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CONTROLLING Pseudomonas aeruginosa BIOFILMS AND PERSISTER CELLS BY MANIPULATING CELL-CELL SIGNALING

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Abstract

Bacterial cells often form sessile biofilms that are up to 1,000 times more resistant to antimicrobial agents than free-living cells. Meanwhile, bacteria produce a small subpopulation of slow-growing or non-growing persister cells that exhibit high tolerance to antibiotics. Both biofilms and persister cells play important roles in the recalcitrance of chronic infections. Recent studies have shown that bacterial cell-to-cell communication, named quorum sensing (QS), is involved in the biofilm and persister formation.

In this study, we investigated the effects of quorum sensing signals *N*-(3-oxododecanoyl)-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butyryl homoserine lactone (C_4-HSL) on isolated *Pseudomonas aeruginosa* PAO1 and PDO300 persister cells. Interestingly, 3-oxo-C_{12}-HSL was found to increase antibiotic susceptibility of isolated *P. aeruginosa* PAO1 and PDO300 persister cells to ciprofloxacin in a dose-dependent manner, although such synergistic effect was not observed with ofloxacin, tobramycin, tetracycline, carbenicillin, and gentamicin. In contrast, C_4-HSL did not affect the susceptibility of *P. aeruginosa* PAO1 and PDO300 persister cells to ciprofloxacin.

Additionally, we engineered polydimethylsiloxane (PDMS) surfaces by supplementing with (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-25(*H*)-one (BF8) to reduce *P. aeruginosa* PAO1 biofilm formation and biofilm-associated persister cells. Compared with the PDMS surface

without BF8, *P. aeruginosa* PAO1 biofilm formation and biofilm-associated persister cells were significantly reduced on the PDMS surfaces containing BF8. For example, after 24 h, the number of biofilm cells and biofilm-associated persisters was inhibited by 98% ± 0.4% and 99% ± 0.5% on the PDMS surface containing 1800 μg/mL BF8, respectively. These results further support that bacterial cell-to-cell signaling is a possible target for controlling biofilm formation and persistence.

Key words: biofilm, persister cell, quorum sensing, antibiotic tolerance.

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CHAPTER 1

LITERATURE REVIEW

1.1 Acquired antibiotic resistance

Since the discovery of penicillin in 1928 [1], antibiotics have been widely used for the treatment of numerous bacterial infections such as those associated with tuberculosis and cystic fibrosis. Antimicrobial agents can be classified into two types: bactericidal drugs that kill bacteria, and bacteriostatic drugs that inhibit growth of bacteria [2, 3]. There are three major classes of bactericidal antibiotics, for instance, fluoroquinolones that inhibit the ligation of DNA gyrase and topoisomerase, aminoglycosides that obstruct protein synthesis, and β-lactams that interfere with peptidoglycan synthesis [4-6]. Although these bactericidal antibiotics attack different targets, they all cause bacterial cell death by producing hydroxyl radicals that are capable of damaging DNA, lipids, and proteins [2, 7, 8].

However, due to the overuse or incorrect usage of antibiotics, antibiotic resistant bacterial infections have become very pressing public health problems throughout the world [4, 6, 9]. According to the U.S. Centers for Disease Control and Prevention (CDC), more than 2 million Americans suffer from antibiotic resistant infections per year [10] leading to more than $20 billion per year in excess health care costs and up to $35 billion per year in societal costs [11]. Bacteria can acquire antibiotic resistance in different ways such as chromosomal mutation and horizontal gene transfer [4, 12, 13].

The mechanisms of acquired bacterial multidrug resistance have been extensively studied including alteration of the antibiotic target site, prevention of drug access to bacterial targets, decreased permeability to antibiotics, enzymatic inactivation of antibiotics, and drug extrusion by efflux pumps [1, 4, 13]. The prevalence of antibiotic resistance has been found in many bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis*,

Mycobacterium tuberculosis, and *Pseudomonas aeruginosa* [14]. MRSA is resistant to all β-lactams such as penicillin, carbepenem, and cephalosporin [13]. This is due to the *mecA* gene that encodes a new penicillin binding protein (PBP), which has a low affinity to β-lactams [13]. Vancomycin is still an effective antimicrobial agent to treat MRSA [12, 15, 16]. However, the elevated usage of this agent has caused other species, e.g. *Enterococci*, to be vancomycin-resistant [13]. *P. aeruginosa* is a common Gram-negative pathogen that causes infections in patients with cystic fibrosis and compromised immune systems. Multidrug resistance of *P. aeruginosa* was attributed to overexpression of multidrug efflux pumps, genetic mutations, or horizontal gene transfer of antibiotic resistance genes [17-19].

1.2 Intrinsic antibiotic resistance

In contrast to acquired antibiotic resistance, bacteria can also tolerate antibiotics through mechanisms that are not specific to certain classes of antimicrobials.

1.2.1 Biofilms

Biofilms are highly-structured microbial communities attached to surfaces and enclosed in a matrix of extracellular polymeric substance (EPS) [20]. EPS generally contains polysaccharides, proteins, and extracellular DNA [20, 21]. The presence of EPS allows the biofilms to contain open water channels for transport of nutrients into the biofilm and waste products out of the biofilm [20]. It has been well documented that bacteria in the biofilm are up to 1,000 times more resistant to antimicrobial agents than planktonic cells [22]. According to the National Institute of Health, more than 60% of nosocomial infections are associated with biofilms [12, 22-24].

A well characterized model organism for biofilm study is *P. aeruginosa* that causes lung infection in cystic fibrosis patients. The development of *P. aeruginosa* biofilms occurs in the following five stages: 1) initial and reversible attachment of free-floating cells to the surface; 2) irreversible attachment; 3) production of the EPS matrix and early development of biofilm architecture; 4) maturation of biofilm architecture; 5) dispersion [20, 25]. Extracellular DNA (eDNA) has been found important to the initial attachment of *P. aeruginosa* biofilms [21]. Additionally, one finding suggested that eDNA is similar to chromosomal DNA based on the polymerase chain reaction (PCR) and Southern analysis results [26].

A number of studies have been reported in attempt to understand the genetic basis of biofilm formation. Whiteley et al. [25] found that 73 genes are differentially expressed in *P. aeruginosa* biofilm cells compared with free-living cells based on DNA microarray results. Among them, 34 genes were upregulated while 39 genes were downregulated. Interestingly, seven genes coding for flagella and pili were repressed in mature biofilm populations, which means that flagella and pili may not be necessary for mature biofilm development [25]. In addition, the *rpoS* gene that encodes an RNA polymerase σ subunit was also repressed in the microarray data [25]. Within 4 h, the isogenic *rpoS* mutant of *P. aeruginosa* produced 2-fold more biofilm biomass than the wild-type strain [25]. In addition, biofilms of the *rpoS* mutant were much more resistant to tobramycin compared to wild-type *P. aeruginosa* biofilms [25]. These results show that the *rpoS* gene plays important roles in biofilm formation and tobramycin susceptibility [25].

It is speculated that biofilm-associated drug resistance is due to low growth of biofilm [27], drug efflux [28], and impaired diffusion of drugs into cells due to the matrix material [29-31]. However,

Lewis et al. [32] found that the majority of cells in the biofilm can be killed by antibiotics except for a small subpopulation, known as persister cells.

1.2.2 Persister cells

In 1944, J. W. Bigger defined persister cells when he studied incomplete killing of *Staphylococci* by penicillin [33]. He found that these dormant persister cells make up only a small fraction of the bacterial population, which neither grow nor die in the presence of antibiotics. When persister cells are inoculated into a fresh medium, they can revert to normal cells and be killed by antibiotics. Thus, persister cells are not antibiotic-resistant mutants but rather phenotypic variants, which may play important roles in chronic infections [5]. *E. coli*, *P. aeruginosa*, and *M. tuberculosis* are well characterized for their persister formation, and it is speculated that virtually any bacterial culture may contain persister cells [34-37].

1.2.2.1 Formation of persister cells

Persister formation increases in biofilms and stationary phase cultures [5, 38]; biofilms of *E. coli* and *P. aeruginosa* contain 0.1%-1% cells as persister cells. The mechanisms of persister formation are still poorly understood. No negative mutants of persister formation were found after screening the transposon insertion libraries of *E. coli* [39, 40] and *P. aeruginosa* PAO1 [41], suggesting the redundancy in persister genes. However, a number of factors have been linked to persister formation [42].

1) Persisters and heterogeneous growth

The finding that persister cells only make up a small percentage of the population suggests the heterogeneity in growth rates of a given bacterial population [43-45]. Actively growing bacterial cells are more susceptible to antibiotics than non-growing and dormant cells. In 2004, Balaban et al. [43] studied the persistence of individual bacterial cells of *E. coli* high persistence (*hip*) mutants using transparent microfluidic devices. The results indicate persistence is associated with preexisting heterogeneity in bacterial populations due to the phenotypic switch between actively growing cells and persister cells with reduced growth rates [43].

2) Persisters and SOS response

SOS response is a global response system in bacteria, in which DNA replication and cell division are arrested and DNA repair is induced [46]. More than 40 genes are involved in SOS response including the genes *recA* and *lexA* [46, 47]. SOS response was proposed to induce several TA systems containing *tisAB/istR*, *symER*, *yafN/yafO*, and *hokE* [48-50]. The connection between SOS response and persistence has been studied in recent years. One finding showed that the fluoroquinolone antibiotic ciprofloxacin (Cip), which targets gyrase and topoisomerase and results in double-strand breaks, induced the persister formation in *E. coli* by activation of SOS response [47]. Additionally, another independent study suggested that the TisB toxin controlled the persister formation induced by Cip in *E. coli* [36]. The TisB toxin is a membrane-acting peptide that disrupts the proton motive force (pmf) and results in reduced ATP levels [36]. As a result, the TisB toxin induced dormancy and multidrug tolerance in *E. coli* by the shutdown of antibiotic targets [36].

