91	ns
PA14_67560	*typA*	regulatory protein TypA	-2.93	ns
PA14_52340			-3.04	ns
PA14_27370			-3.52	ns
PA14_15970	*rpsP*	30S ribosomal protein S16	-3.55	ns
PA14_70180	*rpmG*	50S ribosomal protein L33	-3.59	ns
PA14_15990	*trmD*	tRNA (guanine-N1)-methyltransferase	-3.60	ns
PA14_39060			-4.14	ns

a. Where no gene name is provided it is an uncharacterized gene with a conserved hypothetical gene product.

b. Values are the mean of 2 biological replicates.

c. Rows in bold are genes involved in metabolism.

d. No significant fold change (ns).

4 THE SMALL RNA, SrbA, IS IMPORTANT FOR BIOFILM FORMATION AND PATHOGENICITY

Within the past decade, non-coding, small RNA (sRNA) transcripts have been found to have important effects on biofilm formation and virulence in a host[134,188,199,236]. Specifically in *P. aeruginosa*, there have been multiple studies investigating its transcriptome[181,186,187]. Investigations of the genomes and transcriptomes of bacteria have found a vast number of novel, putative transcripts. Of particular interest are novel sRNAs, which potentially have regulatory roles in complex biological activities such as biofilm formation. Recent transcriptomic work adds a large amount of new information to the field, but there have been few follow-up studies taking the next step to determine what biological role these novel sRNAs have in bacteria.

One novel sRNA, SrbA (**S**mall RNA **r**egulatory of **b**iofilms **A**), was found in three independent studies of the *P. aeruginosa* transcriptome[181,186,187]. Of these studies, I was involved in the work by Gill et al (2018), where I found SrbA to be highly expressed in *P. aeruginosa* PAO1 biofilms. I hypothesize that SrbA has a role in biofilms. In this chapter, I present my work that demonstrates SrbA is important for biofilm formation and pathogenicity in *P. aeruginosa* PA14.

Portions of the work presented in this chapter has been published previously in the peer-reviewed journal PLoS One[201]. For this work, Anthony Van Kessel kindly provided a great deal of time and assistance working with *C. elegans* and helping perform the slow-killing assays with *P. aeruginosa* strains. Tony Roenspies patiently trained Anthony and me to work with *C. elegans* and synchronized the life cycles of worms for our experiments. Otherwise, all experiments were performed by myself. Work that has not been peer-reviewed include the experiments of phase-contrast and fluorescence microscopy with Δ*srbA*, swarming assays with Δ*srbA*, and the in silico predicted structures of SrbA.

4.1 Genetic Characteristics of *srbA* and Expression of SrbA

Previous transcriptomic studies that found SrbA designated it as PA2633.1[186], pant235[181] and PA14sr_067[187]. Through use of the Pseudomonas Genome Database[93] and Pseudomonas Browser[187], I confirmed that *srbA* is encoded in an intergenic region and is conserved across all sequenced strains of *P. aeruginosa* (Figure 14). I found PA14 *srbA* to have 100% sequence identity with its homolog in PAO1 that was used in previous work where I initially characterized expression of SrbA[186]. In PA14, SrbA is encoded from nucleotides 2,604,298 to 2,604,536 from the origin of replication[93]. The *srbA* gene is 239 bp in length and encoded between genes *aceA* (isocitrate lyase) and PA14_30070 (uncharacterized, hypothetical protein) (Figure 14). Other notable characteristics of *srbA* architecture is a lack of rho-independent terminators and no recognizable Shine-Dalgarno sequence. By use of in silico methods, I was able to determine that there are no peptides predicted to be translated from the SrbA transcript[226]. The browser based application, ExPASy, assesses whether there are putative proteins expressed from a transcript by simulating translation in the three possible reading frames occurring both forwards and backwards.

Figure 14. Diagram of the *srbA* locus in the PA14 chromosome. Reference scale of 100 base-pairs (bp) length is provided.

Previous studies have investigated SrbA expression in planktonic, swarming, and biofilm modes of growth in the *P. aeruginosa* wild type strain PAO1[181,186], while work for this thesis used the wild type strain PA14. Although a homologous sequence of *srbA* was found in PA14, I first needed to confirm that SrbA is in fact expressed in PA14. Through use of qPCR, I found that SrbA

was up-regulated 45-fold in biofilm cells compared to planktonic cells in exponential phase growth cultures (Figure 15). I also compared expression between in stationary phase and exponential phase cells and found that SrbA was up-regulated 4-fold in planktonic stationary phase cells (Figure 15).

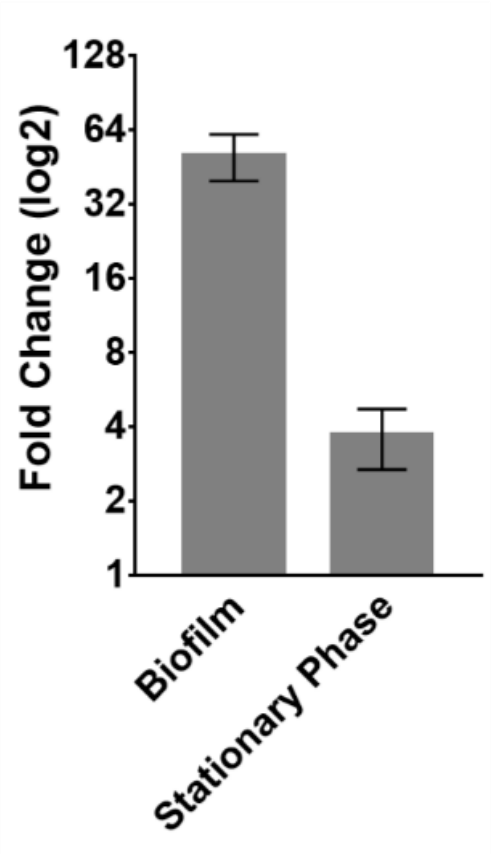

Figure 15. Expression of SrbA in biofilm and stationary cultures. Expression of SrbA was determined from performing qPCR using whole cell RNA isolated from PA14. Fold changes for both biofilm and stationary phase cultures are relative to planktonic exponential phase cultures for 3 separate experiments. The housekeeping gene *rpoD* was used for normalization between samples. Errors bars are the standard error of the mean.

4.2 Generation of the Deletion Strain Δ*srbA*

After finding that SrbA was significantly up-regulated in biofilms, I was interested in what effect it may have on biofilm formation in PA14. I constructed a strain with a chromosomal deletion as described in the Materials and Methods (Chapter 2). Having generated isolates, I confirmed these strains did carry a deletion of *srbA* through negative selection on sucrose media to ensure the pEXΔ*srbA* suicide plasmid was eliminated. I also checked for a chromosomal deletion of *srbA* by use of sequencing and PCR amplification (Figure 16). I generated multiple isolates, however, chose one for use in this study and designated it Δ*srbA*. As shown in Figure 16, I made multiple DNA extractions of Δ*srbA* and through use of PCR, I checked the deletion site to ensure that the strain was indeed a monoculture with the deletion as seen in lanes 3 and 5 of Figure 16.

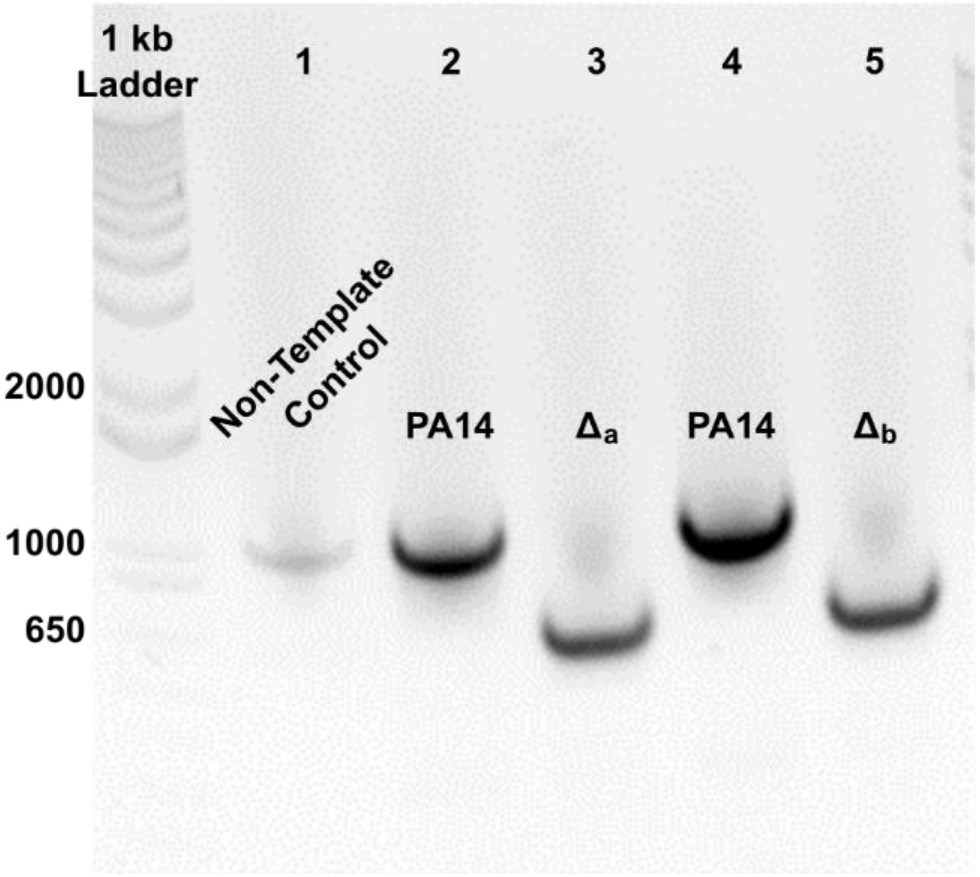

Figure 16. PCR amplification of Δ*srbA*. A region of 1 kilobase (1 kb) in the wild type which includes the *srbA* gene was amplified for PA14 (lanes 2 and 4) and 2 separate isolations of Δ*srbA* (lanes 3 and 5). Δ*srbA* isolations are ~300 bp smaller than PA14 confirming deletion of *srbA*. Lane 1 is product of a control PCR reaction which did not have any template DNA added.

To confirm that the chromosomal deletion in Δ*srbA* resulted in loss of expression of SrbA, I also performed qPCR by isolating RNA from Δ*srbA* and using primers specific to *srbA*. By comparing the expression of *srbA* in the wild type and Δ*srbA* strains, I was able to confirm that the chromosomal deletion in Δ*srbA* was sufficient to eliminate SrbA expression in PA14 (Figure 17). To confirm that biological effects observed in further experiments were due to the loss of SrbA, I also set out to create a SrbA complementation strain. I was able to achieve this and restore expression through cloning SrbA into the pUCP18 vector (pUC*srbA* in strain *srbA*$^{+}$) which expressed the sRNA constitutively (Figure 17).

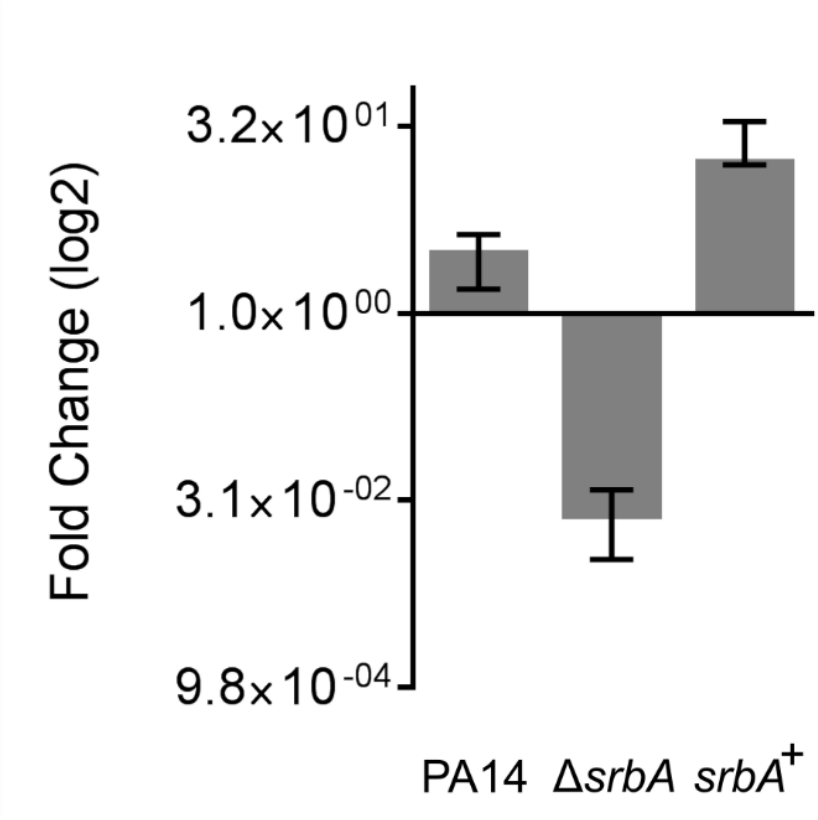

Figure 17. Complementation restores expression of SrbA. Use of semi-quantitative PCR (qPCR) compared biofilm cultures to stationary growth cultures. Loss of expression was confirmed in Δ*srbA* and restoration of expression in the complement strain (*srbA*$^{+}$). Higher than wild-type expression in *srbA*$^{+}$ is due to the pUCP18 vector being high-copy and constitutively expressed in *P. aeruginosa*. Three biological replicates are represented in the graph and error bars are the standard error of the mean.

While *srbA* is not a part of any previously characterized operon, I still thought it was important to determine if the chromosomal deletion of *srbA* had any polar effects on *aceA*, the gene immediately adjacent and downstream of *srbA*. I found that expression of *aceA* was not significantly affected when comparing expression of *aceA* in Δ*srbA* to that of PA14 (Figure 18).

