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Examining interactions between Pseudomonas aeruginosa and Staphylococcus aureus from cystic fibrosis lung infections

Libertucci, Josie

UNIVERSITY OF CALGARY

Examining interactions between *Pseudomonas aeruginosa* and *Staphylococcus aureus* from cystic fibrosis lung infections

by

Josie Libertucci

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Abstract

Pseudomonas aeruginosa and *Staphylococcus aureus* are two common cystic fibrosis (CF) pathogens, thought to interact within the lung and influence disease progression. This study on interspecies interactions revealed that *P. aeruginosa* mediates antagonistic interactions with *S. aureus*. Further analysis was completed to identify the mechanism of negative interactions between an *S. aureus* CF isolate, C105, and twenty seven *P. aeruginosa* CF isolates – nine of which were identified as inhibitors. It was demonstrated that an inhibitory factor is secreted by *P. aeruginosa;* is less than 5 kDa, can induce tobramycin resistance in C105 and reduce hemolytic activity suggesting the formation of small colony variants (SCVs). GC-MS analysis revealed that the primary inhibitory factor was not 4-hydroxy-2n-heptlyquinolone-*N*-oxide (HQNO) rather inhibition of *S. aureus* by *P. aeruginosa* is complex and involves the secretion of multiple factors. This research suggests that *P. aeruginosa* produces multiple anti-staphylococcal agents that play a paradoxical role as they inhibit growth yet allow for aminoglycoside resistance in *S. aureus*.

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To my love, Alessandro

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
AHL	Acyl homoserine lactone
ANOVA	Analysis of variance
Bcc	*Burkholderia cepacia* complex
BHI	Brain Heart Infusion
C4-HSL	*N*-butyryl-L-HSL
C8-HSL	*N*-octanoyl-L-HSL
3-oxo-C12-HSL	*N*-3-oxo-dodecanoyl-L-HSL
CF	Cystic Fibrosis
CFTR	Cystic fibrosis transmembrane regulator
CFU	Colony forming unit
^{o}C	Degree Celsius
Da	Dalton
DKPs	Diketopiperazines
g	Gram
GC	Gas chromatography
HAQs	4-hydroxy-2-alkylquinolines
HHQ	4-hydroxy-2-heptylquinoline
HSL	Homoserine lactone
HQNO	4-hydroxy-2*N*-heptylquinolone-N-oxide
kDa	Kilodalton
l	Liter
LB	Lysongeny Broth
LBA	Lysongeny Broth agar
mg	Milligram
MHB	Muller Hinton Broth
MS	Mass spectrometer
MSA	Mannitol Salt Agar
MSB	Mannitol Salt Broth
MSA	Manitol Salt agar
N-acyl HSL	*N*-acyl homoserine lactone
OD_{600}	Optical density at a wavelength of 600nm
PQS	*Pseudomonas* quinolone signal
QS	Quorum sensing
r	Correlation coefficient
RPM	Rotations per minute
SCV	Small colony variants
SD	Standard deviation
SACF	Southern Alberta CF
TSB	Tryptic soy broth

Chapter One: Introduction

1.1 Cystic Fibrosis: overview

Cystic fibrosis (CF), an autosomal recessive disease, is one of the most common fatal genetic diseases in Canada with an incidence of 1 in 2500 live births (O'Sullivan *et al.*, 2009). The sole etiology of CF is a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan *et al.*, 1989), which is located on the long arm of chromosome 7 (Rommens *et al.*, 1989). There are currently 1893 cited CFTR mutations that allow for the onset of CF (CFMD, 2011) the most common mutation being ΔF508 (Riordan *et al.*, 1989; Bobadilla *et al.*, 2002). The CFTR gene encodes for a phosphate regulated chloride ion channel, located on the apical membrane of epithelial cells in organs across the body, making CF a multi-systemic disease (Welsh and Smith, 1993).

The CFTR mutation disrupts the function of chloride ion channels, inhibiting the transport of chloride ions across the epithelial membranes (Anderson *et al.*, 1991). Inhibition of chloride ion transportation in the lungs draws water into the lumen leading to dehydrated hyperviscous mucus and impairing mucocillary clearance (Donaldson and Boucher, 2003). Disrupted mucocillary clearance creates an ideal environment for bacteria to colonize, which eventually leads to impaired lung function (Chmiel and Davis, 2003). Once bacteria colonize within the mucus, the infections persist throughout the patient's life and slowly reduce lung function. Persistent CF pulmonary bacterial infections are often found to be in the form of biofilms and are difficult to eradicate as they are inherently resistant to antibiotics (Costerton *et al.*, 1999). These chronic bacterial infections cause a persistent robust inflammatory immune response, and result in marked lung damage

(Amadori *et al.,* 2009) leading to morbidity and mortality in CF patients (Gibson *et al*., 2003; Chmiel and Davis, 2003; Rogers *et al.*, 2003).

1.2 Microbial Diversity in the CF lung

Traditionally, CF microbial dogma stated that only certain pathogens were present in the lung and consequently only a few species were the cause of lung function decline (Gilligan, 1991). The common pathogens included *Pseudomonas aeruginosa, Staphylococcus aureus, Haemophilus influenza,* and the *Burkholderia cepacia* complex (Bcc) (Gilligan, 1991) and as a result, antibiotic treatment was based on the identification of these species in a patient (Ramsey, 1996). However, with the advent of high throughput pyrosequencing and modern culturing techniques, the image of the CF microbiota has vastly changed. The microbes in these infections are extremely diverse and the infection is likely polymicrobial rather than due to a single pathogen (Harris *et al.,* 2007; Sibley *et al.,* 2008; Guss *et al.,* 2011). It also appears that patients with CF can have high fungal as well as bacterial loads within their airways (Delhaes *et al.,* 2012). These studies underline the importance of understanding the entire microbial community and its interactions. The bacterial pathogens that are commonly isolated from CF patients, *S. aureus, P. aeruginosa* and *Burkholderia* spp, form chronic infections that are difficult to treat and they cause the most damage in the CF lung. As our understanding grows on the polymicrobial nature of these chronic infections it is clear that more research is needed to understand how the bacterial interactions and population dynamics shape the infection and ultimately disease progression.

Currently, CF dogma is undergoing a shift as more research is supporting the notion of microbial community based treatment rather than species specific treatment (Sibley *et*

al., 2008). This concept is based on the observations that in some cases pathogen loads may not change during antibiotic treatment, but the patients' conditions improve. This might suggest that other members of the community may be playing a larger role in disease states than the pathogens (Sibley and Surette, 2011). Many factors need to be considered to understand bacterial community influence on disease progression. Factors such as CF oropharyngeal microbiota have been shown to influence the virulence of *P. aeruginosa* in a rat lung model (Duan *et al*., 2003). Antibiotic treatment has the ability to decrease a specific microbial community which would aid in differential species pathogen colonization (Evanno *et al.,* 2009) in addition to host factors. The research conducted in our lab is largely focused on understanding and examining the interactions (both intra- and inter-species) that may occur between isolates of *P. aeruginosa* and other CF isolates.

1.3 *P. aeruginosa* as a CF pathogen

Currently, *P. aeruginosa* is one of the most studied CF pathogens as this species is reported to infect 50% of adolescent patients and 80% of adult patients (CFC, 2009). Once an infection has been established, *P. aeruginosa* will colonize the lung as eradication from the lung is rarely possible (Hoiby *et al.,* 2000). Further, antibiotic treatment has been shown to encourage development of resistance mechanisms that make the bacterium even harder to eradicate (Hancock and Speert, 2000). Its ability to adapt in the CF lung environment to changes in nutrients and stress are the fundamental factors that aid in its success as a CF pathogen (Hauser *et al.,* 2011). One reason that *P. aeruginosa* has gained such attention as a CF pathogen is due to its capacity to facilitate damage within the lung. Young children who tested for positive cultures of *P. aeruginosa* have a 2.6 fold increase in mortality over the subsequent 8 years compared to children who test negative (Emerson

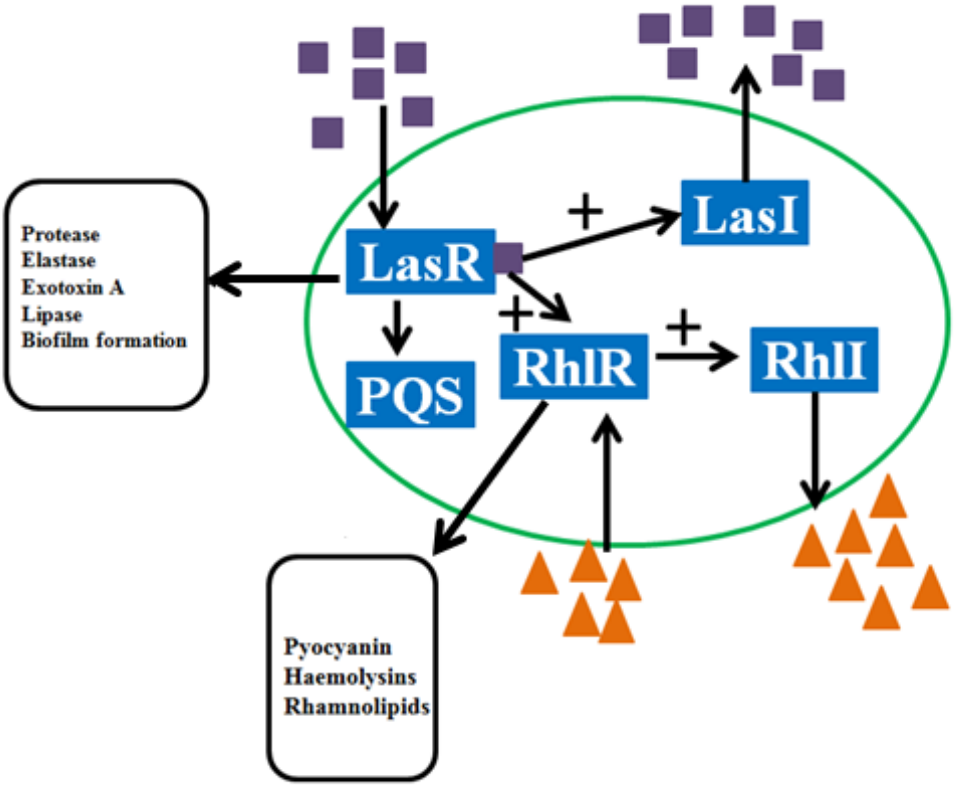
et al., 2010). *P. aeruginosa* colonization has also been associated with lower FEV_1, lower weight percentile and increased hospitalization due to more frequent exacerbations in both children and adult patients (Emerson *et al.,* 2010; Navarro *et al.,* 2001). Taken together *P. aeruginosa* is an important CF pathogen as it infects the majority of patients and causes severe lung dysfunction – an ability that can be attributed to its intricate quorum sensing systems as it controls the production of virulence factors and ultimately host virulence.

1.3.1 Quorum sensing in *P. aeruginosa*

Bacteria communicate intracellularly and can receive information from other species through a cell density cell-to-cell signalling system known as quorum sensing (QS) (Nelson and Hastings, 1979). In gram-negative bacteria the QS system is composed of LuxIR homologues also known collectively as an autoinduction system (Gambello and Iglewski, 1991). The system consists of an autoinducer – acylated homoserine lactone signal molecule – and an R protein which is an autoinducer dependent transcriptional activator protein (Pesci *et al.,* 1999). At low cell densities, a low concentration (basal level) of the autoinducer accumulates inside and outside the cell as the molecule is permeable to the cell membrane. As cell density increases, the concentration of autoinducer increases concurrently inside the cell until a threshold concentration is reached, at which point the autoinducer will bind to protein R and the autoinducer-R complex triggers the expression of specific target genes (Pesci *et al.,* 1999).

Quorum sensing in *P. aeruginosa* (Figure 1) controls the expression of multiple virulence factors such as elastase (LasB) (Gambello and Iglewski, 1991), protease (LasA) (Toder *et al.,* 1991) and exotoxin A (Gambello *et al.,* 1993). *P. aeruginosa* has two sets of

Figure 1: *P. aeruginosa* quorum sensing systems are controlled by the global regulator lasR. The global regulator LasR controls the expression of many virulence factors in *P. aeruginosa*. LasR regulates the transcriptional regulator RhlR in addition to another QS system, PQS. The expression of *lasIR* and *rhlIR* systems are dependent on cell density, as cell density increases the systems are activated.



3-oxo-C12-HSL

C4-HSL

genes that are homologous to the LuxIR system (found in *Vibrio fisheri*) known as the *las* and *rhl* system (see figure 1) – both systems being under the control of their respective autoinducer; *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butyryl-L-homoserine lactone (C_4-HSL) (Pearson *et al.,* 1993). In the *las* system, the autoinducer 3-oxo-C_{12}HSL is produced by LasI which binds to and activates the transcriptional activator protein LasR (Passador *et al*., 1993). The *rhl* system works by the same autoinducer principle as RhlI synthesizes the autoinducer signalling molecule C_4-HSL which binds and activates the transcriptional regulator RhlR (Ochnser *et al.,* 1994). The *las* and the *rhl* system are organized in a hierarchical manner with the *las* system maintaining transcriptional control over the *rhl* system (Latifi *et al.,* 1996). In *P. aeruginosa* social behaviour is controlled via QS which allows for cooperation and social cheating to arise (Ng and Bassler, 2009).

In addition to the *lasIR* and *rhlIR* systems, *P. aeruginosa* produces and secretes a 4-quinolone signal molecule identified as 2-heptyl-3-hydroxy-4(1H)-quinolone known as the Pseudomonas quinolone Signal (PQS) (Pesci *et al.,* 1999). It has been shown that PQS is regulated by both the *las* and *rhl* systems placing the PQS system at the bottom of the hierarchical QS systems in *P. aeruginosa* (Diggle *et al.,* 2003). PQS is as well integrated into the QS regulatory circuitry as it has been shown that as LasR regulates the production of PQS, an accumulation of exogenous PQS induces expression of *lasB, rhlI* and *rhlR* (McKnight *et al*., 2000). Five structural genes are required for PQS production (*pqsABCDE*) which is controlled by a transcriptional regulator (*pqsR* also known as *mvfR)* and PqsE catalyzes the final steps for the synthesis of PQS (Pesci *et al.,* 1999; Gallagher *et al.,* 2002). Overall, *lasR* stimulates the production of MvfR (PqsR) which in turn initiates

transcription of the *pqsABCDE* operon (Wade *et al.,* 2005). This operon controls the expression of HHQ, a precursor to PQS (Deziel *et al.,* 2004). PQS is not involved in cell density signalling since it is produced later in the growth cycle but it has been linked to pyocyanin production (Diggle *et al.,* 2003). Deleting *mvfR* results in reduced production of pyocyanin, elastase, exoprotein, 3-oxo-C_{12}-HSL and no expression of *pqsABCDE* or AQ biosynthesis (McKnight *et al.,* 2000).

QS systems in *P. aeruginosa* are not only regulated by HSL containing small molecules, *P. aeruginosa* produces non-HSL molecules capable of interacting with a *N*-acylhomosensor lactone (AHL) biosensor (Holden *et al.,* 1999). These compounds are characterized as cyclic molecules called diketopiperazine (DKPs) and *P. aeruginosa* produces four DKPs: cyclo(ΔAla-$_L$-Val), cyclo(L-Pro-LTyr) and cyclo(L-Phe-L-Pro) and cyclo(Leu-Pro) (Holden *et al.,* 1999). The role of these DKPs has yet to be determined, but there is speculation that these molecules may partake in cross-talk between the other QS regulatory systems (Holden *et al.,* 1999).

1.3.2 Virulence factors and pathogenicity of *P. aeruginosa* in the CF lung

P. aeruginosa is an environmental organism which is commonly found in many moist environments including soil and water. Following inhalation of *P. aeruginosa* into the CF lung, this opportunistic pathogen colonizes the lung with ease due to its pathogenic capabilities and a prime colonization environment because of disrupted mucocilary clearance. *P. aeruginosa* possess adhesins including pili and flagella which allow the bacterium to bind to receptors found either in secretions or on cell surfaces (Tang *et al.,* 1995). Once colonization has been established these structures are lost due to downregulation of these specific genes – a process specific to the lungs of patients with CF

(Saiman and Prince, 1993). Following the cessation of adherence structures *P. aeruginosa* must survive in the lung by evading the immune system, antibiotic treatment and by adapting to a harsh environment.

The common phenotypic switch that occurs in *P. aeruginosa* (specific to only CF infections) is the conversion to the mucoid phenotype (Dogget *et al.,* 1964; Hoiby *et al.,* 1977). The mucoid phenotype has been associated with severe lung infections in CF patients (Hoiby, 1974 in Lam *et al.,* 1980). Overproduction of the expolysaccharide alginate (occurs as a result of the mucoid phenotype) surrounds the individual bacterium creating a protective barrier from host immune system and causes a hyperimmune response in the host and overall decreased prognosis (Hoiby, 1974 in Lam *et al.,* 1980).

P. aeruginosa produces a multitude of exoproducts, secreted to the surrounding environment, which aid in host immune system evasion including elastase, alkaline protease, lipase, exotoxin A and pyocyanin. Elastase and alkaline protease are both metalloproteases, active at alkaline pH and work by degrading immunoglobulins, gelatin, casein, laminin, complement components and cytokines (Kharazmi *et al*., 1984). Lipases, produced by many CF isolates (Lutter, 2008), have been shown to significantly inhibit macrophages and monocytes which are important cells for host defence (Jaeger *et al.,* 1991). *Pseudomonas* exotoxin A is a highly toxic substance that belongs to the family of-ADP-ribosyltransferases and is capable of catalyzing ADP ribosylation of the eukaryotic elongation factor 2 (eEF-2) that severely affecting protein synthesis in host cells (Iglewski and Kabat, 1975). The siderophore, pyocyanin, inhibits the immune system by impairing lymphocyte proliferation (Sorenson *et al.,* 1983) and supports *P. aeruginosa* virulence through inhibiting mammalian cell respiration (Stewart-Tull *et al.,* 1972) and epidermal

cell growth (Cruickshank and Lowbury 1953). Pyocanin is able to inhibit respiration of other microbes (Hassan and Fridovich, 1980) as well as hydrogen cyanide (Castric, 1975).

P. aeruginosa is intrinsically resistant to antibiotics and possesses a number of different mechanisms capable of extruding antibiotics; it also mutates quickly resulting in multi drug resistance. The outer membrane of *P. aeruginosa* is somewhat impermeable and works as a barrier to antibiotics (Scott *et al*., 1999) with an uptake, that is approximately 10 to 100 fold lower than other pathogens (Hancock and Speert, 2000). Two efflux pumps, MexAB-OprM and MexX-MexY, contribute to intrinsic resistance (Zhao *et al.*, 1998; Aires *et al.*, 1999); when these are mutated susceptibility to quinolones, tetracyclines, aminoglcosides and some β-lactams increases. Resistance is easily attainable as mutations in regulatory machinery for either efflux pump, DNA gyrase (Mouneimne *et al*., 1999), or β-lactamase (Hancock and Speert, 1996) results in multi-drug resistance.

1.3.3 *P. aeruginosa* biofilms

It is believed that *P. aeruginosa* infections within the CF lung exist in the form of biofilms (Lam *et al*., 1980; Hoiby, 1977: Singh *et al.*, 2000; Wagner *et al.*, 2003). Biofilms aid in the survival of bacteria in a hostile environment, such as the CF lung, by allowing for greater antibiotic tolerance (Ceri *et al.*, 1999) and through dispersal of planktonic cells (Costerton *et al.*, 1999). Some researchers consider the formation of biofilms to be analogous to the development of a multicellular organism due to intricate intercellular signalling that regulates growth and differentiation throughout formation (Harrison *et al.*, 2005). Most biofilms begin as free swimming planktonic cells until absorption and attachment to a surface occurs. From this stage, growth and division will occur resulting in increased cell density and the initiation of cell to cell communication. This communication

between cells will trigger formation of extracellular polymeric substance (EPS) which acts as a shield around the microcolony and promotes continuous growth. A matrix around the microcolony begins to form that consists of EPS and DNA. Microcolony growth leads to formation of biofilms, at which point cells will begin to detach, become planktonic and reattach at a different location continuing the chronic infection (Lawrence *et al.,* 1991; Costeron *et al.,* 1995).

Since *P. aeruginosa* QS cell signalling systems exhibits their effects at high cell density, it is hypothesized that the *lasIR* and *rhlIR* systems are not involved in the initiation of biofilm formation, but rather that these systems are required for biofilm differentiation (Davies *et al*., 1998). This was proven through the use of *P. aeruginosa* prototypical strain PAO1 and a double QS mutant (*lasIRhlI*) PAOJP2. Microscopic observations showed that both strains could adhere to a glass surface however only PAO1 could form mature microcolonies and achieve true biofilm architecture. It was also shown that the singular *rhlI* mutant could form biofilms similar to WT however the *lasI* mutant was significantly different to WT which suggests 3-oxo-C_{12}-HSL, produced by LasI, is an important signalling molecule.

1.4 *Staphylococcus aureus* as a CF pathogen

Staphylococcus aureus is a common CF pathogen and is routinely isolated from children as it is considered to be one of the first colonizers within the CF lung (Bauernfeind *et al.*, 1987; Burns *et al.,* 1998; Stone and Saiman, 2007; Kahl, 2010; Dasenbrook, 2011; Goss and Muhleback, 2011). It is commonly but not always replaced by *P. aeruginosa* in adolescent children (Sibley *et al.,* 2011). In the pre-antibiotic era *S. aureus* was the leading cause of mortality in CF patients (Anderson, 1949). The incidence of methicillin-resistant

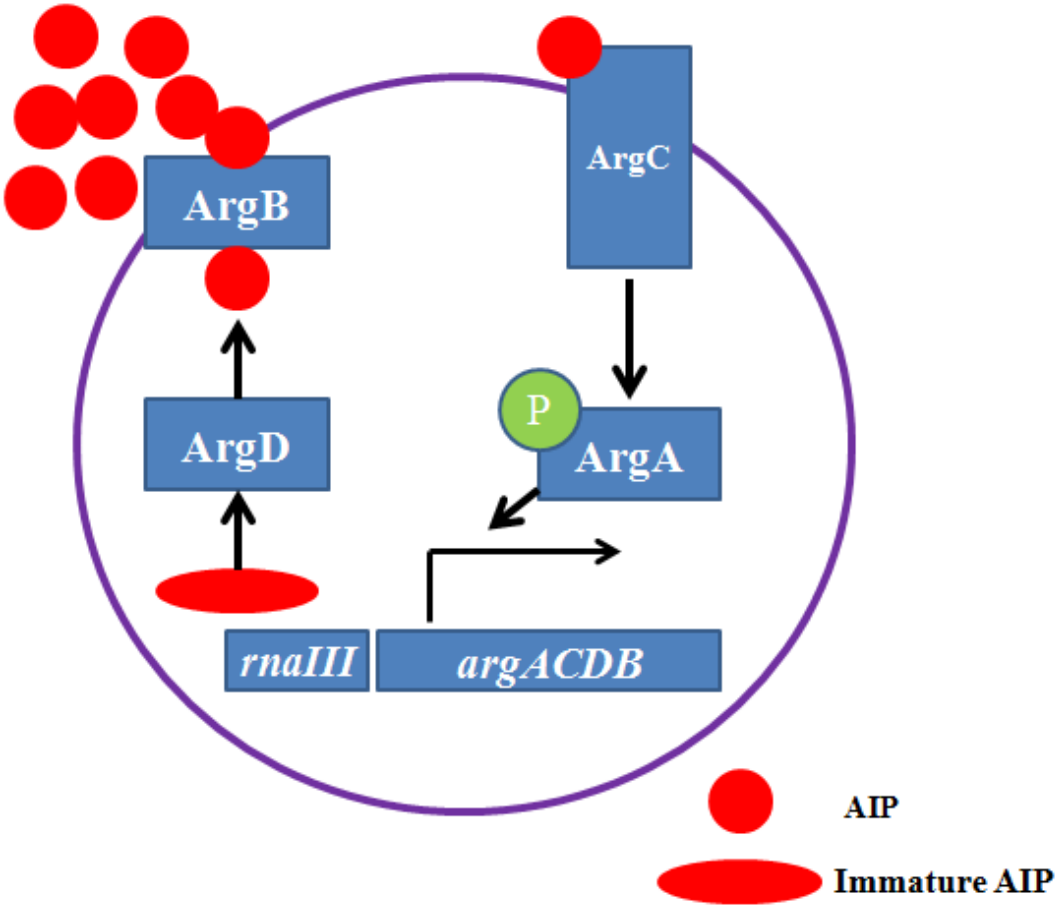
S. aureus (MRSA) has increased over the past years in the United States reaching a 25.7% frequency across CF patients (CFF, 2011). As methicillin sensitive *S. aureus* (MSSA) and MRSA are rarely distinguished from one another in CF clinics, the prognosis of each strain is unclear but MRSA has been associated with higher antibiotic use and an increased mortality rate in patients (Boxerbaum *et al.,* 1988; Dasenbrook *et al.,* 2010). In a recent study, out of 419 patients with CF, 53.9% had chronic MSSA infection with a greater prevalence of MSSA in children rather than adolescence, 17.4% were colonized with MRSA and 66.3% had *P. aeruginosa* colonization (Hubert *et al.,* 2013). In addition to this, patients with MRSA showed significantly decreased FEV_1 values with co-colonized with *P. aeruginosa* (Hubert *et al.,* 2013).

1.4.1 Quorum sensing in *S. aureus*

Gram-positive bacteria also communicate in response to cell density, with the use of secreted peptides as autoinducers as opposed to HSLs which are used in many gram-negatives (Miller and Bassler, 2001). The peptide is secreted outside the cell and accumulates until a certain density is reached and the threshold value is obtained. Peptides are sensed via a two-component sensor kinase detector that undergoes a series of phosphorelay events (for review see Hoch and Silhavy, 1995). The phosphorlation events eventually lead to the activation of a response regulator protein that initiates transcription of virulence factors (Miller and Bassler, 2001).

In *S. aureus* the AgrC/AgrA cell density cell-to-cell communication system is used to activate virulence genes (figure 2) (Novick, 1999). The Arg system is mostly conserved throughout the *Staphylococcus* group since the ability of AIP to bind to its receptor is highly specific (Novick, 2003). The operon *agrBDCA* encodes components of the QS

Figure 2: **Virulence factor production in *S. aureus* is controlled by a two-component regulatory system.** The Arg system in *S. aureus*is activated later in its growth cycle as it is triggered by cell density. The AIP protein is processed via the ArgD protein and exported out of the cell through ArgB. Once AIP has accumulated to its threshold value, it binds to ArgC. AIP:ArgC complex phorphorlates ArgA which activates transcription of virulence factors (Miller and Bassler, 1991).


ArgB
ArgC
ArgD
P
ArgA
rnaIII
argACDB
AIP
Immature AIP

regulatory system (Morfeldt *et al.,* 1988) and is upstream to the *hld* gene which codes for the effector protein, RNAIII molecule, whose function is to regulate the expression of the virulon (Janzon and Arvidson, 1990). ArgD is the precursor to the final autoinduction peptide (AIP) and is processed by the ArgB protein to become the fully activated AIP (Mayville *et al.,* 1999) by processing ArgD at both the N and C terminal to create a thiolactone ring (Ji *et al.,* 1997). ArgC acts as the sensor kinase of the two-component system whereas ArgA functions as the response regulator and when phosphorlayted increases expression of RNAIII (Janzon and Arvidson, 1990). The ArgC/AgrA system controls the expression many virulence factors important for pathogenesis including, toxic shock syndrome toxin, coagulase, cytolytic toxins and enterotoxins (Smith, 1979).

1.4.2 *S. aureus* small colony variants (SCVs)

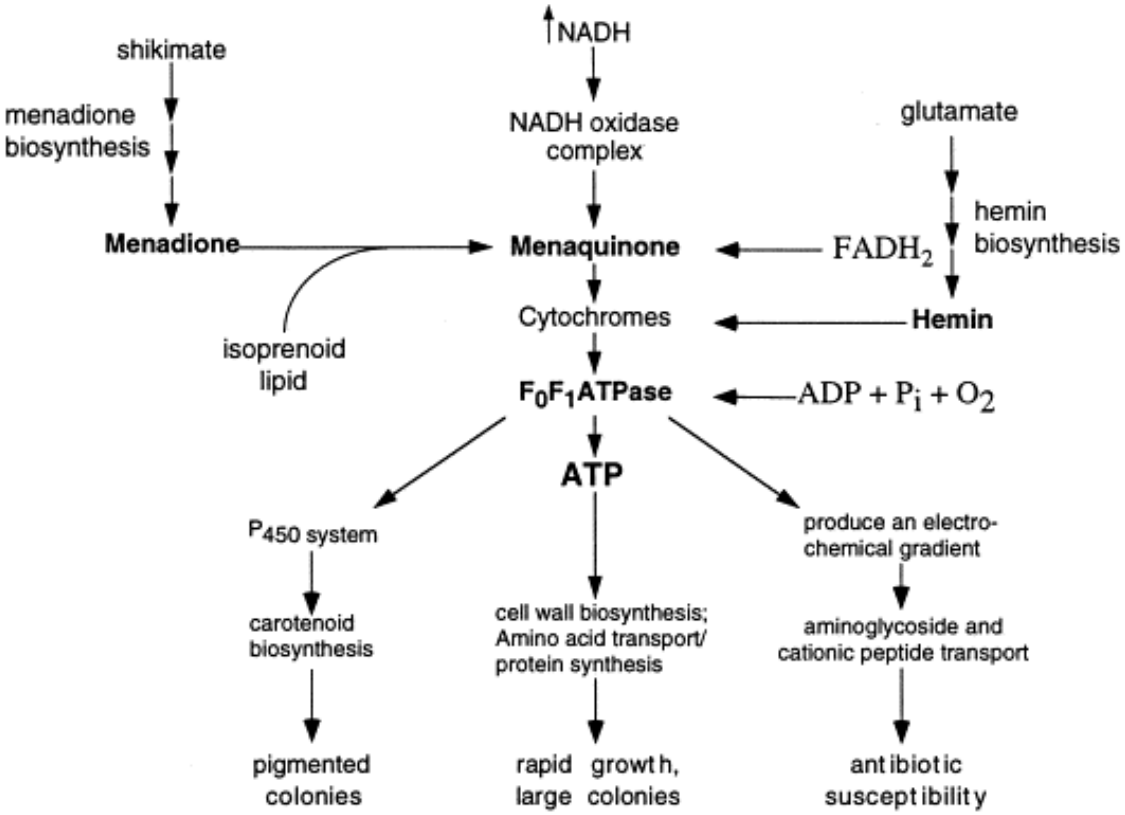
Canadian and American patient databases indicate that *S. aureus* is a common pathogen among children patients; with increasing age, *P. aeruginosa* becomes the dominant pathogen within the lung (CFF 2011; CFC 2011). However, this observation has been challenged through the use of modern classification techniques, which support the notion that *S. aureus* colonizes the lung at a similar frequency to *P. aeruginosa* in adult patients (Sibley *et al.,* 2011). This recent insight has been attributed to *S. aureus's* ability to persist within CF infections by switching phenotypes to a small colony variant (SCV) form (Mitchell *et al.,* 2011a; Mitchell *et al.,* 2011b; Yagci *et al.,* 2013). Normal or non-SCV *S. aureus* exhibits standard colony size (1-2 mm), pigmentation (white) and hemolysis (beta) on Columbia agar plates as opposed to the characteristics of SCVs – nonpigmented, nonhemolytic and 0.1 mm in size (Kahl *et al.,* 1998). *S. aureus* SCV frequency is high amongst children (Gibson *et al.,* 2003), but emergence of SCVs in adults also occurs

frequently as age advances, one reason being intense antibiotic therapy resulting in selection for a persister phenotype (Yagci *et al.,* 2013).