3) Persisters and toxin/antitoxin modules

Toxin/antitoxin (TA) modules are ubiquitous and have been found on the chromosomes and plasmids of bacteria [51]. TA systems have been extensively studied for their roles in plasmid maintenance [51]. However, the role of chromosomally encoded TA systems remains unclear [52]. Typically, a TA module is composed of two genes in an operon which encodes a stable protein toxin that interrupts important cellular functions and a labile antitoxin degraded by a specific protease such as Lon, ClpAP, and ClpXP [53]. To date, gyrase and mRNA have been found as two major targets of TA toxins [54]. Antitoxins are small RNAs in type I (that inhibit toxin translation) and type III (that inhibit toxin activity) TA modules or proteins in type II (that inhibit toxin activity) TA modules [55]. Antitoxin can bind to its cognate toxin and then form a stable and non-toxic complex which functions as a repressor of the TA operon [54].

Recent work suggests that TA modules are involved in persister formation [53, 55, 56]. Based on biochemical and bioinformatics analyses, at least 36 putative TA modules have been identified in *E. coli* K-12 [57]. Among them, eight TA modules have been well studied including *hipBA*, *relBE*, *mazEF*, *chpBIK*, *yafNO*, *mqsAR*, *yefM-yoeB*, and *dinJ-yafQ* [57]. It suggested that more persister cells are formed when toxins such as HipA and RelE are overexpressed [36, 53, 58]. However, due to the redundancy in TA modules, only a few TA systems cause a sharp reduction in persistence when their toxin genes are deleted [36, 53]. The *hipA* (high persistence) gene, located in the *hipBA* operon, was the first recognized persistence gene in *E. coli* K-12. It encodes a 440-amino-acid inhibitor of cell growth [59]. Compared to the wild-type strain, the *hipA* mutant exhibited a 1,000-fold increase in the level of persister cells [60].

In *M. tuberculosis*, more than 60 putative TA modules have been identified including *relBE* and *mazEF* [57]. For *P. aeruginosa* PAO1, only three TA loci have been described to date including

higBA, *relBE*, and *parDE* [51, 61]. Williams et al. [51] showed that *higBA* and *relBE* TA systems were present and transcribed in clininal isolates of *P. aeruginosa*.

1.2.2.2 Current methods of persister control

Due to the high level antibiotic tolerance of persister cells, it is important to develop new antimicrobials for persister control. However, eradicating persister cells remains challenging. Allison et al. [62] suggested that specific metabolites such as mannitol, glucose and fructose could potentiate the killing of *E. coli* and *S. aureus* persister cells by aminoglycosides. This approach depends on the generation of proton motive force by these sugars which enhance the uptake of aminoglycosides. This study showed that metabolic activities can be a good target for persister control.

Using a different approach, Conlon et al. [63] found that a compound (acyldepsipeptide antibiotic, ADEP4) can eradicate actively growing, stationary and biofilm populations of *S. aureus* in combination with rifampicin. ADEP4 activates and dysregulates caseinolytic protease P (ClpP) that is characterized as the proteolytic core of bacterial protein degradation machinery in an ATP-dependent process [64]. Interestingly, ADEP4-activated ClpP enables the degradation of over 400 proteins in the absence of ATP [63, 65]. The cell division protein FtsZ was proposed to be a major target of ADEP4-activated ClpP [66]. Thus, ADEP4 primarily inhibits bacterial cell division and eventually causes cell death.

In addition, 3-[4-(4-methoxyphenyl)piperazin-1-yl]piperidin-4-yl biphenyl-4-carboxylate (C10), has been reported to reduce *E. coli* and *P. aeruginosa* persister formation in combination with

fluoroquinolone antibiotics [67]. C10 appeared to revert persister cells to antibiotic-susceptible cells at growth non-inhibitory concentrations.

Recent work has suggested that bactericidal antibiotics promote the generation of reactive oxygen species (ROS) and thus result in bacterial cell death [7]. As reported by Grant et al. [7], decrease in dissolved oxygen (DO) of bacterial media facilitated the persister survival. On the contrary, all cells could be killed by antibiotics via sufficient ROS if DO concentration is maintained at high levels. It has been found that thiourea, the hydroxyl-radical scavenger, rescued *M. smegmatis* persister population even though DO was maintained at high levels. It should be noted that this relationship between oxygen and antibiotic sensitivity not only exists in mycobacteria but also in *P. aeruginosa* and *E. coli*.

An alternative approach has been proposed to control *P. aeruginosa* PAO1 persister cells by using weak electrochemical currents alone or in combination with tobramycin [68]. For example, 98% of *P. aeruginosa* PAO1 persister cells was reduced by the treatment with 70 $\mu A/cm^2$ direct currents using stainless steel for 1 h compared to the untreated control.

It also has been suggested that TA modules could be considered as potential targets for the development of novel antibiotics because the released toxins would kill bacterial cells [51, 69, 70]. Compounds that induce toxin activation might be useful as novel antibiotics [69].

1.3 Quorum sensing

Quorum sensing (QS) is an important cell-to-cell communication system in bacteria, which regulates the expression of target genes in response to the cell population density [71-73]. It involves the formation and detection of signaling molecules termed autoinducers. When the

autoinducers reach a certain threshold concentration, the expression of target genes will be altered, such as those involved in the virulence factors, motility, competence, and biofilm formation [73-76].

1.3.1 Quorum sensing in Gram-negative bacteria

In general, Gram-negative bacteria employ acylated homoserine lactones (acyl-HSLs or AHLs) as autoinducers. All AHLs have a basic structure of HSL ring and differ in the length of the R-group side-chain ranging from 4 to 18 carbon atoms [77]. In AHL-mediated QS systems, the AHL signals are produced by autoinducer synthases (termed I proteins) and are recognized by cognate transcriptional regulatory proteins (termed R proteins) [77, 78]. QS System was first discovered in the Gram-negative bacterium *Vibrio fischeri* [72]. Three AHL signals have been found in *V. fischeri*, 3-oxo-hexanoly-HSL (produced by the LuxI protein and sensed by the LuxR protein), *N*-hexanoyl-L-HSL (produced by the LuxI protein and sensed by the LuxR protein), *N*-octanoyl-HSL (produced by AinS protein and sensed by the LuxR protein) [78-80].

Two AHL QS systems have been found in *P. aeruginosa*: the *las* system and *rhl* system. The *las* system consists of the transcriptional regulatory protein LasR and autoinducer synthase LasI, which is responsible for the production of *N*-(3-oxododecanoyl)-HSL (3-oxo-C_{12}-HSL) [81, 82]. The chemical structure of 3-oxo-C_{12}-HSL is shown in Figure 1-1. This QS signal can freely diffuse into *P. aeruginosa* cells, while its transport to the extracellular environment relies on efflux pumps [83]. As the cell population density increases, the concentration of the autoinducer increases accordingly. When the autoinducer reaches a critical threshold concentration, the autoinducer binds to LasR protein and then forms LasR-autoinducer complex. This complex then triggers a series of target genes including genes encoding a number of virulence factors (for

example, genes *toxA*, *lasA*, *lasB*, and *aprA*) and other genes such as *xcpR* and *xcpP* [81, 83, 84]. Besides these genes, LasR-autoinducer complex also induces the expression of the *lasI* gene, which generates a positive feedback loop for signal production [81, 85]. It should be noted that the catabolite repressor homolog Vfr specifically binds to the *lasR* promoter and activate *lasR* transcription [86, 87]. Interestingly, RsaL protein encoded by the *rsaL* gene directly represses the transcription of the *lasI* gene, which generates a negative feedback loop and maintain 3-oxo-C_{12}-HSL homoeostasis [88, 89]. Meanwhile, RsaL protein also represses the production of virulence factors such as pyocyanin and hydrogen cyanide [88, 89].

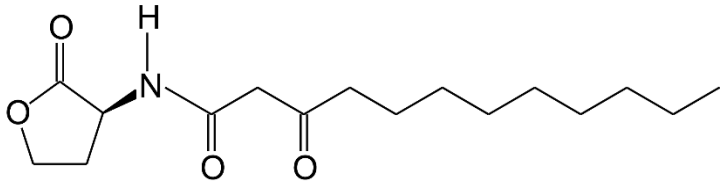

Figure 1-1. Chemical structure of 3-oxo-C_{12}-HSL.

The *rhl* system consists of the RhlR protein and the autoinducer synthase RhlI, which is involved in the production of *N*-butyryl HSL (C_4-HSL) [81]. The chemical structure of C_4-HSL is shown in Figure 1-2. Unlike 3-oxo-C_{12}-HSL, C_4-HSL can freely diffuse both in and out of *P. aeruginosa* cells [83]. Analogous to the LasR-autoinducer complex, RhlR-autoinducer complex activates a number of genes such as *rhlI*, *rpoS* (which encodes the stationary phase sigma factor), *lasB*, *rhlAB*, and genes involved in pyocyanin biosynthesis [72].

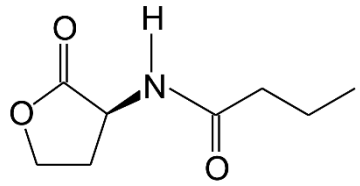

Figure 1-2. Chemical structure of C_4-HSL.

As mentioned above, both the *las* system and the *rhl* system regulate the genes involved in virulence and biofilm formation. As shown in Figure 1-3, these two QS systems are not independent but hierarchically correlated because the LasR protein/3-oxo-C_{12}-HSL could also positively regulate the *rhlR* and *rhlI* genes [81, 82]. It's worth noticing that 3-oxo-C_{12}-HSL competes with C_4-HSL for binding to the receptor RhlR when the concentration of 3-oxo-C_{12}-HSL is higher than that of C_4-HSL [81].