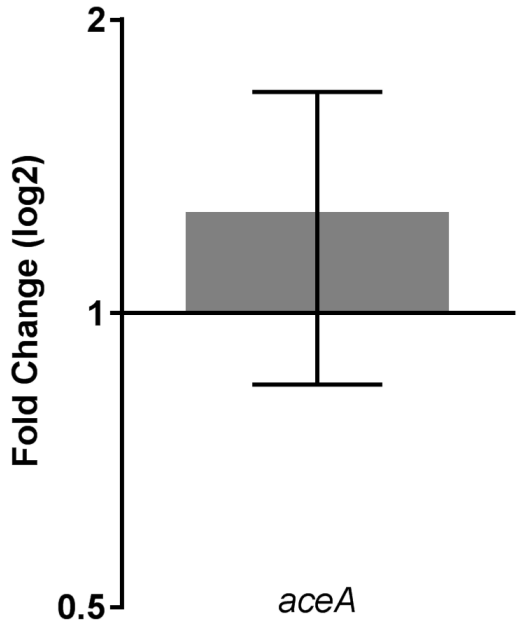

Figure 18. Assessment of polar effects in Δ*srbA*. The expression of *aceA* immediately downstream of *srbA* was not significantly affected in Δ*srbA*. Expression of *aceA* was compared between biofilm cultures of PA14 and Δ*srbA* using qPCR. The column is the mean of 3 biological replicates and error bars are the standard error of the mean.

4.3 Characterization of Δ*srbA* Biofilms

Having confirmed that Δ*srbA* contains a clean chromosomal deletion of *srbA*, I was interested in whether there was an observable difference in its biofilm physiology. I first used crystal violet staining to characterize any differences in the ability of Δ*srbA* to form biofilms. Interestingly, I found Δ*srbA* biofilms displayed a significant reduction in staining by crystal violet relative to PA14. Δ*srbA* produced only 34% of the amount of biofilm mass compared to PA14 as measured by crystal violet staining (Figure 19A).

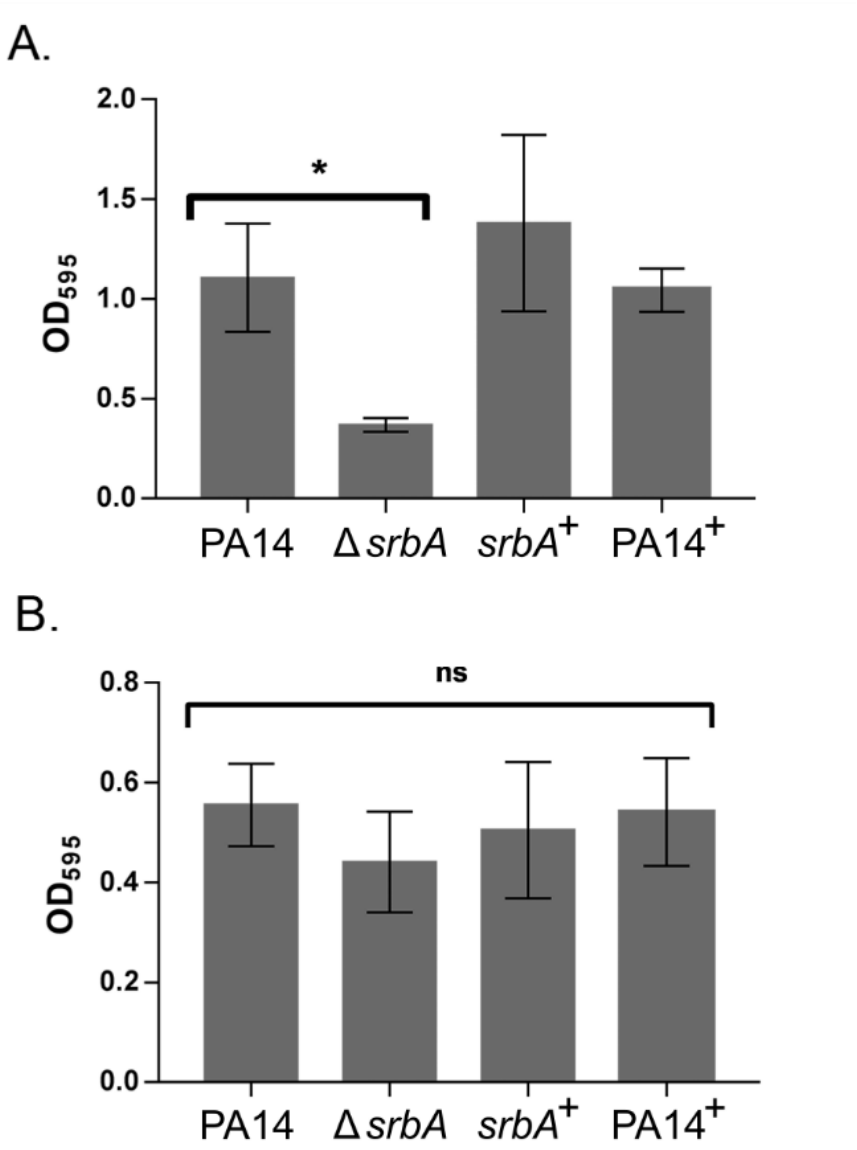

Figure 19. Crystal violet staining of PA14 and Δ*srbA* biofilms. (A.) Static biofilms grown for 24 h after which biofilms were stained with 0.1% crystal violet. Amount of crystal violet taken up into biofilms was measure by optical density at 595 nm (OD_{595}). A Bonferroni-Dunn test was used to determine significance where * represents $p < 0.05$. (B.) Crystal violet staining for rapid attachment of cells beginning biofilm formation. A one-way ANOVA was performed to determine no significance (ns). PA14 and Δ*srbA* for these

experiments carried empty pUCP18 vectors. PA14^{+} and *srbA*$^{+}$ carried the pUC*srbA* construct. All strains were grown with 100 μg/ml carbenicillin in media. Columns represent the mean of 4 biological replicates and error bars represent the standard error of the mean.

I also performed a rapid attachment assay to gain a better understanding whether early biofilm development or later stages of biofilm maturation were affected in Δ*srbA*. Rapid attachment assays are performed in the same manner as crystal violet staining of biofilms, however, incubation of the cultures occurs only for 30 min. This short incubation allows for cells to irreversibly attach to the well but not undergo any significant biofilm growth. Both flagella and type IV pili play a necessary role in *P. aeruginosa* adherence to surfaces and subsequent establishment of biofilms[109,132,237]. Rapid attachment assays use crystal violet to stain adherent cells early after inoculation of cultures (~30 min). Within this early time-frame, cells have had the opportunity to irreversibly attach, undergoing the first step in forming biofilms. While mature biofilm mass after roughly 24 h is significantly reduced in Δ*srbA*, early attachment of cells is unaffected relative to PA14 (Figure 19B).

After having measured a significant reduction in biofilm mass through crystal violet staining, I was interested in directly observing Δ*srbA* biofilms. The following microscopy experiment is presented for the first time in this thesis and has not been through peer-review. Using fluorescence microscopy, I performed "live-dead" staining of biofilms. For these assays, to observe as natural a biofilm morphology as possible, I opted for growth of biofilms using a flow cell system. I grew biofilms for 72 h under continuous flow of M63-glucose. After growth of biofilms, I stained flow cells with Syto 9 for live cells and propidium iodide (PI) for dead/permeable cells. Under phase-contrast, I observed that Δ*srbA* had a dramatically different biofilm morphology compared to that

of PA14. The PA14 wild type displayed a phenotype of large biofilm macrocolonies that are characteristic of *P. aeruginosa* grown with glucose as a carbon source (Figure 20A). In contrast, Δ*srbA* was only capable of developing small microcolonies after 72 h of growth in flow cells (Figure 20B). This indicated that SrbA does indeed have a role in the later stages of maturation and development in biofilms of *P. aeruginosa*. Live cells were easily visible in PA14 and Δ*srbA* with Syto 9 staining in fluorescence microscopy. However, I observed that PA14 macrocolonies displayed greater PI staining than Δ*srbA* (Figure 20). The adherent nature of the biofilm EPS matrix retains dead cells and is itself comprised of contents such as eDNA[65,138]. While both PA14 and Δ*srbA* had healthy cell populations, the biofilm formation deficiency of Δ*srbA* resulted in little retention/generation of dead cells or matrix components. The continuous movement of fluid in the flow cell assay probably washed away dead cells that could not be retained in the Δ*srbA* microcolonies.

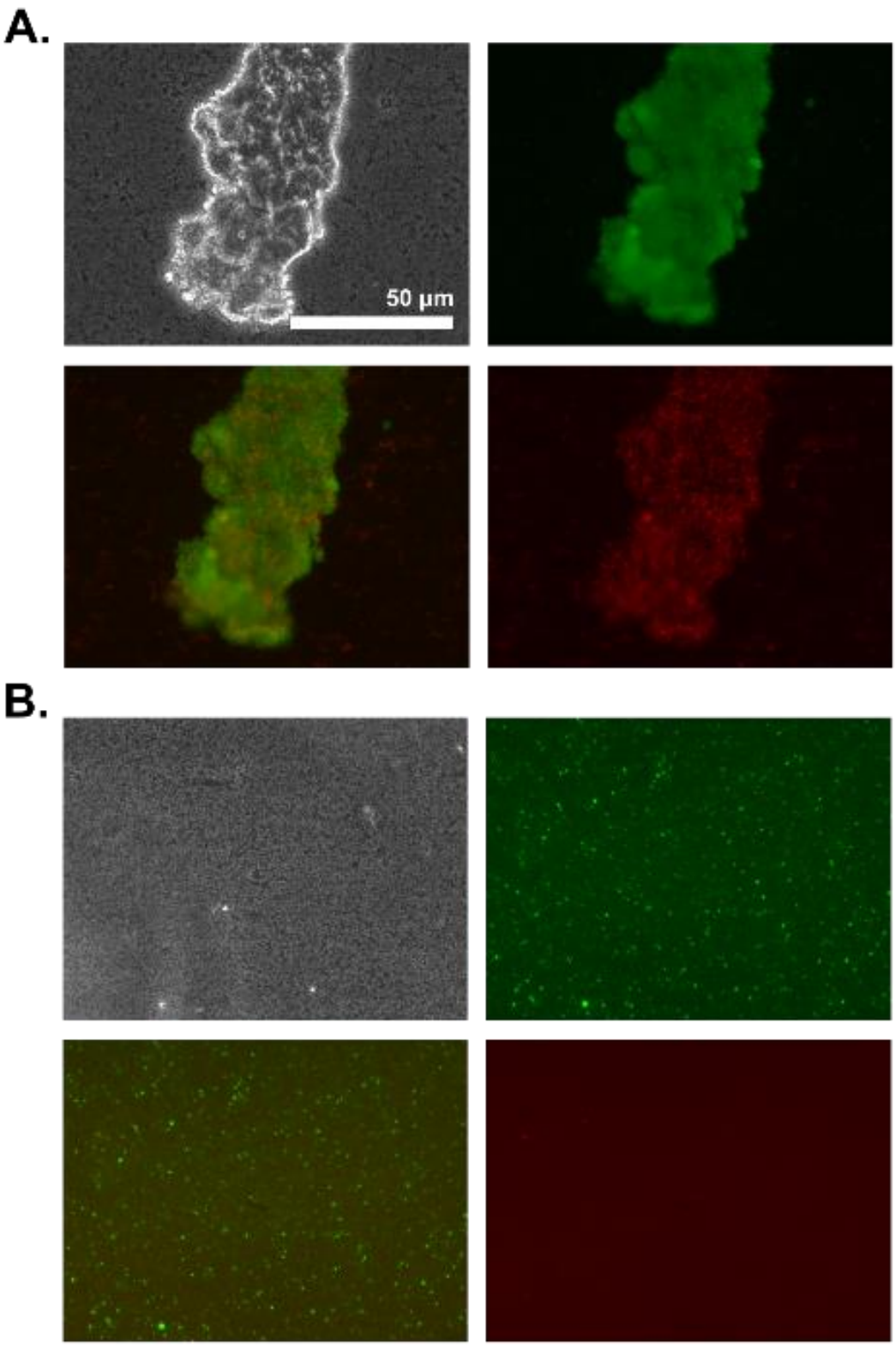
A.
50 µm
B.

Figure 20. Fluorescence microscopy of Δ*srbA* biofilm physiology grown on flow cells. For PA14 (A.) and Δ*srbA* (B.) images of (clockwise from top left) phase contrast, Syto 9 fluorescence, propidium iodide (PI) fluorescence, and merged fluorescence were utilized to observe biofilm morphologies. Images used are from one of three biological replicates. For each replicate 30 fields of view were observed for each strain.

4.4 Swarming Motility in Δ*srbA*

For this work, I largely focused on the role SrbA has in biofilms. However, I was also curious whether motility, and specifically swarming motility, was affected in Δ*srbA*. These swarming assays have not previously undergone any peer-review. Swarming cells often have many traits that are in common with biofilm cells: intercellular communication occurring in the form of quorum sensing and secretion of specific signalling molecules as well as heterogeneity of cellular populations[56,238–241]. These two modes of growth can also be considered opposite ends of a spectrum. Biofilms are sessile conglomerates while swarm cells are highly motility. Biofilm cells downregulate many virulence factors while swarm cells will highly express virulence factors and antibiotic resistance mechanisms[56,216,242]. When testing Δ*srbA*, I found that it is completely deficient in swarming motility (Figure 21). In the biofilm formation assays reported above, I was able to restore wild-type levels of biofilm formation in Δ*srbA* through complementation with pUC*srbA*. For swarming motility, I was unable to restore wild-type levels of swarming in Δ*srbA* through use of any high (pUC*srbA*), low (pBBR1MCS-5::*srbA*), or inducible (pMQ*srbA*) expression systems for *srbA*. Under the same conditions, PA14 consistently displayed characteristic tendril formation of motile swarm colonies. Considering this swarming deficiency in addition to the inability of Δ*srbA* to form mature macrocolonies it is possible that the strain is

capable of expressing flagella and pili but the systems are inactive or dysregulated in their functionality

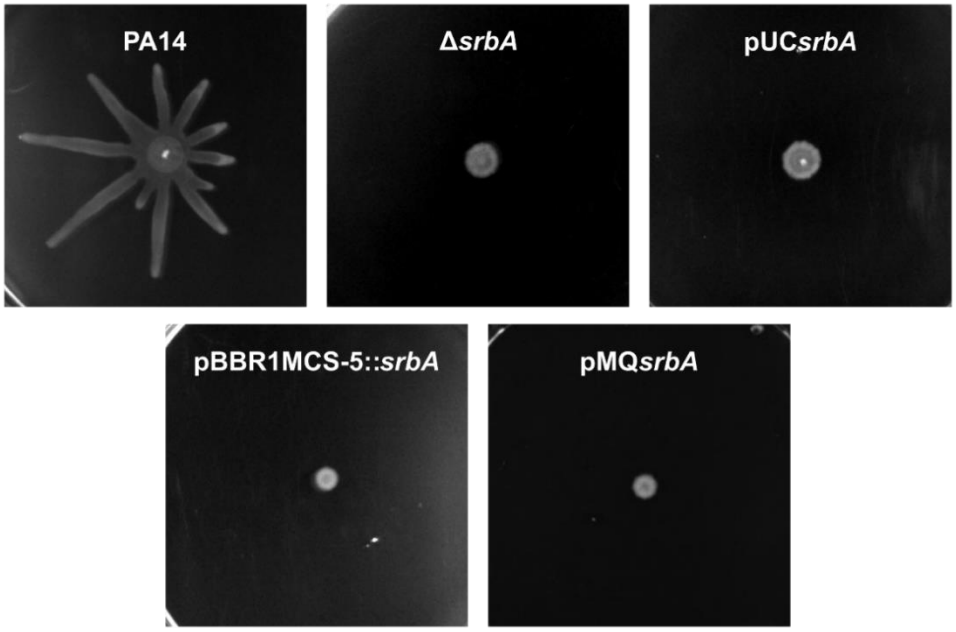

Figure 21. Swarming motility in Δ*srbA*. PA14 displayed motility on swarm agar. Complementation of *srbA* expressed from high-copy (pUC*srbA*), low-copy (pBBR1MCS-5::*srbA*), and inducible (pMQ*srbA*) vectors did not restore wild-type swarming.