In addition to antibiotic selection pressure, SCVs can form in response to a *P. aeruginosa* exoproduct, 4-hydroxy-2-heptylquinolone-*N*-oxide HQNO (as described in section 1.5). HQNO binds to the enzyme, cytochrome bo_3, which acts as a respiratory oxidase in the cytoplasmic membrane in *Escherichia coli*. In gram positives, specifically *S. aureus*, HQNO interferes with terminal respiration by inhibiting oxidation of cytochrome b by cytochrome c (Lightbrown and Jackson, 1955). *S. aureus* slow growing SCVs are auxotrophic for menadione (necessary for menaquinone production) or hemin or both – figure 3 depicts *S. aureus* respiration (Acar *et al.,* 1978; Balwit *et al.,* 1994). Menaquinone and heme are required for the transfer of electrons as menaquinone is the first electron acceptor that receives electrons from $FADH_2$ and hemin receives electrons from menaquinone (McNamara and Proctor, 2000). Exogenous menadione and hemin restore function to the electron transport chain and reverse the SCV phenotype (Goldenbaum *et al.,* 1975).

S. aureus SCVs are intrinsically resistant to antibiotics as many antibiotic targets are non functional in SCVs – antibiotic resistance can be directly related to an impaired electron transport chain (Proctor and Peters, 1998). Aminoglycosides require the formation of an electrochemical gradient, specifically the transmembrane potential ($\Delta\Psi$), to enter the cell as they are positively charged and become associated with the cellular membrane (Mates *et al.,* 1983). Therefore if *S. aureus* is grown anaerobically or has a dysfunctional electron transport chain it become resistant to aminoglycosides as the antibiotic cannot be internalized. The result of this reduction in uptake leads SCVs to acquire a 4-8 fold

Figure 3: Respiration in SCVs of *S. aureus* are auxotropic for hemin and menadione. Respiration in SCVs is impaired as menadoine and hemin are not produced causing a reduced electrochemical gradient, inhibited growth, reduced virulent factor production, and antibiotic resistance. This figure depicts proper respiration in *S. aureus.* Figure taken from McNamara and Proctor, 2000. Figure use kindly permitted by Elsevier.


NADH
shikimate
menadione biosynthesis
NADH oxidase complex
glutamate
hemin biosynthesis
Menadione
Menaquinone
$FADH_2$
Cytochromes
Hemin
isoprenoid lipid
F_0F_1ATPase
$ADP + P_i + O_2$
ATP
P_{450} system
produce an electro-chemical gradient
carotenoid biosynthesis
cell wall biosynthesis; Amino acid transport/ protein synthesis
aminoglycoside and cationic peptide transport
pigmented colonies
rapid growth, large colonies
antibiotic susceptibility

increase in the MIC of aminoglycosides, lantibiotics and protamine (Kahl *et al.,* 1998). Antibiotics that require a smaller electron potential are able to penetrate the cell since glycolytic ATP produces 60-70% of the total membrane potential (McNamara and Proctor, 2000).

1.4.3 *S. aureus* biofilms

The first step in forming biofilms is adhesion to a surface – just as in *P. aeruginosa* biofilms. Adhesion to a surface in *S. aureus* is dependent on a group of proteins known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that have been shown to play a pivotal role in initial adhesion (Patti *et al.,* 1994). The cell accumulation phase for *S. aureus* biofilms has been shown to be dependent on the polysaccharide intercellular adhesion (PIA) protein which is controlled by the *icaADBC* operon (Heilmann *et al.,* 1996). Most *S. aureus* strains do have a functional *ica* operon but when some regions of this operon are non-functional, biofilm formation will be skewed from normal (Beenken *et al.,* 2003). This operon is controlled by a repressor which is encoded by *icaR* found upstream from this operon (Conlon *et al.,* 2002). The *arg* QS system has been shown to regulate biofilm formation – specifically the production of MSCRAMMs – once the two component system is activated MSCRAMMs are repressed (Vuong and Otto, 2000). *S. aureus* strains that produce greater than normal levels of *arg* will have reduced biofilm production (Beenken *et al.,* 2004). The staphylococcal accessory regulator (*sarA*) is also involved in the regulation of biofilms as it has been shown to regulate transcription of the *ica* operon (Valle *et al.,* 2003).

1.5 LasA, HQNO and the mediation of interspecies interactions

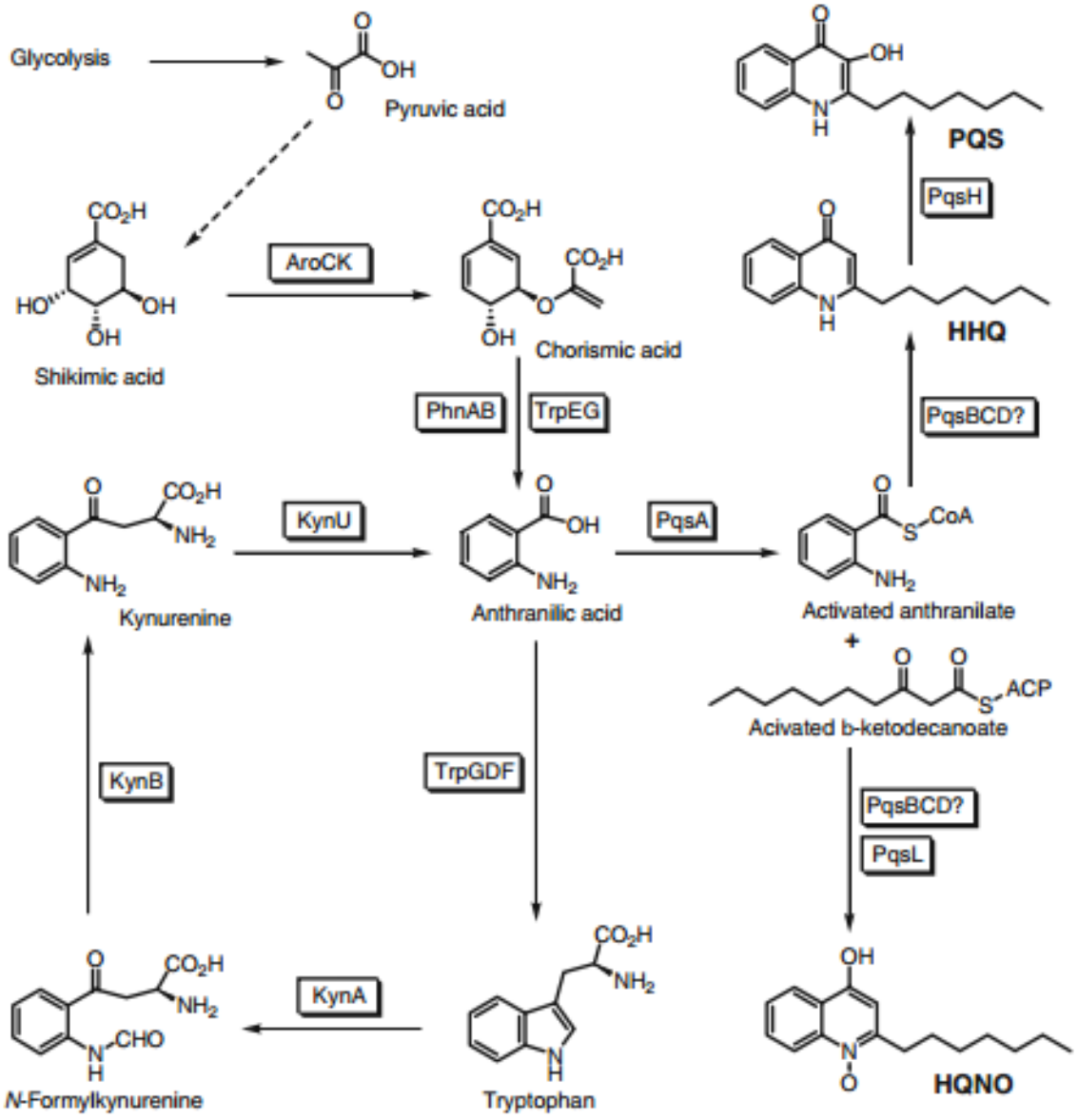
The pathogenesis of *P. aeruginosa* is largely due to the secretion of proteases (Kharazmi, 1989) and these virulence factors may give *P. aeruginosa* an advantage in a CF infection. In terms of interactions between *S. aureus* and *P. aeruginosa,* a particular protease that may affect interactions between these bacteria is the LasA protease, or staphylolysin, a 20kDa protein that is secreted by *P. aeruginosa* and functions as a staphylolytic endopeptidase (Kessler *et al*., 1993). The LasA protease works by disrupting the pentaglycine cross-links in the peptidoglycan of *S. aureus* by cleaving peptide bonds following the Gly-Gly pair in proteins (Kessler *et al.*, 1998). An aspect of the role of LasA activity in the CF lung that we are interested in examining is whether *P. aeruginosa* uses LasA to mediate antagonistic interactions with *S. aureus* in to gain a competitive advantage.

P. aeruginosa is also able to inhibit the growth of *S. aureus* through expression of HQNO (Machan *et al.,* 1992). HQNO is active against many *S. aureus* strains including strains that are methicillin-resistant (Machan *et al*., 1992). However, in the CF lung, HQNO has been shown to have a paradoxical role (Hoffman *et al*., 2006). Since HQNO inhibits the growth of *S. aureus* by inhibiting its electron transport, it provides a protective effect on *S. aureus* to aminoglycosides, such as streptomycin and dihydrostreptomycin, (Hoffman *et al.,* 2006) in addition to its inhibiting affect. HQNOs` paradoxical effect has led researchers to question the relationship between this molecule and small colony variants (SCVs) of *S. aureus* in the CF lung. *S. aureus* SCVs are persistent slow growers commonly found in CF patients that have been co-colonized with both *S. aureus* and *P. aeruginosa* (Proctor *et al*., 2006). *P. aeruginosa* has evolved to restrain competing

microorganisms metabolically (Hogan and Kolter, 2002) and induce *S. aureus* into SCVs (Hoffman *et al.,* 2006). Therefore in a CF lung environment, long exposure of HQNO induces aminoglycoside resistant *S. aureus* SCVs—a potential advantage for the bacterium to persist in this environment (Hoffman *et al.,* 2006).

HQNO synthesis is regulated by the *pqsABCDE* operon, whose expression is controlled by the transcriptional regulator *mvfR*, ultimately controlled by the global regulator LasR (Figure 4) (Wade *et al*., 2005). HHQ is not a precursor to HQNO (Lepine *et al.,* 2004), as was previously thought but as PQS positively regulates the *las* and *rhl* system HHQ does have a role in HQNO production. Genes from the *pqs* operon, *pqsA* and *pqsL,* are essential for HQNO production since if they are not present, biosynthesis of HQNO will cease (Hofmann *et al.,* 2006). Biosynthesis of HQNO starts from glycolysis and the production of pyruvic acid which synthesizes shikimic acid (Luckner and Ritter, 1965; Ritter *et al*., 1971). From this, *AroCK* will initiate the production of chorismic acid at which time *phnAB* and *TrpEG* will help produce anthranilie acid (Luckner and Ritter, 1965; Ritter *et al*., 1971). Anthranilic acid will be activated by the products of PqsA. Activated anthanilate acid will react with activated b-ketodecanoate and with the help of PqsL will finally form HQNO (Luckner and Ritter, 1965; Ritter *et al*., 1971).

Figure 4: Biosynthetic pathway for the production of HQNO in *P. aeruginosa*. HQNO is regulated by the PQS system which is ultimately controlled by the global regulator LasR. The genes *pqsA* and *pqsL* are essential for the production of HQNO as if they are mutated no HQNO will be produced. HHQ, even though it is very similar structurally, is not a precursor to HQNO production. HHQ does have an involvement in the quantities of HQNO produced however it is not necessary. Figure taken from Chapter 2: 2-Alkyl-4(1H)-Quinolone Signalling in *Pseudomonas aeruginosa* by Matthew P. Fletcher, Stephan Heeb, Siri Ram Chhabra, Stephen P. Diggle, Paul Williams and Miguel Cámara. Found in: Juan L. Ramos and Alain Filloux *Pseudomonas* Volume 6: Molecular Microbiology, Infection and Biodiversity10.1007/978-90-481-3909-5_2. Use kindly provided by Springer Science+Business Media B.V.


Glycolysis
Pyruvic acid
Shikimic acid
AroCK
Chorismic acid
PhnAB
TrpEG
Anthranilic acid
KynU
Kynurenine
PqsA
Activated anthranilate
CoA
ACP
Acivated b-ketodecanoate
PqsBCD?
PqsL
HQNO
PqsH
HHQ
PQS
PqsBCD?
TrpGDF
Tryptophan
KynA
N-Formylkynurenine
KynB

1.6 Community interactions

Polymicrobial infections involve complex interactions between colonizing microbes, the microbiota, and the host (Sibley *et al*., 2008). In the past decade a *Drosophila melanogaster* model was developed to examine the role of various bacterial factors in an infection (Chugani *et al*., 2001). In 2008, Sibley *et al* used this fly feeding model to investigate different interactions that can occur between microbes – specifically between a lab strain of *P. aeruginosa*, PAO1, and oropharyngeal flora (OF) isolated from CF patients. The results indicated that the microbes can interact in one of three ways and they classified these into three classes. The first class was named virulent, the OF microbes in this class were found to kill flies on their own and also increased killing when mixed with PAO1. The second class was identified as avirulent as these OF microbes are not able to kill flies alone or with PAO1. And the third class was called synergistic, as these OF microbes were not able to kill flies on their own but in combination with PAO1 were found to be just as virulent as class I. Interestingly, these authors did not find any antagonistic or competitive interactions between these groups of microbes.

Previous work in our lab has highlighted interactions between sub-populations of *P. aeruginosa* which revealed that these sub-populations were capable of synergistic, neutral or antagonistic interactions (Lutter *et al*., 2008). This means that CF microbes can work together to increase virulence (synergistic interaction) as was seen in Sibley *et* al., 2008 or compete with each other to decrease virulence (antagonistic interaction or competition) (Lutter, 2008). From this work, two *P. aeruginosa* CF isolates, 14672 and 14651, were found to inhibit the virulence and pathogenesis of other CF *P. aeruginosa* isolates and also *Burkholderia cenocepacia* CF isolates (Lutter, 2008; Purighalla, 2011).

The interactions between *P. aeruginosa* isolate 14672 and species of the *Burkholderia cepacia* complex was further investigated and it was suggested that small signalling molecules were mediating this interaction (Purighalla, 2011). Furthermore, it was shown that these signalling molecules were diketopiperazines (DKPs). Our lab has also examined the competitive interactions between isolate 14651 and subpopulation isolates of *P. aeruginosa*. It appears that pyocins are playing a role in the interactions of isolate 14651 (MacLean, 2012). Taken together, the research on these two factors suggests that they may be influencing the competitiveness of the isolates (14672 and 14651) and this may have a role in shaping the CF lung population. We have now become interested in whether or not these mechanisms may also be involved in interactions between *S. aureus* and *P. aeruginosa*. For instance, do these factors give *P. aeruginosa* isolates a competitive advantage over *S. aureus* and so allow *P. aeruginosa* to out compete *S. aureus* in the lungs of CF patients?

1.6 Project overview

Chronic lung infections associated with CF are dominated by bacterial pathogens, such as *P. aeruginosa* and *S. aureus*, against a polymicrobial backdrop. Microbes within polymicrobial infections have been shown to interact in a cooperative manner to enhance the virulence of mixed infections. Microbes can also exhibit antagonistic interactions, which might give certain members of the lung population a competitive advantage that can shape the composition of the infection. Although some studies have reported antagonistic interactions can occur, little is known regarding the mechanism that controls interactions between microbes in CF infections. The importance of polymicrobial infections in CF is understood, but the mechanism(s) that controls antagonistic interactions and possibly

enhance persistence of the infection are not clearly defined and understanding this is necessary for developing treatments to treat polymicrobial infections. The goals of this study were to survey interactions between *P. aeruginosa* and *S. aureus* and underline the mechanisms that control this interaction. This work expands on previous findings in out laboratory that showed *P. aeruginosa* displays intraspecies antagonistic interactions in addition to displaying interspecies antagonistic interactions with the BCC complex.

1.6.1 Hypothesis

We hypothesize that antagonistic interactions between *P. aeruginosa* and *S. aureus* may allow one species to dominate in an model infection and who may also dominate in the CF lung population. These interactions, which are controlled by specific mechanisms, may shape the population dynamics of the chronic lung infections and may also impact the overall progression of lung disease in CF.

1.6.2 Research Objectives

1. **Examine interactions between a *S. aureus* CF isolate (C105) and a group of thirty *P. aeruginosa* CF isolates – in vitro.**
2. **Identify potential mechanism(s) of interaction between C105 and *P. aeruginosa.***
3. **Examine a broader range of CF *S. aureus* and *P. aeruginosa* interactions from different types of infection in CF.**

Chapter Two: Materials and Methods

2.1 Strains and growth conditions

Bacterial strains and isolates used in this study are listed in Table 1. Freezer stocks of bacterial cultures were made from lawns of bacteria and mixed with 10% sterile skim milk and stored at -80ºC. Both *S. aureus* and *P. aeruginosa* CF isolates were cultured in a number of different media including Lysogeny Broth (LB) [10 g NaCl (EDM Chemicals), 5 g yeast extract (EDM chemicals) and 10 g tryptone (EDM Chemicals) per litre L] , Tryptic Soy Broth (TSB) [30 g of TSB powder (Difco) per L], Peptone Tryptic Soy Broth (PTSB) [50 g peptone (Difco) and 2.5 g TSB powder per L], Brain Heart Infusion (BHI) [37 g of BHI powder (Difco) per L], Mannitol salt broth (MSB) [96 g of MSB powder (VWR) per L], Muller Hinton Broth (MHB) [22 g of MHB powder (Difco) per L]. Commonly, bacteria were cultured on solid agar which consisted of the appropriate broth supplemented with 1.5% select agar (Invitrogen) unless indicated otherwise. All liquid media and solid agar powders were mixed with deionised water and subsequently autoclaved. Liquid bacterial cultures were prepared by inoculating a single colony taken from an agar plate into the appropriate liquid medium for 16-18 hours at 37ºC shaking at 200 revolutions per minute (rpm). Liquid cultures always consisted of 90% air 10% culture to allow for maximum growth.

Table 1: Bacterial strains and isolates used in this study

Strain	Description	Reference
Pseudomonas aeruginosa		
PAO1	Prototypical wild type laboratory strain	(Holloway *et al.*, 1979)
PAOJP2	PAO1 *lasI/rhlI* mutant	(Pearson *et al*., 1997)
PAO214	*lasI*:FRT, unmarked deletion	(Hoang *et al.*, 2000)
PDO100	*rhlI*::Tn*501*	(Brint and Ohman, 1995)
PDO111	*rhlR*::Tn*501*	(Brint and Ohman, 1995)
PA103	Hypertoxigenic non mucoid CF isolate, *lasR*	(Liu, 1966)
14683	Non-mucoid CF isolate, Patient 92, isolated on 05/13/98	(Lutter *et al.*, 2008)
14684	Non-mucoid CF isolate, Patient 92, isolated on 05/13/98	(Lutter *et al.*, 2008)
14685	Non-mucoid CF isolate, Patient 92, isolated on 05/13/98	(Lutter *et al.*, 2008)
14670	Non-mucoid CF isolate, Patient 90, isolated on 05/07/98	(Lutter *et al.*, 2008)
14671	Non-mucoid CF isolate, Patient 90, isolated on 05/07/98	(Lutter *et al.*, 2008)
14672	Non-mucoid CF isolate, Patient 91, isolated on 05/07/98	(Lutter *et al.*, 2008)
14673	Non-mucoid CF isolate, Patient 91, isolated on 05/07/98	(Lutter *et al.*, 2008)
14715	Non-mucoid CF isolate, Patient 38, isolated on 05/26/98	(Lutter *et al.*, 2008)
14716	Non-mucoid CF isolate, Patient 38, isolated on 05/26/98	(Lutter *et al.*, 2008)
14717	Non-mucoid CF isolate, Patient 38, isolated on 05/26/98	(Lutter *et al.*, 2008)
5585	Non-mucoid CF isolate, Patient 88, isolated on 10/24/88	(Gallant *et al.*, 2000)
5588	Non-mucoid CF isolate, Patient 34, isolated on 10/24/88	(Gallant *et al.*, 2000)
5552	Mucoid CF isolate, Patient 34, isolated on 10/17/88	Gallant *et al.*, 2000

Table 1: Bacterial strains and isolates used in this study continued

Strain	Description	Reference
7307	Non-mucoid CF isolate, Patient 29, isolated on 11/18/91	(Lutter *et al.*, 2008)
14655	Non-mucoid CF isolate, Patient 29, isolated on 04/30/98	(Erikson *et al.*, 2002)
14656	Non-mucoid CF isolate, Patient 29, isolated on 04/30/98	(Lutter *et al.*, 2008)
14649	Non-mucoid CF isolate, Patient 89, isolated on 04/29/98	(Lutter *et al.*, 2008)
14650	Non-mucoid CF isolate, Patient 29, isolated on 11/18/91	(Lutter *et al.*, 2008)
14651	Non-mucoid CF isolate, Patient 29, isolated on 11/18/91	(Lutter *et al.*, 2008)
14690	Non-mucoid CF isolate, Patient 93, isolated on 04/14/98	(Lutter *et al.*, 2008)
6106	Mucoid CF isolate, Patient 26, isolated on 01/10/90	(Erikson *et al.*, 2002)
14660	Non-mucoid CF isolate, Patient 57, isolated on 05/04/98	(Lutter *et al.*, 2008)
14661	Non-mucoid CF isolate, Patient 57, isolated on 05/04/98	(Lutter *et al.*, 2008)
4384	Non-mucoid CF isolate, Patient 35, isolated on 02/02/87	(Raivio *et al.*, 1994)
5166	Non-mucoid CF isolate, Patient 35, isolated on 02/18/88	(Raivio *et al.*, 1994)
5154	Non-mucoid CF isolate, Patient 91, isolated on 02/11/88	(Raivio *et al.*, 1994)
14703	Non-mucoid CF isolate, Patient 33, isolated on 05/20/98	Lutter *et al.*, 2008
Staphylococcus aureus		
ATCC 13709	Wild type, phage type 44A/42E, haemolytic, coagulase positive, virulent in mice	(Morse, 1960)
ATCC 25923	Wild type, clinical isolate, application: antibiotic susceptibility testing, CF isolate	(Boyle *et al.*, 1973)
ATCC 29213	Wild type, wound isolate, application: susceptibility disk testing	(Ceri *et al.*, 1999)
C105	CF isolate, Patient 94	This study

Table 1: Bacterial strains and isolates used in this study continued

Strain	Description	Reference
VL6	CF isolate, Patient 95, exacerbation, chronic SA infection, isolated on 3/29/2010	This study
VL7	CF isolate, Patient 95, exacerbation, chronic SA infection, isolated on 11/1/2010	This study
VL8	CF isolate, Patient 95, exacerbation, chronic SA infection, isolated on 3/14/2011	This study
VLF	CF isolate, Patient 95, routine clinic, chronic SA infection, isolated on 12/1,/2004	This study
VLM	CF isolate, Patient 95, routine clinic, chronic SA infection, isolated on 7/23/2008	This study
VLR	CF isolate, Patient 95, routine clinic, chronic SA infection, isolated on 3/7/2012	This study
RMF	CF isolate, Patient 96, routine clinic, chronic SA infection, isolated on 1/23/1995	This study
RMM	CF isolate, Patient 96, routine clinic, chronic, SA infection, isolated on 10/21/1998	This study
RMR	CF isolate, Patient 96, routine clinic, chronic, SA infection, isolated on 1/5/2000	This study
RPF	CF isolate, Patient 97, routine clinic, chronic SA infection, isolated on 2/14/1995	This study
RPM	CF isolate, Patient 97, routine clinic, chronic SA infection, isolated on 10/1/2003	This study
RPR	CF isolate, Patient 97, routine clinic, chronic SA infection, isolated on 3/12/2012	This study
AKK	CF isolate, Patient 98, routine clinic, replaced by PA, isolated on 12/20/2006	This study

Table 1: Bacterial strains and isolates used in this study continued

Strain	Description	Reference
LAM	CF isolate, Patient 99, routine clinic, replaced by PA, isolated on 12/16/2008	This study
LL	CF isolate, Patient 100, routine clinic, replaced by PA, isolated on 1/17/1990	This study
TPP	CF isolate, Patient 101, routine clinic, replaced by PA, isolated on 6/22/2006	This study
BAG	CF isolate, Patient 102, routine clinic, co-infection, isolated on 2/15/1989	This study
JSI	CF isolate, Patient 103, routine clinic, co-infection, isolated 2/23/2009	This study
EPP	CF isolate, Patient 104, routine clinic, co-infection, Isolated on 12/23/1987	This study

2.2 *S. aureus* inhibition Assays

2.2.1 Supernatant *S. aureus* inhibition assay

Interactions were assessed by examining growth inhibition of *S. aureus* caused by either *P. aeruginosa* culture or culture cell-free supernatant. To do so *S. aureus* isolates were grown in MSB overnight at 37ºC while shaking and standardized to an optical density (OD_{600}) equal to 0.005 in 5 mL of molten agar (1% peptone and 0.5% select agar). Molten agar containing *S. aureus* was poured on top of MSA plates and allowed to dry for a minimum of 30 minutes. *P. aeruginosa* isolates were grown in TSB or PTSB overnight using the same conditions as *S. aureus*. *P. aeruginosa* supernatant was collected via centrifugation at 7,000 rpm for 10 minutes and subsequently filtered sterilized using a 0.2 μm filter (Millipore, Canada) to ensure no cells were left in the supernatant. *P. aeruginosa* supernatant was spotted on top of the overlay plates at a volume of 5μl and placed into the incubator overnight at 37°C. Zones of inhibition were measured and recorded. To test for the effect of whole cells on *S. aureus* one amendment was made, 5 μl of *P. aeruginosa* culture was spotted onto the overlay plates.

2.2.2 Molecular weight cut off and heat-treated supernatant assay

Size fractions and heat treatment was used to deduce if the inhibiting factor were a small molecule or a protein. Overlay plates containing *S. aureus* were prepared as in section 2.2.1. To prepare the supernatant 10 mL of *P. aeruginosa* culture was filtered sterilized following centrifugation. Total supernatant was subdivided into two groups – heat and non heat treated total supernatant. One mL of total supernatant was heat treated by placing supernatant contained in a microcentrifuge into a water bath at temperature 90°C

for 10 minutes. One mL of total supernatant was taken and labelled as non heat treated total supernatant. Then 5 mL of total cell-free supernatant was added into a 5,000 Dalton (5 kDa) spin concentrator cut-off column (Agilent, Canada) and concentrated for a minimum of 1 hr at 3,000 rpm. Supernatant was then divided into less than and greater than 5 kDa fractions. Each fraction was then further subdivided into heat and not heat treated fractions. All fractions (total supernatant, heat treated total supernatant, > 5 kDa non heat treated, > 5 kDa heat treated, < 5 kDa non heat treated and < 5 kDa heat treated) were spotted (5 µl) onto overlay plates and incubated overnight at 37°C. Zones of inhibition were recorded the next day.

2.2.3 HQNO concentration inhibition assay

Synthetic HQNO (Enzo Life Sciences) was spotted onto overlay plates (prepared as in section 2.2.1) to test for a dose dependent response. One mg of HQNO was dissolved in 1 mL of dimethyl sulfoxide (DMSO) which resulted in a 1 mg/mL concentration. This stock concentration was further diluted to concentrations of 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.12 µg/mL, 0.5 µg/mL in LB and spotted directly (5 µL) onto the overlay plate. Five µL of DMSO was spotted onto the overlay plates as a control.

2.3 Growth Curves

Growth curve experiments were conducted with bacterial cultures in one of three ways. First, viability of C105 was assessed by growth in MHB standardized to an OD_{600} of 0.02. This standardized culture was added to a Starsted 96 well microtitre plate in volumes of 75 µl per well and an additional 75 µl of ddH_2O was added to each well to mimic the effects of spent medium on culture growth. Note that with the addition of ddH_2O at equal volumes to C105 the final concentration of C105 becomes 0.01. Second, the effects of *P.*

aeruginosa supernatant on C105 was tested by adding 75 µl of C105 standardized culture and 75 µl of filtered sterilized *P. aeruginosa* supernatant to each well. Third, the effects of synthetic HQNO (Enzo Life Sciences) was compared to the effect of *P. aeruginosa* supernatant on the viability of C105 by adding equal volumes of standardized culture of C105 and standardized HQNO (10 ug/mL) to each well. As a control, an equal volume (75 µl each) of C105 and dimethyl sulfoxide (DMSO, sigma) was used. In all conditions, 30 µl of mineral oil was added to each well to prevent desiccation of C105 which resulted in a final volume of 180 µl. The OD_{600} of C105 was taken by placing the 96 well plate into a Perkin Elmer Victor X4 model 2030 multilabel counter, the protocol was set to take a measurement every other hour for a duration of 24 hours.

2.4 Tobramycin induced SCV assay

P. aeruginosa supernatant was tested for behaviour similar to synthetic HQNO by testing for tobramycin resistance of *S. aureus* when treated with *P. aeruginosa* supernatant. MHA plates were supplemented with tobramycin at the following concentrations: 0.1 µg/mL, 0.2 µg/mL, 0.3 µg/ mL, 0.4 µg/mL, 0.5 µg/mL, 0.6 µg/mL, 0.7 µg/mL, 0.8 µg/mL, 0.9 µg/mL and 1.0 µg/mL taken from a stock solution. The tobramycin stock solution was made by weighing out 10 mg of tobramycin and adding 1 mL of DNase RNase free H_2O which was filtered sterilized. C105 was grown overnight in TSB and a 1:1000 dilution was made the following day. Lawns of C105 were made on the tobramycin plates by dipping a cotton swab into the dilution and streaking the plate entirely with the swab. Lawns were allowed to dry for a minimum of 30 minutes at which time 5 µl of *P. aeruginosa* supernatant (as prepared in section 2.2.1) was spotted onto the plates. Plates were placed in the incubator overnight at 37°C.

2.5 Hemolysis plate assay

A single colony of *S. aureus* was taken from a LBA plate using a cotton swab and streaked onto a Columbian blood agar plate. The plates were placed in an incubator at 37°C for 24 hours. Degradation of red blood cells was analyzed and recorded.