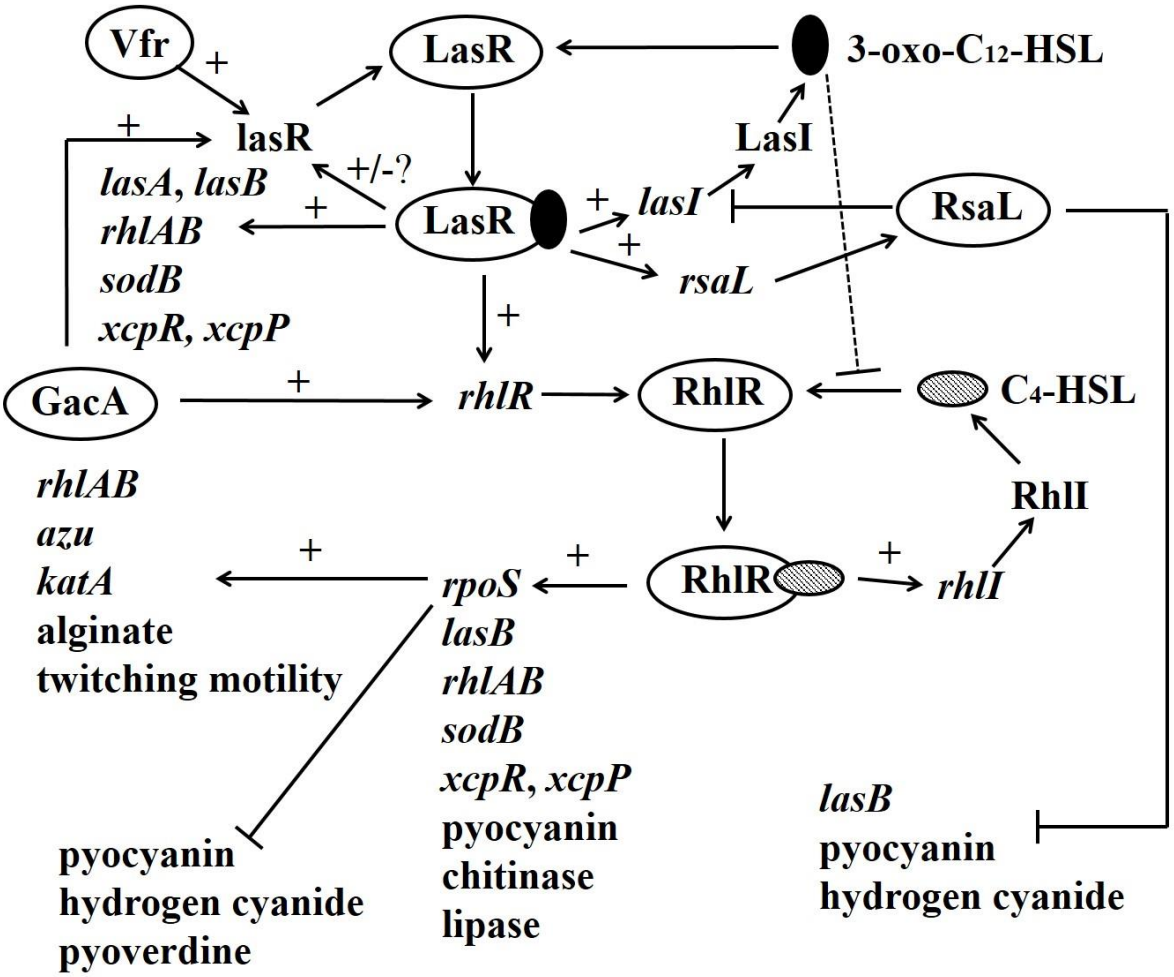

Figure 1-3. The *las* and *rhl* QS systems in *P. aeruginosa*. Plus symbols represent transcriptional activation of the genes at the end of an arrow. The symbol of "+/-?" represent the effect of the LasR/3-oxo-C_{12}-HSL complex on *lasR* is unknown. The symbols of "-|"represent transcriptional repression of the genes.

In *P. aeruginosa*, another QS system has been discovered that uses 2-heptyl-3-hydroxy-4-quinolone (PQS) as the signaling molecule [90]. LasR protein is required for the production of the PQS autoinducer. Meanwhile, the PQS signal activates the transcription of the *rhlI* gene.

Therefore, the PQS QS system provides an additional link between the *las* system and the *rhl* system [72].

1.3.2 Quorum sensing in Gram-positive bacteria

Peptide signals are commonly used in QS systems of Gram-positive bacteria [75]. Unlike the AHL-mediated QS, peptide-mediated QS depends on two-component signal transduction systems. Peptide signals cannot diffuse across the cell membrane freely and their release relies on the dedicated peptide exporters [75]. A precursor peptide is produced and then modified and processed into mature peptide. ATP-Binding Cassette transporter complex can help the release of the mature peptide to the extracellular environment [73]. The concentration of QS peptides increases with the cell density. At high cell density, the mature peptides bind to the two-component signal transduction system. Subsequently, a response regulator is phosphorylated and then binds to specific target promoters to regulate the expression of target genes.

1.3.3 Quorum sensing and biofilm formation

The role of QS systems in biofilm formation of Gram-negative bacteria has been extensively studied. QS signals are involved in the organization of *P. aeruginosa* biofilm structures [91]. Compared to the wild-type *P. aeruginosa* PAO1, double *lasI-rhlI* knockout mutant forms much thinner biofilms and the cells within biofilm are more densely packed [91]; these defects were rescued by adding the QS signal 3-oxo-C_{12}-HSL.

Burkholderia cepacia, a Gram-negative bacterium commonly found in the patients with cystic fibrosis, uses the *cep* QS system consisting of the transcriptional regulatory protein CepR and autoinducer synthase CepI [92]. Huber et al. [92] showed that the *cep* QS system plays an important

role in the maturation of *B cepacia* H111 biofilms. However, this QS system has no effect on the initial attachment of this strain [92].

In comparison, the role of QS systems in biofilm formation of Gram-positive bacteria is less understood. Petersen et al. [93] showed that exogenously added the competence stimulating peptide CSP significantly increases the production of *S. intermedius* biofilms.

1.3.4 Quorum sensing inhibitors

Since QS system is involved in the regulation of virulence factors and biofilm formation, interfering with QS system may be a promising strategy in the treatment of chronic infections. A variety of methods have been described to disrupt QS system such as interfering with the autoinducer receptors, inactivating or degrading the autoinducers, and disrupting the synthesis of QS autoinducers [78, 94, 95].

1.3.4.1 Halogenated furanones

More than 20 different brominated furanones are natural quorum sensing inhibitors (QSIs) produced by the marine red alga *Delisea pulchra* [96]. These furanones are located in the vesicles on the surface of the algea and exhibit a high level antifouling activity. They have chemical structures similar to AHLs, and thus were thought to disrupt QS completing with cognate AHL receptor proteins [97, 98].

To date, the most extensively studied natural furanone is (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone (BF1). It has been shown that natural furanone is a QS signal antagonist

and inhibit biofilm formation and swarming of *E. coli* [99]. BF1 also exhibits inhibition activity on the biofilm formation of Gram-positive bacterium *Bacillus subtilis* [100].

A number of halogenated furanones have been synthesized and studied for the inhibition of biofilm formation by several bacterial species, such as *S. aureus*, *E. coli*, *P. aeruginosa*, *S. epidermidis*, and *S. enterica* serovar Typhimurium [37, 99-104]. Hentzer et al. [105] developed a novel synthetic furanone compound, called furanone C-30, which could inhibit *P. aeruginosa* QS, QS-regulated virulence factors, biofilm development, and persistence of infecting bacteria in a pulmonary mouse model. Specifically, about 80% of the QS-regulated genes were remarkably repressed by this synthetic furanone [105]. In addition, a synthetic QSI, (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one (BF8), has been reported to sensitize *P. aeruginosa* PAO1 and *E. coli* persisters to antibiotics [37, 101]. Furthermore, this chemical was found to inhibit biofilm formation and biofilm-associated persister cells of *P. aeruginosa* PDO300 [106].

1.3.4.2 AHL analogues

Based on the chemical structure of AHLs, several strategies have been described for the development of AHL analogs including modifications of the acyl side chain and alterations of HSL ring [94, 95, 107]. Persson et al. [108] reported that a series of sulfide AHL analogues significantly inhibit the transcriptional regulator LuxR and/or LasR. Smith et al. [109, 110] designed a library of synthetic AHL analogs in which the HSL group was modified to amines and alcohols. By screening this library, one compound with the HSL ring replaced a cyclohexane ring, showed significant inhibition of the production of virulence factors and biofilm formation [107, 109].

1.3.4.3 AHL-degradation enzymes

A handful of enzymes that can degrade AHLs have been recently reported such as the AHL lactonase, the AHL acylase, and oxidoreductases [111, 112]. The first identified AHL lactonase, which could hydrolyze the lactone ring, is synthesized by the protein AiiA from *Bacillus sp.* 240B1 [113]. AHL lactonases have also been characterized in other species such as *Agrobacterium tumefacies*, *Bacillus thuringiensis*, *Bacillus anthracis*, and *P. aeruginosa* PAO1 [114-117].

Another AHL-degrading enzyme is the AHL acylase that hydrolyzes the amide bond between HSL and acyl side chain. An AHL acylase encoded by AiiD of *Ralstonia* is capable of hydrolyzing long-chain AHLs such as 3-oxo-C_8-HSL, 3-oxo-C_{10}-HSL, and 3-oxo-C_{12}-HSL [111, 117]. In *P. aeruginona* PAO1, two AHL acylases encoded by the *pvdQ* gene (PA2385) and the *quiP* gene (PA1032) have been reported recently [117, 118]. These two enzymes also have the specific degradation activities for the long-chain AHL.

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CHAPTER 2

EFFECTS OF *N*-(3-OXODODECANOYL)-*L*-HOMOSERINE LACTONE ON *Pseudomonas aeruginosa* PERSISTER CELLS

2.1 Abstract

Pseudomonas aeruginosa is a common Gram-negative pathogen that causes persistent lung infections in patients with cystic fibrosis. Here, we report that the quorum sensing signal *N*-(3-oxododecanoyl)-homoserine lactone (3-oxo-C_{12}-HSL), can sensitize *P. aeruginosa* (both the wild-type PAO1 and its mocoid mutant PDO300) persister cells to ciprofloxacin (Cip), but not gentamicin, tobramycin, carbenicillin. Also, the synergistic effects were only observed for 3-oxo-C_{12}-HSL, but not another quorum sensing signal *N*-butyryl homoserine lactone (C_4-HSL). DNA microarrays and qPCR were used to understand the effects of 3-oxo-C_{12}-HSL on gene expression of *P. aeruginosa* PAO1 persister cells.

2.2 Introduction

Bacterial persistence is a phenomenon in which a small subpopulation of slow-growing or non-growing cells, known as persister cells, is refractory to antibiotic treatment [1]. This phenomenon has been found in most bacterial species such as *E. coli* [2], *P. aeruginosa* [3], *S. aureus* [4], and *M. tuberculosis* [5]. A variety of factors has been linked in persister formation, including toxin-antitoxin modules [6], starvation [7], heterogeneous growth [8], oxidative stress [9], SOS response [10], and phosphate metabolism [11, 12].