Due to this inability of Δ*srbA* to swarm even when complemented in multiple different ways, I was then curious whether there was a general growth deficiency present as a result of mutagenesis. After performing growth assays in rich (LB) and minimal (1% tryptone and M63-glucose) media I found there was no growth deficiency present in Δ*srbA* (Figure 22).

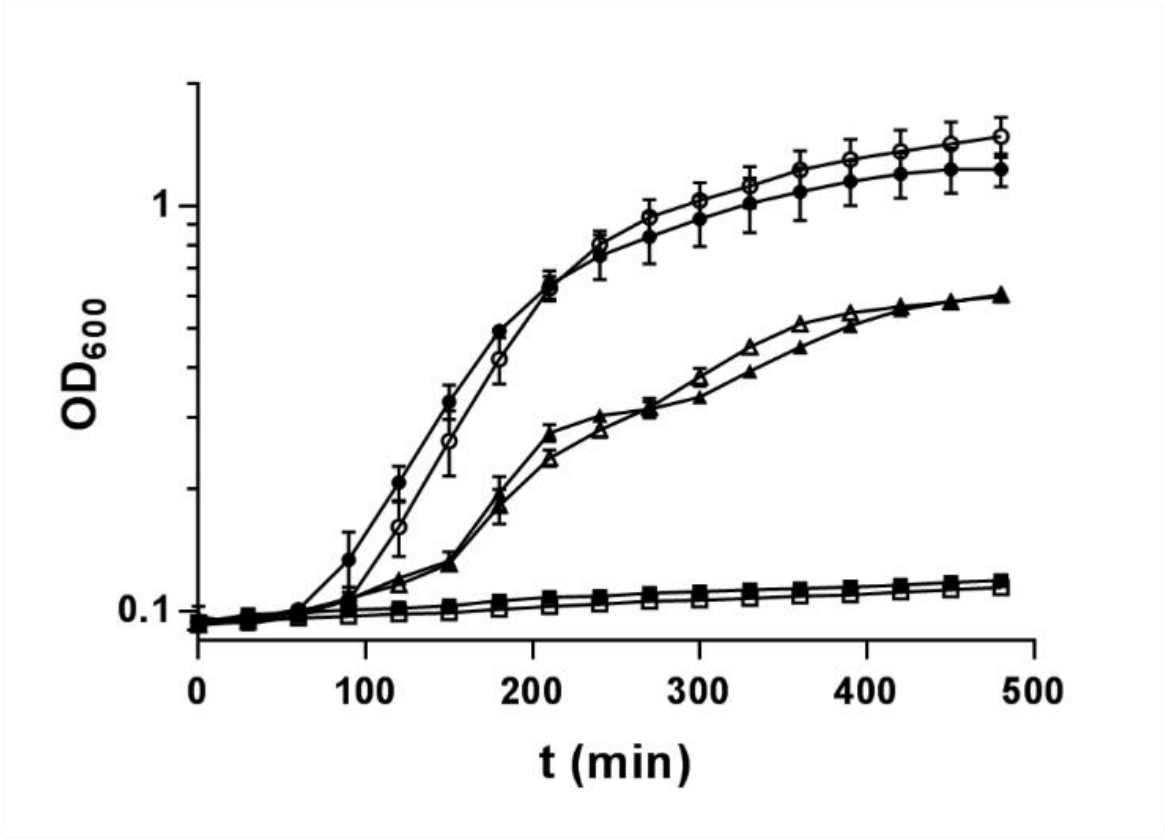

Figure 22. Assessment of Growth in Δ*srbA*. PA14 (solid fill data points) and Δ*srbA* (open data points) were cultured in rich medium LB (circles) as well as minimal media 1% tryptone (triangles) and M63-glucose (squares). Samples were taken every 30 min for optical density as a measure of culture growth. Data points are the mean of 3 biological replicates. Error bars are the standard error of the mean.

Finally, to further test phenotypes of Δ*srbA,* I was curious whether there were any effects on its resistance to antibiotics. I tested ciprofloxacin, gentamicin, and tobramycin against Δ*srbA*. The only differences I found across MICs was in ciprofloxacin which displayed a 2-fold decrease in Δ*srbA*. It is unlikely this can account for the significant differences observed in biofilm formation and swarming motility (Table 5).

Table 5. MICs and MBC-Ps for Δ*srbA*.

	MIC (μg/ml)[a]		MBC (μg/ml)[a]	
Antibiotic	PA14	Δ*srbA*	PA14	Δ*srbA*
Ciprofloxacin	0.1	0.05	0.8	0.8
Gentamicin	2	2	16	16
Tobramycin	2	2	16	16

a. Data listed are the mode of 5 biological replicates.

The swarming motility phenotype of Δ*srbA* (Figure 21) contrasts with growth assays (Figure 22), antibiotic challenges (Table 5), and the fact that Δ*srbA* is not deficient in its ability to adhere to surfaces (Figure 19). I was curious as to whether there may be any dysregulation of flagella in Δ*srbA*. I chose *fliK* and *flgJ* as representatives for the expression of their respective operons. Each are members of separate gene clusters for the production of the flagellar apparatus and secreted products in *P. aeruginosa*[93]. Through use of qPCR, I investigated whether there was dysregulation of flagellar genes *fliK* and *flgJ* but found there was no significant difference between Δ*srbA* and PA14 (Figure 23). Here the gene *pslE* was also included as an important gene for biofilm formation but not swarming motility. It is part of the Psl polysaccharide synthesis operon which is an important part of the EPS matrix. I found that *pslE* was moderately up-regulated in Δ*srbA* (Figure 23). However, it is unlikely that this change in expression could alone explain the drastic reduction biofilm formation in Δ*srbA* and *pslE* has no role in regulating swarming motility. This led me to consider whether the biofilm deficiency in Δ*srbA* is due to dysregulation of the flagellar apparatus. It may be possible that the apparatus is being expressed but is not active in Δ*srbA*. This could be a result of other signalling mechanisms being dysregulated in Δ*srbA* and this idea will be explored further in the Discussion (Chapter 5).

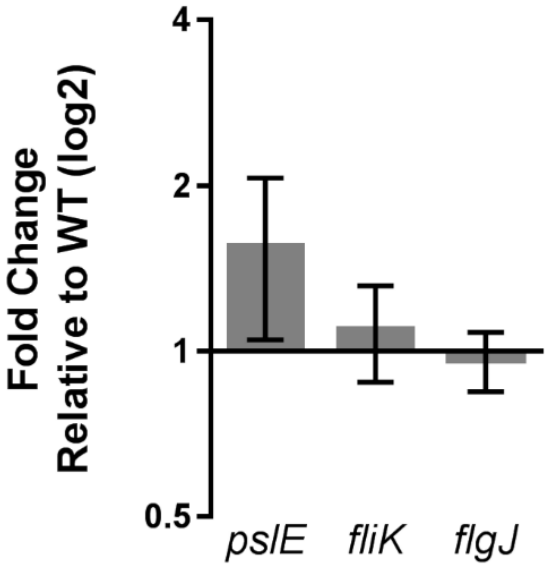

Figure 23. Expression of flagellar genes in Δ*srbA*. qPCR was used to determine the presence of dysregulation in flagellar genes in Δ*srbA*. *fliK* and *flgJ* were used as representatives from two large gene clusters for flagellar genes in the PA14 chromosome. *pslE* was chosen as a representative for Psl synthesis genes for biofilms. Columns are the mean of 3 biological replicates. Error bars are the Standard error of the mean.

4.5 Characterization of Δ*srbA* Effect on Colonizing the Gut of *Caenorhabditis elegans*

Having observed that Δ*srbA* has a distinct biofilm deficiency, I further questioned whether there was an effect on its ability to establish infections. I hypothesized that SrbA may also be involved in pathogenicity of *P. aeruginosa* through its role in biofilm formation. Biofilms are an important part of *P. aeruginosa* successfully colonizing a host and to its success as a pathogen. I therefore set out to determine if the biofilm deficiency of Δ*srbA* led to a reduced ability to persist in an animal host. To test this hypothesis, I used a slow-killing assay in *C. elegans* which was

developed previously[204,243]. *C. elegans* were placed on lawns of either PA14 or Δ*srbA* that worms then proceeded to feed on resulting in *P. aeruginosa* gut infections. These *P. aeruginosa* infections are inevitably fatal to the worms within 72 hours and a measure of mortality can be made from this slow-killing assay. Counts of dead worms were taken every 24 h and after 72 h, I found that worms feeding on Δ*srbA* had a mortality rate of 39% which was significantly lower than a mortality rate of 78% observed in worms feeding on PA14 (Figure 24). For this assay, the *E. coli* strain OP50 was also tested as a control. It is non-virulent in *C. elegans* and the worms use OP50 as food under laboratory conditions. I observed there was no mortality in worm plates that fed on *E. coli* OP50. Through use of a *C. elegans* model, I was able to determine that indeed SrbA has a role in *P. aeruginosa* pathogenicity likely due to its involvement in biofilm formation. The slow-killing of *C. elegans* replicates a scenario of chronic biofilm infection. However, it is still possible that virulence factors and swarming motility characteristic of acute infections may have an impact on this slow-killing model. As Δ*srbA* was found to also be swarming deficient, it is possible this may also be contributing to the attenuation in pathogenicity observed here.

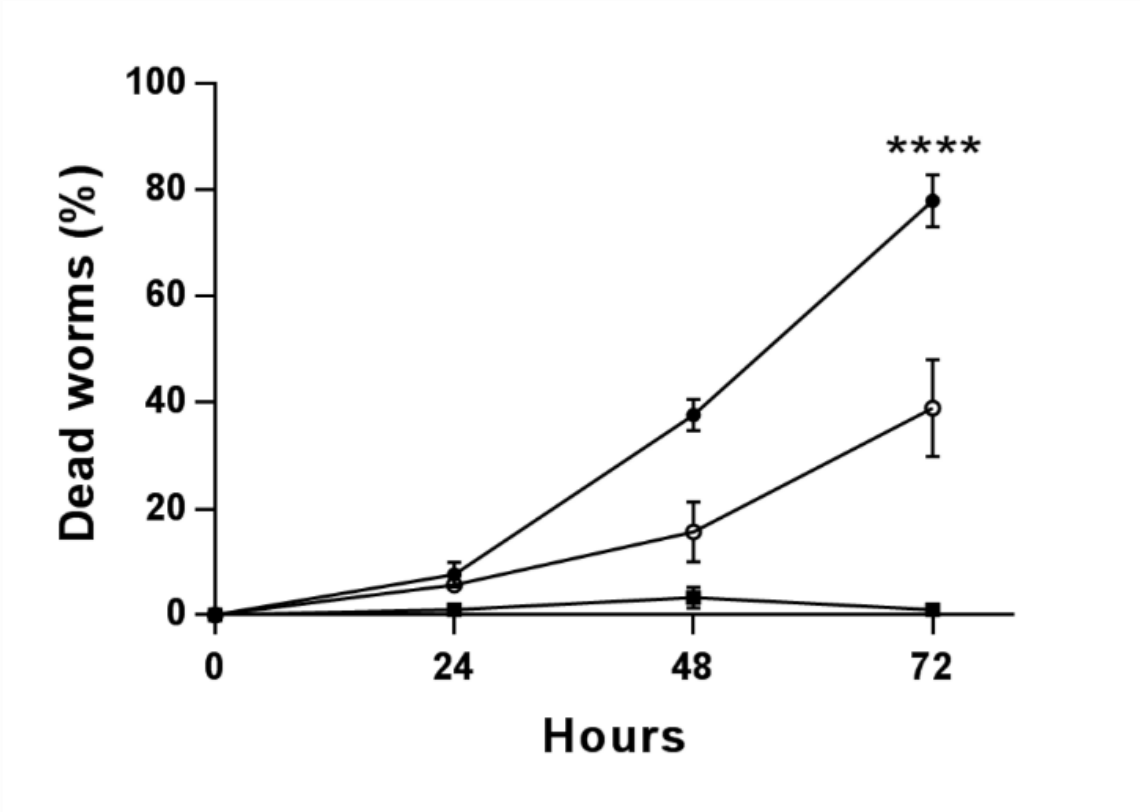

Figure 24. Slow-Killing of infected *C. elegans*. Worms were incubated for 72 h at room temperature in a humid environment and left to feed on a bacterial lawns. Bacterial strains tested include PA14 (closed circles), Δ*srbA* (open circles) and negative control for mortality, *E. coli* OP50 (closed squares). Data points each 24 h are the mean of 3 experiments. The error bars are the standard error of the mean. A Bonferroni-Dunn test was performed for PA14 and Δ*srbA* to determine statistical significance where **** represents $p < 0.0001$.