2.6 Gas Chromatography – Mass spectrometry analysis

2.6.1 Solvent extraction and preparation for GC-MS of culture supernatants

An organic extraction of the inhibiting factor produced by PAO1 (PAOJP2 was used as a negative control) and other *P. aeruginosa* CF isolates was prepared from 20 mL of bacterial culture at mid-log phase. Pellets were harvested via centrifugation at 7, 000 rpm for 10 min at 4°C. Supernatants were then acidified through the addition of hydrochloric acid (HCl) to bring the pH down to less than 2. Ethyl acetate was added to the acidified supernatant at an equal volume, shaken and added to a separatory funnel which allowed the aqueous and solvent layer to separate. The aqueous layer was drained and discarded. The solvent layer was drained into sodium sulfate contained in filter paper and collected in order to repeat the extraction process twice more. Between each extraction, sodium sulfate was rinsed thoroughly with ethyl acetate to ensure all solvent had been removed. Following extraction the collected ethyl acetate was concentrated using rotary evaporation to a volume approximately the size of a quarter. This was transferred into a 4 mL glass vial and derivatized with N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) by adding 0.5 µl and placing the vial into a water bath at 60°C for 15 minutes. The samples were then stored at room temperature in the dark until injected into the GC-MS.

2.6.2 Identification of the secreted factor via GC-MS

Similar methods were used as in Purighalla, 2011. Briefly, GC-MS analysis was completed using a GCMS-7980 A Plus gas chromatograph- mass spectrometer (Agilent Technologies). The GC column used was a DB-1 MS capillary column (50m x 0.32 mm x 0.52 μm) coated with 5% Ph Me siloxane and was operated in the spiltless mode. Analysis was conducted to obtain retention times and fragment ion patterns of the molecule.

2.6.3 Identifying concentration of HQNO secreted by *P. aeruginosa* supernatants

Area under the curve for the molecular peak was obtained via MSD Chem Station Data Analysis Application (Agilent). A standard curve was plotted (concentration of HQNO vs. area under the curve) and concentration values of HQNO for each individual isolate were extrapolated.

2.7 Biofilms

2.7.1 Single species biofilms

Overnight cultures of *S. aureus* CF isolates were prepared in TSB and standardized to an OD_{600} of 0.1 the following day. One hundred and eighty μl of standardized bacterial culture was added to each well in a flat bottom 96 well NUNC plate and a MBEC lid was placed on top. The edges of the lid and plate were parafilmed together and placed on a gyrotary shaker at 37°C with 95% humidity for 48 hours. Biofilm formation was later analyzed using crystal violet assay (2.8) or bacterial cell enumeration from the peg (2.9).

2.7.2 *S. aureus* biofilms with *P. aeruginosa* supernatant

S. aureus and *P. aeruginosa* inoculum was prepared and standardized or supernatants filtered sterilized as in section 2.7.1 and 2.2.1 respectfully. Biofilms were grown in the same device as in section 2.7.1, but instead of adding 180 μl to each well, 90

µl of *S. aureus* culture and 90 µl of *P. aeruginosa* supernatant (ddH_2O was used for the control) was added.

2.8 Crystal violet assay

Biofilms were prepared as in section 2.7.1 or 2.7.2 and following incubation, lids were removed and allowed to dry by a flame for 10 minutes. Pegs were then stained with 1% crystal violet for 10 seconds followed by 3 washes in 1 x PBS for 3 seconds by dipping the lid in a trough filled with the appropriate substances. The pegs were then destained in 100% methanol by dipping the lid into a flat bottom plate for 10 seconds with 175 µl methanol in each well. The OD_{600} was then read using our Perkin Elmer Victor X4 model 2030 multilabel counter.

2.9 Viable bacterial enumeration (CFU/mL)

Biofilms were prepared as in section 2.7.1 or 2.7.2 and following incubation cells on the peg were calculated. A flat bottom 96 well plate was filled with 200 µl in the first row (A) and 180 µl in rows B – H. Pegs were removed using pliers and placed into the first row containing 200 µl. The 96 well plate was then placed into the sonicator for 5 minutes to allow all cells to fall into suspension. Following sonication pegs were removed using needle nose tweezers and discarded. Serial dilutions were then performed by removing 20 µl from each row and adding it to the next row until the last row. One hundred µl was taken from each well and spread plated onto the appropriate agar plate.

2.10 *Drosophila melanogaster* virulence model

***2*.10.1 *D. melanogaster* fly stock maintenance**

D. melanogaster, Oregon R wild type, was maintained on standard cornmeal-yeast-agar-sucrose-glucose medium [6 g select agar (Invitrogen), 28 g dried brewer's yeast

(Sigma), 77 g cornmeal agar (Sigma), 27 g sucrose, 53 g glucose, 3.5 mL propionic acid and 0.3 mL phosphoric acid in 1L of water] in plastic 250 mL vials (Applied Scientific) at room temperature. Once larvae and pupae started to heavily accumulate in the vial, flies were transferred to new vials. After 3 to 5 days, newly hatched male flies were used to complete the fly feeding assay (2.5.2).

2.10.2 *D. melanogaster* fly feeding assay

Three to five day old male flies were taken the day of experiment and starved for 5 hours. While the flies were starving, sucrose agar was prepared (1.5 mL 20% sucrose, 0.13 g agar per vial) and 5 mL was added to each vial. The agar was allowed to harden for a minimum of an hour and filter paper was placed onto the sucrose agar in each vial. Bacterial cultures were normalized to an OD_{600} of 3, pellets were harvested and resuspended with 175 µl of 5% sucrose. Bacterial cultures were added on top of the filter paper and allowed to dry for 45 minutes. Flies were transferred into the vials and monitored for 14 days.

2.12 Statistical analysis

All data were plotted and analysed using graph pad prism version 5.0a. One-way ANOVA was performed under non-parametric conditions in addition to a Dunns post test.

Chapter Three: *P. aeruginosa* CF isolates may use an HQNO like molecule in addition to HQNO to gain a competitive advantage over *S. aureus* in the CF lung environment

3.1 Introduction

In an environment where microorganisms share the same pool of nutrients, three scenarios could occur between species; co-evolution, synergism or competition (Harrison, 2007). In any of those three scenarios, microorganisms must develop mechanisms to drive those interactions (Hibbing *et al.,* 2010). Microorganisms have developed diverse and in some cases, complicated mechanisms that allow species to dominate, co-evolve or become synergistic in a population. In the CF lung, where nutrient resources are scarce, microorganisms either adapt to gain a neutral benefit or compete with one another in order to survive. Understanding the mechanisms that drive competitive interactions between *P. aeruginosa* and *S. aureus* in the CF lung will be explored in this section.

The aims of this chapter were first to screen for interactions between *P. aeruginosa* and *S. aureus* and then to identify the underlining mechanisms that are responsible for driving interspecies interactions. Twenty seven *P. aeruginosa* adult CF isolates were screened for competitive interactions against 3 ATCC *S. aureus* strains and one *S. aureus* adult CF isolate, C105. All *P. aeruginosa* CF isolates were taken from 13 different patients who attended the southern Alberta CF clinic at Foothills Hospital in Calgary, Alberta. The 27 isolates have been well characterized as their genotypic and phenotypic diversity have been researched (Lutter, 2008; Faria, 2009).

3.2 Results

3.2.1 Supernatant from some *P. aeruginosa* CF isolates are able to inhibit growth of *S. aureus*

P. aeruginosa can produce virulence factors that are able to inhibit the growth of *S. aureus* such as the protein LasA (Kessler *et al*., 1993) and the small molecule HQNO (Hoffman *et al.,* 2006). It is well established that strains PAO1 and PA14 of *P. aeruginosa* can produce these virulence factors (Kessler *et al.,* 1993; Hoffmann *et al.,* 2006) but no research study has focused on whether or not CF isolates can produce anti-staphylococcal agents. Recently, research has shown that HQNO was found in sputum samples taken from CF patients, meaning this molecule is present in the CF environment (Hoffman *et al.,* 2006). However, these studies do not directly link *P. aeruginosa* CF isolates to the production of HQNO or any other *S. aureus* inhibitory factor. As well, insights regarding the population dynamics and social behaviour (such as cheating) of *P. aeruginosa* inside the CF lung have not been addressed with regards to the production of an anti-staphylococcus agent. Given that *P. aeruginosa* can produce and secrete inhibitory *S. aureus* actors, it was our initial hypothesis that all *P. aeruginosa* CF isolates would be able to mediate competitive interactions with *S. aureus*.

In order to determine if all 27 *P. aeruginosa* CF isolates mediate competitive interactions with *S. aureus* an initial screen was first completed using an overlay assay which only examines growth inhibition in general. *P. aeruginosa* supernatant taken from late log phase to early stationary phase was collected and used to test for the presence of an anti-staphylococcal agent. Inhibitory profiles of each *P. aeruginosa* isolate were compared

against three *S. aureus* ATCC strains and a *S. aureus* CF isolate C105 to determine if inhibition of *S. aureus* is widespread.

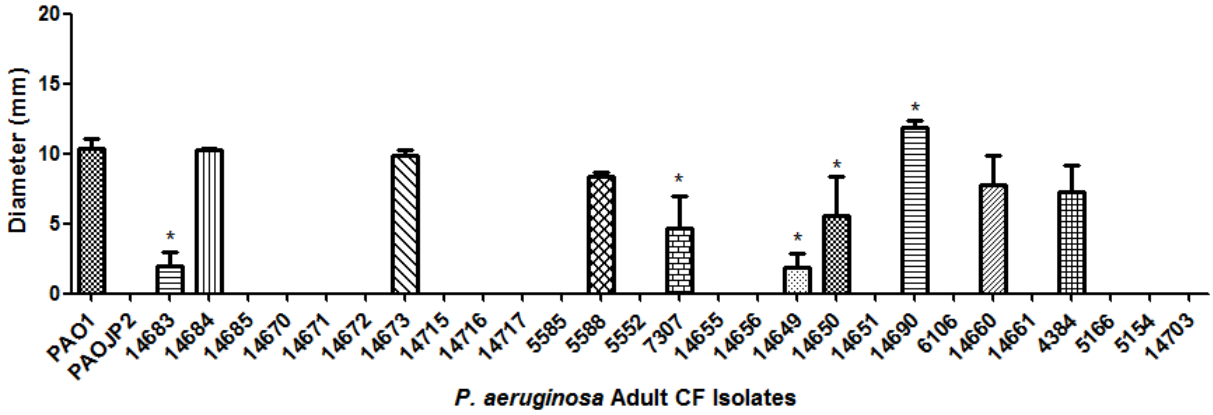
3.2.1.1 Inhibition activity of *P. aeruginosa* CF isolates on *S. aureus* ATCC strains 13709, 25923, 29213 and CF isolate C105

Figure 5a,b,c,d shows that each individual *P. aeruginosa* CF isolate can produce variable levels of a *S. aureus* inhibitory exoproducts ranging from less than, equal to or greater than PAO1. Interestingly, even isolates taken from the same sputum sample collected from the same patient on the same day show different inhibitory activity, which was the case for all 13 patients. An example of this would be isolates 14672 and 14673 taken from the same sputum sample (patient 91). These particular isolates give some insight into phenotypic variability that can exist amongst *P. aeruginosa* CF isolates. Our initial hypothesis stated that, all *P. aeruginosa* CF isolates would be able to inhibit the growth of *S. aureus* however that was not the case as the majority of *P. aeruginosa* isolates could not inhibit any *S. aureus* strains. In addition, each of the *S. aureus* isolates responded slightly differently to the various *P. aeruginosa* isolates (Figure 5). For example *S. aureus* isolate 13709 was inhibited at a level similar to that of PAO1 by 6 of the isolates, was inhibited at a level lower than PAO1 by 4 isolates and not inhibited by 18 isolates. On the other hand isolate C105 was inhibited by 8 *P. aeruginosa* isolates at a level equal to or higher that PAO1, only one isolate had a slight inhibitory effect and 19 of the *P. aeruginosa* isolates did not inhibit C105 at all (Figure 5A and D) (further analysis found in section 3.2.1.2).

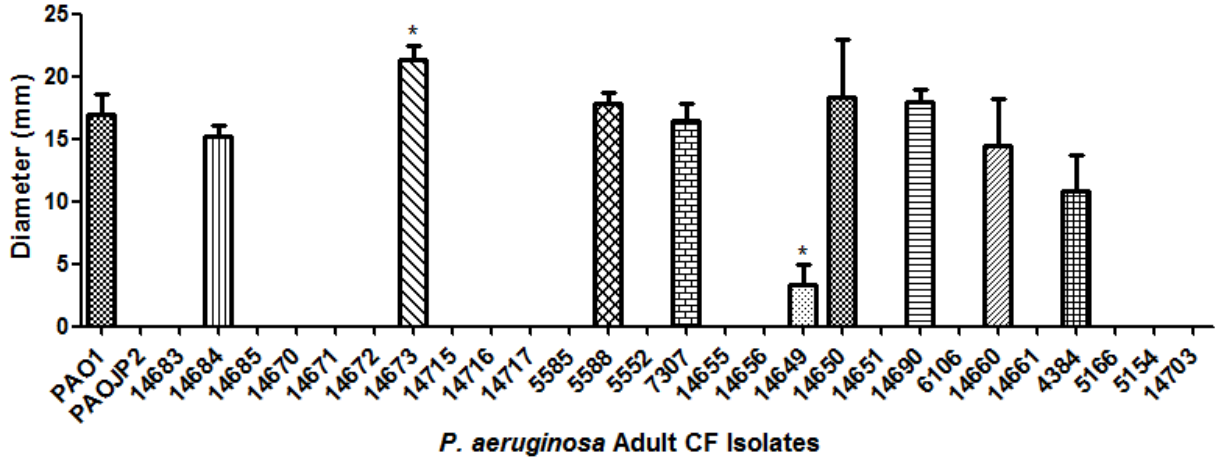
Figure 5: *P. aeruginosa* CF isolates exhibit a diverse range of inhibition activity on *S. aureus* ATCC strains 13709 (A), 25923 (B), 29213 (C) and isolate C105 (D).

Inhibition of *S. aureus* growth caused by *P. aeruginosa* supernatants were determined using molten overlay agar plates containing *S. aureus* on a Mannitol salt agar base. The diameter of the zone of clearance resulted from growth inhibition of *S. aureus* caused by a secreted factor found in *P. aeruginosa* supernatant. PAO1 was used as the positive control for *S. aureus* inhibition while PAOJP2 acted as the negative inhibitory control. Experiments were completed three times in triplicate with error bars representing standard deviation (SD). Data were analyzed using one-way ANOVA and significant variation from results with PAO1 was determined as $p<0.05$ and denoted by *. All isolates with no diameter value differed significantly from results with PAO1.

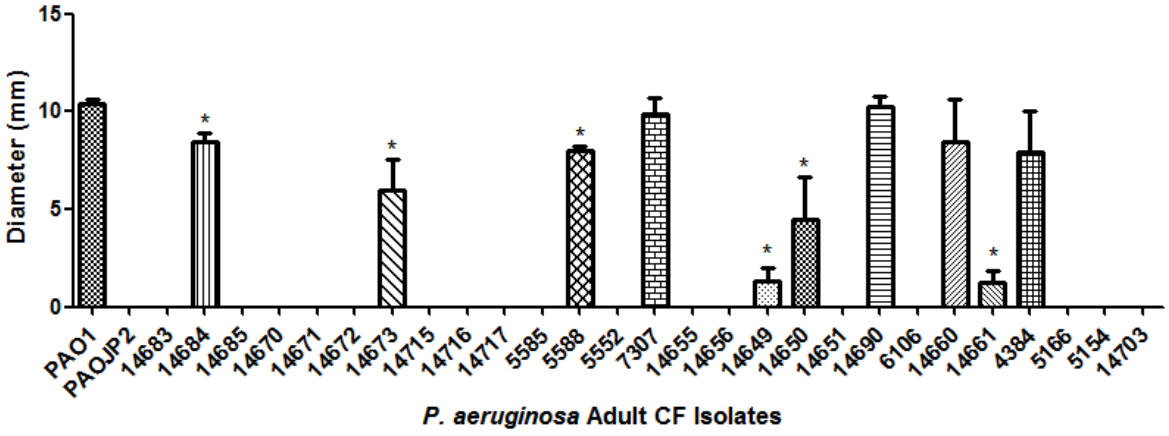
A) 13709



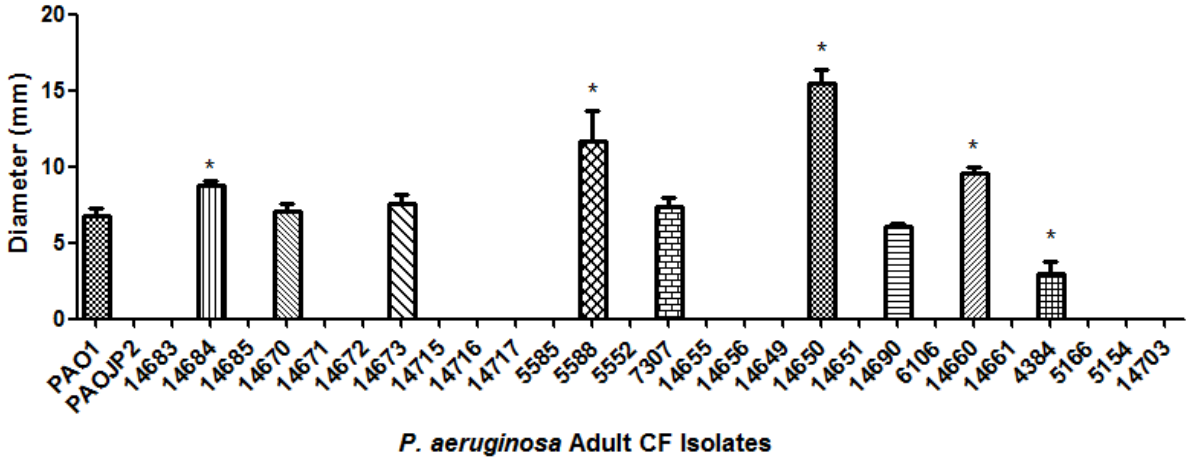
B) 25923



C) 29213



D) C105



3.2.1.2 Overall comparison of inhibition activity of *P. aeruginosa* CF isolates

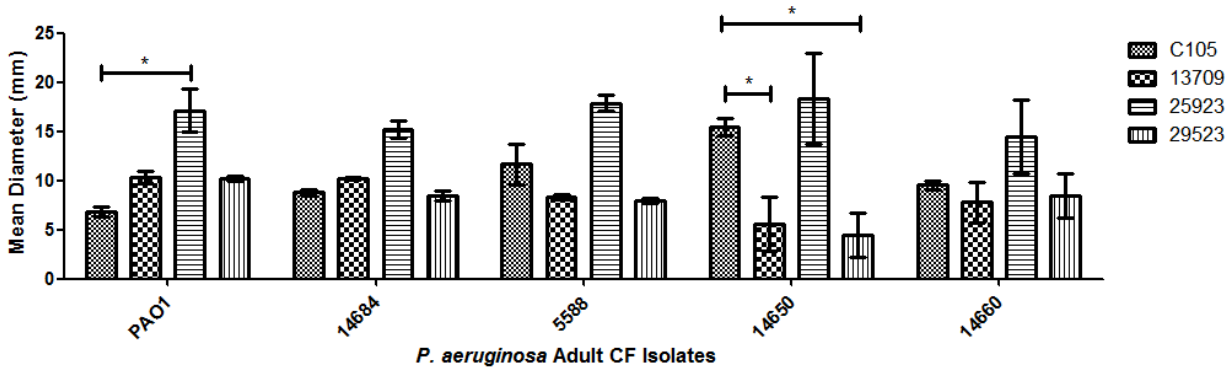
P. aeruginosa CF isolate inhibitors of C105 were organized into three different categories (based on data from section 3.2.1.1): strong C105 inhibitors, intermediate C105 inhibitors and weak C105 inhibitors. This was done for two reasons, the first being, since PAO1 is the prototypical strain we can assume the production of staphylococcal inhibitor being produced is at a standard level and we hypothesize that any CF isolate that produces a greater or lesser amount of staphylococcal inhibitor have a phenotype different than PAO1. Secondly, we wanted to test if inhibition of *S. aureus* would vary depending on the strain or isolate being used. Figure 6A showed that among the strong C105 inhibitors, all four *S. aureus* isolates were sensitive to the factors produced by these strains. ATCC strain 25923 is the most sensitive to PAO1 and the four CF isolates whereas ATCC strain 29523 is the least sensitive.

Among the intermediate C105 inhibitors, *P. aeruginosa* isolate 14670 was able to inhibit C105 whereas it did not inhibit any of the three ATCC strains (figure 6B). In contrast, for the five other *P. aeruginosa* isolates (14673, 5588, 7307, 14690 and 4384) all four *S. aureus* isolates were inhibited to varying degrees. Again ATCC strain 25923 was the most susceptible *S. aureus* isolate to all the *P. aeruginosa* supernatants except for 14670. These first two groups, except for *P .aeruginosa* isolate 14670, were behaving as we might have suspected in that these strains were all able to inhibit all the *S. aureus* strains (Figure 6AB). The third group of *P. aeruginosa* isolates, those that did not inhibit C105, were interesting because the majority of these isolates did not inhibit any of the *S. aureus* strains tested (Figure 6C). The two exceptions were isolate 14683 that only

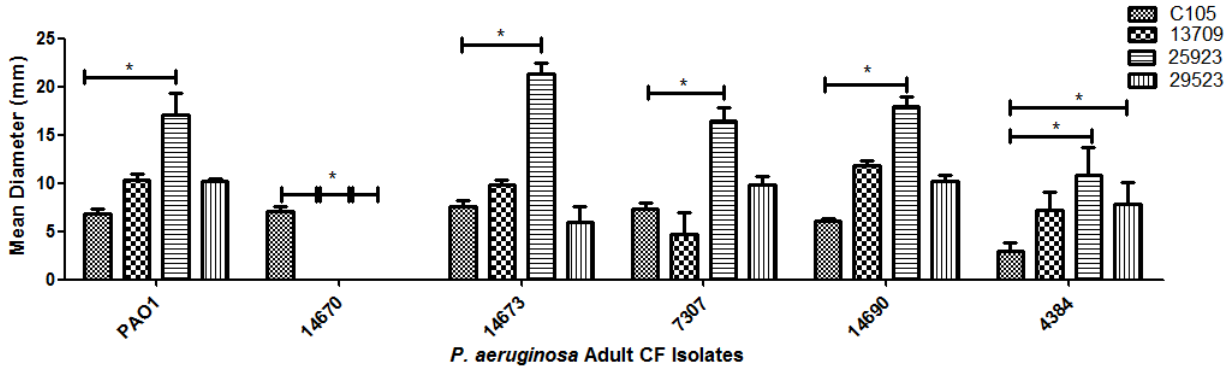
Figure 6: Inhibition zones of the *S. aureus* isolates in response to *P. aeruginosa* CF isolates based on initial inhibition of *S. aureus* isolate C105.

Data from section 3.2.1.1 were organized into three different categories A) strong C105 inhibitors B) intermediate C105 inhibitors and C) non-inhibitors of C105. Variation of inhibition intensity of each individual *P. aeruginosa* CF isolate was tested against C105 inhibition through one-way ANOVA analysis where significance is denoted by * and represents a p value <0.05.

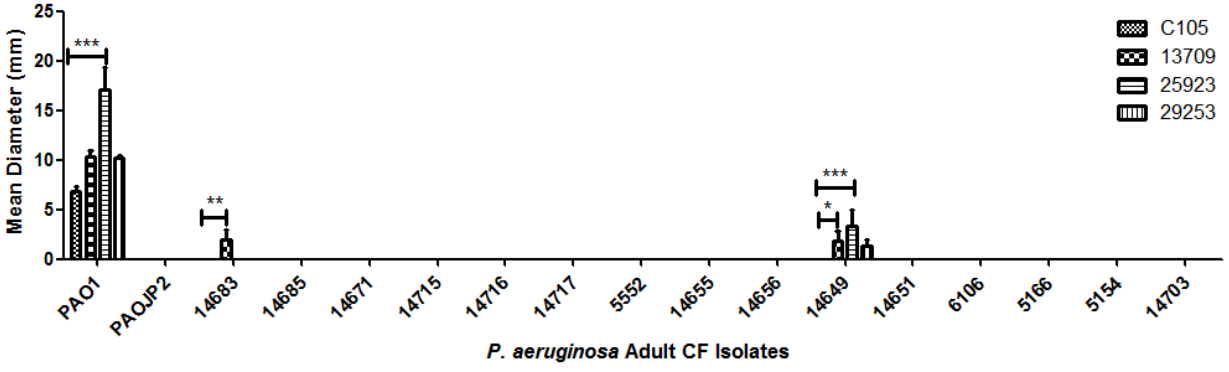
A) Strong Inhibitors



B) Intermediate Inhibitors



C) Non Inhibitors



inhibited *S. aureus* strain 13709 and isolate 14649 that inhibited all the ATCC *S. aureus* isolates. The variability of these two strains *P. aeruginosa* isolates 14649 and 14683 coupled with isolate 14670 suggested that some *S. aureus* strains may have evolved mechanisms to resist respiratory toxins produced by *P. aeruginosa*.

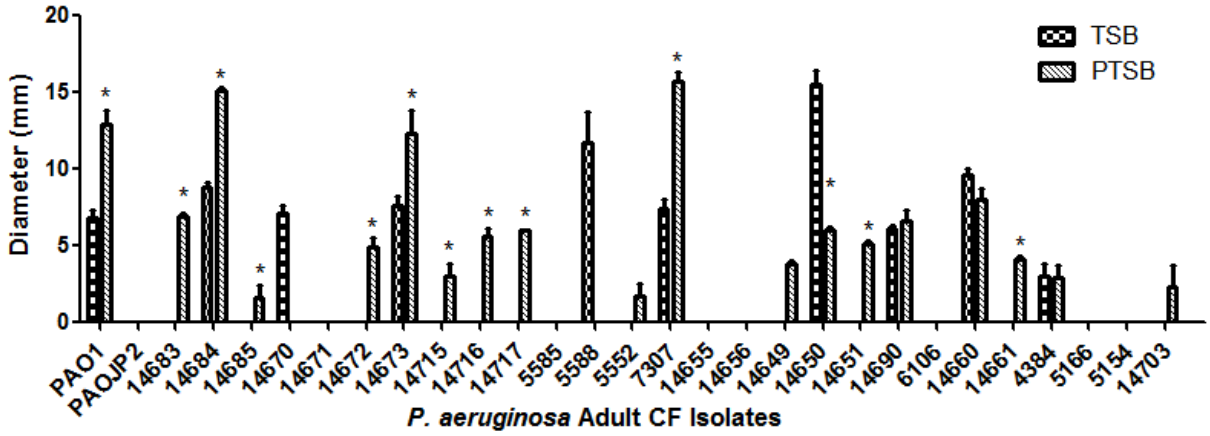
3.2.1.3 Growth medium can influence anti-staphylococcal agent production in *P. aeruginosa* CF isolates against C105

As shown in Figure 5 and 6, PAOJP2, a QS null strain, did not produce any factors that inhibited the four *S. aureus* strains tested. This suggested that the inhibitory factors might be regulated by the quorum sensing system of *P. aeruginosa*. To test this possibility we grew the *P. aeruginsoa* isolates in PTSB, a medium that has been shown to optimize the production of elastase (Ohman *et al.,* 1980); gene, *lasB*, is controlled by the *las* and *rhl* QS systems in *P. aeruginosa* (Gambello and Iglewski, 1991). Testing for QS regulation of the anti-staphylococcal agent is in line with current literature as LasA and HQNO are both controlled by the global regulatory system in *P. aeruginosa*, and as a result we hypothesized that inhibition profiles of *P. aeruginosa* would differ when grown in PTSB as compared to TSB.

For some *P. aeruginosa* C105 inhibitors, zones of clearance increased when grown in PTSB as seen in Figure 7 – this was the case for PAO1, 14684, 14673 and 7307. Other inhibitor profiles were unchanged when grown in PTSB such as 14690, 14660 and 4384. Interestingly, many *P. aeruginosa* CF isolates that did not inhibit C105 when grown in TSB have the ability to do so when grown in PTSB. This was the case for 14683, 14685, 14672, 14715, 14716, 14717, 5552, 14649, 14651, 14661 and 14703. However, the trends

Figure 7: PTSB increases inhibitor production in *P. aeruginosa* against *S. aureus* CF isolate C105.

Inhibition of *S. aureus* C105 growth caused by *P. aeruginosa* supernatant was determined using molten overlay agar plates containing *S. aureus* on a MSA base. *P. aeruginosa* CF isolates were grown overnight in PTSB. Cell free supernatants were then spotted on the overlay plates with *S. aureus.* The diameter of the zone of clearance resulted from growth inhibition of *S. aureus* caused by a secreted factor found in *P. aeruginosa* supernatant. PAO1 was used as the positive control for *S. aureus* inhibition while PAOJP2 acted as the negative inhibitory control. Experiments were completed three times in triplicate with error bars representing standard deviation (SD). Data were analyzed using one-way ANOVA and significant variation ($p<0.05$) to the TSB value was denoted as *.


20
15
10
5
0
Diameter (mm)
TSB
PTSB
PAO1
PAOJP2
14683
14684
14685
14670
14671
14672
14673
14715
14716
14717
5585
5588
5552
7307
14655
14656
14649
14650
14651
14690
6106
14660
14661
4384
5166
5154
14703
P. aeruginosa Adult CF Isolates

were not absolutely clear as two isolates grown in PTSB, 5588 and 14670 did not inhibit C105 even though the TSB supernatants did inhibit *S. aureus* isolate C105. Taken together the data suggested that the inhibtory factors involved were at least partially regulated by the *P. aeruginosa* QS system.

3.2.1.4 Inhibition of *P. aeruginosa* CF isolates on C105 is observed in liquid culture

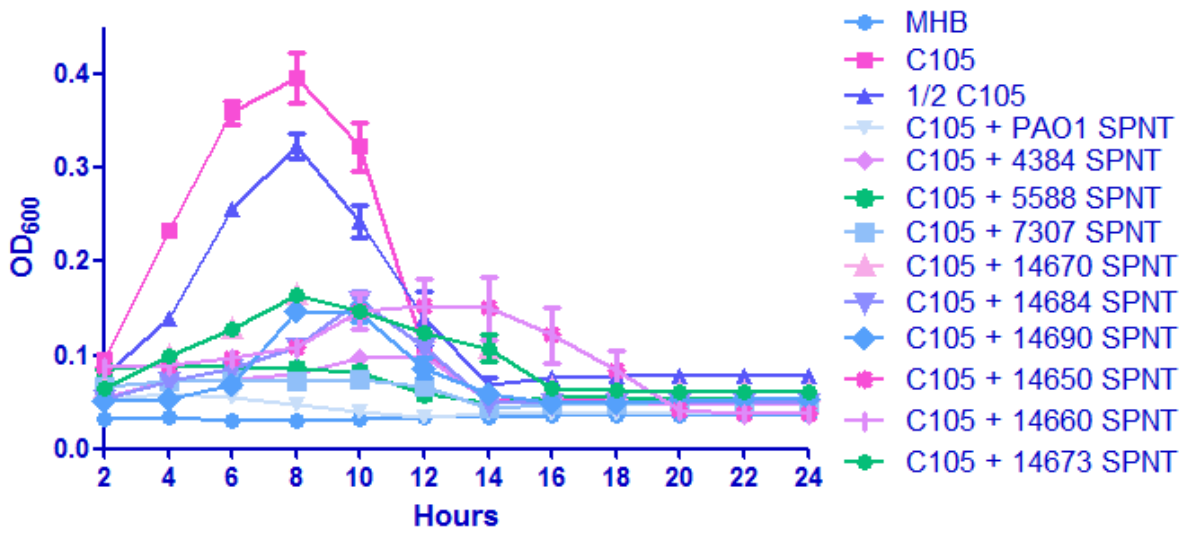
Cultures of C105 were grown with inhibiting supernatants to confirm *P. aeruginosa* antagonistic interactions would be mediated in a liquid state. Figure 8 shows antagonistic interactions were observed with the addition of *P. aeruginosa* cell free supernatant to C105 culture and grown for 24 hours in order to observe inhibitory interactions. Equal concentrations of C105 (1/2 C105 with medium) and C105 with *P. aeruginosa* supernatant show a significant reduction in liquid culture growth (Figure 8a). PAO1 supernatant with C105 showed the most significant reduction in growth by exhibiting a 3 fold difference in growth curves to the control (1/2 C105) which was similar to the MHB uninoculated control. This is contrary to the plate assays as 14684, 5588, 14650 and 14660 were stronger inhibitors than PAO1 (Figure 5d) whereas in this experiment PAO1 is the strongest inhibitor. After PAO1, the next strongest inhibitors were 4384, 5588, 7307 and 14690 as they all reduced C105 growth by 2 fold leaving 14670, 14684, 14650, 14660 and 14673 as the weakest inhibitors by reducing growth by only 1 fold.