P. aeruginosa is a common Gram-negative pathogen that causes lung infections in patients with cystic fibrosis and compromised immune systems [13]. According to the CDC National Nosocomial Infections Surveillance system from 1986-2003, *P. aeruginosa* is the fifth most commonly isolated organism responsible for 9% of hospital-acquired infections in the U.S. [14, 15]. Treatment of infectious diseases caused by *P. aeruginosa* has become more difficult due to rapid development of antibiotic-resistant strains. *P. aeruginosa* becomes multidrug resistant through different mechanisms such as overexpression of multidrug efflux pumps, genetic mutations, and horizontal transfer of drug resistance genes [16-18]. Besides these intrinsic resistance mechanisms, *P. aeruginosa* can also acquire multidrug tolerance by forming highly structured biofilms and dormant persister cells, which make the antibiotic treatment even more difficult.

P. aeruginosa PDO300 (PDO300) is an isogenic *mucA22* mutant of non-mucoid wild-type PAO1 [19]. The phenotypic difference between PDO300 and PAO1 is the overproduction of alginate in PDO300. This alginate overproduction was thought to influence the biofilm architecture and enhance the resistance of biofilm bacteria to antimicrobial agents [20].

Quorum sensing (QS) is an important cell-to-cell communication system in bacteria, which regulates the expression of target genes in response to the cell population density. QS systems play a major role in the production of virulence factors and biofilm formation. In *P. aeruginosa*, there are two acylated homoserine lactone (acyl-HSL or AHL)-mediated QS systems: the *las* system and the *rhl* system. The *las* system consists of the transcriptional regulatory protein LasR and autoinducer synthase LasI, which produces the autoinducer *N*-(3-oxododecanoyl)-homoserine lactone (3-oxo-C_{12}-HSL) [21]. This autoinducer binds to LasR and then forms LasR-autoinducer complex, which triggers a series of target genes including *toxA, lasA, lasB, aprA, xcpR,* and *xcpP* [22]. In addition, the LasR-autoinducer complex also induces the expression of *lasI*, which generates a positive feedback loop for signal production. Analogous to the *las* system, the *rhl* system is composed of the RhlR and the autoinducer synthase RhlI, which produces the signal *N*-butyryl HSL (C_4-HSL). The RhlR-autoinducer complex activates a number of genes such as *rhlI, rpoS, lasB, rhlAB,* and some genes involved in pyocyanin biosynthesis [23, 24]. Two QS systems are hierarchically correlated because the LasR protein/3-oxo-C_{12}-HSL complex could also induce *rhlR* and *rhlI* [22, 24]. It's worth noticing that 3-oxo-C_{12}-HSL competes with C_4-HSL for binding to the receptor RhlR when the concentration of 3-oxo-C_{12}-HSL is higher than that of C_4-HSL [22].

Recently, Möker N et al. [25] reported that 3-oxo-C_{12}-HSL significantly enhances the persister formation in exponential cultures of *P. aeruginosa* PAO1 and PA14 but not *E. coli* or *S. aureus*. In the present project, the effects of 3-oxo-C_{12}-HSL and C_4-HSL on *P. aeruginosa* PAO1 and PDO300 persister cells were tested. Our results show that 3-oxo-C_{12}-HSL can enhance the susceptibility of PAO1 and PDO300 persister cells to Cip. In contrast, C_4-HSL did not show any effect on the susceptibility of PAO1 and PDO300 persister cells to Cip.

2.3 Materials and methods

2.3.1 Bacterial strain and growth media

The wild-type *P. aeruginosa* PAO1 strain, *lasR* mutant, and *rhlR* mutant were purchased from the Manoil library at University of Washington [26]. PDO300 strain was obtained from Dr. Parseck at University of Washington. These strains and PDO300 were grown in the Luria-Bertani (LB) medium containing 10 g/L NaCl, 10 g/L tryptone and 5 g/L yeast extract, and cultured with shaking at 200 rpm at 37 ℃ for 18 h.

2.3.2 Chemicals

The QS signals of 3-oxo-C_{12}-HSL and C_4-HSL were ordered from Sigma-Aldrich Co. (St. Louis, MO, USA) and Cayman Chemical Co. (Ann Arbor, MI, USA), respectively. These two chemicals were dissolved in dimethyl sulfoxide (DMSO) (MP Biomedicals, LLC, Santa Ana, CA, USA) to the concentration of 5 mg/ml.

2.3.3 Persister isolation

Overnight culture of PAO1 or PDO300 persister cells were isolated by killing the normal cells with 200 μg/mL Cip for 3.5 h [27, 28]. After the treatment, the surviving persister cells were washed three times with 0.85% NaCl solution and then resuspended in 0.85% NaCl solution. The isolated persister cells were then treated with different AHLs shown as below. The cells after each treatment were further challenged with 200 μg/mL Cip for 3.5 h. Then the samples were washed three times with 0.85% NaCl solution and plated on LB agar plates (1.5% agar) after a series of lot dilution to determine the colony forming units (CFU) [29].

2.3.4 Effects of 3-oxo-C_{12}-HSL on isolated PAO1 or PDO300 persister cells

Persister cells were isolated from overnight cultures of PAO1 or PDO300 as described above. The isolated persister cells were diluted by 50 times with 0.85% NaCl solution and then treated with different concentrations of 3-oxo-C_{12}-HSL. The amount of DMSO was adjusted to be the same in all samples to eliminate any solvent effect. All samples were incubated at 37 ℃ for 2 h with shaking at 200 rpm. After the 2 h treatment, 1 mL of each sample was withdrawn and washed three times with 0.85% NaCl solution. Then cells were diluted and plated on LB agar plates. Meanwhile, the remaining portion of each sample was further challenged with 200 μg/mL Cip for 3.5 h. Each sample was then tested to determine the CFU as described above. This experiment was conducted with two biological replicates. The effects on QS mutants were tested using the same method.

2.3.5 Effects of 3-oxo-C_{12}-HSL on antibiotic susceptibility of isolated PAO1 or PDO300 persister cells

PAO1 or PDO300 persister cells were isolated by killing the normal cells with 200 μg/mL Cip for 3.5 h and then diluted by 50 times with 0.85% NaCl solution. PAO1 persisters were treated with different concentrations of 3-oxo-C_{12}-HSL. After 2 h of incubation, 1 mL of each sample was washed three times with 0.85% NaCl solution and plated to count CFU. The remaining portion of each sample was further challenged with 5 μg/mL ofloxacin (Ofl), 200 μg/mL tobramycin (Tob), 500 μg/mL carbenicillin (Car), 200 μg/mL gentamicin (Gen), or 200 μg/mL tetracycline (Tet) for another 3.5 h. Then each sample was tested to determine the CFU as described above. This experiment was conducted with two biological replicates.

2.3.6 Effects of C_4-HSL on isolated PAO1 or PDO300 persister cells

This experiment was conducted by following the same protocol as described for the test of 3-oxo-C_{12}-HSL. The QS signal C_4-HSL was tested at 0, 5, 10, 15, and 30 μg/mL. This experiment was conducted with two biological replicates. The effects on QS mutants were tested using the same method.

2.3.7 RNA extraction and microarray analysis

Overnight cultures of PAO1 were grown with agitation (200 rpm) in LB medium at 37 ℃ for 18 h. Persister cells were isolated from overnight cultures as described above. The isolated persisters were diluted by 5 times with 0.85% NaCl solution and then treated with 100 μg/ml 3-oxo-C_{12}-HSL at 37 ℃ for 1 h. After 1 h treatment, cells were pelleted by centrifugation at 2 ℃ for 2 min at 10,000 rpm and the supernatant was discarded. The cell pellets were frozen instantly in a dry ice-ethanol bath and then stored at -80 ℃.

Total RNA from the persister cells was extracted using RNeasy Mini Kit (Qiagen, Austin, TX, USA) with on-column DNase I treatment (RNase-Free DNase Set, Qiagen) by following the manufacturer's instructions. The integrity of RNA samples was determined by gel electrophoresis. RNA samples were stored at -80 ℃ until sent to the DNA Microarray Facility at SUNY Upstate Medical University (Syracuse, NY, USA) for hybridization on GeneChip *P. aeruginosa* Genome Array (*P. aeruginosa* Genome Array, Affymetrix, Santa Clara, CA, USA) which contained probes for all the 5570 predicted PAO1 genes. This experiment was conducted with two biological replicates.

2.3.8 Quantitative PCR analysis

The DNA microarray results were validated by quantitative PCR (qPCR) analysis. The *rpoD* gene was used as the house-keeping gene as described previously [30]. Total RNA was extracted as mentioned above and then reverse transcribed into cDNA by using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). All reactions were conducted in a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the following steps: 25 ℃ for 5 min, 42 ℃ for 30 min, 85 ℃ for 5 min, and holding at 4 ℃. The concentration of cDNA was determined using the Evolution201 UV-Visible spectrophotometer (Thermo Scientific, Waltham, MA, USA). Then cDNA was stored at -20 ℃. Based on genomic sequence of PAO1 listed in NCBI, qPCR primers for genes of interest were designed using OligoAnalyzer 3.1 software (Integrated DNA Technologies, Coralville, IA, USA). Primers were designed to yield PCR products of 225-275 bp with melting temperature around 60 ℃. The selected genes and primer sequences were listed in Table 2-1. To verify primer specificity, regular PCR reactions using Taq polymerase were run at 94 ℃ for 2 min followed by 30 cycles of 94 ℃ for 15 s, 60 ℃ for 15 s, and 68 ℃ for 30 s; and one final extension step at 68 ℃ for 5 min. iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) was used to detect any double-stranded DNA. All reactions were conducted in white 96-well plates. A negative control (cDNA samples without forward and reverse primers) was also included in all qPCR runs. The qPCR reactions were run using a Mastercycler ep *realplex* (Eppendorf, Hauppauge, NY, USA) with the following thermo cycling program: initial DNA denaturation at 95 ℃ for 2 min, followed by 40 cycles of denaturation at 95 ℃ for 15 sec, annealing at 60 ℃ (or 61 ℃) for 1 min, and extension at 50 ℃ for 30 sec. qPCR was performed with three biological replicates. Data analysis was performed using the LinRegPCR program.

Table 2-1. Genes and specific primers used for qPCR analysis.