4.6 Putative Target Complementarity of SrbA

sRNAs are capable of acting on multiple different mRNA targets[184,185,189]. I was interested in determining what potential targets SrbA may be interacting with that may explain its role in biofilms. Biofilm formation and regulation is complex and involves a large number of pathways

in *P. aeruginosa*[25,134,244–246]. sRNAs typically act on their targets through short stretches of complementarity to mRNA transcripts. This affects levels of translation through interactions with the ribosomal binding site (RBS) or affecting mRNA stability via recruitment of RNase E[182,183,185,189]. I searched for putative targets by querying the whole SrbA transcript against the UCBPP-PA14 reference genome using TargetRNA2 and BLASTn. TargetRNA2 is a browser based tool specifically built to search for mRNA targets of sRNAs[223]. Targets found via BLAST searches were manually curated by myself. I checked alignments that were returned by BLAST to determine that complementary, antisense base-pairing interactions could occur between SrbA and the putative target. I finalized a list of putative targets by selecting hits that occurred using both TargetRNA2 and BLAST searches. In total there were 61 putative targets that I found in both methods. I generated a multiple sequence alignment of these putative targets that is represented in Figure 25.

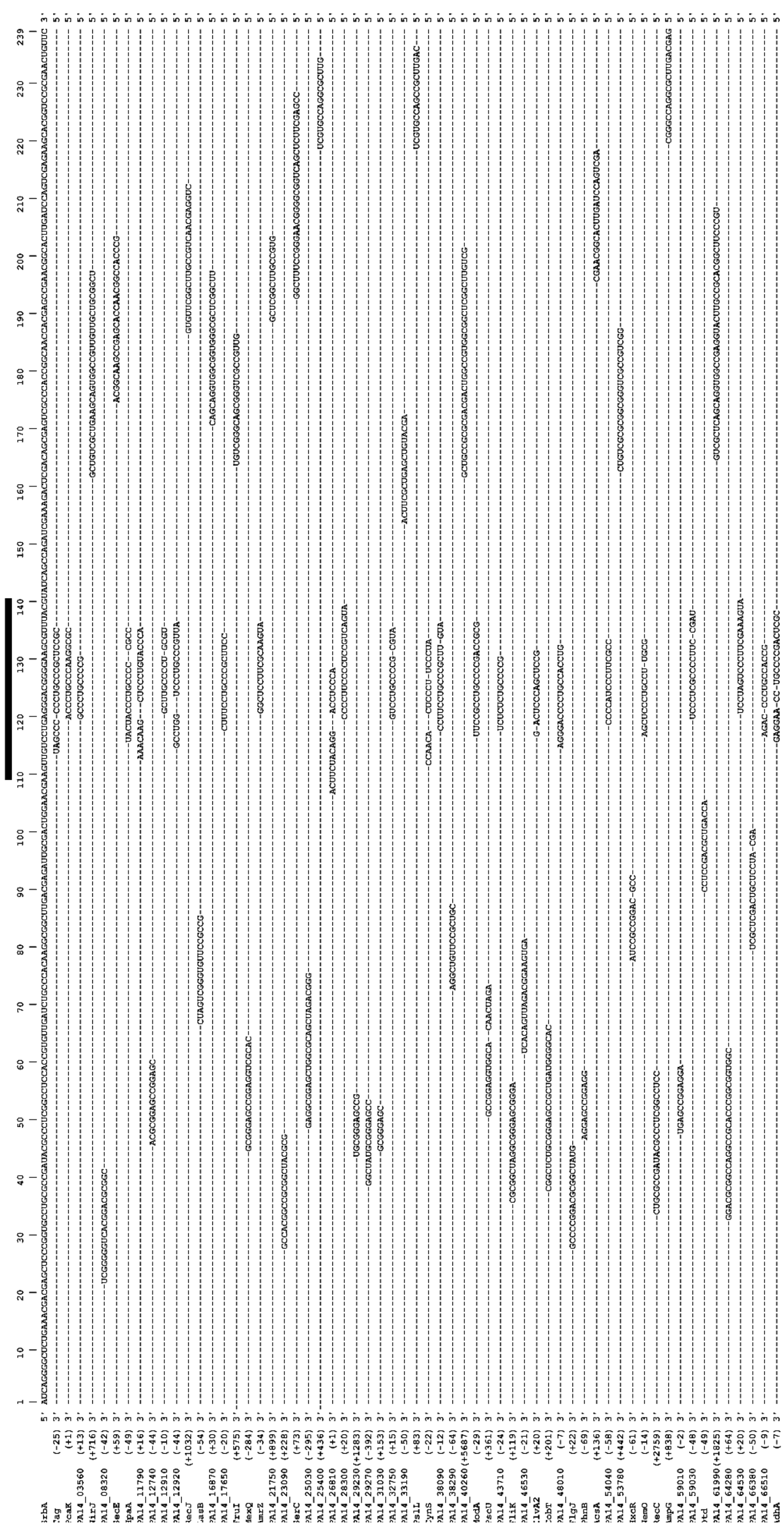

Figure 25. Multiple sequence alignment of putative targets with SrbA. A FASTA format alignment was generated using Clustal Omega. Transcript length of SrbA is indicated by the top row. Relative 5' to 3' orientations are provided for SrbA and putative targets. Black bars flanking sequence alignments indicate an area of higher density of putative targets. The bracketed numbers indicate where the alignment occurred relative to the translational start site of the putative target. If the gene has not been characterized, the PA14 gene designation is provided.

Using the list of putative targets generated, I performed qPCR comparing Δ*srbA* to PA14 under biofilm conditions to determine whether there was any noticeable effects on the expression of these putative targets. I found that 26 putative targets displayed an increase (2 by ≥4-fold, 7 more by ≥2-fold) or decrease (12 by ≥4-fold and 5 more by ≥2-fold) relative to PA14. The fold-changes I observed here may be due to altered stability of these mRNA transcripts due to dysregulation of RNase E recruitment in the absence of SrbA (Table 6).

Table 6. Expression levels in Δ*srbA* biofilm cultures of putative targets of SrbA in the UCBPP-PA14 genome.

Gene Annotation	Gene Name	Gene Function	Fold Change Difference[a]
Metabolism			
PA14_00110	*tag*	DNA-3-methyladenine glycosidase I	-1.81 ±0.21
PA14_06670	*nirJ*	Heme d1 biosynthesis	+6.64 ±6.56
PA14_11000	*hpaA*	4-Hydroxyphenylacetate 3-monooxygenase large chain	+2.17 ±1.56
PA14_21750		Putative acetyltransferase	-1.38 ±0.33

PA14_23090		Putative 2-Keto-3-deoxy-6-phosphogluconate aldolase	-1.90 ±0.09
PA14_23270	*serC*	3-Phosphoserine aminotransferase	-2.16 ±0.09
PA14_25400		Putative phosphodiesterase	-3.25 ±0.15
PA14_37965	*cynS*	Cyanate hydratase	+1.06 ±0.33
PA14_38090		Putative pseudouridylate synthase	+1.33 ±0.71
PA14_47100	*ilvA2*	Threonine dehydrastase	+1.83 ±0.88
PA14_47670	*cobT*	Cobalamin biosynthesis	+2.58 ±1.85
PA14_48010		Putative semialdehyde dehydrogenase	+2.82 ±2.69
PA14_51350	*phnB*	Anthranilate synthase component II	-1.39 ±0.22
PA14_52800	*acsA*	Acetyl-coenzyme A synthetase	+1.52 ±0.60
PA14_54040		Putative amino acid permease	+1.08 ±0.58
PA14_55580	*nemO*	Heme oxygenase	-8.94 ±0.02
PA14_60100	*dtd*	Deoxycytidine triphosphate deaminase	-1.82 ±0.24
PA14_68670		Putative carboxypeptidase	-2.03 ±0.35
PA14_71630	*adhA*	Alcohol dehydrogenase	-3.64 ±0.11
Transport and Secretion			
PA14_02900	*pcaK*	4-Hydroxybenzoate transporter	-1.17 ±0.66
PA14_08695	*secE*	Protein secretion across cytoplasmic membrane	+7.82 ±5.74
PA14_11790		Putative amino acid transporter	-1.90 ±0.27
PA14_12920		Putative taurine ABC transporter periplasmic protein	+1.00 ±0.59
PA14_16870		Probable ATP-binding component of ABC transporter	-1.46 ±0.45
PA14_18250	*fruI*	Phosphotransferase system transporter	-3.11 ±0.16
PA14_25020		Probable ATP-binding component of ABC transporter	-1.23 ±0.21
PA14_31030		Putative cation efflux system protein	-1.43 ±0.29
PA14_40390	*modA*	Molybdate binding precursor	+1.75 ±1.38

PA14_53780		Probable major facilitator superfamily transporter	-2.29 ±0.37
PA14_55440	*hxcR*	Type II secretion system protein	-5.72 ±0.13
PA14_64280		Probable permease of ABC transporter	-5.37 ±0.17
PA14_66380		Putative potassium/proton antiporter	-4.19 ±0.12
Virulence Associated Factors			
PA14_16250	*lasB*	Metalloproteinase	+2.99 ±2.29
PA14_35600	*pslL*	Exopolysaccharide synthesis	-1.29 ±0.09
PA14_42660	*pscU*	Translocation protein in type III secretion	-1.82 ±0.38
PA14_45830	*fliK*	Flagellar hook-length control	-1.19 ±1.63
PA14_50380	*flgJ*	Flagellar structural component	+1.18 ±1.13
Antibiotic Resistance Associated Factors			
PA14_18780	*mexQ*	RND efflux transporter	-1.95 ±0.47
PA14_57100	*ampG*	Permease signal transducer involved in β-lactam resistance	-2.74 ±0.18
Regulation and Signaling			
PA14_20290	*amrZ*	Transcriptional regulator of type VI secretion	+2.03 ±0.48
PA14_26810		Putative two-component sensor	-1.10 ±0.49
PA14_29260		Probable transcriptional regulator	+1.02 ±0.15
PA14_40260		Probable transcriptional regulator	+1.54 ±0.75
PA14_43710		Putative methyl-accepting chemotaxis transducer	+1.56 ±0.43
PA14_66510		Putative MFS transporter	-1.60 ±0.57
Genetic Maintenance and Repair			
PA14_16220	*recJ*	Single-stranded DNA specific exonuclease	-2.06 ±0.14
PA14_55690	*recC*	Exodeoxyribonuclease V gamma chain	-4.31 ±0.12
Unknown Function, Hypothetical Protein			
PA14_03560			-4.52 ±0.13
PA14_08310			-1.07 ±0.31
PA14_12740			+1.08 ±0.45

PA14_12910			-1.67 ±0.38
PA14_17650			-1.75 ±0.38
PA14_28300			-4.17 ±0.03
PA14_29230			-4.85 ±0.11
PA14_32750			-10.63 ±0.07
PA14_33190			-1.13 ±0.66
PA14_38290			+1.29 ±1.09
PA14_46530			+2.38 ±1.17
PA14_59010			-1.79 ±0.26
PA14_61990			-16.80 ±0.04
PA14_64530			+1.01 ±0.67

a. Results from qPCR are presented as the linear fold-change difference of transcript levels in Δ*srbA* relative to PA14 grown as biofilms. Values are the mean of 3 biological repeats and the standard error of the mean is uncertainty.

Through an in silico approach, I additionally generated predicted folded structures of SrbA (Figure 26). This data on the secondary structure of SrbA has not been peer-reviewed. I used the web server based application RNAfold to create structures[227,247]. These were generated with either an emphasis on contribution of minimum of free energy (MFE) or with a primary constraint on the smallest base-pairing distances possible (centroid structure). The MFE structure of SrbA was calculated to have a theoretical MFE of -82.40 kcal/mol (Figure 26A) and the centroid structure has a theoretical MFE of -70.90 kcal/mol (Figure 26B). Both structures are highly similar with the greatest variation roughly occurring at nucleotides 30-100 and 215-239. With the exception of the hairpin loops in those regions, there is the lowest probability of nucleotide base-pairing within SrbA as indicated by colouration of probabilities provided (Figure 26). Both structures predicted and conserved all hairpin loops in SrbA. These hairpin loops contain nearly every nucleotide that

has a probability of 1 for base-pairing interactions. This gives confidence that these secondary structures are likely occurring in the in vivo folded structure of SrbA. Furthermore, these regions with predicted hairpin loops align with many of the putative mRNA targets found (Table 6 and Figure 25). This gives insight into the actual structure as well as interaction targets of SrbA may and will be explored further in the Discussion (Chapter 5).

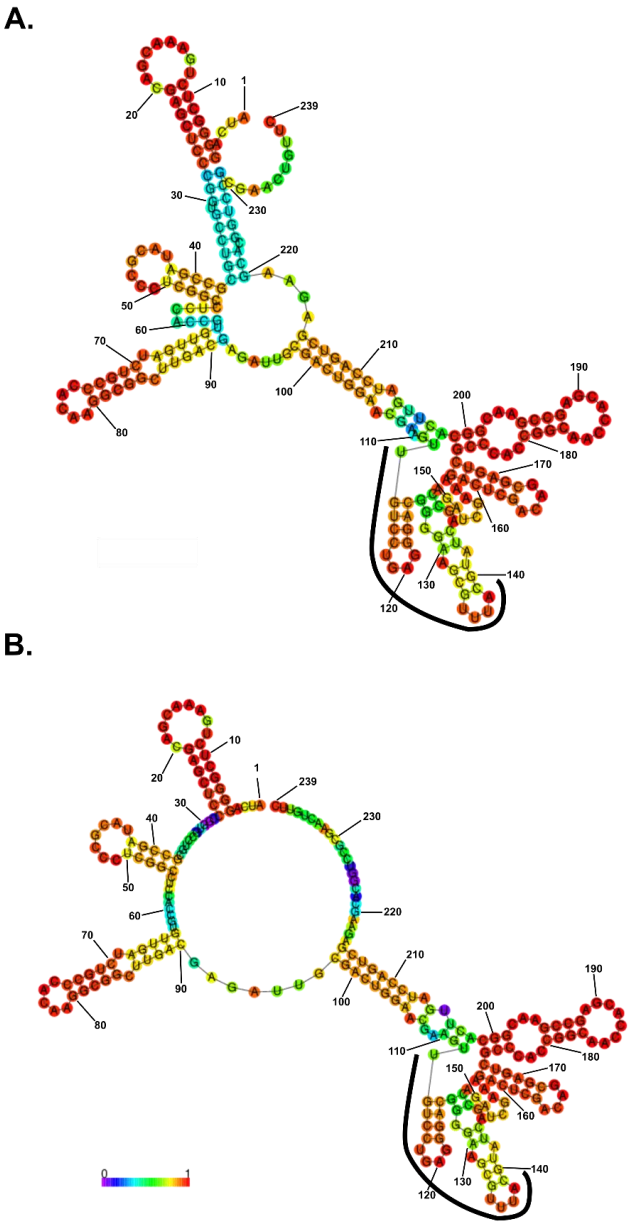

Figure 26. Predicted secondary structures of SrbA. Structures were generated using algorithms from the ViennaRNA package[227] giving preference to constraints on the contribution of minimum free energy (MFE) (**A.**) and minimum base-pairing distances (**B.**). Black lines (nucleotides 110 to 140) indicatea conserved secondary structure of hairpin loops. These regions are also indicated as a high density region of alignment in Figure 25. Nucleotides of the whole coding sequence of SrbA (denoted for every 10 bp) were also measured for their probabilities to potentially be involved in either intra- or inter-RNA molecule base-pairing. Probabilities of 0 to 1 are represented by colours in the spectrum provided.