To ensure only inhibiting *P. aeruginosa* isolates can mediate antagonistic interactions three negative controls were utilized PAOJP2, PA103 and 14655 (Figure 8b). PA103 is deficient for the global regulator *lasR* (Gambello and Iglewski, 1991) which would stop production of most virulence factors and therefore a good negative control. The *P. aeruginosa* CF isolate 14655 was chosen as a negative control since it did not inhibit

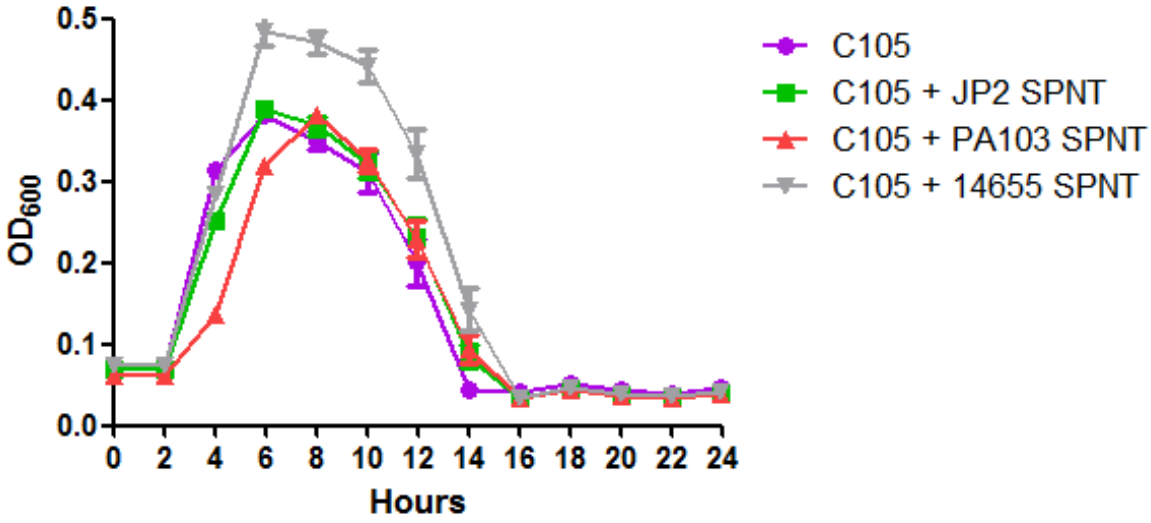
Figure 8: *P. aeruginosa* supernatants were able to inhibit growth of C105 in liquid culture.

Growth curves of C105 (grown in MHB) with *P. aeruginosa* supernatant were completed using the Victor X4 in 96 well plates containing 75 µl of normalized C105 culture and 75 µl of *P. aeruginosa* cell free supernatant with an overlay of 30 µl of sterile mineral oil. The plate was incubated at 37°C for 24 hours. Graph A represents growth curves of C105 culture with supernatant from all *P. aeruginosa* CF isolate inhibitors of C105. Graph B represents C105 culture with supernatant from the negative controls PAOJP2, PA103 and 14655. Experiments were performed in triplicate three times with error bars representing SD. Area under the curve was calculated and fold differences were used to compare each condition.

A)


MHB
C105
1/2 C105
C105 + PAO1 SPNT
C105 + 4384 SPNT
C105 + 5588 SPNT
C105 + 7307 SPNT
C105 + 14670 SPNT
C105 + 14684 SPNT
C105 + 14690 SPNT
C105 + 14650 SPNT
C105 + 14660 SPNT
C105 + 14673 SPNT
OD600
0.0
0.1
0.2
0.3
0.4
2
4
6
8
10
12
14
16
18
20
22
24
Hours

B)


C105
C105 + JP2 SPNT
C105 + PA103 SPNT
C105 + 14655 SPNT
OD600
0.0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
8
10
12
14
16
18
20
22
24
Hours

any *S. aureus* strains or isolate in any condition. All negative controls did not inhibit growth of C105. These data support my previous data that suggested that only certain *P. aeruginosa* isolates can inhibit *S. aureus* and that the inhibitory factor produced by *P. aeruginosa* may be controlled through QS. However, it is still unclear, at this point, what the inhibitory factor(s) are.

3.2.2 *P. aeruginosa* CF isolates mediate inhibitory interactions with C105 through a secreted factor that is less than 5 kDa and resistant to heat

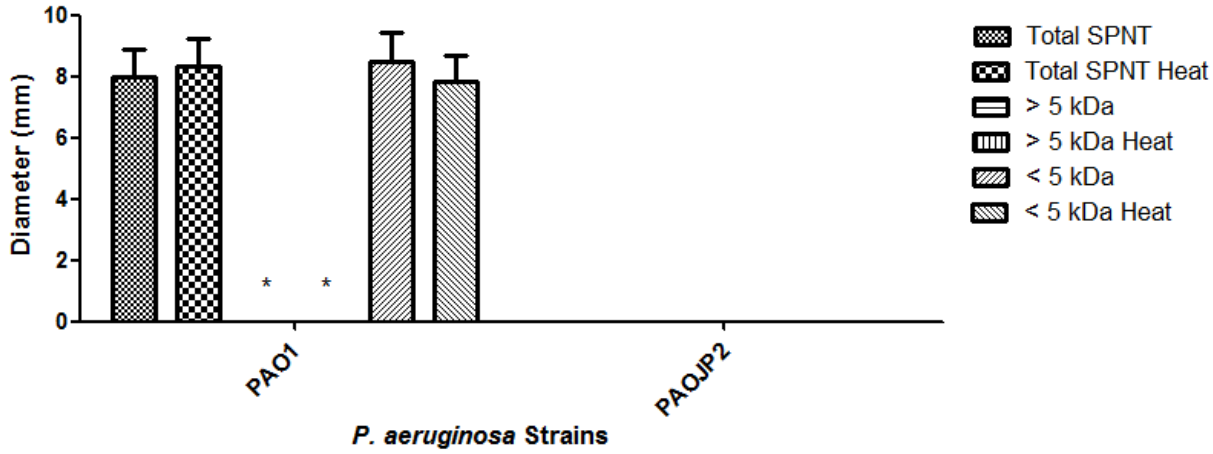
In section 3.2.1 it was established that the supernatants of 9 *P. aeruginosa* CF isolates can mediate antagonistic interactions with a *S. aureus* CF isolate, C105, likely via a secreted product. *P. aeruginosa* secretes a wide range of virulence factors and secondary metabolites that can influence inhibitory effects (Harrison *et al.,* 2007). The mechanism that controls this particular interaction between these CF isolates is unknown and the purpose of this section was to investigate if the inhibitory agent is a protein or small molecule since two known anti-staphylococcal agents are the protein LasA (Kessler *et al.,* 1993) and a small molecule HQNO (Machan *et al.,* 1992) in addition to hydrogen cyanide and pyocyanin (Voggu *et al.,* 2006).

The cell free supernatant was separated using a molecular weight cut off column of 5 kDa. Doing so would place most, if not all, proteins in the greater than 5 kDa fraction and all small molecules in the less than 5 kDa fraction. Since LasA is 20 kDa (Kessler *et al.,* 1993) it would be in the greater than 5 kDa fraction. In addition to size fractionation of the supernatant, by size it was also heat treated for 10 minutes at 90°C to denature and inhibit the activity of any proteins that may act as an anti-staphylococcal agent. Most small molecules would not be affected by the heat treatment and would retain activity. All *P.*

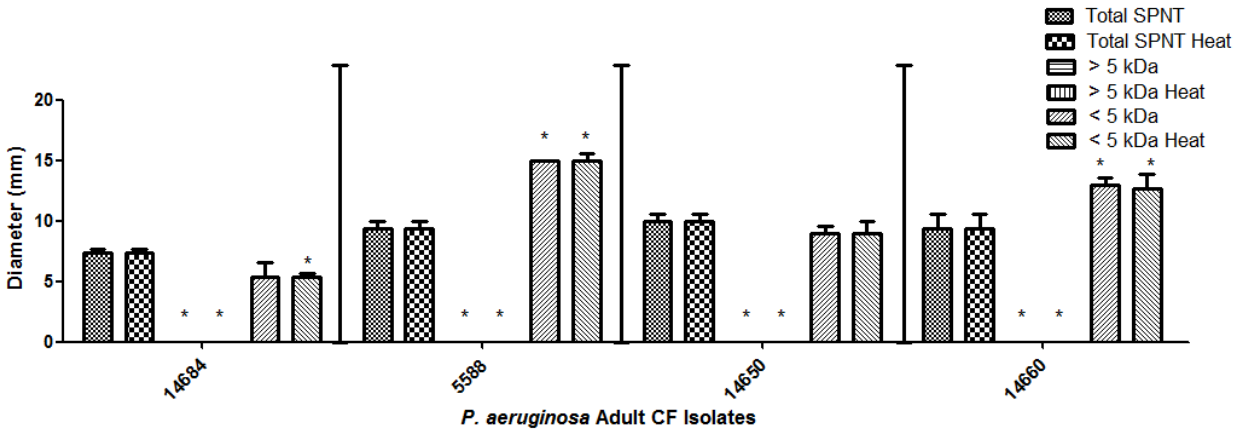
Figure 9: The secreted inhibitory factor produced by *P. aeruginosa* retains its effect after heat treatment and is found in the less than 5 kDa fraction.

Activity of the anti-staphylococcal factor was tested using MSA overlay plates containing standardized C105. *P. aeruginosa* cell free supernatants were heat treated, at 90 ºC for 10 minutes, and/or additionally subjected to fractionation into a >5kDa and a <5kDa. Panel A) depicts *P. aeruginosa* controls, B shows inhibition levels of C105 strong inhibitors and C shows C105 intermediate inhibitors. Experiments were preformed in triplicate, twice. Error bars indicate standard deviation. Data were analyzed using one-way ANOVA and significant variation ($p<0.05$) to the total supernatant value was denoted as *.

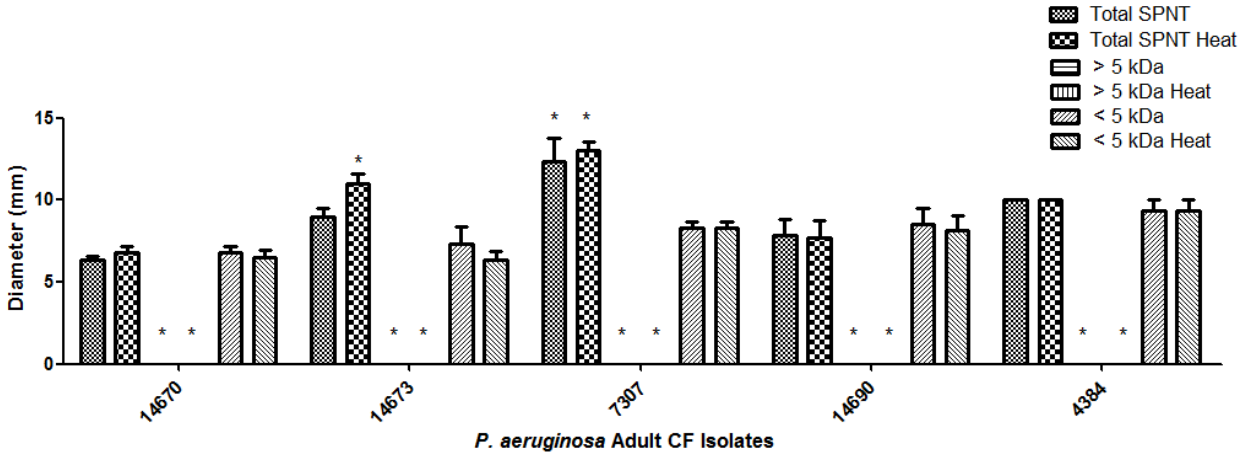
A) *P. aeruginosa* Controls



B) C105 Strong Inhibitors



C) C105 Intermediate Inhibitors



aeruginosa inhibitory CF isolates including the prototypical PAO1 displayed inhibitory activity in the less than 5 kDa fraction and this was not affected by heat treatment (Figure 9a, b, c). Interestingly, no activity was found in the greater than 5 kDa fraction for all inhibiting isolates. In Figure 9a, there is no significant difference between the total supernatant and the heat treated fractions nor the <5 kDa fractions suggesting the inhibitory factor maybe present in these factions. This was the case for all inhibitory CF *P. aeruginosa* isolates.

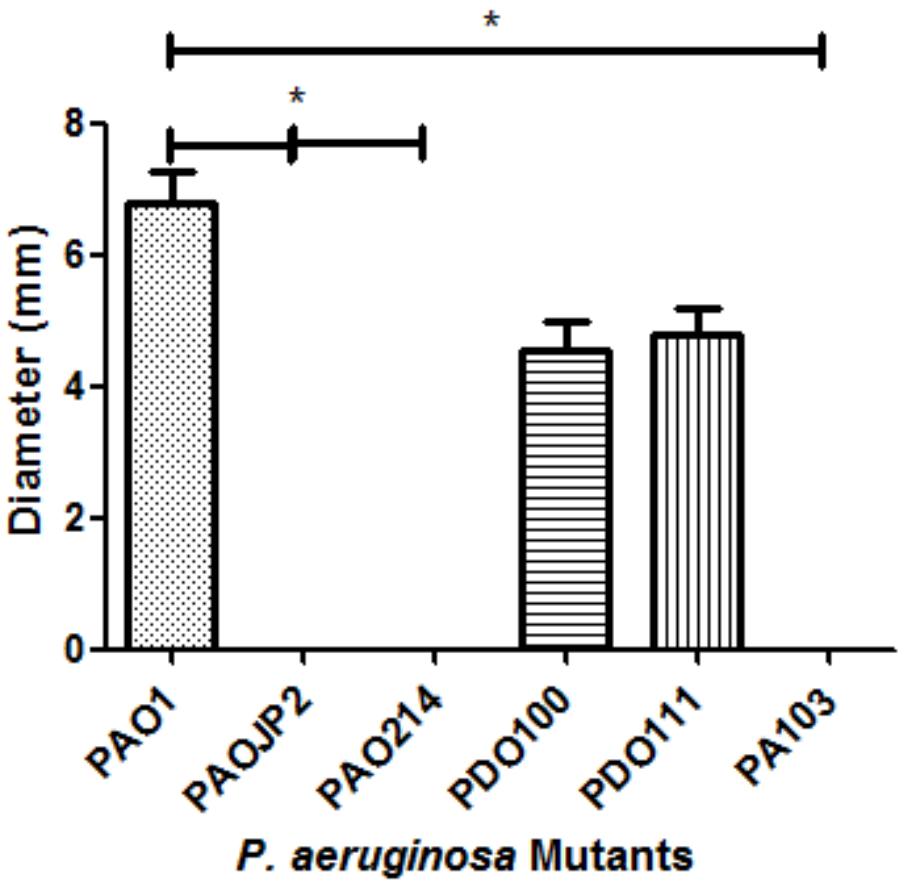
3.2.3 The global regulatory system, LasIR, in *P. aeruginosa* plays an important role in mediating inhibitory interactions with C105

The *las* system in *P. aeruginosa* is considered to be at the top of the hierarchy in terms of QS regulation (as reviewed in section 1.3.1). Since many of *P. aeruginosa* virulence factors are controlled by this system (section 1.3.2) and HQNO, a known antistaphylococcal agent, is also ultimately controlled by *lasR* (section 1.3.3) we wanted to test for inhibition of *S. aureus* growth using different QS mutants. We hypothesized that the inhibitory factor would be controlled by the las system and thus wanted to test inhibition using *P. aeruginosa* QS mutants.

Five QS mutants were used to test this hypothesis PAOJP2, PAO214, PDO100, PDO111 and PA103 (for information on their mutations see Table 1 in chapter 2). Strains that carry mutations in the *rhl* QS system (PDO100 AND PDO111) were able to inhibit the growth of *S. aureus* as shown in Figure 10. The intensity to which those strains inhibited *S. aureus* was slightly less than the parental strain PAO1, but not significantly less, and was supported using one-way ANOVA analysis. This suggested that the *rhl* system does not play a central role in controlling the inhibitory factor. The *las* system, however, may play a

Figure 10: PAO1 isogenic mutants of *lasI/rhlI* and *lasI* as well as a nonisogenic mutant of *lasR* do not retain an inhibitory effect on C105.

Inhibition of *S. aureus* growth caused by *P. aeruginosa* supernatants was determined using molten overlay agar plates containing *S. aureus* on a MSA base. The diameter of the zone of clearance resulted from growth inhibition of *S. aureus* caused by a secreted factor found in *P. aeruginosa* supernatant. PAO1 was used as the positive control for *S. aureus* inhibition while PAOJP2 acted as the negative inhibitory control. Experiments were completed three times in triplicate with error bars representing standard deviation (SD). Data were analyzed using one-way ANOVA and significant variation to PAO1 was determined as $p<0.05$ and denoted by *.


*
*
8
6
4
2
0
Diameter (mm)
PAO1
PAOJP2
PAO214
PDO100
PDO111
PA103
P. aeruginosa Mutants

central role in the regulation of the inhibitory factor as when either *lasI* or *lasR* were mutated, no inhibitory effect was present. Overall this experiment suggests that the *las* system may play a critical role in inhibitory regulation whereas the *rhl* system does not seem to have any large regulatory effects on the inhibitory factor.

3.2.4 The secreted inhibitory factor produced by *P. aeruginosa* is able to induce a phenotypic change to small colony variants in C105 similarly as synthetic HQNO

Since our inhibitory factor was found to be less than 5 kDa, unaffected by heat, and possibly controlled by the *las* QS system, we hypothesized that the inhibiting factor is most likely HQNO. The phenotypic changes that *S. aureus* acquires when the bacterium interacts with HQNO are well documented (Acar *et al.,* 1978; Balwit *et al.,* 1994; Kahl *et al.,* 1998; Hoffman *et al.,* 2006; Mitchell *et al.,* 2011a; Mitchell *et al.,* 2011b; Yagci *et al.,* 2013). Thus, we were expecting *S. aureus* to switch phenotypes to SCVs when treated with *P. aeruginosa* supernatant. Phenotypic variation to SCVs was tested by examining changes in size, resistance to aminoglycosides and hemolytic activity. Knowing that HQNO can cause a clear phenotypic difference in *S. aureus,* we tested if the supernatant from the 9 *P. aeruginosa* inhibiting CF isolates can cause *S. aureus* to switch phenotypes.

3.2.4.1 *P. aeruginosa* supernatant disrupts the electron transport chain in C105 which causes resistance to aminoglycosides

HQNO is known to disrupt the electron transport chain in *S. aureus* as it is thought to bind to cytochrome bo_3, which in turn decreases the electron potential of the membrane (Proctor, 1998). Decreasing the electron potential inhibits antibiotics that require a high gradient to enter the cell such as aminoglycosides, an example of which is tobramycin (Mates *et al.,* 1983). From the work conducted in Sibley *et al.,* 2011, it is known that *P.*

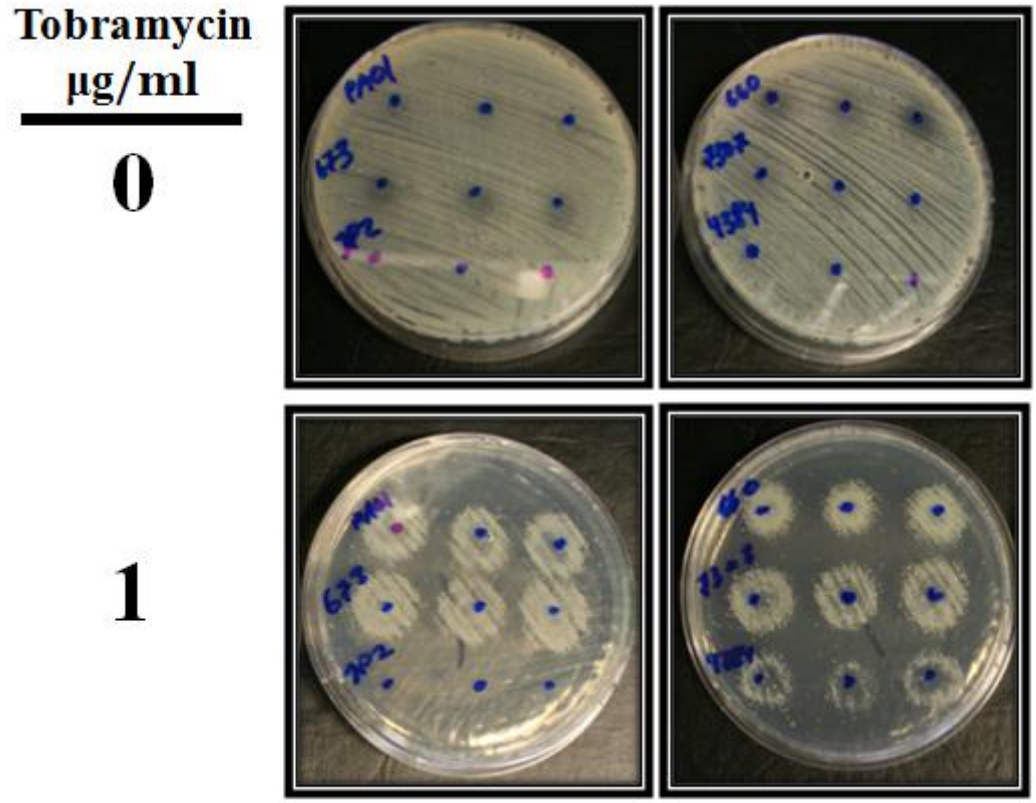
aeruginosa and *S. aureus* frequently co-colonize the adult CF lung, underlining the clinical significance of studying SCVs induction from *P. aeruginosa* CF isolates. To test if the 9 *P. aeruginosa* inhibiting CF isolates can induce SCVs we tested for tobramycin resistance when treated with supernatant (Figure 11).

Figure 11 clearly shows that when *P. aeruginosa* supernatant is spotted onto lawns of C105 on tobramycin plates, growth is observed only where the supernatant was spotted suggesting the inhibitory factor in the supernatant is conferring resistance to tobramycin. Table 2 outlines that all 9 *P. aeruginosa* CF isolates are able to confer tobramycin resistance on C105 as does synthetic HQNO. From this experiment we hypothesize that it is the electron transport chain that is being inhibited and not protein synthesis (which is the target for tobramycin) since the positive control (synthetic HQNO) looks exactly like *P. aeruginosa* supernatant.

Figure 11: C105 becomes resistant to tobramycin once treated with *P. aeruginosa* supernatant.

Lawns of C105 on MHA with or without 1 μg/ml of tobramycin were spotted with *P. aeruginosa* supernatant and incubated at 37° C for 24 hours. Pictures depict plates after 24 hours of incubation. Experiment was completed in triplicate and repeated three times. Panel A depicts supernatants from CF isolates whereas Panel B shows spotting from synthetic HQNO. In Panel 11a isolates are labelled as follows starting from the top left: PAO1, 673 (14673), JP2 (PAOJP2), 660 (14660), 7307 and 4384. And in 11b isolates are labelled as PAO1, PA14, 4383, 14670, synthetic HQNO 10 μg/ml and synthetic HQNO 5 μg/ml.

A)



B)

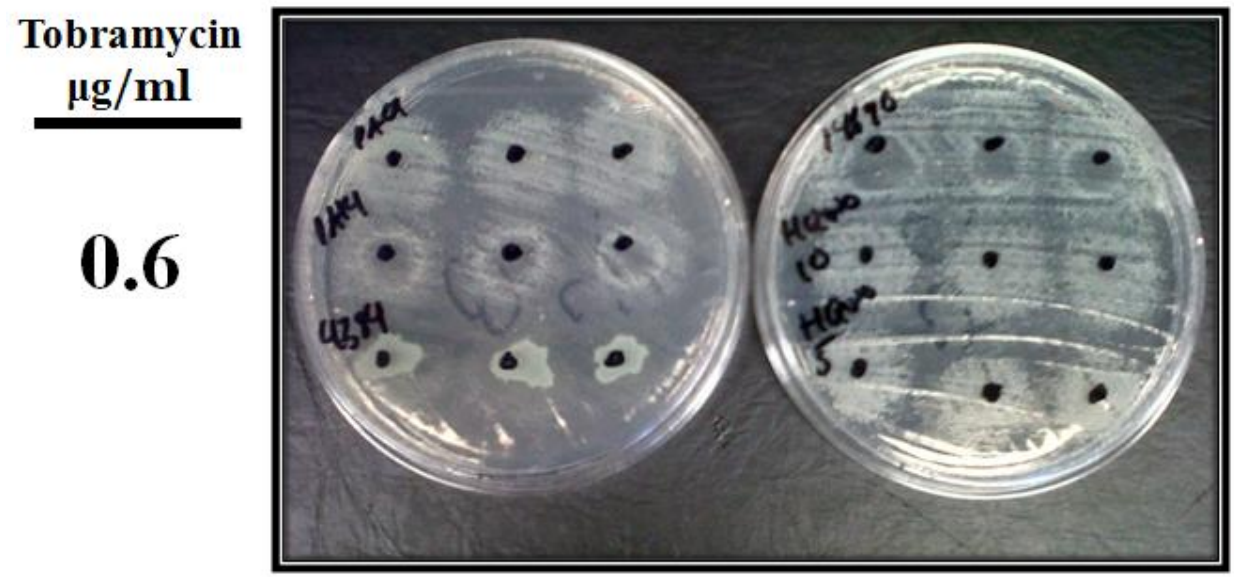


Table 2 : Tobramycin resistance is conferred on C105 by *P. aeruginosa* inhibiting isolates.

Strain, isolate or chemical	1µg/ml Tobramycin Resistant colonies
PAOJP2	-
HQNO	+
PAO1	+
14684	+
14670	+
5588	+
7307	+
14690	+
14660	+
4384	+

3.2.4.2 *P. aeruginosa* supernatant reduces hemolytic activity in C105

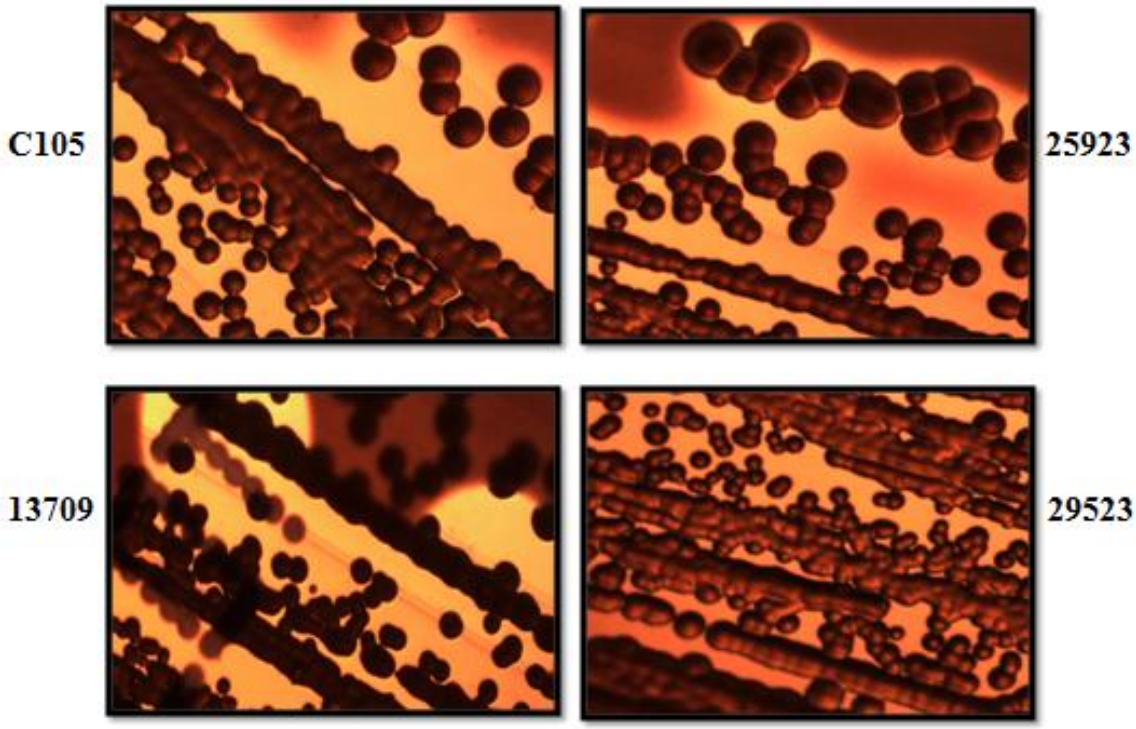
When *S. aureus* is in its SCV state it is not producing virulence factors as the cell is considered to be slow growers (Kahl *et al.,* 1998). Therefore, *S. aureus* SCVs are non hemolytic (Kahl *et al.,* 1998). Hemolytic activity is controlled via the *arg* QS system in *S. aureus.* However, when it differentiates into the SCV phenotype, cells are not communicating with one another. Therefore in order to ensure that the cells from section 3.2.4.2 had switched to a SCV phenotype we tested for hemolytic activity. Figure 12 and Table 3 represents the summary of the hemolytic activity for C105, *S. aureus* ATCC strains 13709, 25923 and 29253. WT *S. aureus* can undergo beta hemolysis; SCVs on the other hand are unable to undergo hemolytic activity. Table 3 shows that the C105 SCV demonstrates only gamma hemolysis, confirming our suspicion of switching phenotypes.

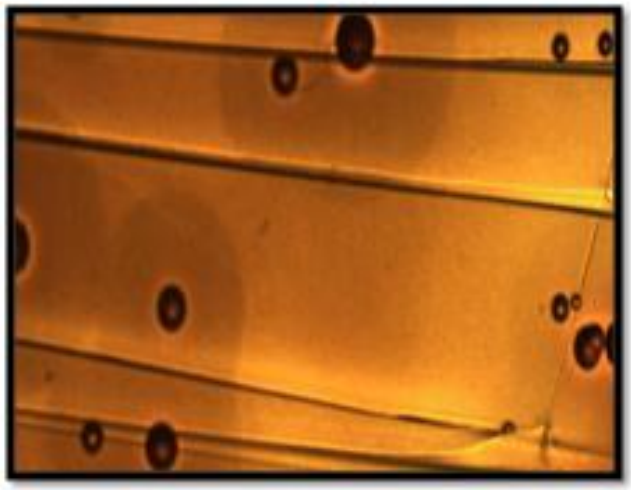
3.2.4.3 *P. aeruginosa* supernatant changes colony morphology of C105

SCVs are one tenth the size of WT *S. aureus* so in order to confirm that the colony morphology had switched to SCV we visualized the colonies taken from a liquid culture of C105 with PAO1 supernatant. Figure 13 shows the comparison between a large *S. aureus* WT colony and a SCV colony

Figure 12: Hemolytic activity in *S. aureus* SCV is reduced.