Gene	Primer sequence (5'-3')	Product size (bp)
rpoD	(+)CGGTCAACCTGAAGGACGAT	253
	(-)ACCAGCTTGATCGGCATGAA	
rsaL	(+)AGCAATGGCTTCACACGAGA	239
	(-)TGATCTTGCCTCTCAGGTCG	
mvaT	(+)TCTACTTACATGCCCGCCAC	229
	(-)CGGATCGAGCAGGGAAATCA	
folD	(+)CCTCTCAGGTCTATGTGGCG	251
	(-)GCCGATGTTGTAGGGATGGA	
phoU	(+)CAGCGGCTCCAGAAGGATTC	226
	(-)GCGCTCCATCTGGTTGATCT	
hmgA	(+)CACCTGGCTGTACCGCATC	256
	(-)GTCGTAGAAGCAACGCTCCA	
pilH	(+)GCATGGTCACCAGGTACTCAA	268
	(-)CAGCAGGGTCTCTTCGTCC	

2.3.9 Statistical analysis

Statistical analysis was conducted using SAS 9.1.3 Windows version (SAS, Cary, NC, USA) to compare the difference between the control and treatment samples. The notes of *, **, and *** indicate that the *p*-value is <0.05, <0.01, and <0.001, respectively.

2.4 Results

2.4.1 Effects of 3-oxo-C_{12}-HSL on PDO300 persister cells

First, we tested if 3-oxo-C_{12}-HSL can affect the viability of PDO300 persister cells and their susceptibility to Cip. As shown in Figure 2-1, 3-oxo-C_{12}-HSL did not affect the viability of PDO300 persister cells (One-way ANOVA, p=0.6879). Interestingly, 3-oxo-C_{12}-HSL increased the susceptibility of isolated PDO300 persister cells to Cip dose dependently (5-30 μg/mL). For instance, 10 μg/mL and 30 μg/mL 3-oxo-C_{12}-HSL sensitized 53% ±32% and 91.1% ±12% of PDO300 persister cells to Cip, respectively.

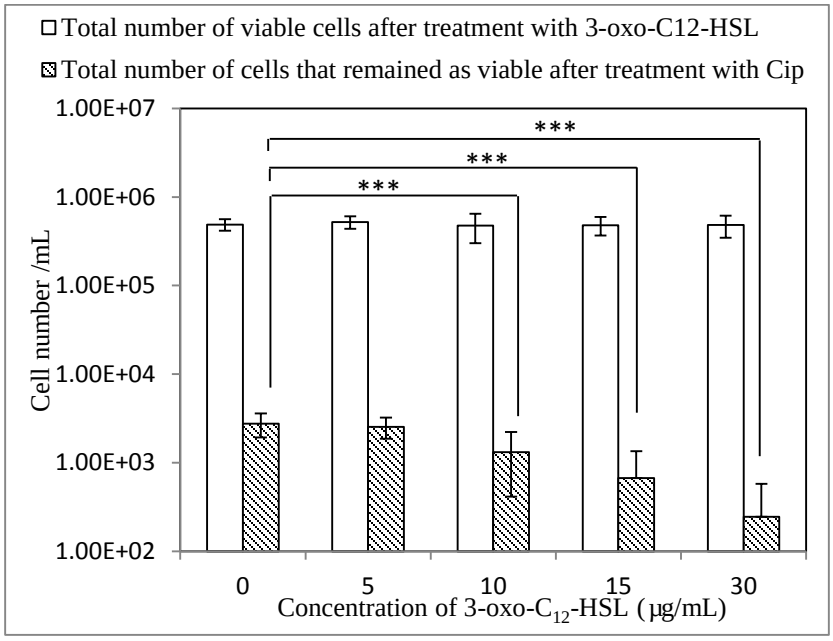

Figure 2-1. Effects of 3-oxo-C_{12}-HSL on PDO300 persister cells. Open bars represent the total number of viable cells after treatment with 3-oxo-C_{12}-HSL. Shaded bars represent the number of cells that remained viable after treatment with 200 μg/mL Cip. The persister cells were treated with 3-oxo-C_{12}-HSL at different concentrations for 2 h. A portion of each sample was further

challenged with 200 μg/mL Cip for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

To determine if 3-oxo-C_{12}-HSL can also sensitize PDO300 persister cells to other antibiotics, we repeated the above experiment by replacing Cip with antibiotics that target protein synthesis (Tet, Tob, and Gen) and cell wall synthesis (Car). As shown in Figures 2-2 to 2-6, we found that 3-oxo-C_{12}-HSL didn't affect the susceptibility of PDO300 persister cells to these four antibiotics. This suggests that the synergy may be specific to a small group of antibiotics.

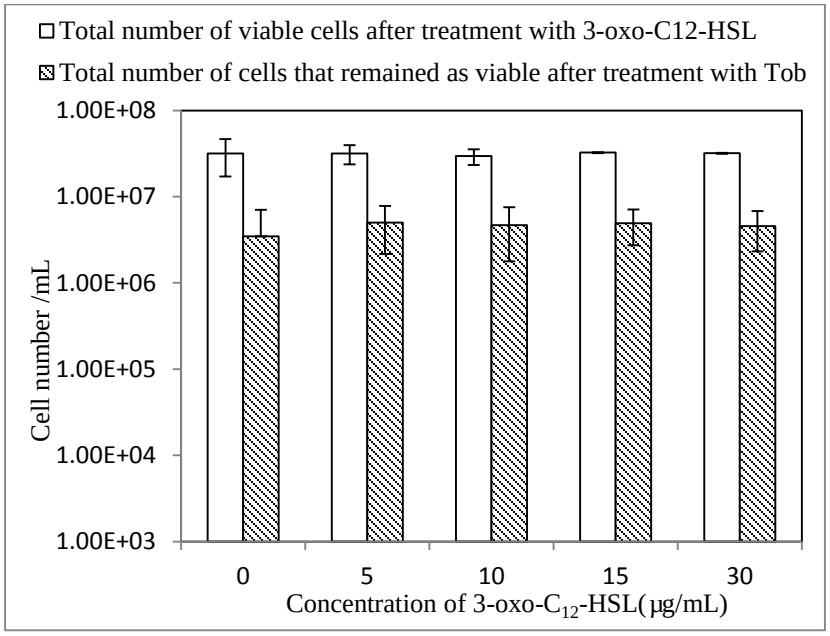

Figure 2-2. Effects of 3-oxo-C_{12}-HSL on PDO300 persister cells by using 25 μg/mL Tob. The persister cells were treated with 3-oxo-C_{12}-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 25 μg/mL Tob for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

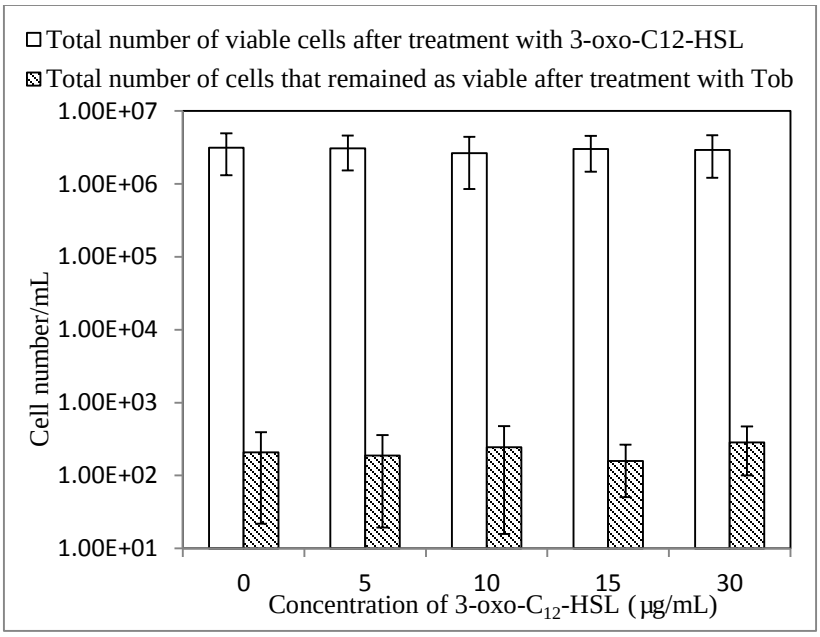

Figure 2-3. Effects of 3-oxo-C_{12}-HSL on PDO300 persister cells by using 200 μg/mL Tob. The persister cells were treated with 3-oxo-C_{12}-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 200 μg/mL Tob for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

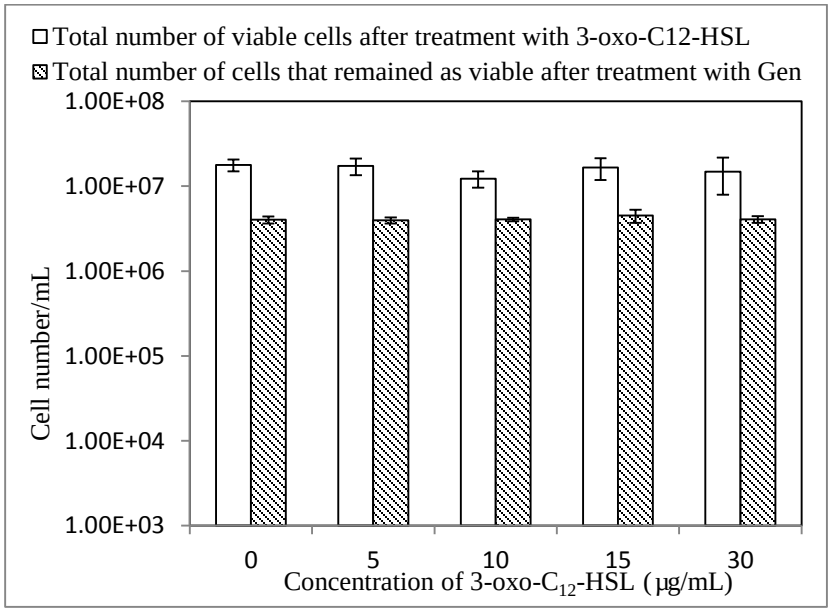

Figure 2-4. Effects of 3-oxo-C_{12}-HSL on PDO300 persister cells by using 200 μg/mL Gen. The persister cells were treated with 3-oxo-C_{12}-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 200 μg/mL Gen for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