5 DISCUSSION

5.1 The Two-Component System TctD-TctE and its Role in Biofilms and Tobramycin Susceptibility in the Presence of Citric Acid

I set out with this project to characterize a biofilm-specific aminoglycoside susceptibility phenotype in Δ*tctED*. Here I have demonstrated that loss of *tctED* results in a heightened accumulation of tobramycin in biofilms, a lack of response to citric acid during biofilm formation, and an inhibition of cell growth in the presence of citric acid. In the sections below, I will explore these findings and discuss them with a focus on the importance of TctD-TctE to biofilm formation and the development of tolerance and resistance in *P. aeruginosa*. With this focus in mind, I will also consider future directions for research that can further our understanding of the roles TctD-TctE has in *P. aeruginosa*.

5.1.1 Accumulation of Tobramycin in Δ*tctED* Biofilms

Previous research in our lab found a biofilm-specific aminoglycoside susceptibility phenotype in Δ*tctED*[68,200]. I was able to further characterize this phenomenon and determined that Δ*tctED* accumulates tobramycin to a greater degree in its biofilms than PA14. However, this heightened accumulation only occurred when citric acid was present in the media (Figure 6). This effect of greater accumulation occurring only in the presence of citric acid is not in itself totally surprising. Products of *tct* genes were originally characterized for their regulation of citric acid uptake into the cell[175,177,178]. This work furthered our understanding of TctD-TctE by characterizing a connection of citric acid regulation and antimicrobial tolerance and resistance in *P. aeruginosa* biofilms. While the broader roles of TctD-TctE in growth inhibition and biofilm morphology are likely contributing factors to this effect on tolerance and resistance, those aspects will be discussed below. First, I will discuss the possible effects of TctD-TctE regulation and how this system may

be affecting antimicrobial tolerance and resistance in *P. aeruginosa* biofilms.

In *P. aeruginosa*, TctD-TctE acts to derepress expression of *opdH* and therefore its product, the OpdH porin[178]. This raises the question of whether there is then an issue of transport into the cell that may be occurring with loss of TctD-TctE. Relative to other Gammaproteobacteria, *P. aeruginosa* has low membrane permeability[248]. This is a contributing factor to its native resistance to many antibiotics and even mild compromises to its membranes can have significant effects that can lead to greater susceptibility in *P. aeruginosa*[249,250]. While tobramycin is taken across the cytoplasmic membrane by active transport, it crosses the outer membrane by mechanisms that are still not fully understood[251–253]. This raises the question of whether tobramycin can be taken up through OpdH, resulting in the higher accumulation observed in Δ*tctED* biofilms.

To address the possibility of tobramycin accumulation within *P. aeruginosa* cells, I over-expressed the drug efflux system PA1875-1877 in Δ*tctED*. PA1875-1877 is a novel efflux system that was previously characterized by the Mah lab[67]. Expression of PA1875-1877 did indeed lead to reduced tobramycin accumulation in Δ*tctED*^{efflux} (Figure 6). However, expression in PA14^{efflux} also resulted in a significant reduction of accumulation. The relative difference between the PA14^{efflux} and Δ*tctED*^{efflux} demonstrated that there was still a significantly greater amount of tobramycin in Δ*tctED* relative to PA14. The expression of the PA1875-1877 efflux system did not alleviate any potential tobramycin build-up within Δ*tctED* cells that might have been occurring. With these findings, I conclude that it is unlikely that loss of TctD-TctE results in greater uptake and accumulation of tobramycin within the cell. It is therefore more likely that mechanisms of tobramycin retention in the extracellular polymeric substance (EPS) matrix is contributing to the heightened accumulation of tobramycin within Δ*tctED* biofilms and this idea will be explored in greater detail below.

5.1.2 Biofilms of Δ*tctED* in the Presence of Citric Acid

This work began investigating a biofilm-specific aminoglycoside susceptibility phenotype in Δ*tctED*. With this project, I was finding that both susceptibility to and accumulation of tobramycin were uniquely affected in Δ*tctED* biofilms when citric acid was present. I began to wonder if biofilm formation itself was affected in Δ*tctED* when citric acid is present in the media. Upon testing biofilm growth in the presence of citric acid, I found that there was indeed a distinct biofilm phenotype in Δ*tctED* where this mutant strain was maintaining high biofilm formation in the presence of citric acid (Figure 9). In this assay, sub-inhibitory concentrations of tobramycin did not appear to exacerbate this phenotype (Figure 7).

P. aeruginosa is highly adaptive to its environment. It has been characterized in the literature that there are specific responses in biofilm morphology when citric acid is present[143]. *P. aeruginosa* forms flat mats without any of the complex domed or "mushroom-cap" colonies with fluid channels when biofilms are grown with citrate as carbon source. I observed through crystal violet staining and phase-contrast microscopy that PA14 did display reduced biofilm formation in the highest concentrations of citric acid tested (10 and 20 mM). In contrast, Δ*tctED* formed thick biofilms under the same conditions of 10 and 20 mM citric acid present in media (Figure 9). Comparison of PA14 and Δ*tctED* biofilms demonstrated no differences in biofilms when citric acid was not present. This agrees with previous work that first characterized the biofilm-specific antibiotic susceptibility of Δ*tctED*[68].

A possible contribution to the reduced biofilm formation of PA14 in high concentrations of citric acid (10 and 20 mM) could be due to an abundance of carbon sources. For these experiments, I used arginine as an alternative carbon source since the inhibition of Δ*tctED* growth due to citric acid (Figure 12) restricted me from using it as a sole carbon source. Effectively, the addition of

higher concentrations of citric acid as a carbon source in addition to arginine created richer media in these experiments. It has been found previously that with an over-abundance of nutrient sources in its surrounding environment there is an effect of nutrient-induced dispersion that has been observed in *P. aeruginosa*[254]. Therefore, in the presence of citric acid there are combined effects on wild type *P. aeruginosa* to develop flat, reduced biofilm morphology and nutrient-induced dispersion. Likely both of these effects are contributing to the sparse and low-level crystal violet staining of PA14 biofilm that I observed here.

The fact that Δ*tctED* maintains high levels of biofilm formation for all concentrations of citric acid tested is possibly due to dysregulation of citric acid uptake. Downstream effects of this would lead to further dysregulation of pathways, signaling, and metabolism that are regulating normal biofilm responses. However, Δ*tctED* does display a moderate increase of biofilm formation in a concentration-dependent manner in the presence of citric acid (Figures 7 and 9). This is suggestive of some other mechanism of sensing or uptake of citric acid that is still active in Δ*tctED* even if by an indirect mean.

From the microarray data (Table 4), it is clear that the regulon of TctD-TctE is much greater than just *opdH* as previously reported[178]. One gene of particular interest that was dysregulated in Δ*tctED* biofilms is *dnr*. This gene encodes the transcriptional regulator Dnr (**D**issimilatory **n**itrate response **r**egulator) which is a major regulator of nitrogen metabolism for anaerobic growth in *P. aeruginosa*[9,255,256]. Dnr and its regulatory targets have been demonstrated to impact biofilm formation in *P. aeruginosa*[257,258]. Dysregulation of pathways such as Dnr in Δ*tctED* may then provide some explanation for the increased biofilm formation observed. For these assays, I grew biofilms as static cultures in 12-well dishes angled at 45°. The visible bands on wells stained with crystal violet in Figure 9 indicate where the majority of cells were adhering and growing as

biofilms. This band was at the air-liquid interface of these static cultures. Without shaking this is where the highest concentration of oxygen is occurring.

In Δ*tctED,* I found that *dnr* is up-regulated 2.06-fold (Table 4). It is possible that Δ*tctED* is expressing nitrogen metabolism pathways for anaerobic respiration to a higher level than PA14, leading to the likelihood that Δ*tctED* is effectively primed for growth in lower oxygen environments. This may account for the higher crystal violet staining observed in Δ*tctED* biofilms (Figures 7 and 9B). As seen in Figure 9B, at 20 mM there is not only a band of stained biofilm at the air-liquid interface, but visible biofilm staining extends across the well which is, in fact, deeper down into nutrient media where oxygen concentrations will be lower than at the air-liquid interface. Higher expression of Dnr and its regulon for anaerobic respiration may actually be enabling Δ*tctED* to grow in conditions with lower concentrations of oxygen. Two different approaches could be used to test whether dysregulated expression of Dnr is contributing to the Δ*tctED* hyper-biofilm phenotype. The first would be to analyze the expression of *dnr* using qPCR when Δ*tctED* is grown in M63-arginine containing citric acid. Secondly, repeating biofilm growth assays under anaerobic conditions could be used to test whether Δ*tctED* maintains a hyper-biofilm phenotype relative to wild-type biofilms. Additionally, constructing a strain with constitutively high expression of Dnr could be used for comparison to Δ*tctED*. It is hypothesized that this high expression of Dnr would have a similar hyper-biofilm phenotype as Δ*tctED* with growth into lower oxygen layers within a liquid medium. Additionally, it would be of interested to determine if the response regulatory TctD may be found to interact with the *dnr* promoter region through use of precipitating TctD incubated with genomic DNA.

5.1.3 Connections in Tobramycin Accumulation and Biofilm Formation of Δ*tctED*

The maintenance of high biofilm formation when Δ*tctED* was grown in the presence of citric

acid led me to consider that this may at least in part explain the heightened accumulation of tobramycin. From my results, it can be argued that the increased biofilm mass of Δ*tctED* is largely due to secretion of EPS matrix. The reasoning for this is due to the fact that the majority of biofilm mass is comprised of EPS matrix[65]. Additionally, I demonstrated here in that Δ*tctED* has decreased cell growth in M63-arginine media that also contains citric acid as a carbon source (Figure 12). It is therefore possible that there is also reduced cell growth in Δ*tctED* biofilms when citric acid is provided as a carbon source. Considering these two observations, it is possible that Δ*tctED* biofilm mass from cultures grown in the presence of citric acid may be due to heightened production of EPS matrix. The increased biofilm production resulting in greater accumulation of tobramycin would create higher localized concentrations of tobramycin within Δ*tctED*. If there is also reduced cell growth within Δ*tctED* biofilms then it is possible that higher localized concentrations of tobramycin would be present around a smaller population of cells. This scenario may be contributing to the tobramycin susceptibility originally observed in Δ*tctED* biofilms. Further support for dysregulation of EPS matrix production comes from the fact that motility in Δ*tctED* was unaffected. Both flagella and pili which are essential for active forms of motility in *P. aeruginosa* are also important for the EPS matrix[109,143]. In swarming and swimming assays, I found no difference between the motilities of PA14 and Δ*tctED* (Figure 8). As discussed above, it is unlikely that high accumulation of tobramycin in Δ*tctED* is due to an abundance within the cell. Heightened secretion of the EPS matrix provides a possible explanation for both the heightened accumulation of tobramycin as well as susceptibility to this antibiotic observed in Δ*tctED*.

It has been well documented that antibiotics such as tobramycin readily diffuse into biofilms with little hindrance[67,88,259]. Furthermore, it has been demonstrated that aminoglycoside antibiotics like tobramycin are chelated by EPS components and are retained within the biofilm[228,229]. This

mechanism is referred to as diffusion-reaction inhibition to confer tolerance and resistance to aminoglycosides[229,260,261]. In a scenario of Δ*tctED* biofilm growth in high concentrations of citric acid, it is possible that heightened EPS secretion is chelating tobramycin and leading to increased accumulation in Δ*tctED* relative to PA14 only in the presence of citric acid, and even when there is greater efflux (Figure 6). Therefore, the greater susceptibility of Δ*tctED* to tobramycin under biofilm conditions may also in part be explained by heightened production of the EPS matrix. As stated above the effect of the EPS matrix helps mitigate the effects of aminoglycosides. However, in this situation of Δ*tctED* growth in citric acid there is also significantly reduced cell growth (Figure 12). It is likely that in Δ*tctED* biofilms there is also a decreased number of cells. The activity of tobramycin may be dampened by the mechanism of diffusion-reaction inhibition but higher accumulation of the antibiotic, and therefore higher localized concentrations within the biofilm, might be overcoming the diffusion-reaction inhibition effect by acting on an already diminished population of cells within the Δ*tctED* biofilm. It was demonstrated here that even in M63 media where arginine was provided as a preferable carbon source there was a reduction in growth of Δ*tctED* in a concentration dependent manner when citric acid was added (Figure 12). A higher localized concentration in this situation could then increase chances of tobramycin uptake and antimicrobial activity within cells through previously characterized mechanisms[249,253,262,263]. It is therefore possible that the increased biofilm formation of Δ*tctED* could in part explain the biofilm-specific aminoglycoside susceptibility observed in previous studies[68,200] as well as the heightened accumulation of tobramycin in biofilms described here.

5.1.4 Citric Acid Growth Inhibition in Δ*tctED*

I observed an increase in biofilm mass for Δ*tctED* as well as a heightened accumulation of tobramycin in Δ*tctED* biofilms. However, I also observed that there is a growth deficiency in

planktonic cultures of Δ*tctED* when citric acid is present. Similar to observations already discussed, this growth deficiency appeared to occur in a concentration dependent manner for citric acid (Figure 12). In fact, Δ*tctED* was rendered completely incapable of growth when citric acid was provided as the sole carbon source (Figures 12 and 14). I observed that this growth deficiency was dependent on only citric acid and was not affected by concentrations of tobramycin (Figure 12) or by the pH of media (Figure 10). Additionally, while there were moderate differences between PA14 and Δ*tctED* for select carbon sources, these were slight and not to the extreme observed with citric acid (Table 3 and Figure 27). I will discuss further the implications of the findings for other carbon sources below. I consider firstly that the growth deficiency of Δ*tctED* is an inhibitory effect in the presence of citric acid.