Colonies of ATCC strains 13709, 25923, 29523 and CF isolate C105 and SCVs of C105 were streaked onto LBA and incubated at 37◦C overnight. The following day, one colony was picked from each plate and streaked onto Columbia blood agar and incubated at 37◦C for 24 hours. Complete degradation of red blood cells (beta hemolysis) was observed by a yellow hue surrounded by *S. aureus* colonies as was seen in C105, 13709, 25923 and 29523. No degradation (gamma hemolysis) or a reduction of degradation was observed in C105 SCVs.





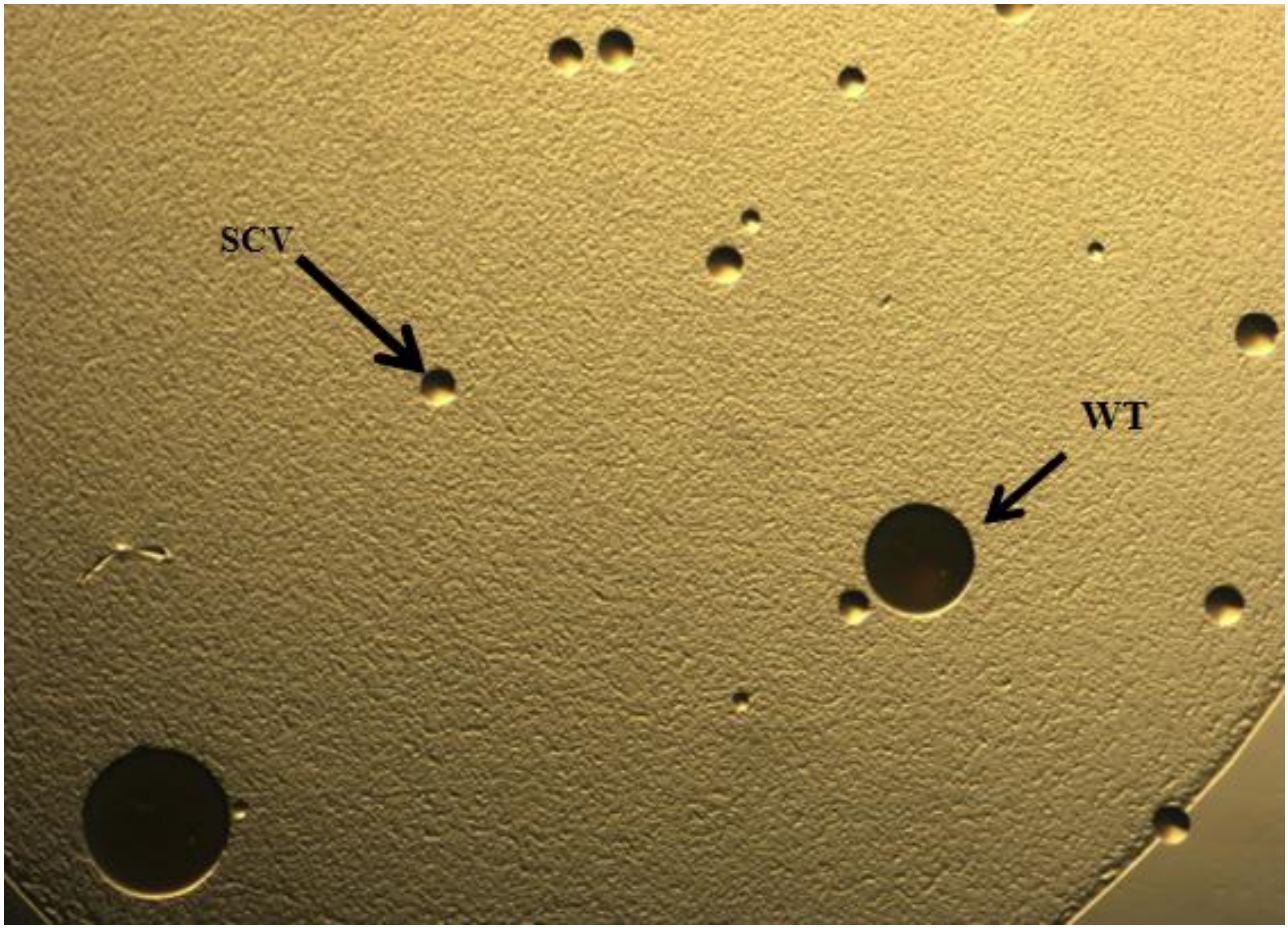
SCV C105

Table 3: Table 3: Hemolytic activity of WT *S. aureus* and SCV

Strain/ Isolate	α	β	γ
WT C105		X	
SCV C105			X
ATCC 29213		X	
ATCC 13709		X	
ATCC 25923		X	

Figure 13: *S. aureus* SCV are one tenth the size of WT *S. aureus*.

C105 was grown with PAO1 supernatant overnight then serial diluted and spotted onto LBA. A mixture of phenotypes WT and SCV was always observed on the plate. The large circular black colony represents a WT *S. aureus* colony. And the colony directly adjacent to this represents a SCV. This experiment was completed in triplicate three times. The colonies were visualized using a disection microscope.


SCV
WT

3.2.5 The inhibitory factor secreted by PAO1, 7307, 14670 and 4384 is the small molecule HQNO

In section 3.2.4 it was made clear that the inhibitory factor behaved similarly to HQNO as supernatant from inhibiting *P. aeruginosa* CF isolates cause C105 to switch into a SCV phenotype. Since it is well documented that HQNO is produced by *P. aeruginosa*, and can inhibit *S. aureus* growth by inducing a SCV phenotype, we hypothesized that the inhibiting factor being produced is HQNO. The final step to confirming that HQNO was present in the supernatant and responsible for the growth inhibition and the induction of SCV was to physically identify the molecule. Molecule identification was completed using gas chromatography mass spectrometry (GC-MS). Previous works had used liquid chromatography mass spectrometry (LC-MS) to identify HQNO (Lepine *et al.,* 2004; Hoffman *et al.,* 2006; Zaborina *et al.,* 2007) putting our methods in line with other research.

The mass spectrum and ion fragment pattern of HQNO was compared to supernatants of PAO1, PAOJP2 and the nine inhibiting *P. aeruginosa* CF isolates (Figures 14, 15, 16 and Table 4). Synthetic HQNO has a retention time of 31.071 and all fragment ion patterns depict peaks similar to HQNO. From the GC-MS data an unexpected conclusion can be made – not all inhibiting *P. aeruginosa* CF isolates produce HQNO yet all are able to induce SCVs. Table 4 summarizes the isolates that are able to produce HQNO which include PAO1, 14670, 7307 and 4384. Interestingly, all C105 strong inhibitors do not produce HQNO whereas less than half of the intermediate C105 inhibitors do produce HQNO. This suggests that an additional small molecule may be produced by *P.*

Figure 14: PAO1 and PAOJP2 both produce the small molecule HQNO.

Overlay of mass spectrum graphs for PAO1, PAOJP2 and synthetic HQNO. The largest peaks represent synthetic HQNO, the second largest is PAO1 and the smallest is PAOJP2. The first peak represents the molecule HQNO without the N-Oxide as this compound is unstable whereas the second peak represent the intact HQNO molecule.

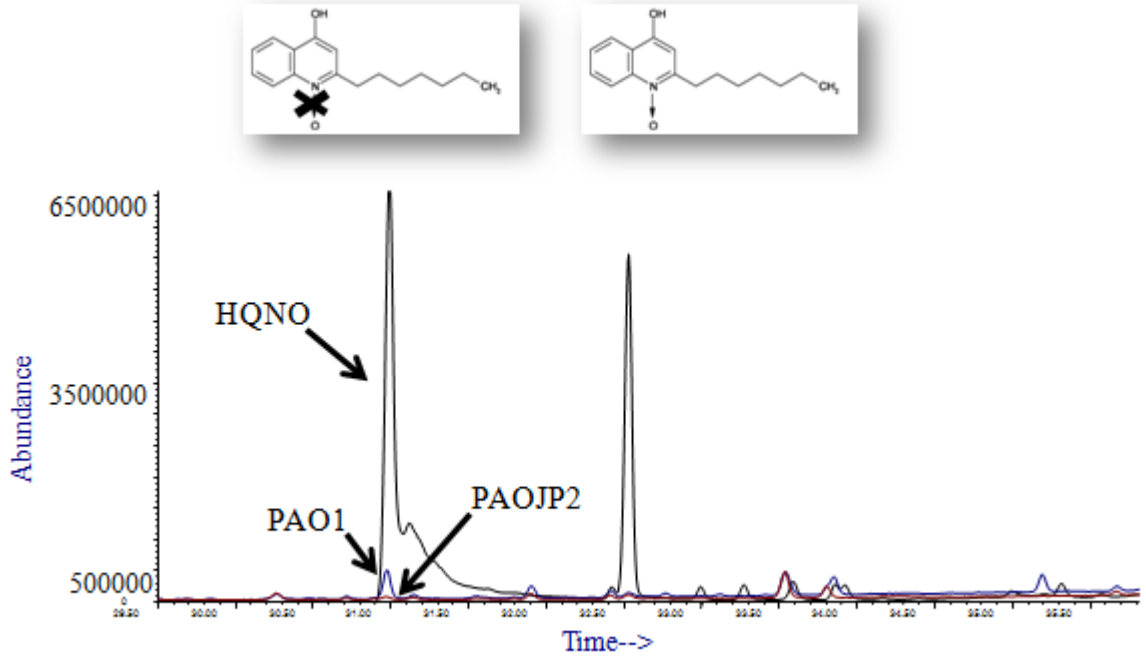
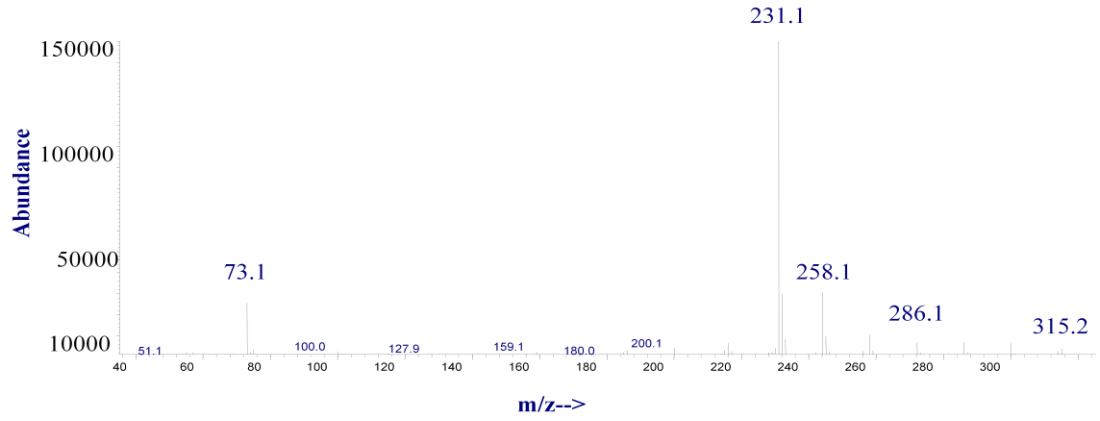

6500000
3500000
500000
Abundance
HQNO
PAO1
PAOJP2
Time-->

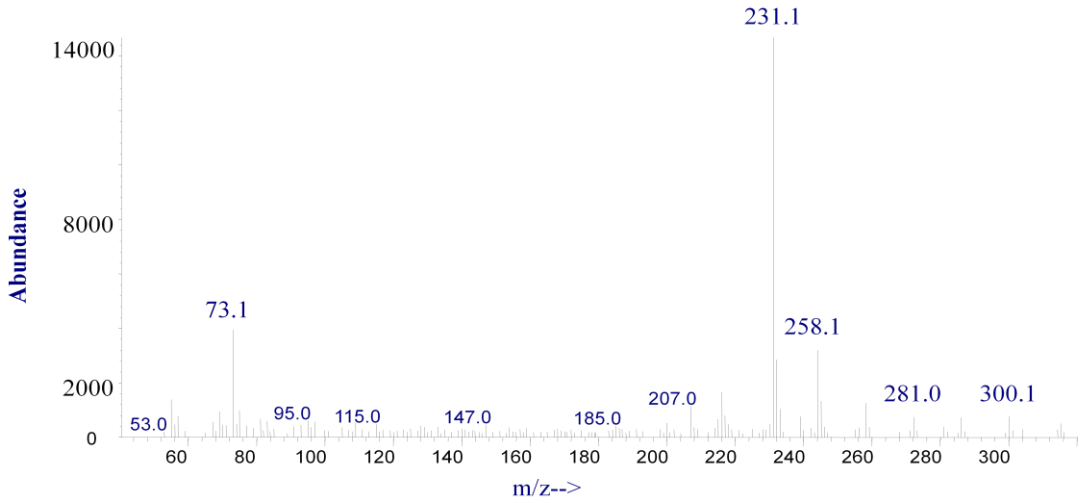
Figure 15: Ion fragment pattern for PAO1, PAOJP2 and synthetic HQNO with retention times 30.959, 30.965 and 31.071, respectively.

Twenty mLs of *P. aeruginosa* supernatant was extracted using equal volumes of ethyl acetate, thrice. The extracted product was derivatized with BSTFA and 1 µl was injected into the GC-MS where A shows the fragment ion pattern for PAO1 and B shows the pattern for PAOJP2 and C is HQNO.

A) PAO1



B) PAOJP2



C) HQNO

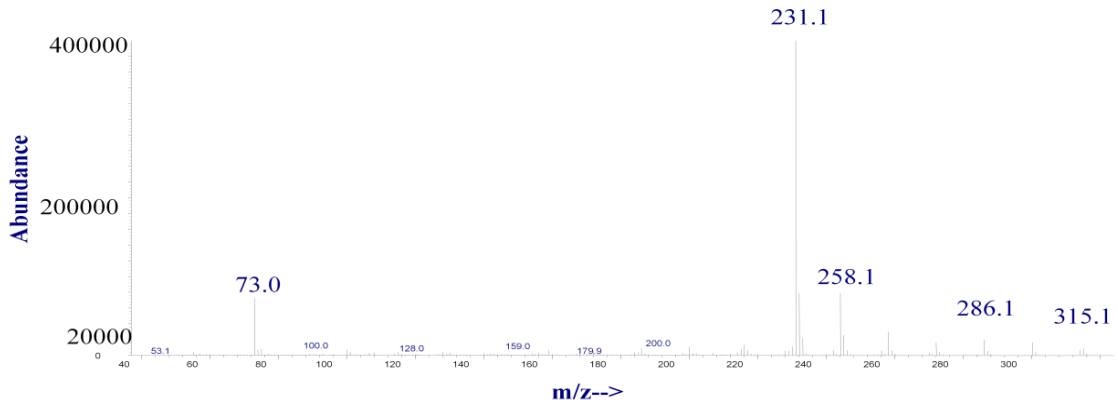
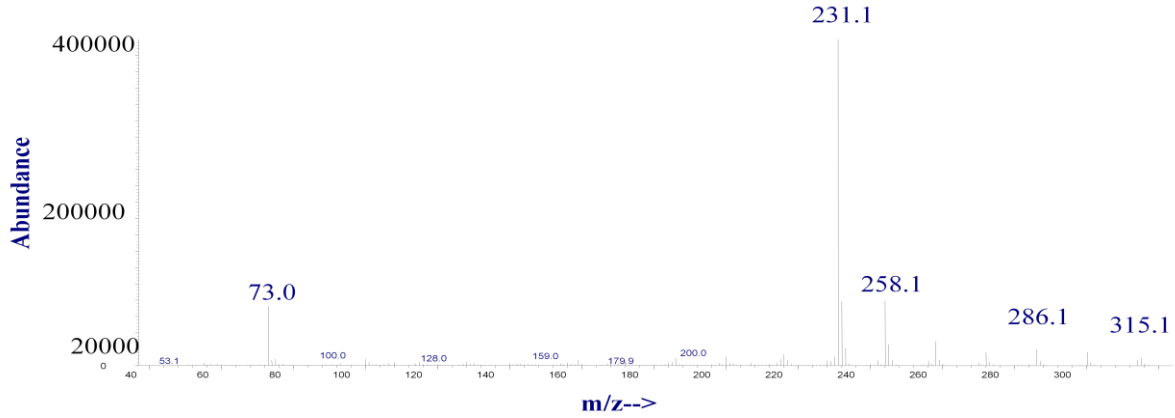


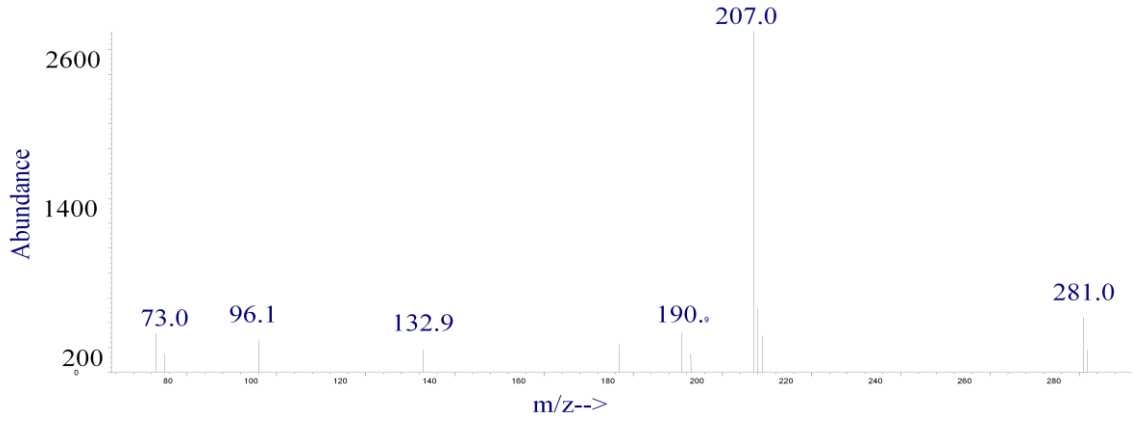
Figure 16: Ion fragment pattern for 7307, 14715 and HQNO with retention times 30.840, 30.338 and 31.071, respectively.

Twenty mLs of *P. aeruginosa* supernatant was extracted using equal volumes of ethyl acetate, thrice. The extracted product was derivatized with BSTFA and 1 µl was injected into the GC-MS where A shows the fragment ion pattern for 7307 and B shows the pattern 14715 and C is HQNO.

A) 7307



B) 14715



C) HQNO

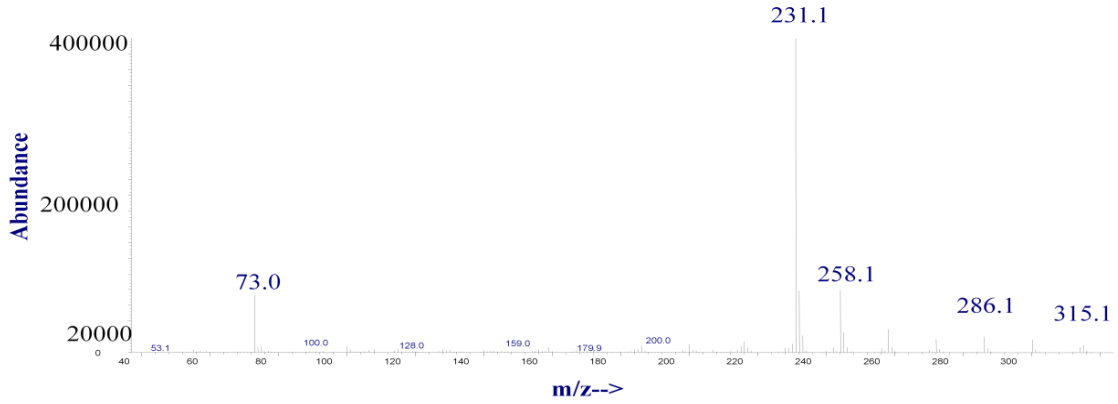


Table 4: Summary of fragment ion patterns for all inhibiting *P. aeruginosa* isolates

Strain/ Isolate	Ion 1	Ion 2	Ion 3	Ion 4	M+	Retention time	Relative Abundance	Inhibit C105	HQNO
HQNO	73	231.1*	258.1	286.1	315.1	31.071	1.8×10^6	+	+
PAO1	73.1	231.1*	258.1	286.1	315.2	30.959	5.0×10^5	+	+
PAOJP2	73.1	231.1*	258.1	281	300.1	30.965	5.0×10^3	-	+
14684	70	207*	244	281	340.9	33.88	-	+	-
5588	73	207*	-	281	340.9	41	-	+	-
14650	70.1	207*	244.1	281	341.1	32.31	-	+	-
14660	70.1	207*	244.1	281	341	33.623	-	+	-
14670	73	231*	258.1	281	300.1	39.903	3.0×10^4	+	+
14673	70	207*	244	281	340.9	33.68	-	+	-
7307	73	231.1*	258.1	286.1	315.1	30.48	1.7×10^5	+	+
14690	73	207*	-	281	340.9	37.502	-	+	-
4384	73	231.1*	258.1	286.1	315.2	30.915	1.0×10^5	+	+
14715	73	132.9	190	207*	281	30.338	-	-	-

* Represents base peak

aeruginosa that can inhibit *S. aureus* and induce SCVs. PAOJP2, which has constantly not inhibited *S. aureus* nor induce SCVs produces a small amount of HQNO (figure 14, 15). Since the relative abundance of HQNO in PAOJP2 is significantly lower (Table 4) than inhibiting isolates we hypothesize that a threshold concentration of HQNO is required for inhibition or HQNO does not play an important role in mediating interactions. As a result of PAOJP2 is not a true negative control, therefore 14715 was used since it does not inhibit C105 and cannot produce HQNO (Figure 16, Table 4).

3.3 Summary

The CF lung environment is dominated by a polymicrobial chronic lung infection. Microbes can mediate interactions within the lung, which results in synergistic, neutral or antagonistic interactions. These interactions are mediated through evolved mechanisms which are important to understand as targeting these mechanisms may aid in novel therapeutic development. *P. aeruginosa* can mediate antagonistic interactions with *S. aureus*. From this research it was found that out of 27 *P. aeruginosa* CF isolates nine are able to mediate antagonistic interactions with 3 ATCC *S. aureus* strains and a *S. aureus* CF isolate C105. Growth medium can effect inhibition as when *P. aeruginosa* was grown in PTSB, the frequency and the number of *P. aeruginosa* CF isolates able to mediate antagonistic interactions increased. Inhibition was also observed in liquid culture in addition to plate inhibition.

The inhibiting factor was characterized to be a small molecule that is unaffected by heat treatment. This result suggested that the inhibiting factor may be HQNO. Since HQNO can induce SCVs in *S. aureus* and SCVs are resistant to tobramycin, small in colony size and unable to hemolyze all of these parameters were tested. It was found that *P.*

aeruginosa supernatant can confer aminoglycoside resistance as well as reduce colony size and reduce hemolytic activity strongly suggesting HQNO was mediating interactions. From the GC-MS work the inhibiting molecule was identified to be HQNO in only 3 of the 9 CF isolates (14670, 7307 and 4384). PAO1 and PAOJP2 can produce HQNO as well. Taken together, these data suggest that there may be alternative inhibitory factors mediating interactions.

Chapter Four: Identifying additional *S. aureus* inhibitory factors, produced by *P. aeruginosa* CF isolates, other than HQNO and LasA

4.1 Introduction

In *P. aeruginosa,* interactions are often mediated by virulence factors that are controlled by the *lasIR* QS system such as LasA (Kessler, 1993), HQNO (Hoffmann *et al.,* 2006), siderophores, lipase and elastase (Van Delden and Iglewski, 1998). Recently, research from our laboratory highlighted an antagonistic interaction between *P. aeruginosa* and *Burkholderia spp.* that was mediated by cyclic peptides not controlled by HSLs (Purighalla, 2011). The cyclic peptides are known as diketopiperazines (DKPs) and are capable of antagonizing *N*-acyl-HSL based QS systems (Holden *et al.,* 1999) in *Burkholderia* spp. (Purighalla, 2011). This section will explore the possibility that mediation of antagonistic interactions between *P. aeruginosa* and *S. aureus* is operated by a non-HSL controlled virulence factor.

Chapter three illustrated that PAOJP2 is capable of producing HQNO in small amounts yet the strain is unable to inhibit C105 in liquid culture or on a plate. Conversely, the majority of inhibitory *P. aeruginosa* CF isolates did not produce HQNO but can inhibit *S. aureus* in liquid culture and on a plate. This finding led to two hypothesises; HQNO is not the sole inhibitory agent being produced by *P. aeruginosa* CF isolates and HQNO may not be as tightly regulated by the *las* and *rhl* systems as it is thought (Hofmann *et al.,* 2006 and Hofmann *et al.,* 2010). As a result this chapter takes a closer look into the regulatory factors that may be mediating these interactions. First we sought to survey a larger *S. aureus* CF library to determine if any additional interactions might occur between *P. aeruginosa* and *S. aureus* CF isolates. The *S .aureus* CF library is taken from patients

who were experiencing different clinical conditions in order to provide insight into whether or not clinical conditions have an effect on the mediation of antagonistic interactions.

4.2 Results

4.2.1 Negative interactions between *P. aeruginosa* and *S. aureus* are widespread among *S. aureus* CF isolates

In chapter three the ability of *P. aeruginosa* CF isolates, including our controls PAO1 and PAOJP2, to inhibit C105 was examined. The goal of that chapter was to gain insight into the mechanism that mediates their interactions. The result has led us to believe that *P. aeruginosa* can produce at least one additional inhibitory factor. However, before we examined the second inhibitory factor being produced by *P. aeruginosa,* we wanted to ensure that the interactions occurring between these two species were not a singular phenomenon that only occurred between C105 and *P. aeruginosa*. Therefore we obtained 19 additional *S. aureus* CF isolates from the CF clinic at Foothills Hospital in Calgary, Alberta. These isolates were taken from patients with different clinical conditions which included patients with chronic *S. aureus* infections, co-infections with *P. aeruginosa* or super-interactions. This section focuses on answering two questions; 1) can *P. aeruginosa* inhibiting isolates mediate antagonistic interactions with all 20 *S. aureus* isolates and 2) do interactions differ depending on the clinical condition. To test this, we surveyed interactions between our 27 *P. aeruginosa* CF isolates and 19 additional *S. aureus* CF isolates using the same methods as in section 3.2.1.

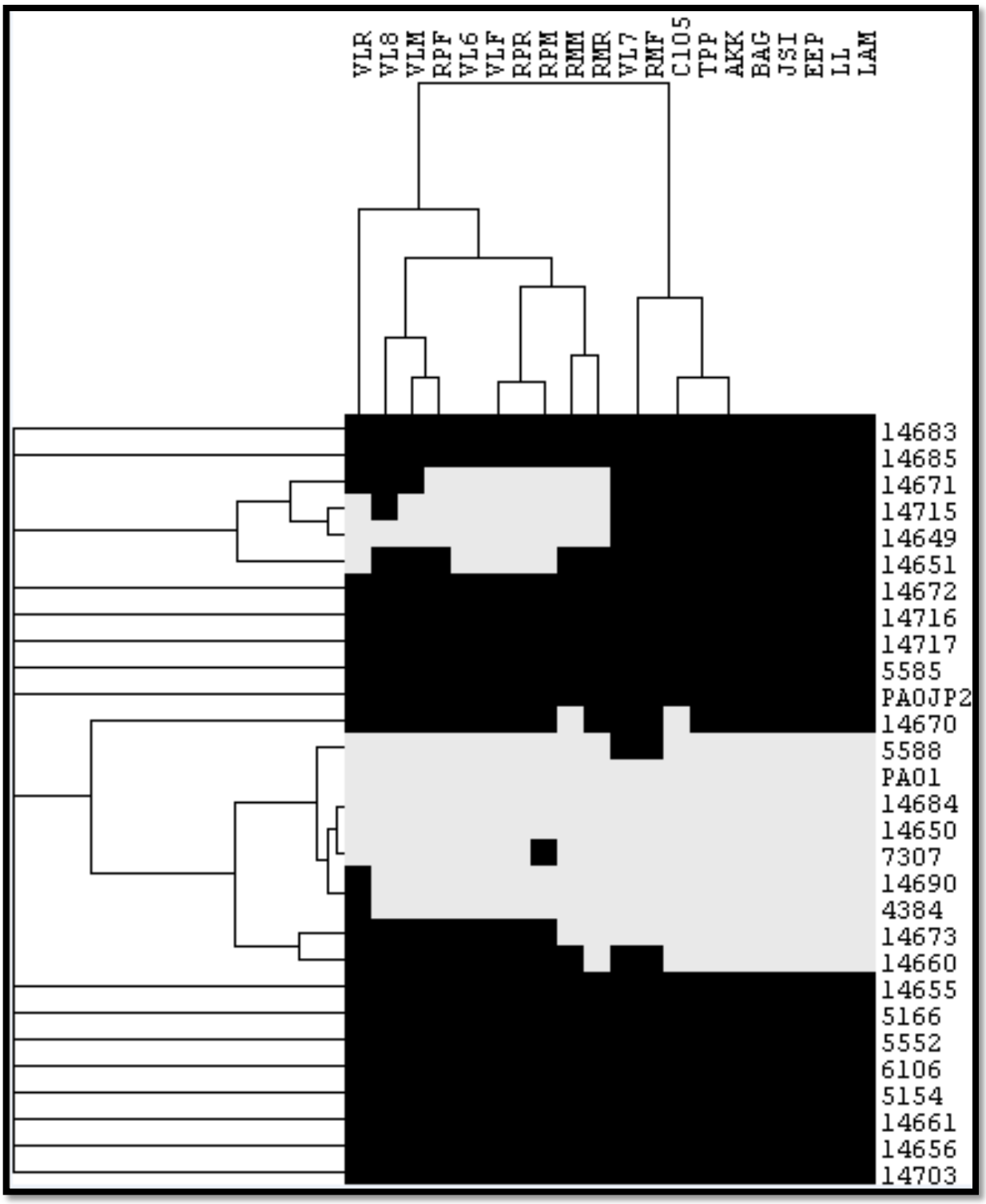
4.2.1.1 Antagonistic interactions mediated by *P. aeruginosa* CF isolates is not specific to only C105

Figure 17 depicts the ability of *P. aeruginosa* CF isolates to inhibit 20 *S. aureus* CF isolates taken from different patients at different clinical conditions. The cluster graph

shown in Figure 17 identifies if the *P. aeruginosa* isolate can inhibit *S. aureus* and does not provide information on the diameter of inhibition. Figure 17 shows that the *P. aeruginosa* strains can be segregated into four groups. The first group is able to inhibit the majority of CF *S. aureus* isolates. This group includes two *P. aeruginosa* CF isolates, 14684 and 14650, that are able to inhibit all 20 *S. aureus* CF isolates; in addition to PAO1. Of these three isolates only PAO1 produces HQNO, therefore we hypothesize that inhibition by PAO1 may involve HQNO as well as other factors but inhibition in CF isolates is not solely mediated by HQNO and at least one other factor is involved. For the second group, 5588, 7307, 4384 and 14673 inhibit C105 and the majority of the other *S. aureus* CF isolates but only 7307 and 4384 produce HQNO. This again suggests that other inhibitory factors are involved. The third group is made up of those *P. aeruginosa* isolates that inhibit about half of the *S. aureus* isolates. This group is made of two sub-groups that inhibit different *S. aureus* isolates with isolates 14671, 14615, 14649, and 14651 representing one sub group that also did not inhibit C105. The other subgroup inhibited C105 and a different set of *S. aureus* isolates and it included 14673, 14660 and 14670. This group contains only one strain, 14670, which only inhibits C105 and one other *S. aureus* isolate, RMM. Interestingly this isolate does produce HQNO. The last group are those that do not inhibit any *S. aureus* strains and these include: PAOJP2, 14683, 14685, 14672, 14716, 14717, 5585, 14655, 5166, 5552 6106, 5154, 14661, 14661, 14656 and 14703. As such it seems like the majority of this set of *P. aeruginosa* isolates are not able to inhibit any of the *S. aureus* CF isolates.