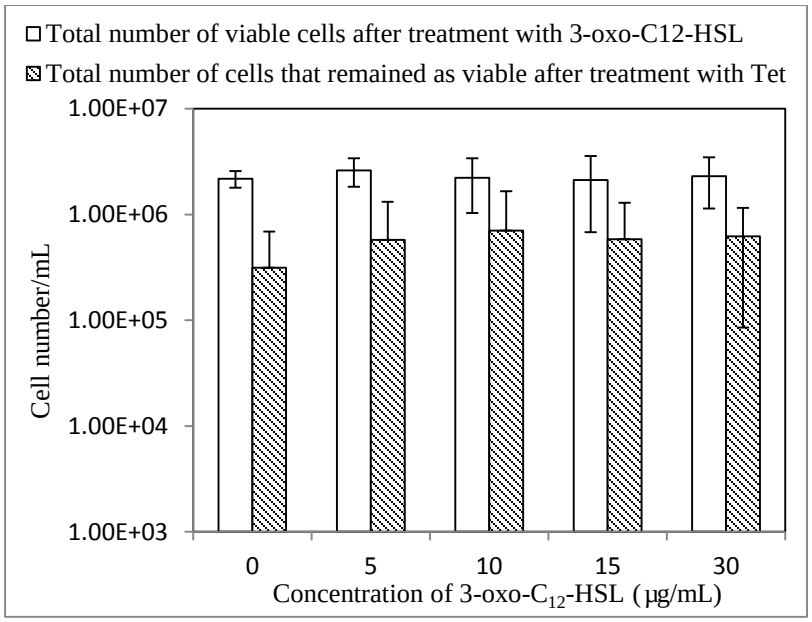

Figure 2-5. Effects of 3-oxo-C_{12}-HSL on PDO300 persister cells by using 25 μg/mL Tet. The persister cells were treated with 3-oxo-C_{12}-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 200 μg/mL Tet for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

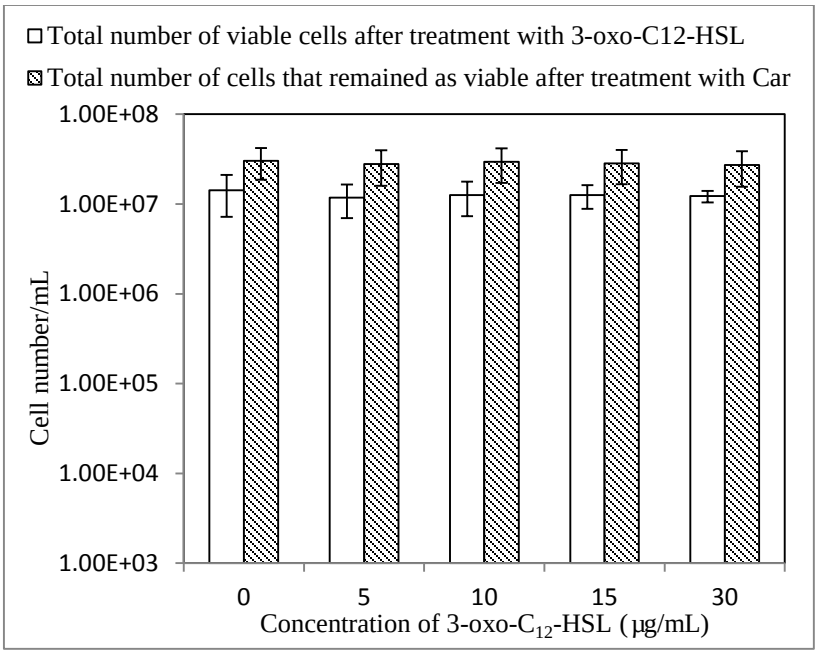

Figure 2-6. Effects of 3-oxo-C_{12}-HSL on PDO300 persister cells by using 500 μg/mL Car. The persister cells were treated with 3-oxo-C_{12}-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 500 μg/mL Car for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

2.4.2 Effects of C_4-HSL on PDO300 persister cells

Unlike 3-oxo-C_{12}-HSL, another QS signal C_4-HSL did not exhibit the same effect. As shown in Figure 2-7, C_4-HSL did not significantly change the viability of PDO300 or its susceptibility to Cip.

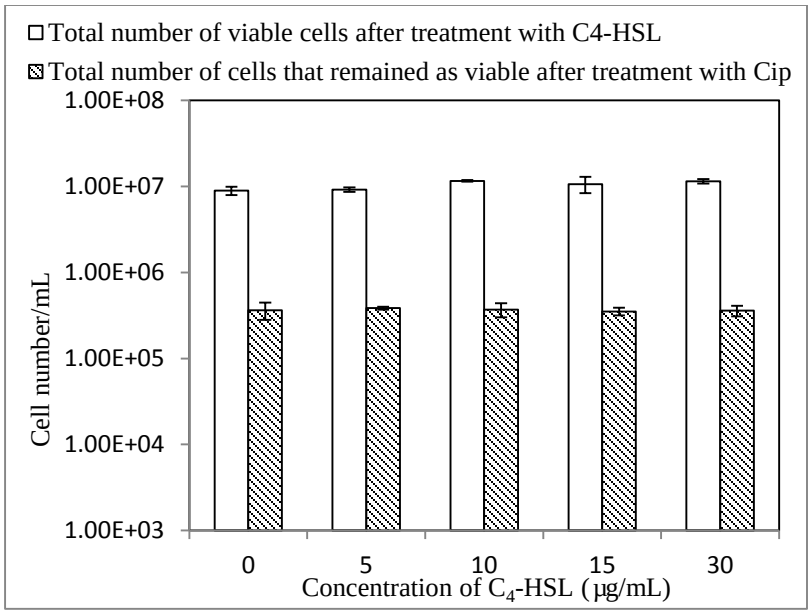

Figure 2-7. Effects of C_4-HSL on PDO300 persister cells. The persister cells were treated with C_4-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 200 μg/mL Cip for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

To corroborate the results, we also tested the mixture of 3-oxo-C_{12}-HSL and C_4-HSL at different ratios. As shown in Figure 2-8, when the concentration of 3-oxo-C_{12}-HSL in the mixture was 15 μg/mL, 66% ±23.9% of isolated PDO300 persister cells were sensitized to Cip (One-way ANOVA, p<0.0001). At the higher concentration of 3-oxo-C_{12}-HSL, e.g., 35 μg/mL, 98.7% ± 1.5% of PDO300 persister cells were sensitized to Cip (One-way ANOVA, p<0.0001).

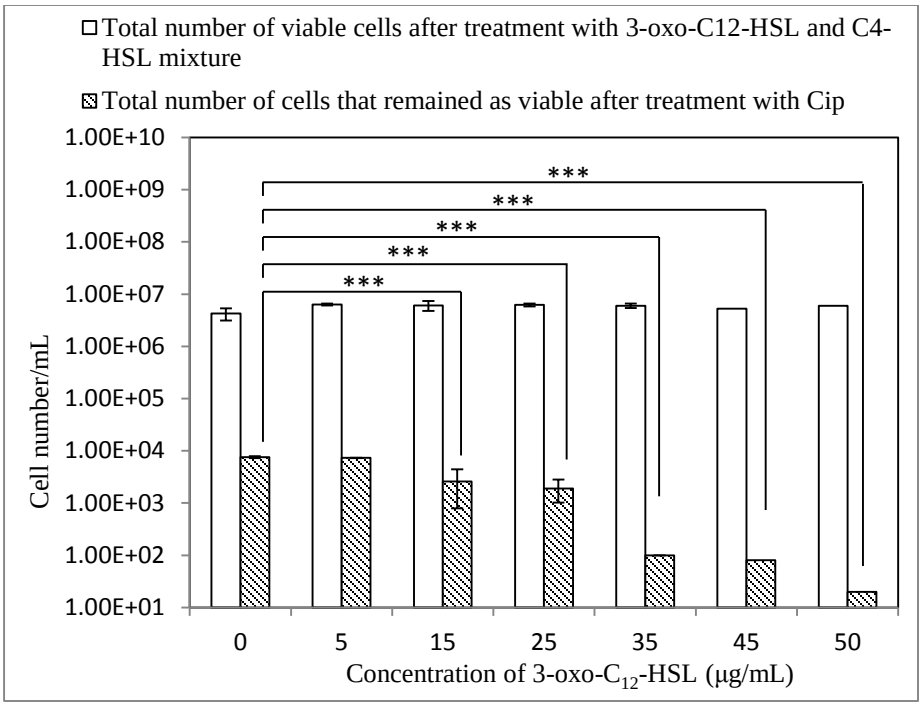

Figure 2-8. Effects of 3-oxo-C_{12}-HSL and C_4-HSL on PDO300 persister cells. The persister cells were treated with the mixture of 3-oxo-C_{12}-HSL and C_4-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 200 μg/mL Cip for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

2.4.3 Effects of 3-oxo-C_{12}-HSL on PAO1 persister cells

Based on the above results, we were motivated to test if 3-oxo-C_{12}-HSL has the similar effect on *P. aeruginosa* PAO1 persister cells. As shown in Figure 2-9, 3-oxo-C_{12}-HSL increased the susceptibility of PAO1 persister cells to Cip in a dose-dependent manner (5-30 μg/mL). For instance, 5 μg/mL 3-oxo-C_{12}-HSL did not affect the viability of persisters but sensitized 72% ± 13% of persister cells to Cip (One-way ANOVA, $p < 0.001$). When the concentration of 3-oxo-

C_{12}-HSL reached 30 μg/mL, 92% ± 8% of PAO1 persisters became susceptible to the treatment of Cip (One-way ANOVA, $p < 0.001$), although the viability of PAO1 persister cells were not significantly affected by 3-oxo-C_{12}-HSL alone.