TctD-TctE has been previously characterized as the sensor-regulator for uptake of tricarboxylic metabolites in *P. aeruginosa*[178]. The study by Tamber et al (2007) found that TctD-TctE was important for regulating expression of the porin as loss of either TctD or TctE led to derepression of *opdH* expression. Therefore, loss of TctD-TctE would maintain expression of OpdH, regardless of concentrations of tricarboxylic acids being present in the external environment. This would likely result in unregulated and constant uptake of tricarboxylic metabolites in Δ*tctED*. It is possible that in Δ*tctED*, concentrations of citric acid would be at an equilibrium between external and intracellular environments. This would therefore be creating a lack of internal regulation and homeostasis of citric acid which would have downstream effects on other metabolic pathways within the cell that are sensitive to citric acid.

Previous studies that have investigated *P. aeruginosa* metabolic activity have found that when relative ratios of citrate metabolites are higher than others, there is a significant reduction in growth[231,232]. High ratios of citrate metabolites relative to other carbon sources inhibited *P.*

aeruginosa growth by reducing its own metabolic activity. My data fits with this model as I found that with increasing amounts of citric acid there was increasingly reduced growth in Δ*tctED* (Figure 12). It is possible that Δ*tctED* is unable to properly maintain internal homeostasis of citric acid thereby causing downstream effects that lead to reduced metabolism and growth. I was able to determine that the growth deficiency on citric acid as a carbon source in Δ*tctED* was due to the loss of TctD-TctE as restoring expression also rescued the ability of the strain to grow on citric acid when it was the sole carbon source (Figure 13). However, this does not address which regulatory pathways are being affected. Additionally, this does not resolve whether these are direct or indirect regulatory effects resulting from loss of TctD-TctE. Having performed a whole genome microarray, it is suggestive that TctD-TctE has a large regulon in *P. aeruginosa* (Table 4).

5.1.5 Altered Growth of Δ*tctED* on Various Carbon Sources

In addition to an inhibition of growth in the presence of citric acid, I also found that Δ*tctED* displayed moderate deviations in growth when cultured on 6 different carbon sources from a diverse set available on phenotypic microarray plates. When Δ*tctED* was grown on α-hydroxy-butyric acid, α-keto-butyric acid, adenosine, carnitine, putrescine, or uridine as sole carbon sources there were moderate but observable differences in growth curves (Table 3 and Figure 27). Besides α-hydroxy- and α-keto-butyric acids, there is no immediately obvious link between these compounds. The only links are some general chemical properties. This includes uridine and adenosine both containing aromatic rings as well as carnitine and the butyric acids sharing chemical similarities in that they each contain a carboxyl group. For these short chain compounds there appears to be no consistent patterns in growth either. Δ*tctED* displayed heightened growth relative to PA14 on α-keto-butyric acid and putrescine while reduced growth on α-hydroxy-butyric acid and carnitine (Table 3 and Figure 27). It is interesting to note that while α-hydroxy- and α-

keto-butyric acids have the same basal chemical structure each has different effects on growth of PA14 and Δ*tctED*. A possible explanation for the increased growth of Δ*tctED* relative to PA14 on putrescine and α-keto-butyric acid could be the fact that putrescine is used as a precursor for butyrate compounds in *P. aeruginosa*[264,265]. For growth on both adenosine and uridine as carbon sources Δ*tctED* displayed an increase relative to PA14. Conclusions are hard to draw from these moderate differences in growth seen in Δ*tctED*. It is also entirely possible that these growth differences between PA14 and Δ*tctED* may be within biological variation on these carbon sources that are not ideal metabolites for *P. aeruginosa*. These differences could possibly be due to specific conditions of my experimental setup. I performed three biological replicates with the phenotypic microarrays and observed these differences in growth. There may be other additional factors such humidity, temperature, or oxygenation that could be contributing to these differences for particular carbon sources where moderate changes between PA14 and Δ*tctED* were observed. By performing further growth assays similar to ones used for testing growth on citric acid, it may be possible to better control experimental conditions than the phenotypic microarrays which are optimized for being high-throughput assays. More experimental runs may be required to determine if growth on these carbon sources with moderate changes are biologically significant.

5.1.6 The TctD-TctE Regulon and Future Directions

The work I performed for my thesis furthered our understanding of biofilm regulation and mechanisms for tolerance and resistance to tobramycin in *P. aeruginosa*. I was able to conclude that deletion of TctD-TctE and growth in the presence of citric acid leads to heightened accumulation of tobramycin in biofilms, increased biofilm formation, and inhibition of cell growth in the presence of citric acid. Through performing both genetic and phenotypic microarrays for this thesis work, I found that TctD-TctE has a much larger regulatory role within the cell other

than just for expression of the OpdH porin and tricarboxylic acid uptake in *P. aeruginosa*. However, there are still aspects of TctD-TctE regulation that are unanswered.

I considered the possibility that heightened production and secretion of the EPS matrix is contributing to the greater accumulation of tobramycin and thick biofilm formation in Δ*tctED* when citric acid is present. Future investigations should aim to determine if this is the case by analyzing the components of the biofilm directly. Through use of mass spectroscopy techniques the components of the EPS matrix could be quantified and compared between PA14 and Δ*tctED*. Another avenue to explore the EPS matrix of Δ*tctED* and whether it is contributing significantly to the accumulation of tobramycin would be to degrade Δ*tctED* biofilms. Repeating accumulation assays while treating Δ*tctED* with proteinase K, periodate, and/or DNase could be used to determine if degrading the EPS matrix can mitigate heightened levels of tobramycin. Finally physically dislodging Δ*tctED* biofilms performing colony forming unit assays could be used to determine cell populations. I found that in planktonic cultures Δ*tctED* has reduced growth when citric acid is present. It would be worthwhile to verify that is also the case for biofilm cultures. Confirming that cell populations are low in Δ*tctED* biofilms would then also strongly suggest that accumulation of tobramycin is occurring in the EPS matrix of Δ*tctED* biofilms.

Given the large number of genes that are dysregulated in Δ*tctED* it is likely that TctD-TctE has multiple effectors that are contributing to aminoglycoside susceptibility and biofilm phenotypes observed in Δ*tctED*. I hypothesize that with the loss of TctD-TctE there is no homeostatic regulation of citric acid as in the proposed model of what is occurring in Δ*tctED* presented in Figure 27. Without TctD-TctE regulation of *opdH*, I believe that OpdH would then be constitutively in the outer membrane regardless of levels citric acid present in the surrounding environment. In this scenario, the Δ*tctED* strain would have no means to restrict uptake of citric

acid into the cell and the internal cellular environment would have similar concentrations as the external one in Δ*tctED*.

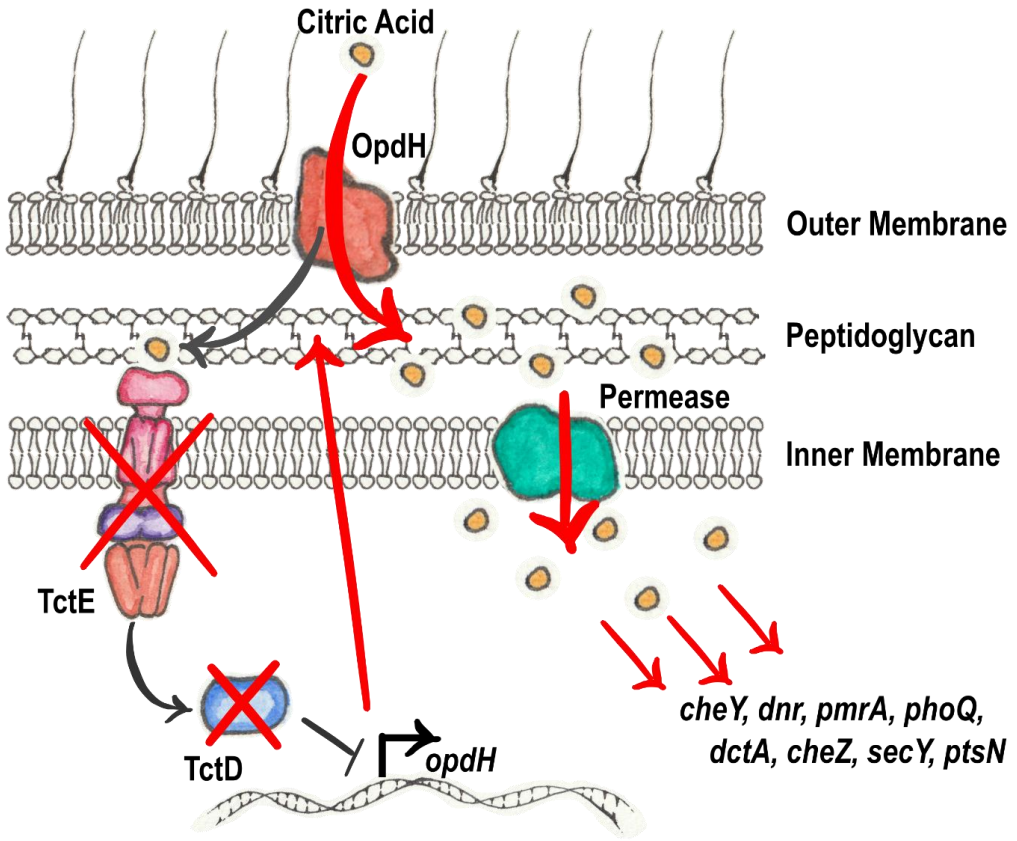

Figure 27. Proposed model for dysregulation of citric acid uptake in Δ*tctED*. Loss of TctD-TctE has been previously demonstrated to derepress *opdH*[178]. Normal regulation by TctD-TctE when citric acid is present in high concentations is represented in the pathway grey arrows. In Δ*tctED* it is hypothesized that pathways in red arrows are occurring. High expression of OpdH would occur in Δ*tctED* and there would be no response to limit citric acid movement into the periplasmic and intracellular spaces which is proposed to then result in downstream indirect effects on additional regulatory pathways for biofilm formation.

It is possible that other factors may then include indirect dysregulation of pathways. Indirect dysregulation would include pathways that are not directly regulated by TctD-TctE but are responsive to levels of citric acid. I demonstrated that TctD-TctE has a larger regulon than has been previously appreciated (Table 4). Genome-wide expression data from the microarrays I performed demonstrated that genes from a multitude of functional roles within *P. aeruginosa* are dysregulated in Δ*tctED* (Table 4). This included a large number of genes that currently have no experimentally determined function in *P. aeruginosa*. This category of genes with unknown function, referred to as genes for hypothetical proteins in *P. aeruginosa,* make up 15 of 23 total dysregulated genes under biofilm conditions and 46 of 94 under planktonic conditions (Table 4). A number of genes that were dysregulated in Δ*tctED* do have known functional roles in *P. aeruginosa*, though. A considerable portion of these genes (all of those with known function in biofilm conditions and 15 of 48 in planktonic conditions) have roles in cellular metabolism. Involvement in metabolism that includes synthesis pathways, transport across membranes, or electron transport chains[92,93]. However, other functional roles are represented. Notably, genes for other TCSs are represented by *cheY*, *phoQ*, and *pmrA* (Table 4 and Figure 27). There is also chemosensory regulator *cheZ*, transporter *dctA*, secretory regulator *secY*, and anaerobic respiration/nitrogen metabolism regulators *dnr* and *ptsN* that are represented (Table 4 and Figure 27). This suggests there is highly complex regulatory control occurring between multiple previously unconnected pathways. While not found in this work, it has been previously determined that TctD is capable of cross-talk with PhoB for regulatory activity over *ompR*[266]. Additionally, as discussed previously, phenotypic microarray data for growth on various carbon sources suggests that the regulatory role of TctD-TctE is not limited to uptake of tricarboxylic compounds.

Future work on TctD-TctE should aim to discern direct and indirect dysregulation occurring

due to loss of TctD-TctE. While many genes are dysregulated in Δ*tctED*, it is of interest to determine which ones may be responding to disrupted citric acid homeostasis within the cell and are subject to regulation by other pathways compared to genes that may be genuine targets of TctD-TctE for regulatory control. A method to investigate this possibility would be to use methods of ChIP-seq applied to bacterial studies[267]. Isolation of DNA-binding targets of TctD would be a means of determining promoter regions it may interact with. Additionally, genome microarrays were done with cultures grown in regular M63-arginine media. It would be of interest to repeat these experiments with Δ*tctED* grown in the presence of citric acid. Further experiments of this kind would also benefit from using RNA-seq methods to determine whole transcriptome changes that may be occurring. The benefit of this would be to also determine if there is dysregulation in non-coding RNAs which have been found to play significant roles in modulating metabolic responses in *P. aeruginosa*[191,198,199]. In this work I was able to determine physiological effects and biofilm roles, however, future work will require focus on resolving signaling pathways and mechanisms of TctD-TctE.

P. aeruginosa biofilm infections display high levels of tolerance and resistance to antibiotic treatment by means that are still not fully understood. Many increases cannot be attributed to antibiotic resistance mechanisms and likely are due to novel systems and from regulatory responses by *P. aeruginosa* that are not known. An involvement of TctD-TctE in tobramycin susceptibility and biofilm formation through a citric acid dependent means provides a potential avenue of investigation for future therapies. Development of novel therapies that take advantage of manipulating bacterial metabolism to extend the efficacy of existing antibiotics.

5.2 The sRNA SrbA is Important for Biofilm Formation and Pathogenicity

In my aim to characterize regulatory mechanisms of biofilm formation in *P. aeruginosa*, I also devoted my thesis work to investigating the roles of sRNAs. Through studying the transcriptome of *P. aeruginosa*, over 500 sRNAs have already been identified[181,186,187,268]. Studies have been undertaken to characterize the functional roles of a handful of novel sRNAs in *P. aeruginosa*[182,185,189,269]. However, this is only a fraction of sRNAs which have been verified to have biological roles within the cell. To contribute to our understanding of regulatory roles that sRNAs have in *P. aeruginosa*, I characterized SrbA, an sRNA I found to be important for biofilm formation and pathogenicity. Here I will discuss my findings of SrbA roles with a focus on biofilm formation, pathogenicity, and regulatory pathways that it is involved in. I will also discuss future directions for research to determine the regulatory targets of SrbA.