Figure 17: Antagonistic interactions between *P. aeruginosa* CF isolates and 20 *S. aureus* CF isolates.

Inhibition of *S. aureus* growth caused by *P. aeruginosa* supernatant was determined using molten overlay agar plates containing *S. aureus* on a MSA base. Inhibiting *P. aeruginosa* strains against the appropriate *S. aureus* isolate were scored as a 1 and are represented as a grey box, whereas non-inhibiting *P. aeruginosa* strains were scored as a zero and are represented by a black box. Left of the cluster graph reads the *P. aeruginosa* CF isolates and to the top of the graph reads the *S. aureus* CF isolates. Experiments were completed three times in triplicate. Cluster analysis was completed using Cluster 3.0 and visualized in Java Treeview.


VLR
VL8
VLM
RPF
VL6
VLF
RPR
RPM
RMM
RMR
VL7
RMF
C105
TPP
AKK
BAG
JSI
EEP
LL
LAM
14683
14685
14671
14715
14649
14651
14672
14716
14717
5585
PAOJP2
14670
5588
PA01
14684
14650
7307
14690
4384
14673
14660
14655
5166
5552
6106
5154
14661
14656
14703

Overall, antagonistic interactions are not specific to only C105 as all the other SA CF isolates are inhibited by at least two CF isolates. This result also suggested that there may be many more inhibitory factors on top of HQNO.

4.2.2 The inhibitory factor secreted by 14671 is the small molecule HQNO

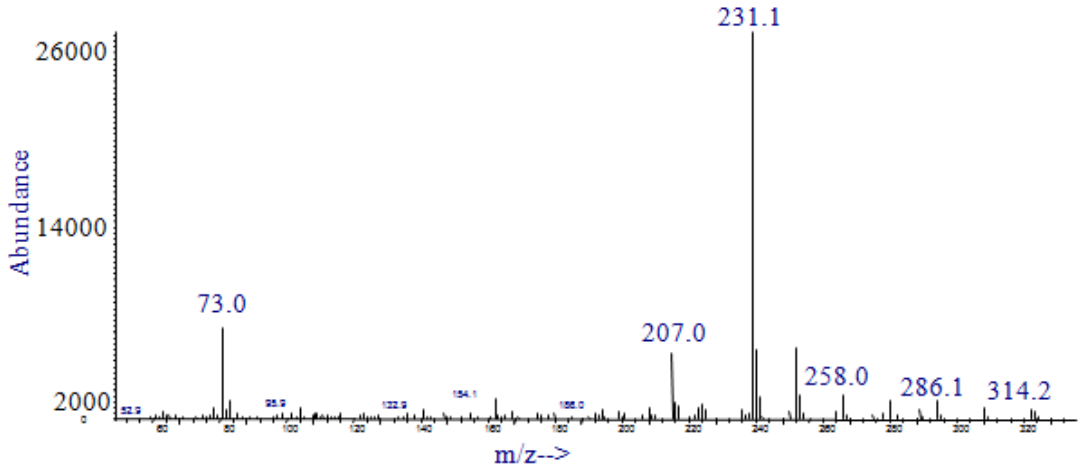
In section 3.2.5 it was shown that not every C105 inhibitor produces HQNO which was contrary to our hypothesis. The survey of an additional 19 *S. aureus* CF isolates as was shown in section 4.2.1, showed that all the strains that inhibited C105 could also inhibit other *S. aureus* CF isolates. Furthermore, additional *P. aeruginosa* CF isolates were identified as inhibitors that were not able to inhibit C105. Those isolates include 14715, 14649, 14651 and 14671. Since chapter three showed that it is possible that antagonistic interactions between these two species may be mediated by more than one small molecule we tested for the production of HQNO in three of the newly identified inhibitors and four non-inhibitors (5585, 14683, 14661 and 6106).

The ion fragment pattern of HQNO was compared to *P. aeruginosa* isolates (Figure 18 and Table 5). Synthetic HQNO has a retention time of 31.071 and all fragment ion patterns depict peaks similar to HQNO. Table 5 summarizes the isolates that are able to produce HQNO which from this set was only 14671. The results were similar to that of chapter three – not every inhibitor produced HQNO. Of the non-inhibitors, all four did not produce HQNO. Again, this result suggests that an additional inhibitor may be mediating antagonistic interactions. This work however raised the question if inhibition is controlled by concentration.

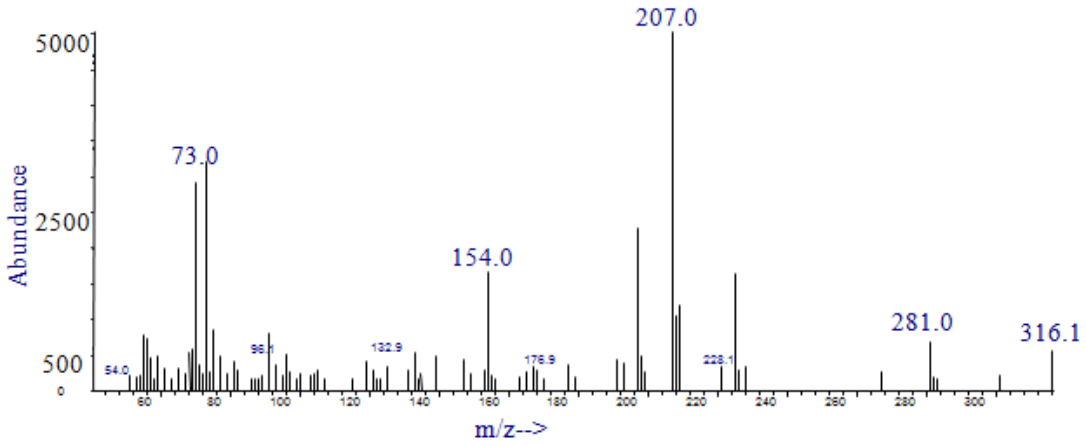
Figure 18: Ion fragment pattern for 14671, 5585 and synthetic HQNO with retention times 30.928, 29.964 and 31.071, respectively

Twenty mLs of *P. aeruginosa* supernatant was extracted using equal volumes of ethyl acetate, thrice. The extracted product was derivatized with BSTFA and 1 µl was injected into the GC-MS where A shows the fragment ion pattern for 14671 and B shows the pattern for 5588 and C shows HQNO.

A) 14671



B) 5585



C) HQNO

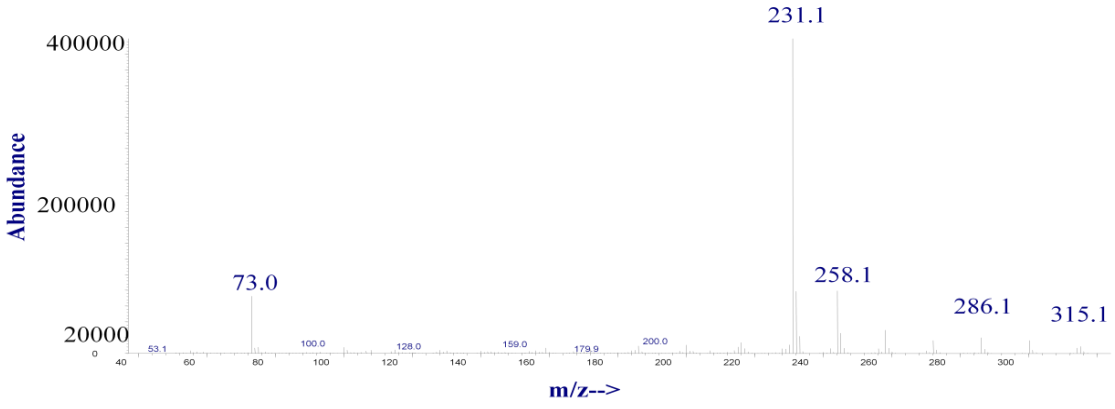


Table 5: Summary of fragment ion patterns for *P. aeruginosa* isolates

Strain / Isolate	Ion 1	Ion 2	Ion 3	Ion 4	M+	Retention time	Relative Abundance	Inhibit SA	HQNO
HQNO	73	231.1 *	258.1	286.1	315.1	31.071	1.8×10^6	+	+
PAO1	73.1	231.1 *	258.1	286.1	315.2	30.959	5.0×10^5	+	+
14715	73	96.1	190	207.0 *	281	30.338	-	+	-
14651	72.9	133.0	154.0	207.0 *	281	30.377	-	+	-
14671	73.0	231.1 *	258.0	286.1	314.2	30.928	2.0×10^5	+	+
5585	73.0	-	154	207*	281	31.103	-	-	-
14683	73	-	-	207*	281	29.989	-	-	-
14661	70.0	154.0 *	207	228.1	280.9	30.321	-	-	-
6106	72.9	-	207	281.0	341.0	30.083	-	-	-

* Represents base peak

4.2.3 Concentration of HQNO plays a small role in the mediation of antagonistic interactions between *P. aeruginosa* and *S. aureus*.

Studies have shown that PAO1 produces a physiological concentration of approximately 20 μg/ml of HQNO at late log phase /early stationary phase (Zaborina *et al.,* 2007) whereas the human isolate PA14 is known to produce half that of PAO1 (Deziel *et al.,* 2004). Previous research has not identified the concentration of HQNO that is produced by CF isolates. As was seen in chapter three, PAOJP2 produced HQNO but did not inhibit any *S. aureus* strains. Further, some strains did not inhibit C105 when grown in TSB but did inhibit these strains when grown in PTSB. Finally, the zones of inhibition varied amongst the different *P. aeruginosa* CF isolates. Considering the variation between PAO1 and PA14 in HQNO production as well as our previous findings, we wanted to examine if inhibition of *S. aureus* is dependent on HQNO concentration. It is possible that a threshold concentration is required to be reached in order for inhibition to occur.

To test concentration of HQNO from our *P. aeruginosa* producers, a concentration curve (concentration vs. area under the curve) using HQNO standards was created in order to extrapolate concentration. The findings are summarized in Table 6. PAO1 was found to produce the highest amount of HQNO (35μg/ml) whereas PAOJP2 produced the lowest which may correspond to the loss of function of LasI and RhlI. This would suggest that the 0.05 ug/ml that PAOJP2 produces is below the threshold needed to inhibit *S. aureus*. The *P. aeruginosa* CF isolates produce a range of HQNO, 14671 produces the highest amount and 14670 produces the lowest. Interestingly, those two isolates were isolated from the same patient yet 14671 was able to inhibit 7 *S. aureus* CF isolates but 14670 was only able to inhibit 2 *S. aureus* CF isolates.

Table 6: Concentration of HQNO from *P. aeruginosa* producers

P. aeruginosa Isolate	Retention Time	Area under the Curve	Concentration of HQNO (µg/ml)
PAO1	**30.959**	**1536382**	**35**
PAOJP2	**30.065**	**194011**	**0.5**
7307	**30.840**	**629883**	**4.5**
4384	**30.915**	**619490**	**4.25**
14671	**30.928**	**853330**	**7.5**
14670	**30.903**	**493580**	**2**

This might suggest variations in sensitivity to HQNO amongst the SACF isolates, such that 14670 produces HQNO at or near the threshold of the most sensitive strains. Thus it can inhibit only two strains. Correlation between concentration and the mediation of antagonistic interactions was calculated (Figure 19). Figure 19A shows a lack of a correlation between HQNO concentration and number of *S. aureus* CF strains inhibited. However if the two outliers were removed the correlation would be better. Figure 19B shows a similar result but a weak correlation between concentration of HQNO, average diameter size and frequency of inhibition was found. Taken together it is possible that the two outlier strains, 4384 and 7307 produce inhibitory factors other than HQNO which might account for their bigger zones of inhibition and the greater number of SACF strains inhibited.

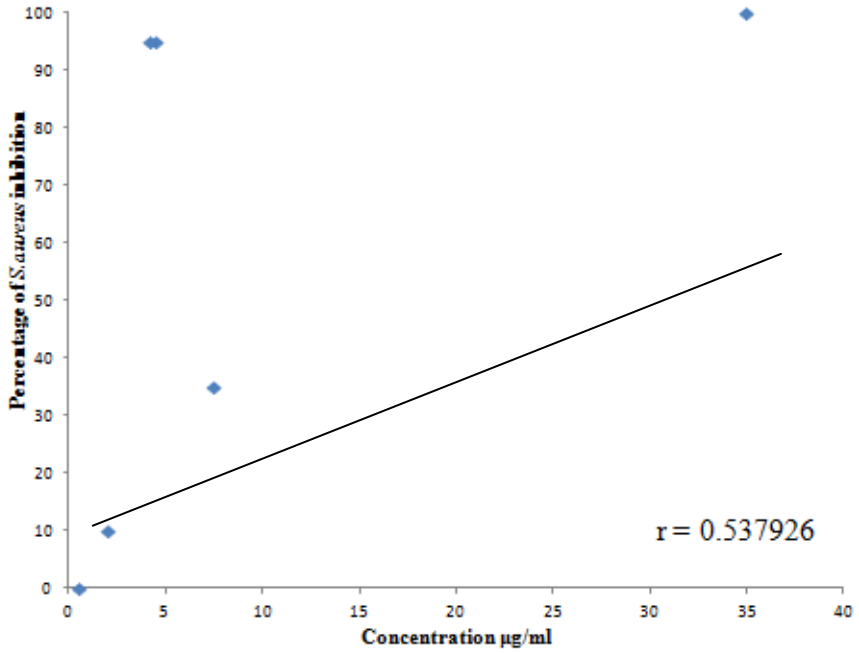
4.2.4 An analysis of the *S. aureus* inhibitory factors produced by *P. aeruginosa* showed that there is a complex interplay between all the factors.

To determine if antagonistic interactions between *P. aeruginosa* and *S. aureus* are dependent on the LasIR, RhlIR or PQS systems, we compared autoinducer production (3-oxo-C_{12}-HSL, C_4-HSL and PQS) (Lutter, 2008) to *S. aureus* percentage inhibition (Tables 7, 8, 9). Moreover, to get a better picture of all the inhibitory factors produced by *P. aeruginosa* we also included in these tables the ability of each isolate to produce pyocyanin and HCN (Lutter, 2008; Voggu *et al.,* 2006). Those *P. aeruginosa* CF isolates able to inhibit greater than 90% of the *S. aureus* CF isolates all produced 3-oxo-C_{12}-HSL, C4 HSL and PQS (Table 7). All but one of these *P. aeruginosa* isolates produced pyocyanin and all but two produced HCN. Only three of the six *P. aeruginosa* strains produced HQNO.

Figure 19: A lack of correlation exists between concentration and *S. aureus* inhibition.

Linear correlation was calculated between concentration and percentage of *S. aureus* isolates inhibited by a *P. aeruginosa* CF isolate (A) and average diameter (B). Linear correlation coefficient (r) was computed using excel. For A, percentage of *S. aureus* inhibition was calculated based on the number of *S. aureus* isolates (out of the 20 CF isolates) able to interact with *P. aeruginosa*. For B, average diameter was calculated based on the zone of clearance that was produced by the *P. aeruginosa* isolate for each 20 CF isolates. Therefore average diameter consisted of an average taken from 120 data points.

A)


Percentage of S.aureus inhibition
Concentration µg/ml
r = 0.537926

B)

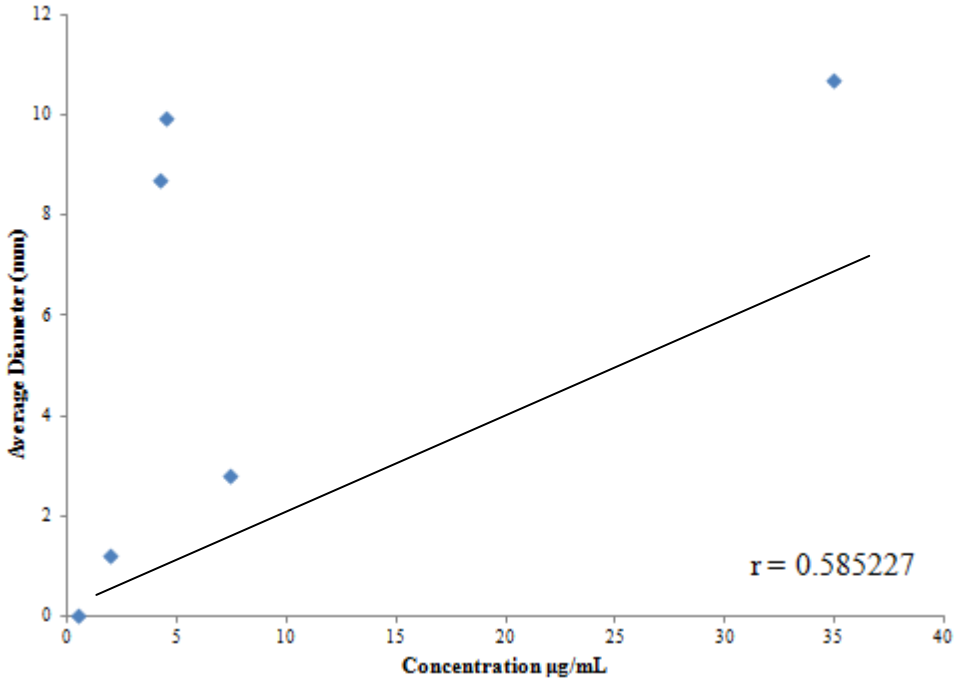

Average Diameter (mm)
Concentration µg/mL
r = 0.585227

Table 7: Potential inhibitory factors produced by *P. aeruginosa* CF isolates able to inhibit ≥90% of the *S. aureus* CF isolates

***P.aeruginosa* isolate**	**Percentage inhibition**	**HQNO**	**HQNO concentration µg/ml**	**3-oxo-C_{12}-HSL**	**C_4-HSL**	**PQS**	**PY**	**HCN**
PAO1	100	+	35	+	+	+	+	+
14684	100	-	-	+	+	+	+	+
14650	100	-	-	+	+	+	+	+
7307	95	+	4.5	+	+	+	+	-
14690	95	-	-	+	+	+	+	+
4384	95	+	4.25	+	+	+	+	+
5588	90	-	-	+	+	+	-	-

Table 8: Potential inhibitors produced by *P. aeruginosa* CF isolates able to inhibit <90% of the *S. aureus* CF isolates

***P.aeruginosa* isolate**	**Percentage inhibition**	**HQNO**	**HQNO concentration µg/ml**	**3-oxo-C_{12}-HSL**	**C_4-HSL**	**PQS**	**PY**	**HCN**
14673	60	-	-	-	+	+	+	-
14649	50	ND	-	+	-	+	+	+
14660	45	-	-	-	+	-	+	-
14715	45	-	-	-	-	-	-	-
14671	35	+	7.5	-	+	+	+	+
14651	25	-	-	-	-	+	-	-
14670	10	+	2	-	+	+	-	-

Table 9: Potential inhibitory factors produced by *P. aeruginosa* CF isolates unable inhibit any *S. aureus* CF isolate

P. aeruginosa isolate	3-oxo-C_{12}-HSL	C_4-HSL	PQS	PY	HCN
PAOJP2	-	-	-	+	-
14683	-	-	+	+	+
14685	-	-	-	+	-
14672	-	-	-	-	+
14716	-	-	-	+	-
14717	-	-	-	+	-
5585	-	-	-	-	-
14655	-	-	-	+	-
5166	+	+	-	+	+
5552	-	-	-	-	-

Table 9: Potential inhibitory factors produced by *P. aeruginosa* CF isolates to unable inhibit any *S. aureus* CF isolate continued

P. aeruginosa isolate	3-oxo-C_{12}-HSL	C_4-HSL	PQS	PY	HCN
6106	-	-	-	-	-
5154	-	-	-	-	-
14661	-	-	+	-	-
14656	+	+	+	+	-
14703	+	+	+	-	-

Taken together, all these isolates except for 5588 produce a full complement or one factor less than a full complement of the *S. aureus* inhibitors and this likely accounts for the wide range of *S. aureus* inhibition. Also noteworthy is the observation that all these strains produce all three autoinducer molecules. Even the one outlier isolate 5588 produces all the autoinducer molecules even though it does not produce any other inhibitors of *S. aureus*. These results suggested that either the autoinducers themselves or something they regulated may be involved in the widespread inhibition of SA CF isolates.

Table 8 represents inhibitory factor production for *P. aeruginosa* isolates that inhibit about 50% of the CF *S. aureus* isolates. As a group these *P. aeruginosa* isolates produce fewer *S. aureus* inhibitors and this likely accounts for the smaller range of *S. aureus* isolates that they inhibit. Unlike the isolates in Table 7, none of these isolates in Table 8 produced all three autoinducers. Interestingly, isolate 14715 did not produce any of the *S. aureus* inhibitory factors and isolate 14651 only produced PQS. These two isolates support the possibility of an additional inhibitory factor that has yet to be reported. However we have not specifically looked into the production of LasA in our *P. aeruginosa* CF isolates.

Table 9 shows those *P. aeruginosa* CF isolates that do not inhibit any of the *S. aureus* CF isolates. One might expect that these isolates would not produce any *S. aureus* inhibitory factors. There were a few isolates (5585, 5552, 6106, and 5154) that did not produce any inhibitory factors but others produced from one to four factors. However, for all the factors except HQNO we have only scored based on production or lack of. Thus, even if these isolates produce a factor like pyocyanin it may be produced at a level below the needed threshold. It is also possible that the isolates in Table 9 are not all true

negatives (*S. aureus* inhibitors) as some of these isolates when grown in PTSB inhibit C105 (Chapter 3, Figure 6).

Overall, my research showed that HQNO is not the sole inhibitory factor in *P. aeruginosa* CF isolates and questions the role that HQNO plays in mediating interactions as the majority of inhibitory isolates do not produce HQNO. Clearly there are many factors involved but the *P. aeruginosa* CF isolates that inhibit most *S. aureus* CF isolates produce the largest array of these factors. Likewise, the isolates that do not inhibit *S. aureus* CF isolates produce the fewest. My data also indicated that there must be an additional inhibitory factor other than those tested.

4.2.5 Diketopiperzines are detected in the supernatants of some of the *P. aeruginosa* isolates but do not correlate to patterns of *S. aureus* inhibition

My GC-MS data on the supernatants of the *P. aeruginosa* CF isolates showed that some isolates produced specific spectral data that did not match HQNO. Previous research in our lab has showed that four DKPs (cyclo Pro-Val, Ala-Val, Leu-Pro, Phe-Pro) were produced by isolate 14672 and have the capability to attenuate virulence in *B. cenocepacia* by inhibiting the CepIR system (Purighalla, 2011). Since these compounds are cyclic molecules the structure is somewhat similar to that of HQNO therefore we hypothesized that DKPs may be able to bind to the cytochrome of *S. aureus* and cause inhibition. In order to test this hypothesis GC-MS was utilized to search for the presence of those four DKPs in *P. aeruginosa* CF isolate supernatant.

Table 10 summarizes the production of DKPs for all inhibitors of *S. aureus* in addition to some non inhibitors of *S. aureus*. Strong inhibitors such as 14684, 14690 and 5588 produce either one or none of the four DKPs. In contrast the other strong inhibitors,

Table 10: Selected DKP production in *P. aeruginosa* inhibitors and non inhibitors or *S. aureus*

P. aeruginosa isolate	*S. aureus* inhibitor	HQNO production	Cyclo (Pro-Val)	Cyclo (Ala-Val)	Cyclo (Leu-Pro)	Cyclo (Phe-Pro)
PAO1	+	+	+	+	+	+
PAOJP2	-	+/-	+	+	+	+
14684	+	-	+	-	-	-
5588	+	-	-	-	-	-
14650	+	-	+	+	+	+
14660	+	-	+	+	+	+
14670	+	+	+	+	+	+
14673	+	-	+	+	+	+
7307	+	+	+	+	+	+
14690	+	-	-	-	-	-
4384	+	+	+	+	+	+
14715	+	-	-	-	-	-
14651	+	-	+	+	+	+
14671	+	+	+	+	+	+
14683	-	-	+	+	-	-
14661	-	-	+	+	+	+
6106	-	-	-	-	-	-

14650, 7307 and 4384 produce all four DKPs. Of the non- *S. aureus* inhibitors 6106 did not produce any DKPs, isolate 14683 produced two DKPs and isolate 14661 produced all 4. Likewise with the intermediate *S. aureus* inhibitors some produce all 4 DKPs and others do not produce any DKPs. Since there is no clear pattern across these isolates my data suggests that DKPs likely do not play a role in the inhibition of *S. aureus* isolates.

4.2.6 Non-HQNO *P. aeruginosa* producers can induce tobramycin resistance in *S. aureus*

Recently, research has shown that *P. aeruginosa* can produce HQNO which is able to inhibit the electron transport chain of *S. aureus* and induce formation of small colony variants (Hoffman *et al.,* 2006). That was the first time it was shown that *P. aeruginosa* can induce small colony variants in *S. aureus.* This interaction has great clinical significance in terms of pulmonary infections in CF patients as these two pathogens frequently co-colonize the lung (Sibley *et al.,* 2011). In chapter three, it was shown that *P. aeruginosa* CF isolates can induce small colony variants in a *S. aureus* CF isolate C105. We originally hypothesized that HQNO was the molecule that was responsible for this interaction, but after further analysis it was revealed that the majority of inhibiting isolates do not produce HQNO. Since data in this chapter emphasize that HQNO is one of many rather than the primary inhibitory molecule we wanted to test if the non-HQNO producers that inhibit *S. aureus* can induce tobramycin resistance in C105. Taking into account all of the data we hypothesized that HQNO does not play a large role in mediating interactions and that non-HQNO producers may be able to induce tobramycin resistance.

Table 11 displays the *P. aeruginosa* isolates that are capable of inducing tobramycin resistance in C105. Note that supernatants from all five of the SA inhibitors

Table 11: Resistance to tobramycin can be induced without the presence of HQNO

P. aeruginosa isolate	Inhibitor	HQNO Producer	Induce Tobramycin Resistance
PAO1	+	+	+
PAOJP2	-	+/-	-
14684	+	-	+
14670	+	+	+
14673	+	-	+
5588	+	-	+
7307	+	+	+
14690	+	-	+
14660	+	-	+
4384	+	+	+
14661	-	-	+
5585	-	-	-
14683	-	-	-
6106	-	-	-
14655	-	-	-
5166	-	-	-
14672	-	-	-
TSB	-	-	-

that do not produce HQNO were able to confer tobramycin resistance. In contrast, of the 7 *P. aeruginosa* CF isolates that are unable to inhibit C105, only one isolate, 14661 can induce tobramycin resistance. This is a very interesting finding since this isolate was not able to inhibit the growth of any of the *S. aureus* CF isolates (Figure 16) unless it was grown in PTSB (Chapter 3, Figure 6). Taken together my data suggested that HQNO was not necessary to induce tobramycin resistance in *S. aureus*. Furthermore, there may be another mechanism that allows the development of tobramycin resistance in *S. aureus* and possibly induce the formation of SCVs.

4.3 Summary

Overall, antagonistic interactions between *P. aeruginosa* and *S. aureus* are widespread and are not limited to one *S. aureus* isolate. It also became very clear in this chapter that interactions between *P. aeruginosa* and *S. aureus* are not solely mediated by HQNO. This was confirmed as additional inhibitors were analyzed via GC-MS and the majority do not produce HQNO. Of the inhibitors that do produce HQNO, concentration was calculated and found to not correlate strongly with either percentage of *S. aureus* isolates that were inhibited or zone of clearance (diameter). This result further supported the notion that HQNO is not the primary molecule mediating antagonistic interactions. In an attempt to classify the second inhibitory factor, the presence of four DKPs was examined. No trend between the production of these four DKPs and inhibition was made. It did become clear, however, that the isolates the can produce 3-oxo-C12HSL, C4-HSL and PQS are the strongest inhibitors. One isolate, 14661, is able to confer tobramycin resistance and yet was classified as a non inhibitor. This finding emphasizes the clinical significance of understanding interactions between these two very common CF pathogens.

Chapter Five: Discussion

P. aeruginosa and *S. aureus* are two common CF pathogens that frequently co-colonize the lung. It is becoming increasingly common for patients (more common in adults) to carry both MRSA and MSSA combined with *P. aeruginosa* and this combination has been found to be extremely deleterious for CF patients (Hubert *et al.,* 2013). Since *P. aeruginosa* and *S. aureus* share a niche, with limited resources, general theory of competitive coexistence suggests one of these organisms will develop mechanisms of spiteful behaviour in order to survive (Chesson, 2000).

P. aeruginosa is capable of developing spiteful behaviour with other *P. aeruginosa* members and with other species. Recently, it was shown that a *P. aeruginosa rhlR* mutant can attenuate virulence of its parental strain in the *D. melanogaster* model (Lutter *et al.,* 2012). *P. aeruginosa* CF isolates are also capable of intraspecific negative interactions (MacLean, 2012). Negative interactions between species have been observed as well, as a *P. aeruginosa* CF isolate can attenuate virulence of *B. cenocepacia* by inhibiting the *cepIR* system (Purighalla, 2011). Interspecific interactions can also affect intraspecific social behaviour in *P. aeruginosa*. When *S. aureus* and *P. aeruginosa* cohabit an environment with limiting resources, specifically iron, *S. aureus* and *P. aeruginosa* compete for that resource. This competition causes *P. aeruginosa* to increase its siderophore production in some strains while the number of *P. aeruginosa* social cheaters increases (Harrison *et al.,* 2007). Taken together we hypothesized that *P. aeruginosa* CF isolates are able to antagonize *S. aureus* CF isolates.

This study provides novel insights into interspecies interactions among *P. aeruginosa* and *S. aureus* CF isolates and provides a better understanding of the dynamics

that may be occurring in pulmonary CF infections. This study demonstrates that *P. aeruginosa* produces an impressive array of *S. aureus* inhibitory factors. Those *P. aeruginosa* strains and isolates that inhibit the widest range of *S. aureus* CF isolates produce the greatest diversity of inhibitory factors. HQNO is one of those inhibitory factors but is not the sole molecule responsible for negative interactions. Furthermore, this research shows the possibility of at least one novel mechanism that can mediate antagonistic interactions. In addition to this, these data showed that *P. aeruginosa* does not depend on the *las* system to mediate antagonistic interactions, although all the strong inhibitors had a functional *lasIR, rhlIR and PQS* system, providing insight into the circuitry that controls spiteful behaviour. The *lasIR* system may be required for PAO1 to produce inhibitors of *S. aureus* but, it may not be a requirement for CF isolates of *P. aeruginosa.* Below is the evidence that supports these conclusions and a proposed novel mechanism for antagonistic interactions between *P. aeruginosa* and *S. aureus*.