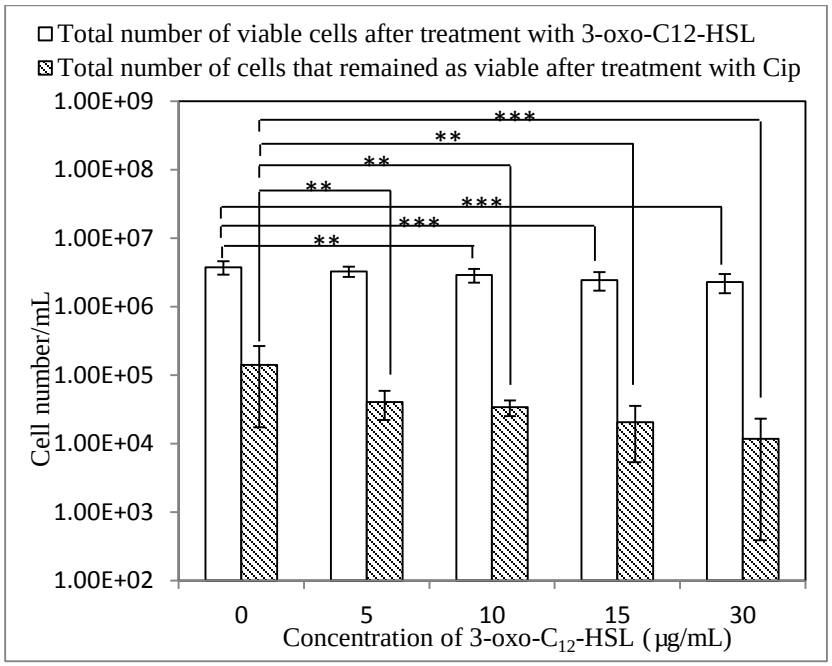

Figure 2-9. Effects of 3-oxo-C_{12}-HSL on *P. aeruginosa* PAO1 persister cells. The persister cells were treated with 3-oxo-C_{12}-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 200 μg/mL Cip for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

To test if 3-oxo-C_{12}-HSL can potentiate PAO1 persister cells to other antibiotics, we tested three types of antibiotics including quinolone (Ofl), aminoglycoside (Tob), and Car. As shown from Figure 2-10 to Figure 2-12, 3-oxo-C_{12}-HSL did not potentiate these hree antibiotics based on the statistical analysis.

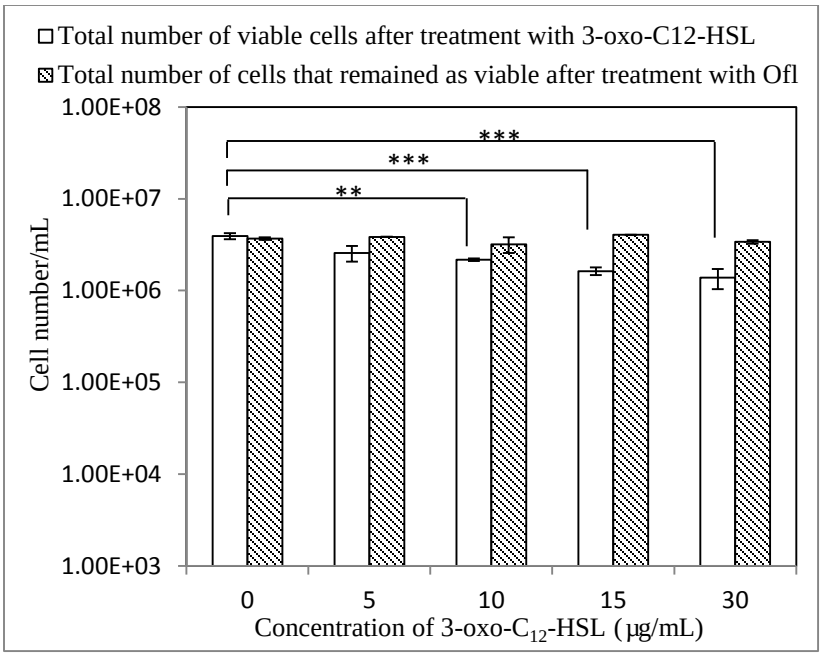

Figure 2-10. Effects of 3-oxo-C_{12}-HSL on *P. aeruginosa* PAO1 persister cells by using 5 μg/ml Ofl. The persister cells were treated with 3-oxo-C_{12}-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 5 μg/ml Ofl for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

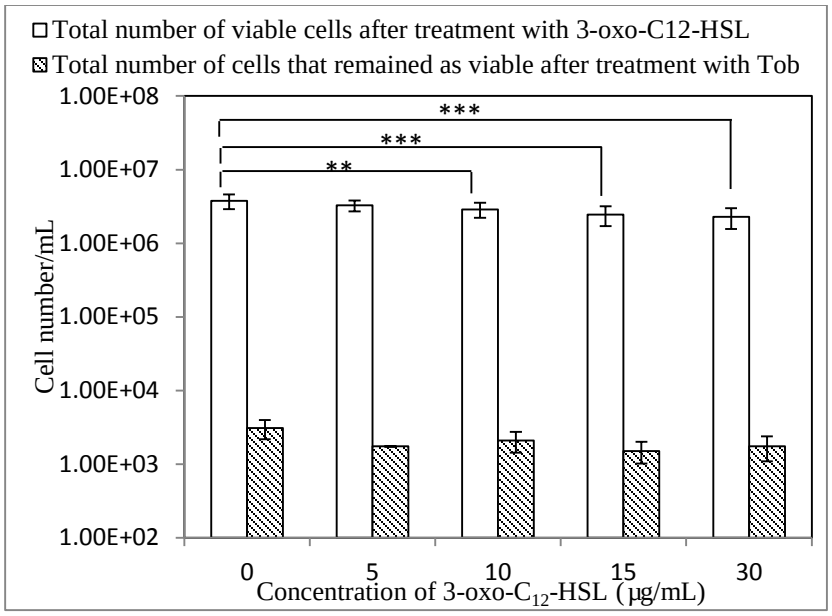

Figure 2-11. Effects of 3-oxo-C_{12}-HSL on *P. aeruginosa* PAO1 persister cells by using 200 µg/ml Tob. The persister cells were treated with 3-oxo-C_{12}-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 200 µg/ml Tob for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

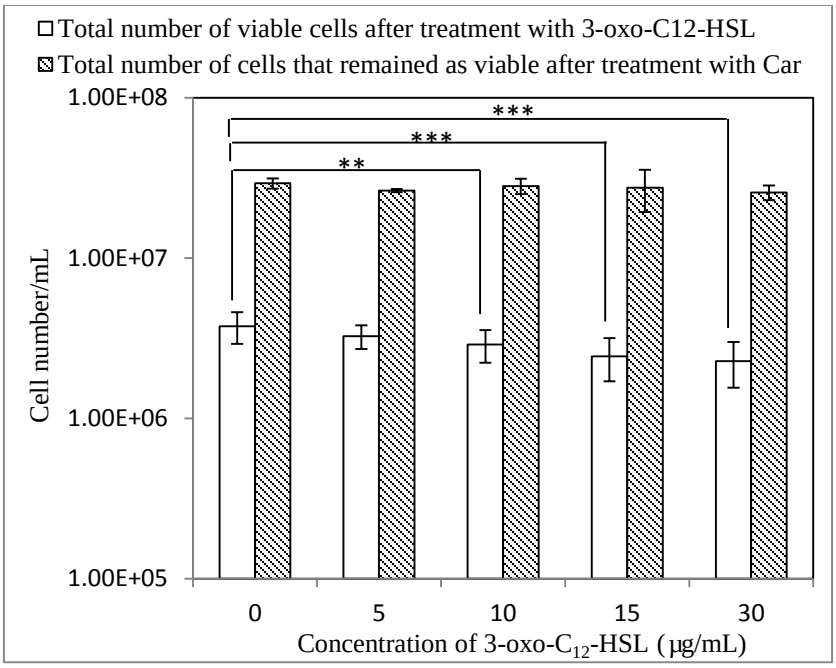

Figure 2-12. Effects of 3-oxo-C_{12}-HSL on *P. aeruginosa* PAO1 persister cells by using 500 µg/ml Car. The persister cells were treated with 3-oxo-C_{12}-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 500 µg/ml Car for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

2.4.4 Effects of C_4-HSL on PAO1 persister cells

Unlike 3-oxo-C_{12}-HSL, C_4-HSL did not sensitize PAO1 persister cells to Cip (Figure 2-13). As described above, this QS signal also did not show any effect on the susceptibility of PDO300 persister cells to Cip.

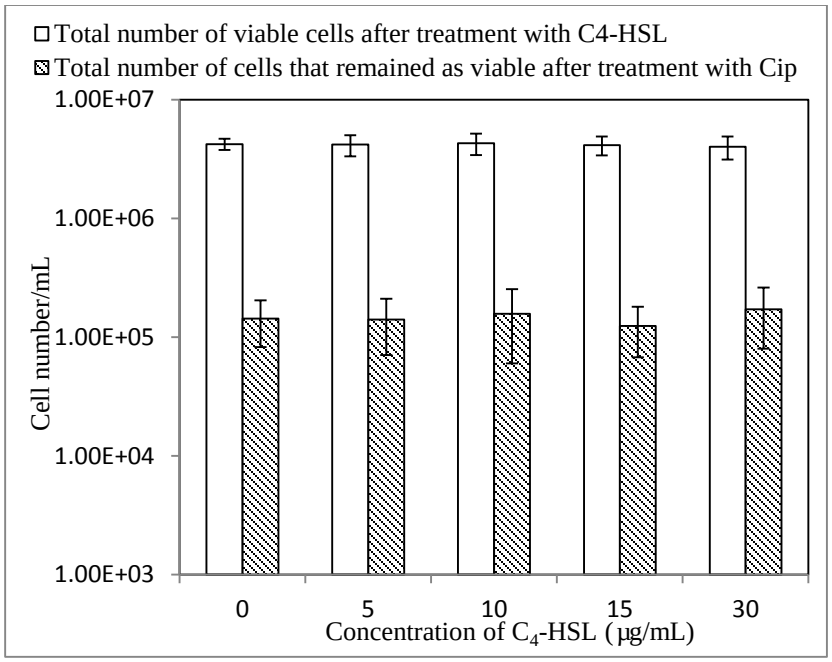

Figure 2-13. Effects of C_4-HSL on *P. aeruginosa* PAO1 persister cells. The persister cells were treated with C_4-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 200 μg/ml Cip for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

2.4.5 Effects of 3-oxo-C_{12}-HSL on persister cells of PAO1 *lasR* and *rhlR* mutants

Since LasR and RhlR are receptors of 3-oxo-C_{12}-HSL and C_4-HSL, respectively, we also evaluated the effects of 3-oxo-C_{12}-HSL on persister cells of PAO1 *lasR* and *rhlR* mutants. As shown in Figure 2-14, 3-oxo-C_{12}-HSL at 30 μg/mL can also sensitize 39.0% ± 46.3% of PAO1 *lasR* mutant persisters to Cip. Similarly, 52.1% ± 10.1% of PAO1 *rhlR* mutant persisters became susceptible to the treatment of Cip (Figure 2-15). However, the synergistic effect on persisters of these two mutants was less than that on wild-type PAO1. These results suggest that the synergistic effect may be through the regulatory proteins LasR and RhlR.