5.2.1 The Role of SrbA in Biofilms

From work I did previously[186,270] as well as for this thesis, I found that SrbA was significantly up-regulated in *P. aeruginosa* biofilms. This led me to investigate whether biofilm growth would be affected with loss of SrbA expression. Biofilm formation was indeed impacted as the deletion strain Δ*srbA* displayed a significant reduction in its ability to grow as a biofilm (Figures 19 and 20). Biofilms are complex and have strict regulation for their formation[62,108,109,121,138,259,260] and I sought to better characterize how SrbA may be involved. Through rapid attachment assays, I found that Δ*srbA* was able to carry out the initial stages of biofilm formation and irreversibly adhere to surfaces (Figure 19B). However, I observed that Δ*srbA* was unable to form biofilm macrocolonies and only maintained small microcolony structures (Figure 20B).

I considered that SrbA may have a role in regulation of flagella and pili in *P. aeruginosa* during biofilm formation. Both flagella and pili are essential for adherence and establishment of biofilms

in *P. aeruginosa*[130,131,133]. However, flagella and pili based motility has also been determine to play a crucial role in forming mature biofilms in *P. aeruginosa*[108,109,143,237]. Here I found that Δ*srbA* was deficient in its ability to swarm, a motility that involves the activity of both flagella and pili[271–273] (Figure 21). While Δ*srbA* displayed a swarming deficiency, I can conclude that the strain must be expressing flagella and pili as it was capable of adhering to surfaces to at least carry out the first stages of biofilm formation (Figure 19B). It is therefore likely that while flagella and pili may be expressed by the Δ*srbA* deletion mutant, there is a defect in the function of one or both of these structures. This would account for the ability of Δ*srbA* to adhere to surfaces and begin biofilm formation but be unable to develop mature macrocolonies and display a deficiency in swarming motility. Additionally, I found that flagellar genes *fliK* and *flgJ* are potential interaction targets of SrbA based on sequence complementarity (Figure 25). It is possible that SrbA directly regulates flagellar synthesis and activity that may be resulting in deficiencies of swarming motility and biofilm formation observed here.

It is interesting to note that regardless of complementation methods to restore expression of SrbA, I was unable to restore swarming motility (Figure 21). Swarming is a complex social behaviour, and in *P. aeruginosa* it has been shown that over 200 genes are active in swarm colonies[242,274]. It is possible that the expression vectors (pUCP18, pBBR1MCS-5, and pMQ72) that I used in this work are insufficient to restore native regulation as well as expression of SrbA. Timing as well as expression levels of sRNA have a significant impact their activity as these systems are important for modulating specific post-transcriptional regulation of their downstream targets[183–185,189]. If the native regulation and levels of expression of an sRNA are not restored, then there will likely be compounding effects as their downstream targets would also be dysregulated. Swarming motility is tightly regulated and it is possible that if SrbA has a role then lacking

restoration of the native regulation over the timing of its expression as well as levels of transcript would be required. Both pUCP18 and pBBR1MCS-5 that I used in attempting to restore swarming motility in Δ*srbA* have constitutive expression in *P. aeruginosa*. Additionally, pMQ72 requires arabinose induction of expression. It is possible that while any of these constructs may have been expressing SrbA, the native timing of expression for SrbA in swarming motility may not have been adequate.

It is also interesting that while complementation of SrbA expression was unable to rescue swarming, it was able to rescue wild-type levels of biofilm formation (Figures 19 and 20). It is worth mentioning that in previous work, I found that SrbA was significantly up-regulated in swarming cultures as well[186]. While not to the same high levels of expression found in PA14 biofilms there was still a higher expression of SrbA in swarming than in mid-log growth phase. Thus, it is likely then that SrbA has roles in both biofilm formation and swarming motility. These observations further suggest that there is some overlap but SrbA likely has distinct regulatory targets under conditions for biofilm formation and for swarming motility. These modes of growth are often considered as either end of a spectrum. Many of the same virulence factors, antibiotic resistance mechanisms, and internal signaling pathways are strictly regulated just in opposite fashions[56].

To gain insight into regulatory pathways that SrbA may be part of in biofilms and swarming motility, I generated a list of putative mRNA targets (Figure 25). I also tested whether there was any significant dysregulation of these putative targets in Δ*srbA* (Table 6). With regard to genes that have previously been found to be involved in swarming motility[274], I found two that were dysregulated in Δ*srbA*. The genes *ampG* (PA14_57100), which encodes a permease for β-lactamase induction[275], and *amrZ* (PA14_20290) which is a transcriptional regulator of T6SSs[276],

displayed fold-changes of -2.74 and +2.03 relative to PA14, respectively. It is not obvious how these genes would directly affect swarming motility or biofilm formation. It is possible they are part of larger regulatory programs that are currently unknown.

With regard to *ampG*, it is worth noting that it was significantly down-regulated in Δ*srbA* biofilms. This could suggest that SrbA has a role in biofilm resistance to β-lactam antibiotics. While I found that there was no change in the MICs of Δ*srbA* compared to the wild type strain for antibiotic classes of fluoroquinolones (ciprofloxacin) and aminoglycosides (tobramycin and gentamicin), I did not test any β-lactam antibiotics (Table 5). For future directions, I am interested in SrbA regulation of β-lactamase activity in *P. aeruginosa* and any resulting changes in biofilm resistance.

In Δ*srbA* biofilms, *amrZ* was significantly up-regulated. Previous research has found that AmrZ acts as a transcriptional regulator for all three T6SSs in *P. aeruginosa*[276]. T6SSs are an important system used interbacterial competition as well as defence against eukaryotes[277,278]. It works in concert with the Rsm sRNA system for regulation of T6SSs. Therefore, it would be of interest to determine if there is any involvement in other sRNA regulators. Specifically, I am interested in whether SrbA is a regulator of T6SSs in *P. aeruginosa*. It is possible that SrbA is acting as a negative regulator of AmrZ translation given the increase in *amrZ* observed in Table 6. Based on the importance that T6SS activity has for inter-species interactions, I am also very interested in how any SrbA regulation could be involved in interactions of *P. aeruginosa* with other prokaryotic and eukaryotic cells[279]. AmrZ has also been found to be important for regulating c-di-GMP synthesis in *P. aeruginosa* and, therefore, is also important for biofilm formation[280]. Future work should focus on expression of T6SS in Δ*srbA* due to the potential importance this may have in further characterizing the role SrbA has in regulating pathogenicity. Additionally,

future work should investigate the expression of c-di-GMP Δ*srbA* and any regulatory connections SrbA with AmrZ.

From further analysis of sequence complementarity, I found that *fliK* (PA14_45830) and *flgJ* (PA14_50380), as well as *pslL* (PA14_35600) were identified as putative targets of SrbA (Figure 25). As discussed above, both *fliK* and *flgJ* are involved in the structural formation of the flagellar apparatus and may explain the swarming deficiency and lack of mature biofilms observed in Δ*srbA*. *pslL* is part of the operon for synthesis of exopolysaccharide which is a major component of the biofilm EPS matrix[139]. However, my qPCR analysis did not identify any of these genes as being significantly dysregulated in Δ*srbA* (Table 6 and Figure 23). This does not rule them out as potential targets of SrbA, though. sRNAs act on the post-transcriptional level and alter protein expression through merely affecting levels of translation and not just mRNA stability[183]. Genes that did not display significant fold-changes in Δ*srbA* but have antisense complementarity with SrbA may still be targets. Reporter assays for levels of protein expression should be used in future work to determine if there is post-transcriptional dysregulation of these putative targets.

While there were only a few clear regulatory connections for biofilm formation based on sequence complementarity and mRNA expression, it does not rule out that there are still other regulatory targets of SrbA. An understudied aspect of sRNAs is the effects of RNA-folding and how secondary and tertiary structures may actually be a significant contributor to sRNA target recognition. It has long been noted that sRNAs such as SrbA use only short seeder sequences that are ~7 nucleotides in length for recognition of targets[183,185,189,281]. This has led to theorizing that structural folding is another major mechanism of target recognition for sRNAs. However, experimental evidence and studies investigating this are still lacking. This is largely due to limitations in our ability to have high-throughput methods of investigation and powerful

computational predictive methods[282–285]. Future work into SrbA targets will require focus on directly assessing translational output to gain a better understanding of regulatory targets. Further work aiming to exhaustively study regulatory targets of SrbA will require whole-proteome analysis in *P. aeruginosa*.

5.2.2 The Role of SrbA in Pathogenicity

After observing the biofilm deficiency of Δ*srbA*, I was curious if this would have any effect on its ability to colonize an animal host and establish an infection. I opted to use a slow-killing model in *C. elegans* as this experimental system generates a chronic infection and can provide conclusions of greater biofilm pathogenicity of *P. aeruginosa*[204,243]. While other animal infection models with *P. aeruginosa* exist, the use of *C. elegans* as a host provided an effective and efficient system to assess any changes to pathogenicity[204]. Future work, however, would benefit from using other animal infection models that could investigate more specific aspects of Δ*srbA* in a host such as immune responses of the host and infections that more closely replicate human infections. Additionally, a significant amount of injury and damage to tissues during *P. aeruginosa* biofilm infections is a result of the host's own inflammatory response[12].

I found that Δ*srbA* had a significantly attenuated pathogenicity in *C. elegans* and displayed a mortality rate of 39% compared to 78% in PA14 (Figure 24). It is very likely the greatest contributor to this is the fact that Δ*srbA* displays such a significantly reduced biofilm phenotype. As discussed above, I found that while Δ*srbA* was readily able to attach to surfaces (Figure 19B) it could only produce biofilms made up of sparse microcolonies (Figure 20B). Without being able to form large macrocolonies like PA14, Δ*srbA* is likely succumbing more readily to the hostile environment of the gut as well as clearance by the immune system of *C. elegans*.

The process of digestion by the host is likely contributing to lower colonization and passage of Δ*srbA*, which is thereby contributing to the attenuated pathogenicity of Δ*srbA*. Biofilm colonies are susceptible to dispersion by shearing forces in a fluid environment[25]. The mechanical processes of muscle contraction from peristaltism and of the host simply moving can be considered shearing forces in this system. With Δ*srbA* biofilms demonstrating a reduced mass they would be more susceptible to being dislodged and passed through the gut and thereby lessening the time to inflict harm on the host resulting in the attenuated pathogenicity observed. Bacterial cells within the reduced biofilms of Δ*srbA* would also be more at risk of exposure to enzymatic and chemical degradation in the *C. elegans* gut. Additionally, destruction of bacterial cells would be a result of the immune system actively subduing pathogens[286,287]. I conclude that the biofilm deficiency in Δ*srbA* is resulting in a severe disadvantage for the strain when colonizing the gut of *C. elegans*. However, this does not rule out that behaviours like swarming may still be contributing factors as Δ*srbA* is also deficient in this ability. Δ*srbA* is more susceptible to shearing stress, digestion, and immune system clearance due to its reduced ability to form biofilms. Through its involvement in biofilm formation, it is likely that SrbA also has a role in pathogenicity in *P. aeruginosa*.

It is still entirely possible that SrbA contributes to pathogenicity through means other than just regulation of biofilm. Referring again to the list and alignments of predicted regulatory targets of SrbA, I found genes *lasB*, *pslL*, *pscU*, *fliK*, and *flgJ* all of which are considered to have roles in virulence or immunogenicity of *P. aeruginosa* (Table 6 and Figure 25)[56,216,288,289]. Additionally, I found *hxcR*, a gene associated with type II secretion systems (T2SSs)[290]. While HxcR has not been directly found to be a virulence factor, it has been characterized that T2SS has a role in virulence of *P. aeruginosa*[288,291,292]. It is likely that the greatest contributor to the attenuated pathogenicity seen in Δ*srbA* is largely due to biofilm formation deficiencies. However, it cannot be ruled out that

SrbA possibly has more diverse regulatory roles and may directly regulate virulence factors in *P. aeruginosa*.

5.2.3 Future Directions in Studying SrbA Regulatory Involvement

In this work, I used in silico methods to find putative targets of SrbA and determine any effects SrbA has on levels of putative mRNA targets in Δ*srbA* through use of qPCR. The putative targets I found in this work represent genes from multiple different roles within *P. aeruginosa*. Putative targets that are part of flagellar synthesis, EPS production, type VI secretion, antibiotic resistance, and virulence have been discussed and will be useful for guiding future work in gaining a clearer understanding of SrbA regulation in *P. aeruginosa*.

I found a total of 61 putative mRNA targets for SrbA that can potentially interact through antisense base-pairing. Interestingly, there was an enrichment of putative interaction sites occurring at nucleotides 110-140 of SrbA (24 of 61 putative targets) as highlighted by the black bars included in Figure 25. There was broad representation of functional roles in these putative mRNA targets. There was no obvious bias towards targets involved in specific biological activities of *P. aeruginosa*. The site of alignments within the sequence of these putative targets occurred at different distances from the translational start site and did not use a shared region within all the putative targets. Most notable is that there are 2 hairpin loops within the structure of this region high density alignment of putative targets with nucleotides 110-140 of SrbA. In Figure 26 I have further drawn attention to these region of high density by outlining the secondary structure at nucleotides 110-140 of SrbA. These hairpin loops were conserved for both minimum free energy (MFE) and centroid predicted structures. Each method of resolving RNA folding predictions. MFE gives principal focus on the lowest requirement of free energy to form hairpin loops while the centroid method maintains a minimum possible distance between nucleotides undergoing base-

pairing[227,247]. While there is variety in the functional roles of the putative targets that align with this region of SrbA (nucleotides 110-140), it is encouraging that there is such a concentration of putative targets and that structure predictions are tightly conserved. I conclude that this predicted structure is likely closely representing the actual folding of SrbA in this region. Many sRNAs have been characterized to have a primary site for their regulatory base-pairing interactions with target mRNAs[183,185,281]. It is possible that this region is the core functional interaction site of SrbA whereby it undergoes hydrogen bonding with mRNA targets to exert effects that may provide access to the RBS, negatively affect ribosomal activity, or have effects on mRNA stability.

However, sRNAs can potentially use any part of their whole length of sequence for activity, and sRNAs often do have multiple interaction sites[281]. It is worth noting that SrbA appears to have two additional regions with higher occurrences of putative target interactions. From nucleotides 40-90 and 160-210, there is a greater concentration of putative targets aligning with SrbA (Figure 25). These additional two regions also contain secondary structures that are conserved in both the predicted structures (Figure 26). It is possible that all three regions of SrbA (nucleotides 40-90, 110-150, and 160-210) are primary sites for its functional activity. A future aim I would pursue is to generate mutant strains of *P. aeruginosa* where these regions of SrbA are altered in sequence to ablate potential secondary structure and target interactions. It would be of interest to use Northern blotting to compare binding of putative targets with wild-type SrbA and any loss of binding in the mutant strains. It would also be of interest to determine if mutant strains with altered sequences of these three regions of SrbA behave similarly to Δ*srbA* with regard to biofilm formation and pathogenicity. Additionally, through use of qPCR it would be of interest to determine if these mutant *srbA* strains had similar expression profiles of putative targets relative to Δ*srbA*.