5.1 Antagonistic interactions occur between *P. aeruginosa* and *S. aureus* CF isolates

The results indicate that a number of *P. aeruginosa* adult CF isolates (taken from our 27 isolate collection from 7 patients) can participate in antagonistic interactions (Figures 5, 6, 7, 8 and 16). All twenty *S. aureus* CF isolates were inhibited by at least one *P. aeruginosa* CF isolate, which indicated that these interactions are widespread among *S. aureus* isolates. It was surprising that not every *P. aeruginosa* isolate could inhibit *S. aureus* which suggests some isolates have developed mechanisms of competition and some have lost that ability. This may be a result of social cheating as *P. aeruginosa* is known to develop social cheaters in the CF lung (Sandoz *et al.,* 2007). Interestingly, some isolates were taken from the same sputum sample on the same day, meaning those isolates shared

the same community however usually only one isolate was able to antagonize *S. aureus*. An example of this was seen in Chapter 3 Figure 5 where isolates 14683, 14684 and 14685 were taken from patient 92 and 14684 was the only isolate able to participate in antagonistic interactions.

What remains unclear from these data is the impact of antagonistic interactions between these two species on host virulence which would provide insight on disease progression. Models are required to study microbe-mircobe interactions in order to gain an understanding in how these interactions would affect the host. The *D. melanogaster* model has proven to be a successful model in displaying the effect of interactions on a host, given that synergism, antagonism and neutral interactions between microbes have been observed using this model (Sibley *et al.,* 2008; Lutter, 2008; Purighalla, 2011; MacLean 2012; Lutter *et al.,* 2012). Moreover, microbes are able to form biofilms in the gut of *D. melanogaster* (Mulcahy *et al.,* 2011) which makes this model suitable to study CF microbe interactions since chronic CF infections are usually in the form of biofilms (Lam *et al.,* 1980).

5.2 HQNO is produced by few *P. aeruginosa* CF isolates

It is well known that *P. aeruginosa* has evolved to develop mechanisms that can mediate antagonistic interactions with *S. aureus* (Kessler *et al.,* 1993; Hoffman *et al.,* 2006). As outlined in section 5.1, a handful of *P. aeruginosa* CF isolates from our collection can inhibit *S. aureus* CF isolates (Chapter 4 Figure 16). As the most well characterized anti-staphylococcal inhibitory factors are LasA and HQNO, we tested if the inhibitory factor could be affected by heat and separated by size. We found that inhibition of *S. aureus* C105 by *P. aeruginosa* supernatant occurred independent of heat treatment and the factor was found to be less than 5 kDa (Figure 9). Since LasA is a 22 kDa anti-

staphylolysin (Peters *et al.,* 1992) and is affected by heat treatment, our data suggested HQNO was possibly the inhibitory factor. Further analysis revealed that out of the nine inhibitors tested for HQNO production, four produced the molecule (Tables 4 and 5).

The lack of production of HQNO from *P. aeruginosa* isolates that inhibit the majority of *S. aureus* isolates was a rather surprising result and contrary to what was shown in Hoffman *et al.,* 2006. HQNO inhibits the growth of *S. aureus* by inducing small colony variants through disruption of its electron transport chain. When HQNO binds to cytochrome bo_3 in *S. aureus* the electron potential reduces significantly and as a consequence aminoglycosides are unable to penetrate the cell. If *S. aureus* is treated with tobramycin in conjunction with HQNO or the supernatant from a HQNO *P. aeruginosa* producer (and inhibitor), *S. aureus* will become resistant to tobramycin. My data showed that all HQNO producers that inhibited *S. aureus* were able to confer tobramycin resistance in *S. aureus*. What was particularly interesting was that all of the *P. aeruginosa* inhibitors that did not produce HQNO were also able to induce tobramycin resistance in C105 (figure 11 and table 11). This finding suggests that there is an additional inhibitory factor that is able to inhibit *S. aureus* and cause tobramycin resistance. It is unclear from these data whether the second inhibitory factor mediates tobramycin resistance using the same mechanism as HQNO. We hypothesize that *S. aureus* small colony variants were produced when treated with PAO1 supernatant containing HQNO; however we have yet to determine if SCVs can be produced by other *P. aeruginosa* CF isolates that inhibit *S. aureus* and do not produce HQNO.

There are three mechanisms that allow *S. aureus* to become resistant to aminoglycosides; a decrease in cell permeability (as is the case for HQNO); modification

in ribosome binding sites or through acquiring aminoglycoside modification enzymes (AME). *S. aureus* produces three types of AMEs, aminoglycoside-3-O-phosphoryltransferase III [aph(3)-III], aminoglycoside- 4-O-phosphoryltransferase I [ant(4)-I] and aminoglycoside-6-N-acetyltransferase/20-O-phosphoryltransferase [aac(6)/aph(2)] (Rouche *et al.,* 1987). AMEs are usually acquired through plasmids and allow for the most resistance since they can modify the antibiotic and they are commonly found in clinical *S. aureus* isolates (Chandrakanth and Patil, 2008). Since AMEs are acquired through plasmids we can speculate that tobramycin resistance in C105 is not a result of AMEs, but rather a result of a decrease in electron potential or modifications to the ribosome binding site. It may be possible that the unidentified inhibitor produced by *P. aeruginosa* targets *S. aureus* protein synthesis by binding to a ribosome and therefore outcompetes tobramycin causing the paradoxical effect of inhibiting growth yet providing aminoglycoside resistance. Further analysis is required to identify the mechanism of aminoglycoside resistance.

5.3 HQNO is not tightly controlled by the *las* system in *P. aeruginosa*

P. aeruginosa produces three signalling molecules, 3-oxo-C_{12}-HSL, C_4-HSL and PQS, whose function is to regulate a number of virulence factors. The PQS system is a non-AHL signalling cascade that controls the regulation and production of HQNO (Fletcher *et al.,* 2000). In 2003 Diggle *et al.,* showed that the PQS system is not controlled by cell density (in contrast to the *las* and *rhl* systems) yet is activated in stationary phase to control multiple virulence factors and plays a large role in regulating the *rhlIR* system. Moreover, it was shown that *lasR* is not essential for the production of PQS and the

activation of the PQS system. The PQS system is able to regulate the *rhl* system independently of LasR (Diggle *et al.,* 2003).

Our data support the notion that PQS is controlled independently of the *lasIR* system. Tables 7, 8, 9 show that two *P. aeruginosa* isolates, 14671 and 14670, produce HQNO but do not produce LasI (synthase for 3-oxo-C_{12}-HSL). Interestingly, 14671 produced a concentration of 7.5 μg/ml of HQNO (table 6), which was the highest concentration among the CF isolates, and does not produce 3-oxo-C_{12}-HSL. This is a clear indication that the PQS system can operate independently of the *las* system and HQNO production is not dependent on a functional *las* system. Since LasR is required for the conversion of HHQ to PQS (Deziel *et al.,* 2003) these data also suggests that *MvfR* is the more important regulator for the PQS operon than large amounts of PQS.

5.4 One proposed mechanism of interactions between *P. aeruginosa* and *S. aureus* CF isolates

Our data suggest that HQNO is not the sole factor but rather one of several factors that can engender antagonistic interactions between *P. aeruginosa* and *S. aureus* CF isolates (Chapter 4). Nine CF isolates were able to inhibit *S. aureus* but, when the inhibiting *P. aeruginosa* supernatants were further analysed using GC-MS only four *P. aeruginosa* isolates produced HQNO (Table 4 and 5). This notion was further confirmed when the correlation coefficient (r) was calculated for comparison between concentration of HQNO to average diameter size and percentage of *S. aureus* isolates that were inhibited by the *P. aeruginosa* isolate (Figure 18 a and b). No correlation was found between HQNO concentration and both of those factors, which strengthens the argument. Which might suggest that there was additional inhibitory factors in addition to HQNO. Originally

we hypothesized that the primary inhibitory factor, if not HQNO, would be a non QS regulated factor since many inhibitory isolates do not produce 3–oxo-C12HSL (tables 7, 8, 9). However, when taking into account the fact that the strongest inhibitory *P. aeruginosa* isolates produced all three signalling molecules, 3-oxo-C_{12}-HSL, C_4-HSL and PQS it may be possible that the inhibitory factor is controlled by QS, or one of the signalling molecules, in *P. aeruginosa*. Taken together, a model has been formed based on these data which identifies an additional inhibitory compound involved in mediating antagonistic interactions between these species.

Long chain HSLs, such as 3-oxo-C_{12}-HSL, are capable of binding to the cytoplasmic membrane of *S. aureus,* which results in an attenuation of virulence by disrupting the *arg* QS system (Qazi *et al.*, 2006). Qazi *et al*., found that spent medium from *P. aeruginosa* isolates that produce 3-oxo-C_{12}-HSL can bind to the membrane. This interaction causes a reduction in growth and virulence factor production such as exotoxin and protein A – hemolytic activity is also reduced. It was found that long chain HSLs bind to the membrane where as short chain HSLs such as C_4-HSL have no effect on growth or virulence (Quazi *et al.,* 2006).

Taken together, we propose that for the strong *S. aureus* inhibitors 3-oxo-C_{12}-HSL may mediate antagonistic interactions between *P. aeruginosa* and *S. aureus* by means of disrupting the electron transport chain. In doing so, the electron potential is reduced and aminoglycoside resistance occurs as a consequence. The *P. aeruginosa* isolates that were able to inhibit the greatest number of *S. aureus* CF isolates (includes PAO1, 14684, 14650, 7307, 14690, 4384 and 5588) could all produce 3-oxo-C_{12}-HSL and their spent supernatants could reduce growth (Figure 8) similar to inhibition data in Qazi *et al.,* 2006.

This suggested that 3-oxo-C_{12}-HSL may be involved in mediating antagonistic interactions. This mechanism is not able to account for those *P. aeruginosa* isolates that are able to inhibit *S. aureus* and do not produce 3-oxo-C_{12}-HSL.

5.5 The ability of *P. aeruginosa* to induce aminoglycoside resistance in *S. aureus* has a high clinical significance in CF

It is becoming more common for *P. aeruginosa* and *S. aureus* to acquire high antimicrobial resistance in the CF lung which results in a more rapid lung function decline in CF patients (Dasenbrook *et al.*, 2010). Aminoglycosides, fluoroquinolones and tetracycline are important therapeutics in CF treatment but have been found to have adverse effects as aminoglycosides can induce bacterial biofilm formation (Hoffman *et al.*, 2006). In addition, *P. aeruginosa* can also stimulate the formation of highly antibiotic resistant biofilms in *S. aureus*, through secretion of HQNO which induces expression of biofilms through the expression of *sigB* (Hoffman *et al.*, 2010). Many *P. aeruginosa* CF isolates have been shown to induce tobramycin resistance in C105 (Figure 11, Tables 2 and 11). Since tobramycin resistance in *S. aureus* is usually a result of small colony variant formation, or at least disruption of the electron transport chain, co-colonization of *P. aeruginosa* and *S. aureus* leads to better biofilms formation and resistant to common CF therapeutics. Taken together novel CF therapeutics are required for successful treatment for pulmonary lung infections. Novel therapeutics should inhibit interactions between species instead of trying to target the growth of bacteria.

5.6 Conclusions

CF lung infections are polymicrobial, characterized by chronic colonization and biofilm growth. *P. aeruginosa* and *S. aureus* are two very common CF pathogens that

cause a large amount of lung damage in CF patients and are usually present throughout the majority of the patient's life. The aim of this study was to survey interactions between *P. aeruginosa* and *S. aureus* CF isolates and determine the mechanism of interactions between the two species. These data show that *P. aeruginosa* can inhibit the growth of *S. aureus* CF isolates in addition to inducing tobramycin resistance in C105. PAO1 supernatant has the capability to alter the phenotype of C105 from WT to small colony variants. We hypothesized that this interaction was mediated by HQNO, but further analysis concluded that the majority of *P. aeruginosa* inhibitors did not produce HQNO. Additional analysis revealed few inhibitors produced HQNO, suggesting that HQNO is not the sole inhibitory molecule. Our data further revealed that *P. aeruginosa* produces multiple inhibitors of *S. aureus*, some of which have not been identified, that can induce tobramycin resistance. Taken together this data provides insight into interactions within the CF lung environment and highlights the need for novel therapeutics.

5.7 Future Directions

5.7.1 Further investigation into the *S. aureus* inhibitory factors

5.7.1.1 Obtain mutants of the PQS system and test for inhibition of *S. aureus*

The first step into further investigating additional inhibitory factors is to test for inhibition of *S. aureus* by *P. aeruginosa* PQS mutants – specifically mutants of *pqsA, pqsL and pqsH* in *P. aeruginosa.* These three genes control production of HQNO, HHQ and PQS. Then after obtaining these three mutants the next step would be to purchase synthetic HHQ and PQS (we already have synthetic HQNO). The experiment will be to first test for inhibition using the three *P. aeruginosa* mutants via an overlay assay as described in section 2.2.1. Then once that has been completed in triplicate the same procedure will be

completed using exogenous PQS, HHQ and HQNO added to the supernatant of the mutants. This experiment will also be completed using tobramycin plates (as in section 2.4) to test for resistance caused by the inhibitors.

5.7.1.2 Testing for induced tobramycin resistance caused by HSLs

Since we hypothesize that long chain HSLs may inhibit the growth of *S. aureus* we would need to confirm that long chain HSLs can induce tobramycin resistance. In order to test this, the experiment would be carried out as in section 2.4 but using synthetic HSLs. Once that experiment has been completed an overlay assay as explained in section 2.2.1 should be completed with synthetic HSLs.

5.7.1.3 Testing for LasA activity in *P. aeruginosa* CF isolates

The method that will be utilized to test for LasA activity will be taken from Oldak and Trafny *et al.,* 2005. Briefly *S. aureus* will be grown overnight and harvested in the morning. The pellet will be resuspended in phosphate buffer to an $OD_{595} = 0.8$. One hundred microliters will be added to 900µl of standardized *P. aeruginosa* culture. Staphylolytic activity will be measured by observing optical density every 5 minutes for an hour.

5.7.2 The effect of interspecies interactions between *P. aeruginosa* and *S. aureus* on virulence using the *D. melanogaster* fly feeding model

From this work it has been shown that *P. aeruginosa* and *S. aureus* CF isolates can participate in negative interactions. The next step in examining interactions between two species is to understand how interactions affect virulence in a host to gain insight on disease progression. Since it has been shown that the *D. melanogaster* model is a suitable model for studying microbe-microbe interactions (Sibley *et al.*, 2008; Lutter, 2008;

Purighalla, 2011; MacLean 2012; Lutter *et al.,* 2012) this method would be employed. The study would include testing interactions using the fly feeding model between C105 (in addition to using the three ATCC *S. aureus* strains) against the *P. aeruginosa* isolates that were able to inhibit the growth of *S. aureus*. Some negative controls would be included as well, this would consist of a few *P. aeruginosa* isolates that were classified as non-inhibitors. Once this has been completed in triplicate three times – other *S. aureus* CF isolates should be put through the system as well. Preliminary experiments were completed using this model (appendix A).

In addition to examining interactions, the formation of small colony variants as a result of a mixed infection should be observed as well. A preliminary experiment was completed by homogenizing the flies on specific days throughout the experiment and testing for small colony variant formation (appendix A). The preliminary data shows that after day four in the fly, no *S. aureus* WT can be recovered from the fly. Small colony variants were tested by plating *S. aureus* on tobramycin plates once extracted from the fly

5.7.3 Mixed species biofilms and tobramycin resistance

Since it is well know that chronic lung infections within cystic fibrosis patients occur in the form of biofilms, and from this research it was shown that antagonistic interactions occur between *P. aeruginosa* and *S. aureus,* the next factor that should be examined is mixed species biofilms. Three questions would be asked from this experiment. The first being, can *S. aureus* small colony variants form when *P. aeruginosa* and *S. aureus* are grown in a mixed species biofilm. The second question that would be addressed would be; do better biofilms form as a result of small colony variants. And the last question that would be addressed is; are mixed species biofilms more resistant or tolerant to tobramycin.

These experiments would be completed using the MBEC device and biofilm growth would be observed using CFU/Peg and CFU/mL. Once CFUs have been calculated, biofilms will be observed using confocal laser scanning microscopy. Since *P. aeruginosa* mediates interactions via a secreted product, *S. aureus* isolates would be grown with *P. aeruginosa* supernatant. The isolates that will be used in this study will include C105, all three ATCC strains and one *S. aureus* CF isolate from each clinical condition (chronic, co-infection and superinfection). Once SCV formation and CFUs were tested, tobramycin resistance will be examined. Since SCVs are resistant to tobramycin, we hypothesize that *S. aureus* SCV biofilms will be more resistant to tobramycin than WT. Tobramycin resistance will be completed by using the MBEC system as well. Preliminary data showing *S. aureus* C105 biofilm growth can be seen in appendix A.

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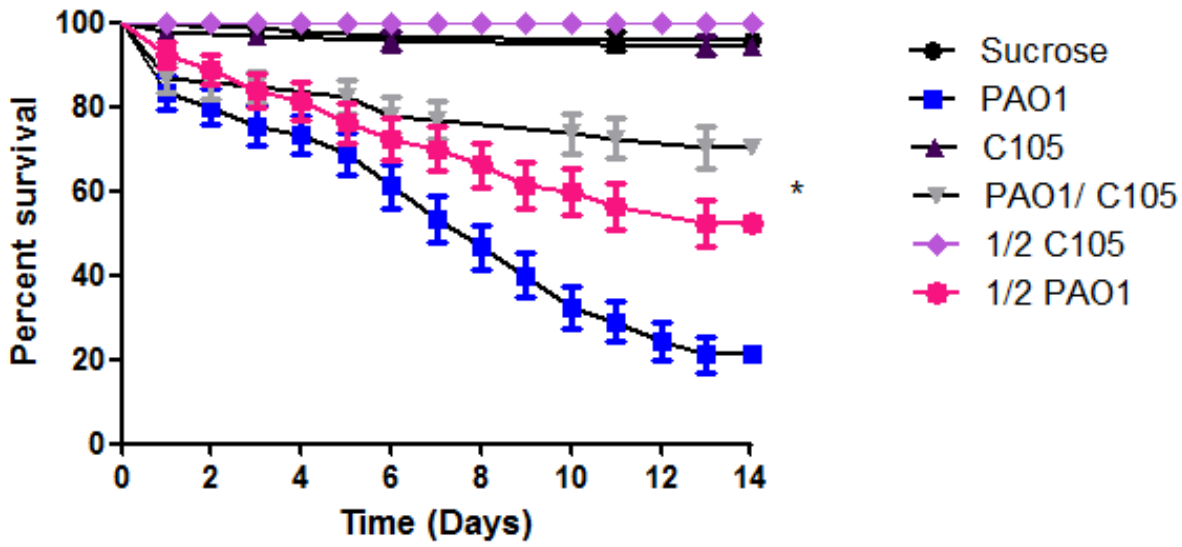
Appendix

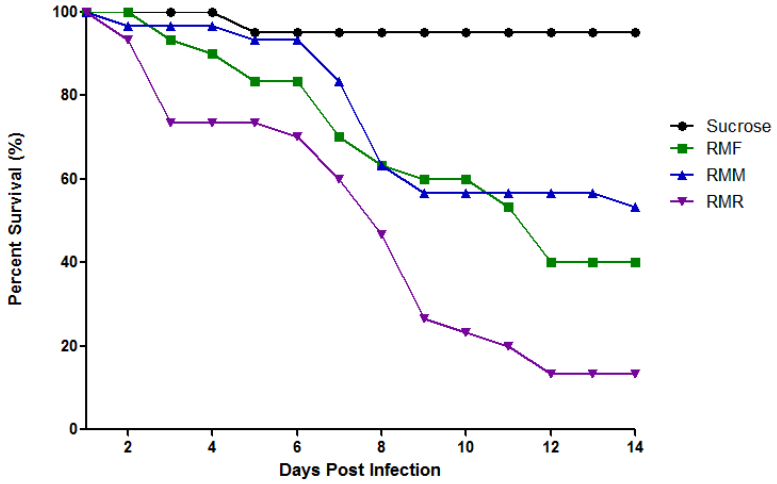
Appendix A: Preliminary Data

A.1 Fly Feeding Data: Kill curves from a fly feeding assay of a mixed infection of *S. aureus* and *P. aeruginos*a CF isolates. Flies were fed the appropriate concentration of bacterial culture and survival was monitored over a 12 day period. Panel A represents a mixed fly feeding assay with PAO1 and C105 whereas B, C and D represents kills curves for a single infection with *S. aureus* CF isolates.

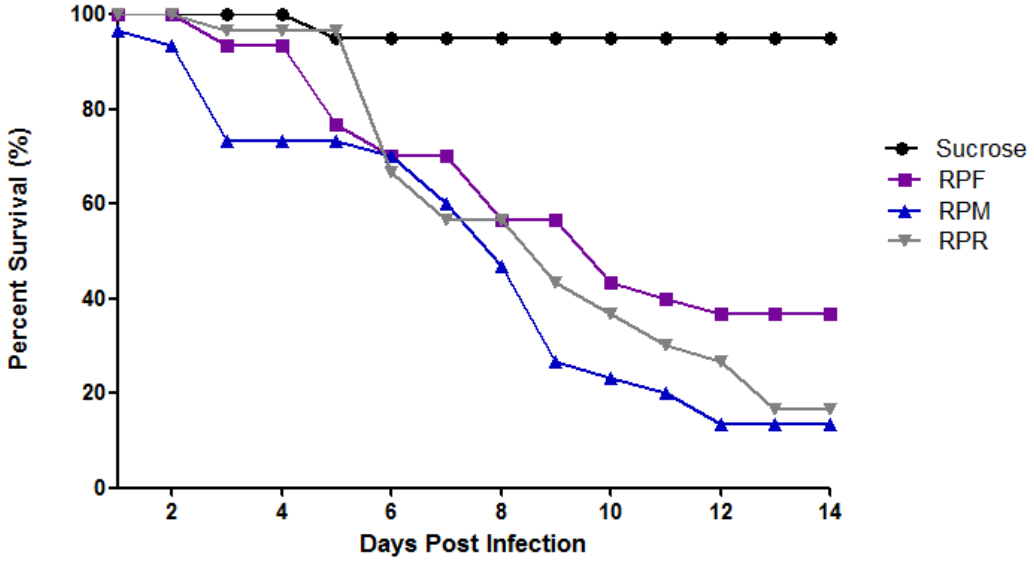
A)



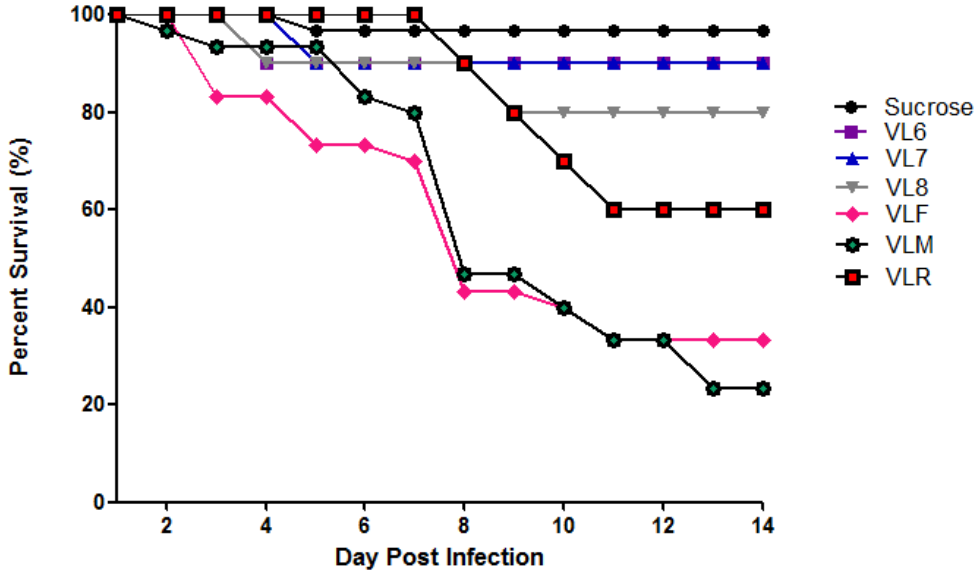
B)



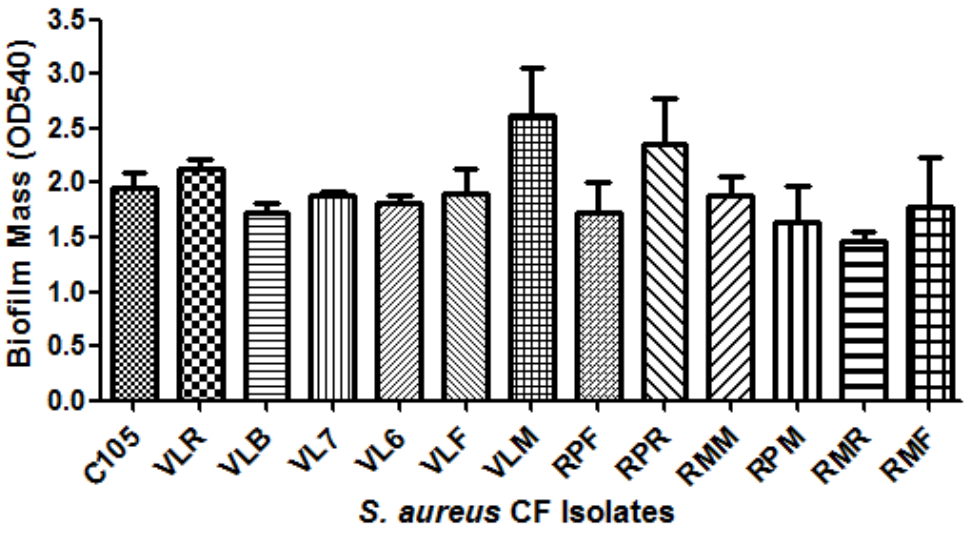
C)


Percent Survival (%)
100
80
60
40
20
0
2
4
6
8
10
12
14
Days Post Infection
Sucrose
RPF
RPM
RPR

D)


Percent Survival (%)
100
80
60
40
20
0
2
4
6
8
10
12
14
Day Post Infection
Sucrose
VL6
VL7
VL8
VLF
VLM
VLR

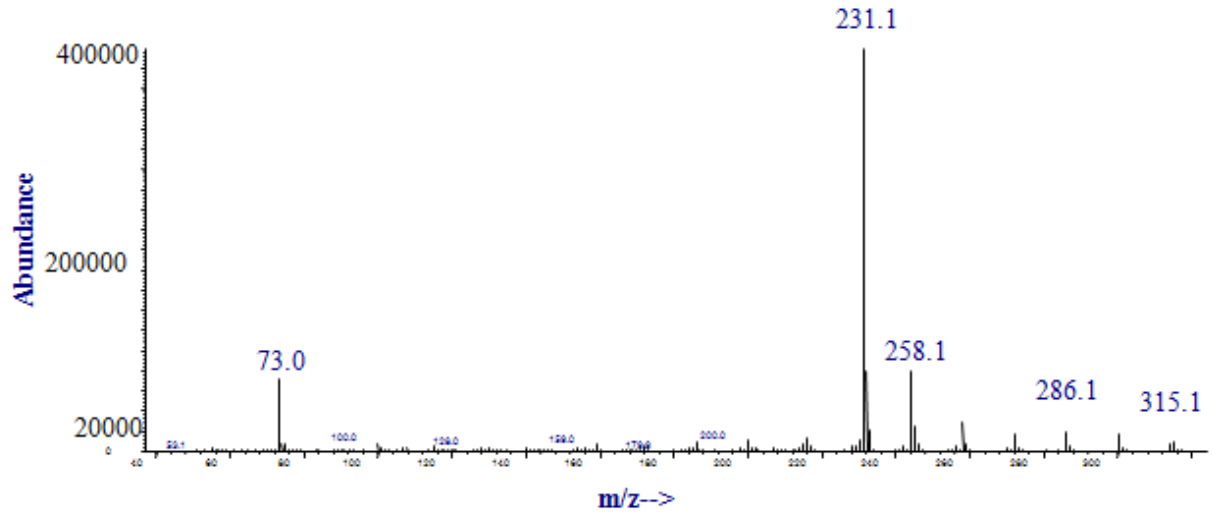
A.2 **Biofilm Data**: Biofilms were grown overnight in TSB and standardized the following day. Biofilms were grown in the MBEC device by adding 180 µl of culture to each well. Biofilms were grown for 48 hours at 37◦C at 180 rpm. Bars represent optical density of each peg stained in cystal violet. Error bars represent standard deviation.



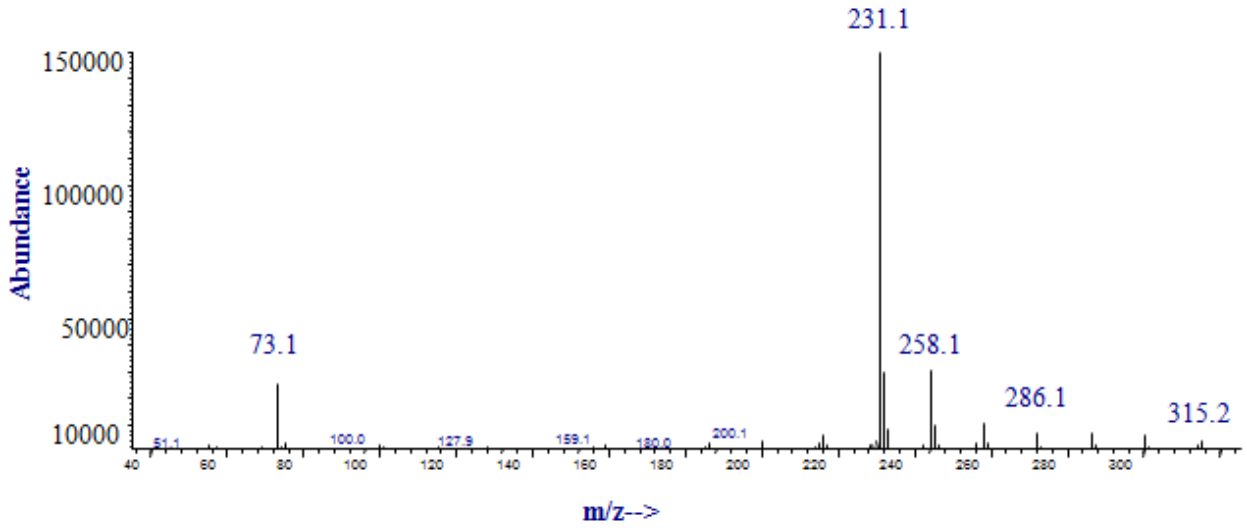
Appendix B: GC-MS Data for *P. aeruginosa* isolates.