The qPCR data showed that 26 putative targets had expression changes greater than 2-fold in Δ*srbA* (Table 6). These results provide insight into the effect SrbA may be having on the stability of these mRNAs. sRNAs are capable of affecting mRNA stability as a mechanism of regulation[183,189]. An sRNA binding to its target can act to provide stability of the mRNA. In other scenarios, the sRNA can act to recruit RNase E for degradation of the mRNA. It is possible that there are significant changes in levels of 26 putative mRNA targets seen in Δ*srbA* due to this mechanism of regulation. It is worth noting that of these 26 putative targets there are 9 of which (PA14_03560, *hpaA*, PA14_26810, PA14_32750, *ilvA2*, PA14_48010, *nemO*, PA14_59030, and *adhA*) that have complementarity in the alignment region of nucleotides 110-140 within SrbA (Figure 25). Future work with purified transcripts and RNase E could be performed to validate this. Again, through use of mutant forms of SrbA with alter sequence in putative interaction regions, I would test whether these putative binding sites affect mRNA stability. It would be of interest to see through use of qPCR if there are mRNA fold-changes in the mutant forms of SrbA that are similar to those in Δ*srbA* suggesting a loss of an interaction site.

This does not rule out that the other 35 putative targets are not targets of SrbA because there was no changes in mRNA expression. The predominant mechanism by which sRNAs exert post-transcriptional regulation is to moderate levels of translation by interacting with the ribosome binding site[183]. In future work, I would further investigate the biological roles of SrbA with regard to specific biofilm components and its effect on metabolic pathways represented in the list of putative mRNA targets in Table 6 and Figure 25. Through use of methods such as electron microscopy and fluorescent tagging, it would be of interest to further study the expression of flagella in Δ*srbA*. Additionally, a future direction for this work would be to use phenotypic microarrays with Δ*srbA* to study growth on various metabolites. There are 19 metabolic genes

represented as putative targets of SrbA and it would be of interest to determine if Δ*srbA* has an altered ability to grow on certain nutrient sources.

Future work into validating targets of SrbA will also help to determine the exact molecular mechanisms used by this sRNA for regulation. Currently, I hypothesize that as a *trans*-sRNA SrbA is acting on its targets via two characterized mechanisms. It is possible that SrbA is either altering accessibility of the ribosome to the mRNA targets, having an effect on mRNA stability through recruitment of RNase E, or it is acting through both these mechanisms[138, 192] (Figure 28). Both mechanisms of action may be possible due to *trans*-sRNAs being capable of having different regulatory effects on distinct mRNA targets. There is support for both these mechanisms in SrbA. As discussed above there are 35 putative targets found in this work that did not have any significant changes in RNA levels in Δ*srbA* which leaves open the possibility that SrbA does indeed interact with these mRNA targets having only effects on translation. Of particular interest for biofilm formation and pathogenicity as discussed above are FliK, FlgJ, PcaK, PslL, and PscU (Figure 28). However, there is also support for SrbA having a mechanism of action whereby it regulates targets in a post-transcriptional manner by affecting the stability of mRNA targets. There are 26 putative targets that are significantly dysregulated in Δ*srbA* (Table 6). Of particular interest are *ampG*, *amrZ*, *lasB*, *hxcR*, *secE*, and *mexQ* (Figure 28) for their potential roles in the biofilm and pathogenicity defects observed in Δ*srbA*. It is likely that based on the high number of putative targets that SrbA utilizes multiple mechanisms of action for its role in biofilm formation and pathogenicity in *P. aeruginosa*.

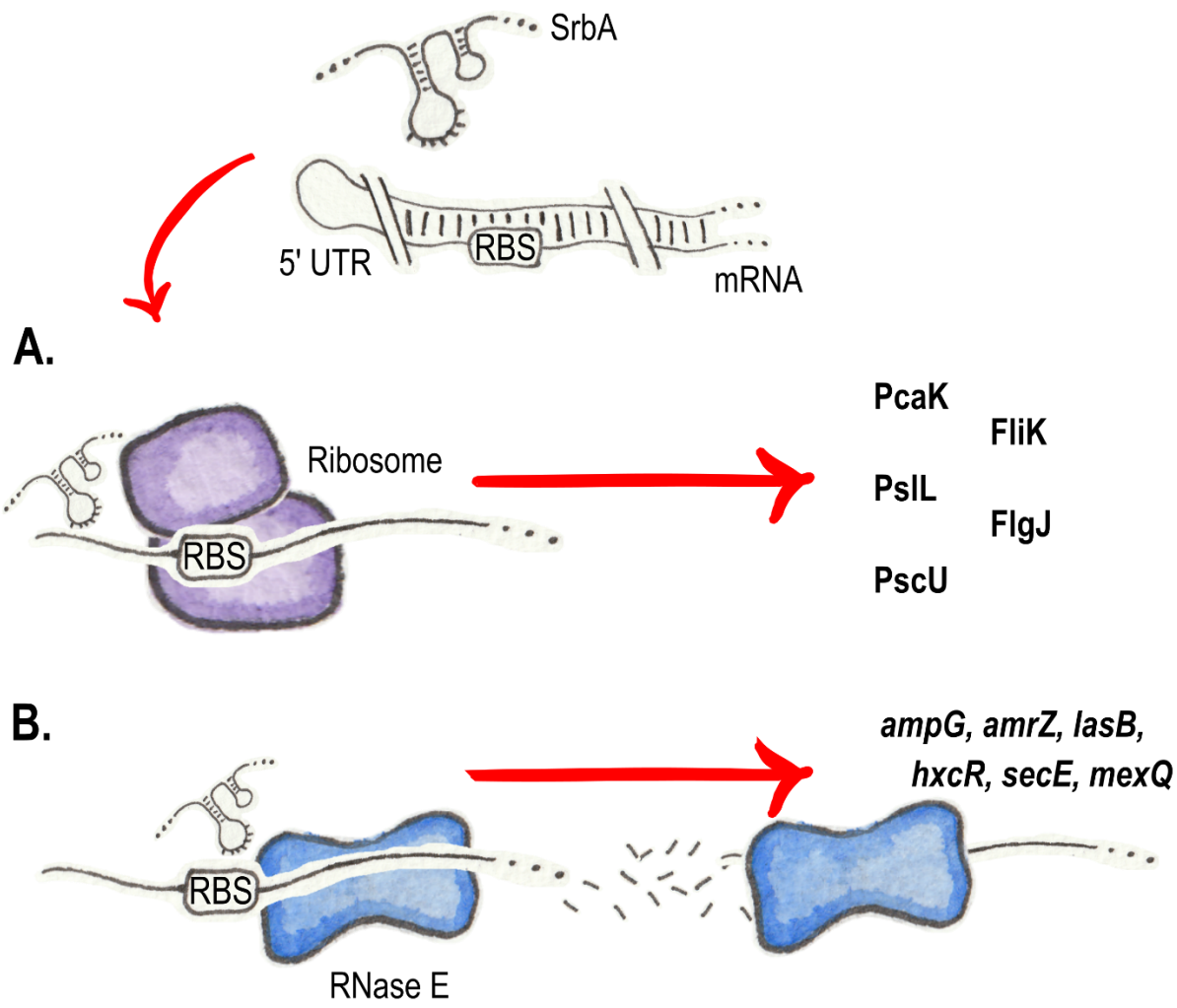

Figure 28. Proposed model of regulatory activity of SrbA on putative targets. Through established mechanisms of actions for *trans*-sRNAs[138,192] that SrbA acts to regulate its targets. SrbA may modulate translation of targets such as those that were found in Figure 25 to have sequence alignment but no changes in expression in Table 6 having effects on levels of protein synthesis (**A.**). Additionally, SrbA may act to regulate recruitment of RNaseE for putative targets that were found to have sequence alignment (Figure 25) and be significantly dysregulated in Δ*srbA* (Table 6) therefore having effects on mRNA stability (**B.**).

These putative target searches lay the ground work for generating hypotheses. A greater understanding of the regulatory roles of SrbA and its mechanisms of action may be gained through use of proteomic approaches, directed mutagenesis, and further investigation of biological phenotypes.

I was able to demonstrate that SrbA is an important regulator in biofilm formation in *P. aeruginosa*. Through deletion of SrbA, I found it is likely that it has a role in regulating the development of mature biofilm macrocolonies and may be acting on the expression and activity of flagella. Due its role in biofilm development, it is also likely that SrbA regulation in biofilms also has an effect on pathogenicity in *P. aeruginosa*. I was able to find numerous putative targets of SrbA but it is possible that there are yet more that cannot be found by in silico methods used here. SrbA is a novel sRNA regulator in *P. aeruginosa* with a significant effect on biofilm formation and pathogenicity. It is therefore a system with great potential to help further our understanding of how *P. aeruginosa* develops difficult to treat infections. *P. aeruginosa* biofilm infections are exceptionally difficult to clear by antibiotic treatment alone. Furthering our understanding of how *P. aeruginosa* regulates the development and maintenance of its biofilms is of significant importance for future therapies. Bacteria utilize sRNAs more extensively than has ever been previously appreciated. Through transcriptomic data we have found that *P. aeruginosa* encodes potentially hundreds of sRNAs. Far more work is needed to determine sRNA regulatory roles that may impact our understanding of the development of virulence, antibiotic resistance, and biofilm formation.

5.3 Concluding Remarks

For over 200 years, a massive portion of studying biology has focused on cataloging new organisms, new species, and new genes. With recent advances in high-throughput methods, we are

continuing this trend with genetics, proteomics, lipidomics, and metabolomics. With these enormous volumes of data being catalogued, there still needs to be an effort to understand the context, the biological roles of this molecular realm has and provide us an even better resolution of the intricacies of living systems.

In conclusion, my thesis work has focused on elucidating biological roles of regulatory systems in *P. aeruginosa*. I was able to further characterize the roles of the TCS TctD-TctE and the sRNA SrbA. There are still outstanding questions around specific pathways that these systems are targeting, to what extent their regulatory roles reach, and their molecular mechanisms of action. However, I did expand on earlier findings of TctD-TctE and initial cataloging of SrbA. I found that each have their own roles in the formation and maintenance of biofilms in *P. aeruginosa*. Understanding the fine details of regulatory systems such as TctD-TctE and SrbA is important for finding new ways to combat antibiotic resistance and virulence in the human pathogen *P. aeruginosa*. Studying the biological roles of regulatory systems such as these is also crucially important for contributions to our understanding of prokaryotic evolution, emergence of behavioural responses, and molecular mechanisms adapted by bacteria.

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APPENDEX I

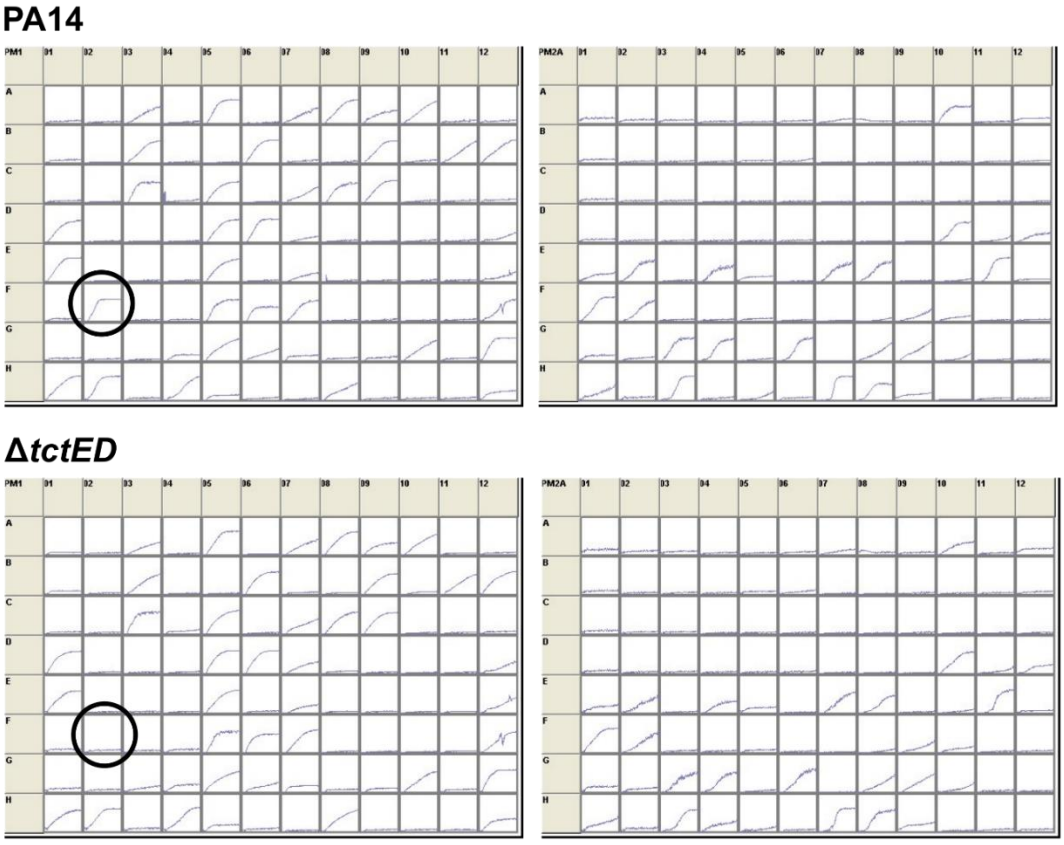

Figure 29. Growth of Δ*tctED* on phenotypic microarray plates with diverse carbon sources. Images are final results of a representative set of phenotypic microarrays performed. Each cell displaying a growth curve is one carbon source tested. PM1 and PM2A each contain separate selected carbon sources in each well. Carbon sources in each plate are available at: https://biolog.com/products-portfolio-overview/phenotype-microarrays-for-microbial-cells/. Wells with no growth curve indicate that a strain was unable to utilize the provided carbon source. Circled (F2) is the well that provided citric acid as a carbon source. Assays were performed for 24 h. Each growth curve is increasing absorbance due to accumulation of the reduced form of a dye due to oxidative phosphorylation. Each plate shown is representative of 3 biological replicates.