Ion fragment pattern for *P. aeruginosa* CF isolates: Twenty mLs of *P. aeruginosa* supernatant was extracted using equal volumes of ethyl acetate, thrice. The extracted product was derivatized with BSTFA and 1 µl was injected into the GC-MS where. Each panel is labeled with the retention time and isolate name.

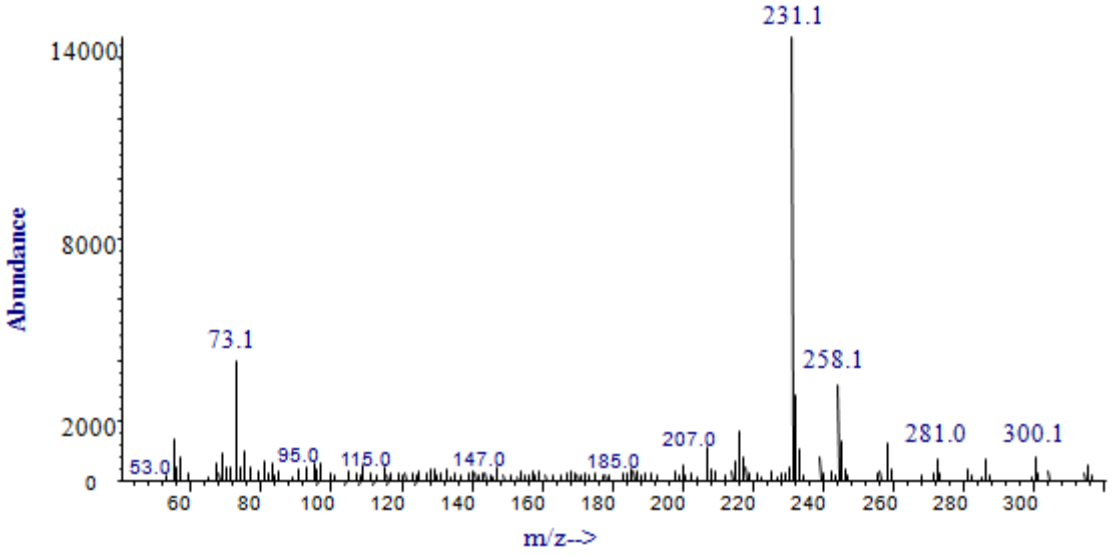
A) HQNO STD. RET Time 31.071



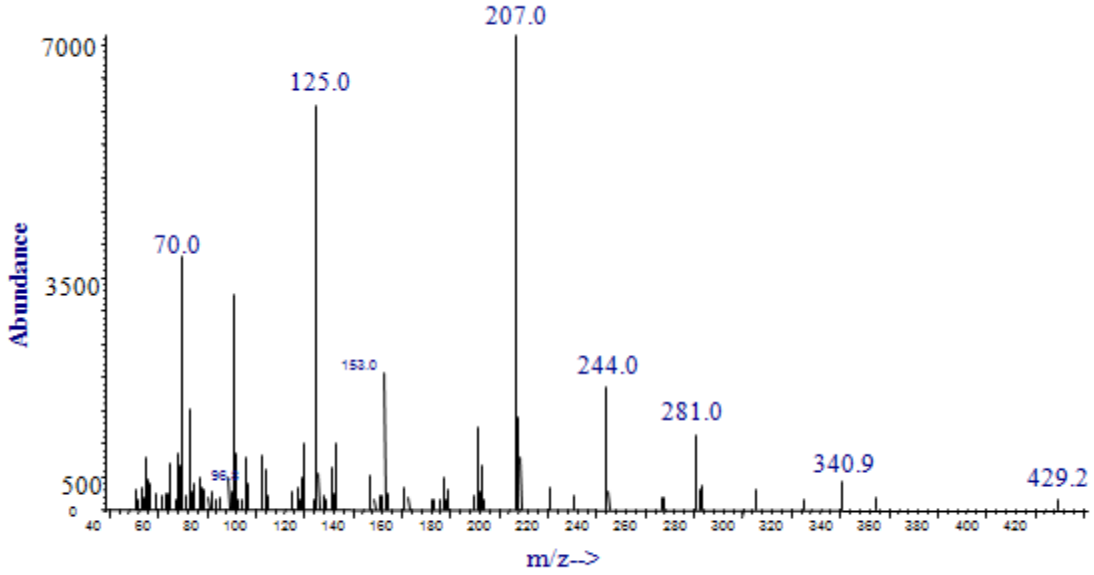
B) PAO1, RET time 30.950



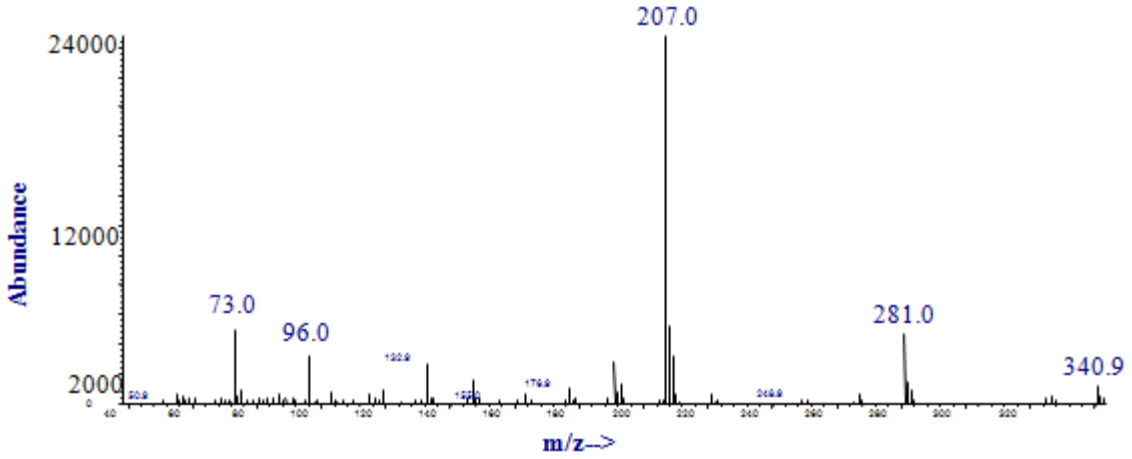
C) PAOJP2 RET Time 30.965



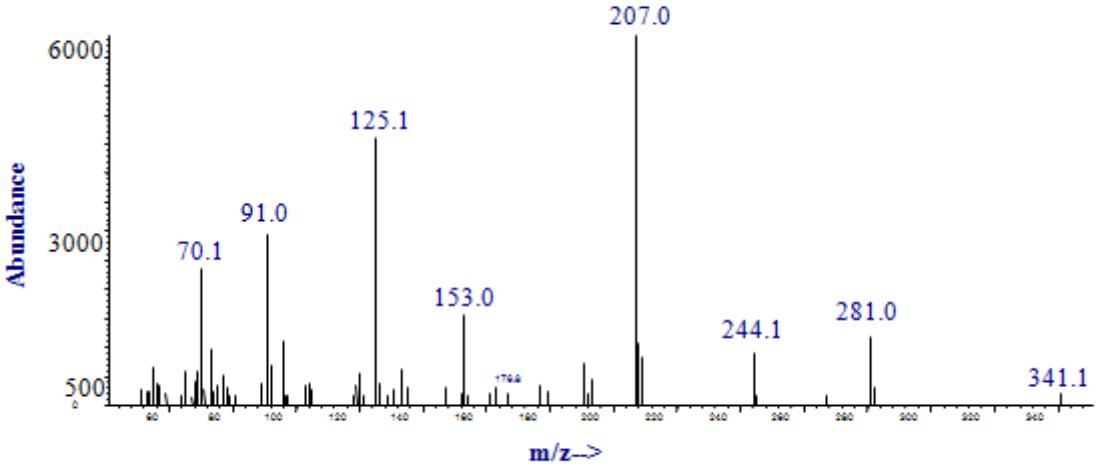
D) 14684 RET time 33.880



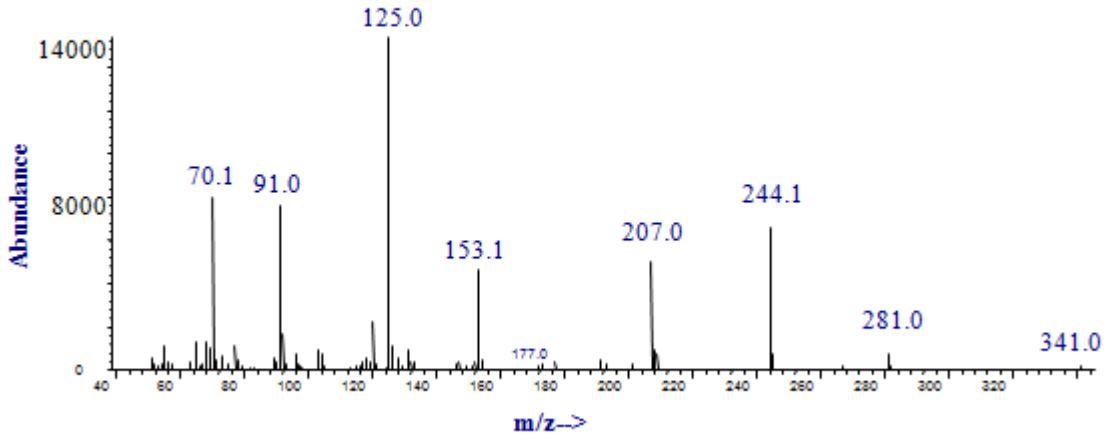
E) 5585 RET Time 41.063



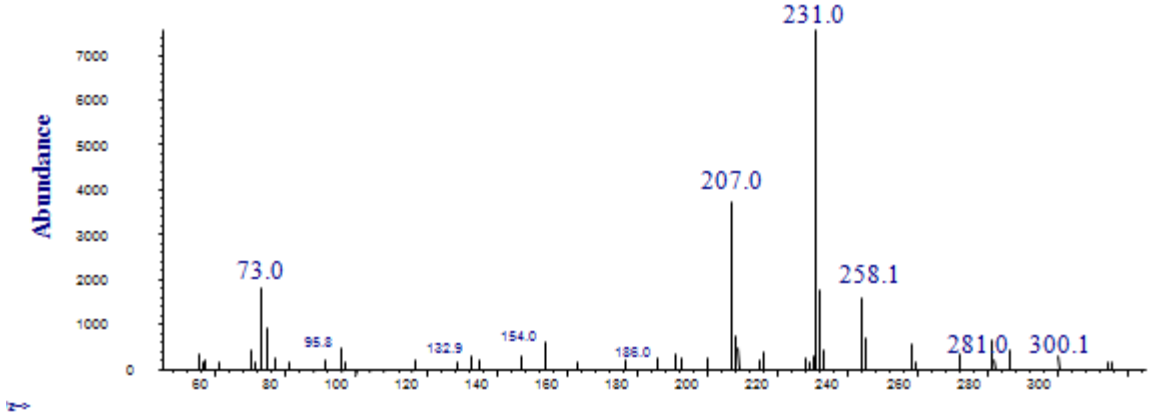
F) 14650 RET Time 32.310



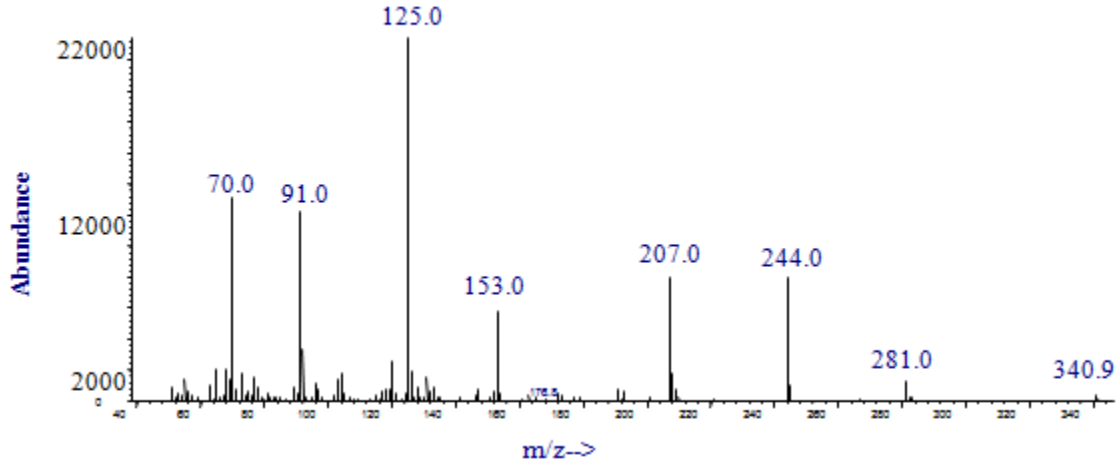
F) 14660 RET Time 33.623



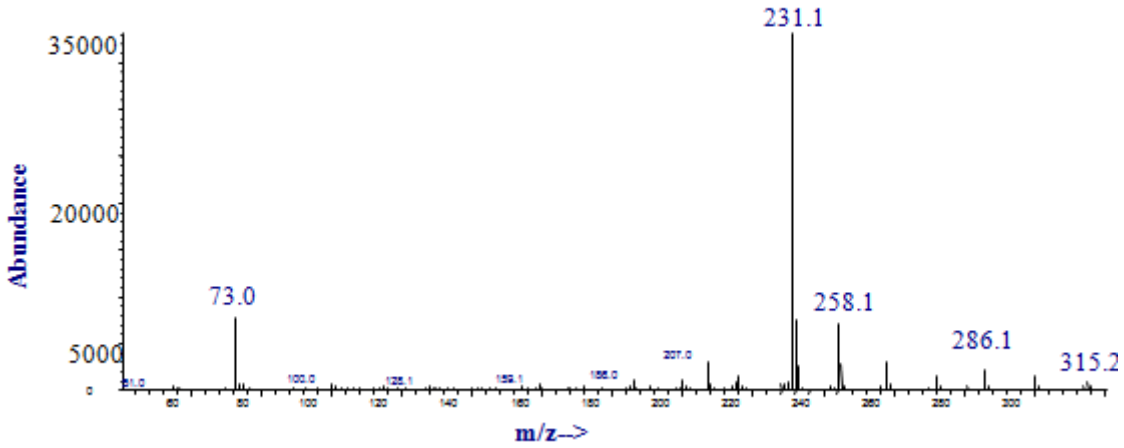
G) 14670 RET Time 30.903



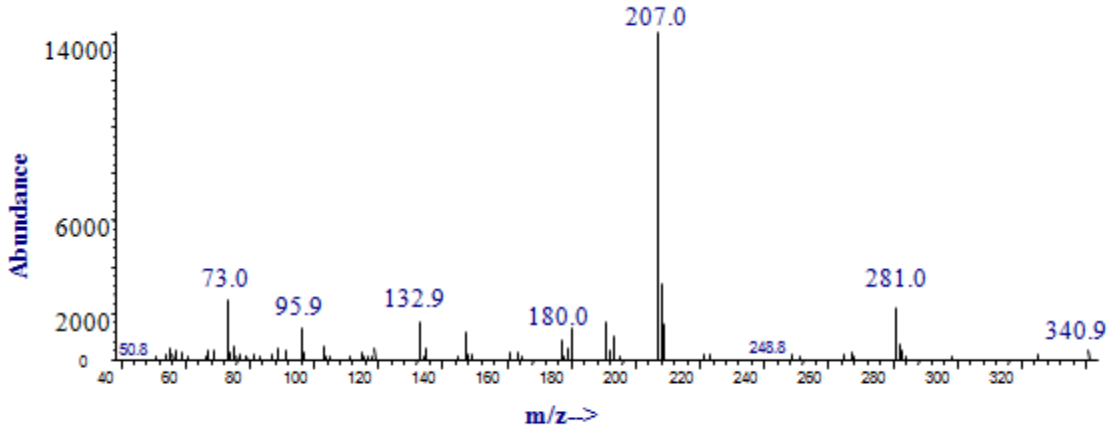
H) 14673 RET Time 33.680



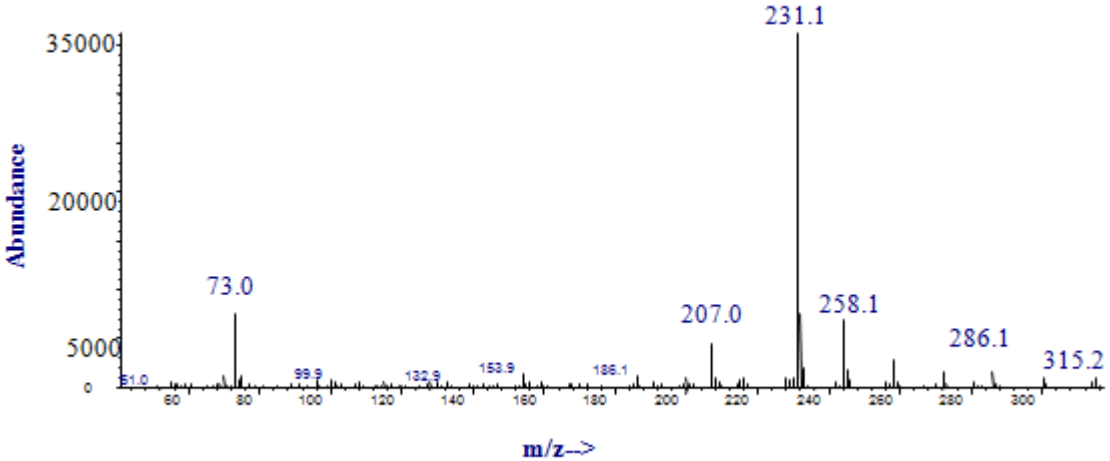
I) 7307 RET Time 30.840



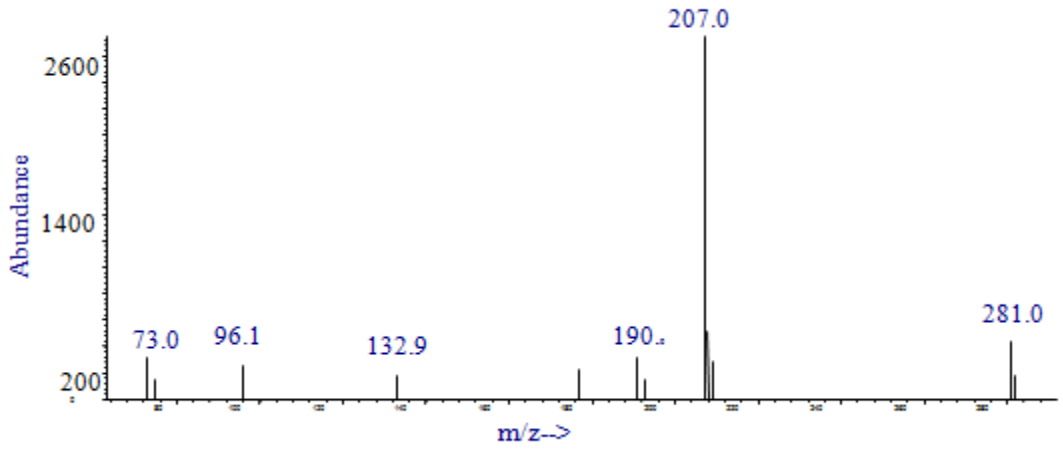
J) 14690 Ret Time 37.502



K) 4384 RET Time 30.915



L) 14715 RET Time 30.338



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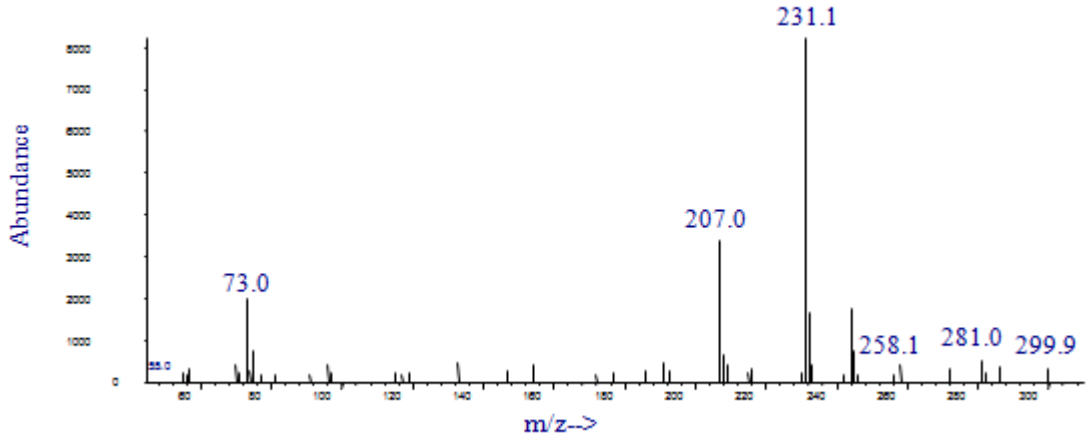

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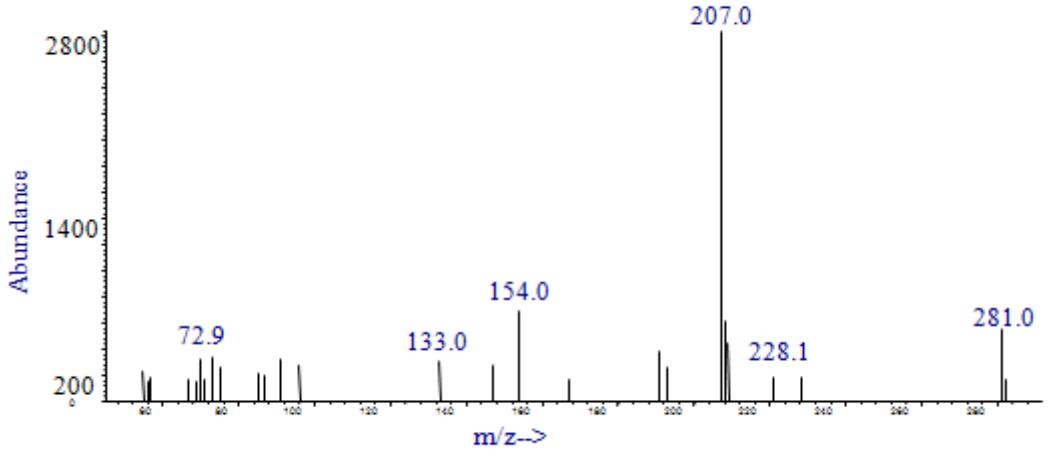

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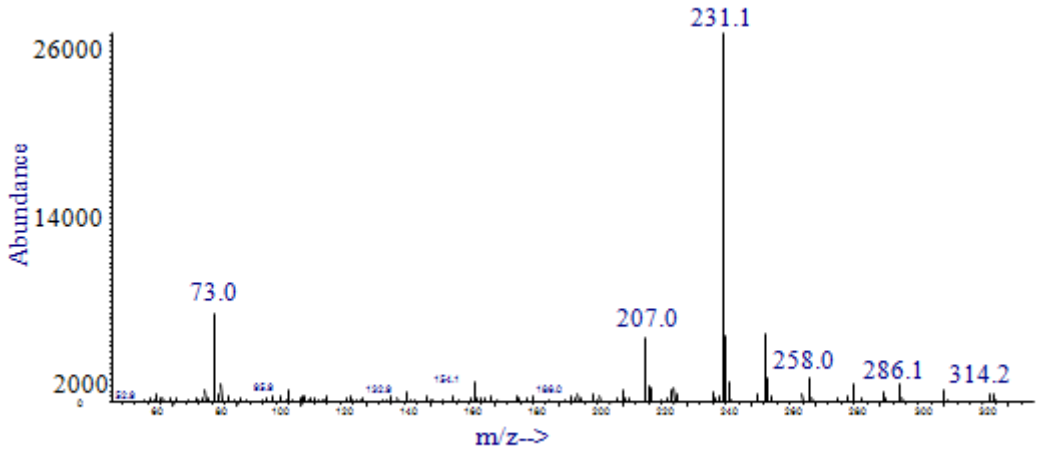

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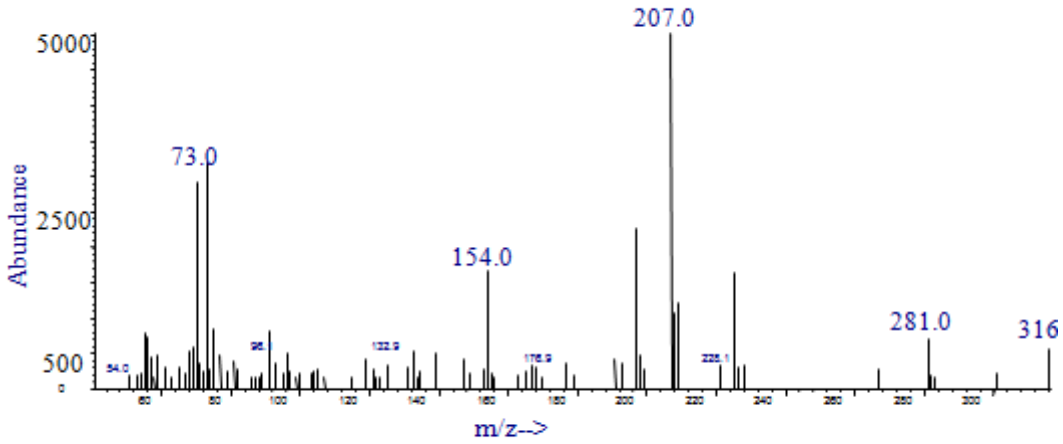

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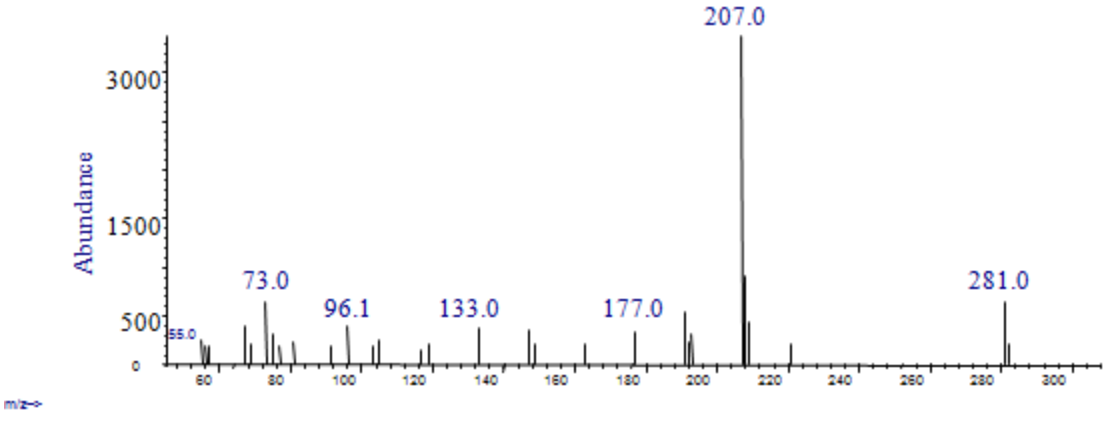

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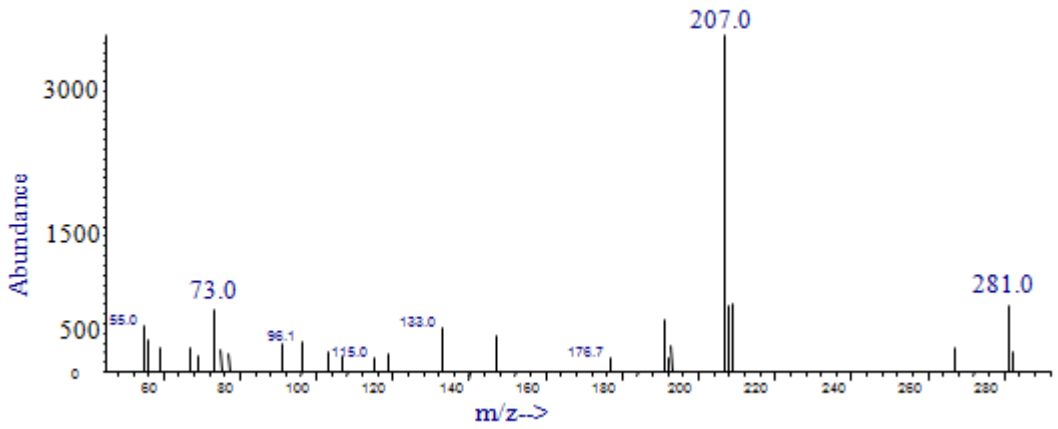

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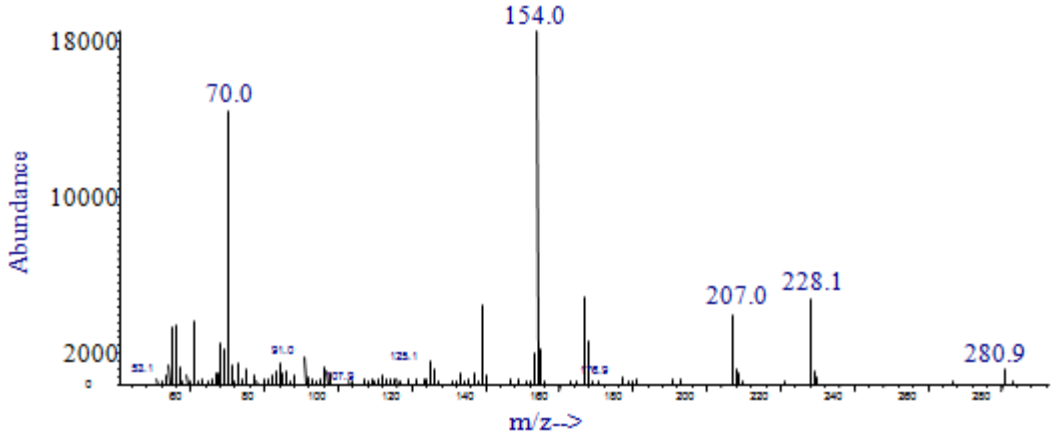

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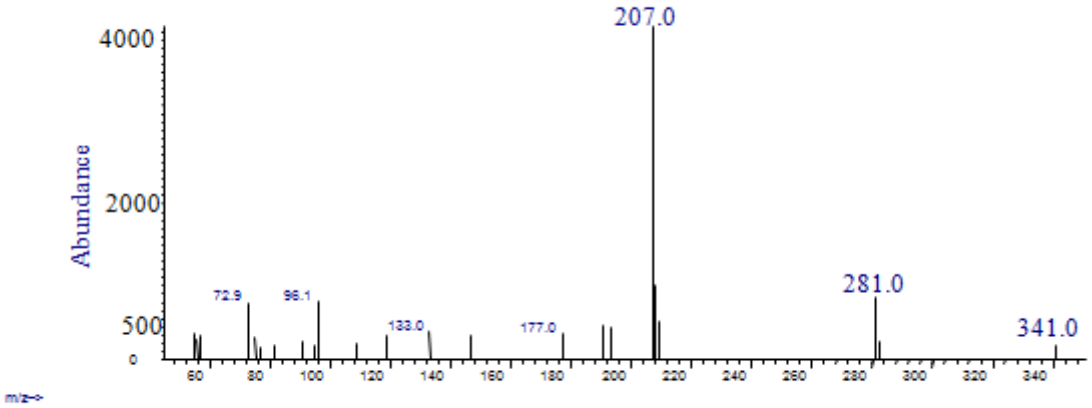

U) TSB RET Time 30.046