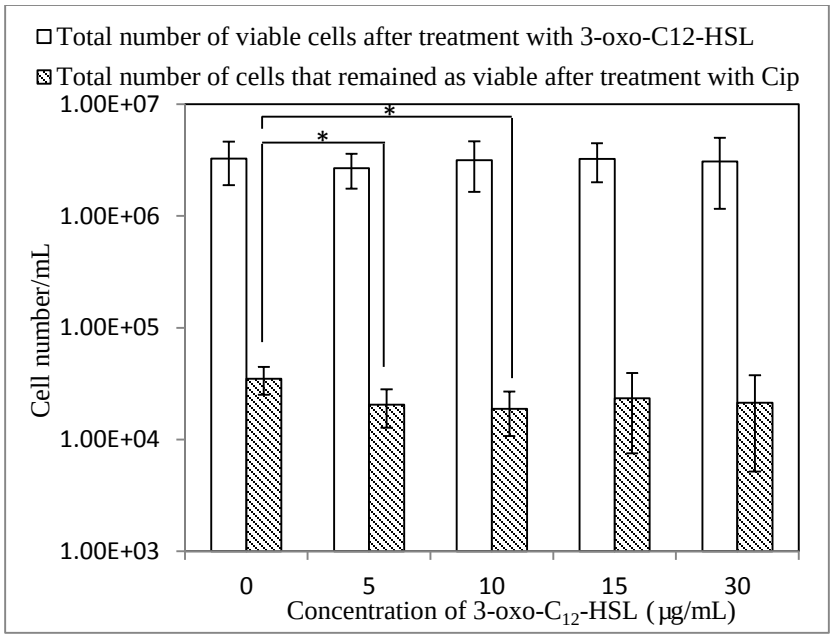

Figure 2-14. Effects of 3-oxo-C_{12}-HSL on persister cells of *P. aeruginosa* PAO1 *lasR* mutant. The persister cells of *lasR* mutant were isolated from an 18 h overnight culture and treated with 3-oxo-C_{12}-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 200 µg/ml Cip for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

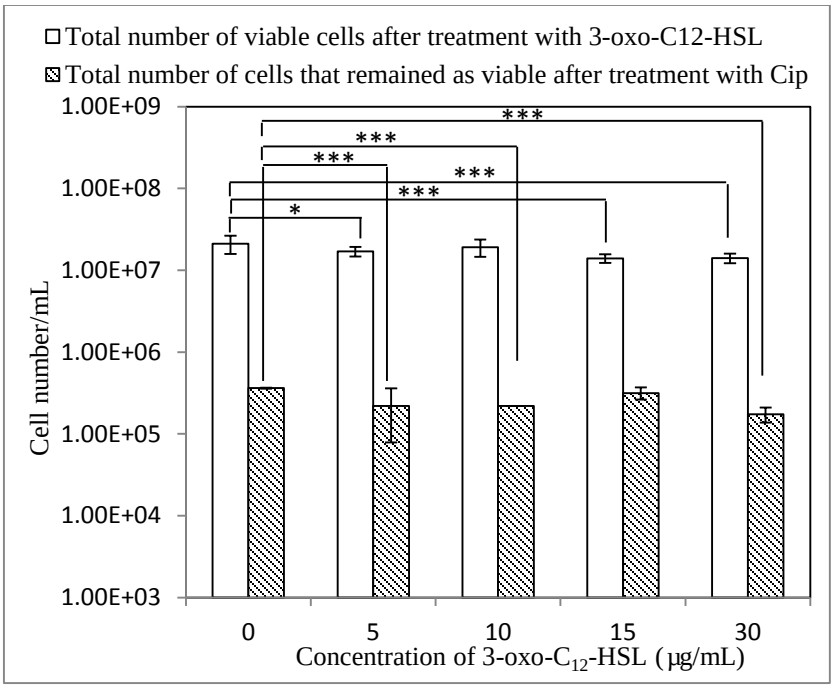

Figure 2-15. Effects of 3-oxo-C_{12}-HSL on persister cells of *P. aeruginosa* PAO1 *rhlR* mutant. The persister cells of *rhlR* mutant were isolated from an 18 h overnight culture and treated with 3-oxo-C_{12}-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 200 μg/ml Cip for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

2.4.6 Effects of C_4-HSL on persister cells of PAO1 *lasR* and *rhlR* mutants

As shown in Figure 2-16 and Figure 2-17, C_4-HSL also showed no effect on the susceptibility of persister cells of the *lasR* and *rhlR* mutants to Cip.

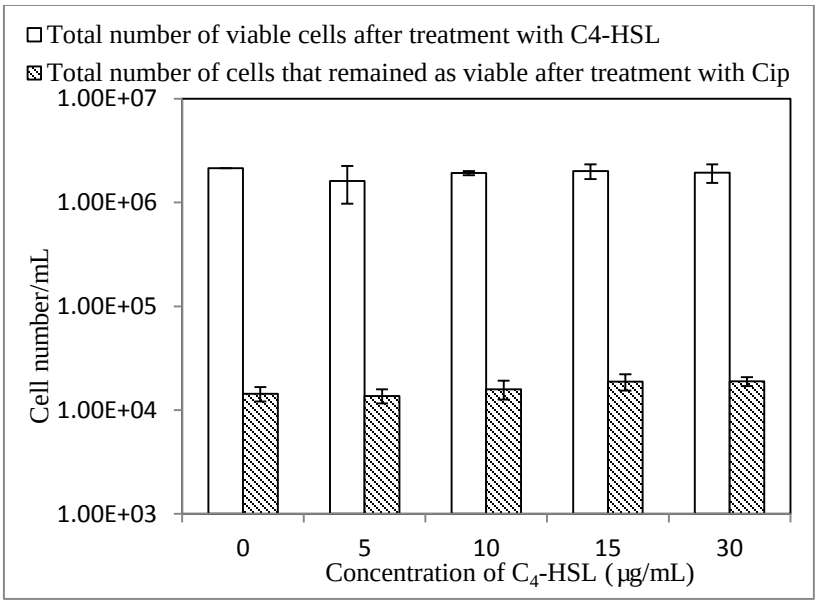

Figure 2-16. Effects of C_4-HSL on persister cells of *P. aeruginosa* PAO1 *lasR* mutant. The persister cells of *lasR* mutant were isolated from an 18 h overnight culture and treated with C_4-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 200 µg/ml Cip for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

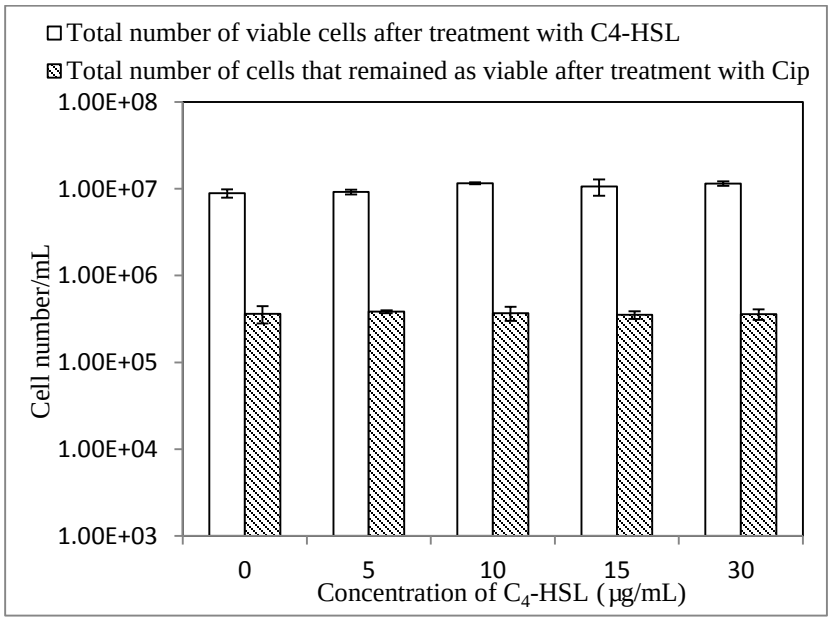

Figure 2-17. Effects of C_4-HSL on persister cells of *P. aeruginosa* PAO1 *rhlR* mutant. The persister cells of *rhlR* mutant were isolated from an 18 h overnight culture and treated with C_4-HSL at different concentrations for 2 h. A portion of each sample was further challenged with 200 μg/ml Cip for 3.5 h to determine the number of viable cells. The number of viable cells of untreated controls were normalized to 100%.

2.4.7 DNA Microarray Analysis

As mentioned above, 3-oxo-C_{12}-HSL only showed the synergistic effect with the antibiotic Cip in killing *P. aeruginosa* PAO1 persister cells, but not the other three antibiotics tested such as Ofl, Tob, and Car. In order to obtain insights into how 3-oxo-C_{12}-HSL rendered PAO1 persister cells sensitive to Cip, we used the DNA microarray to understand the effects of 3-oxo-C_{12}-HSL on gene expression in PAO1 persister cells. *P. aeruginosa* microarrays were employed to compare gene expression in persister cells without or with 3-oxo-C_{12}-HSL. These microarrays include 5570 predicted PAO1 genes. The transcriptome analysis of PAO1 persister cells treated with and without 100 μg/ml 3-oxo-C_{12}-HSL for 1 h was performed in duplicate.

Overall, 144 genes showed a gene expression ratio higher than 1.5 fold in response to 3-oxo-C_{12}-HSL. Among these 144 genes, 59 genes were upregulated and 85 genes were downregulated by the treatment of 3-oxo-C_{12}-HSL. Approximately, 46% of the 144 genes encode hypothetical proteins (Figure 2-18). Some representative induced and repressed genes is shown in Table 2-2. Importantly, the genes *parC* (PA4964) and *parE* (PA4967), which encode the subunit A and B of the topoisomerase IV (one target of Cip) [31], were upregulated in our microarray data.

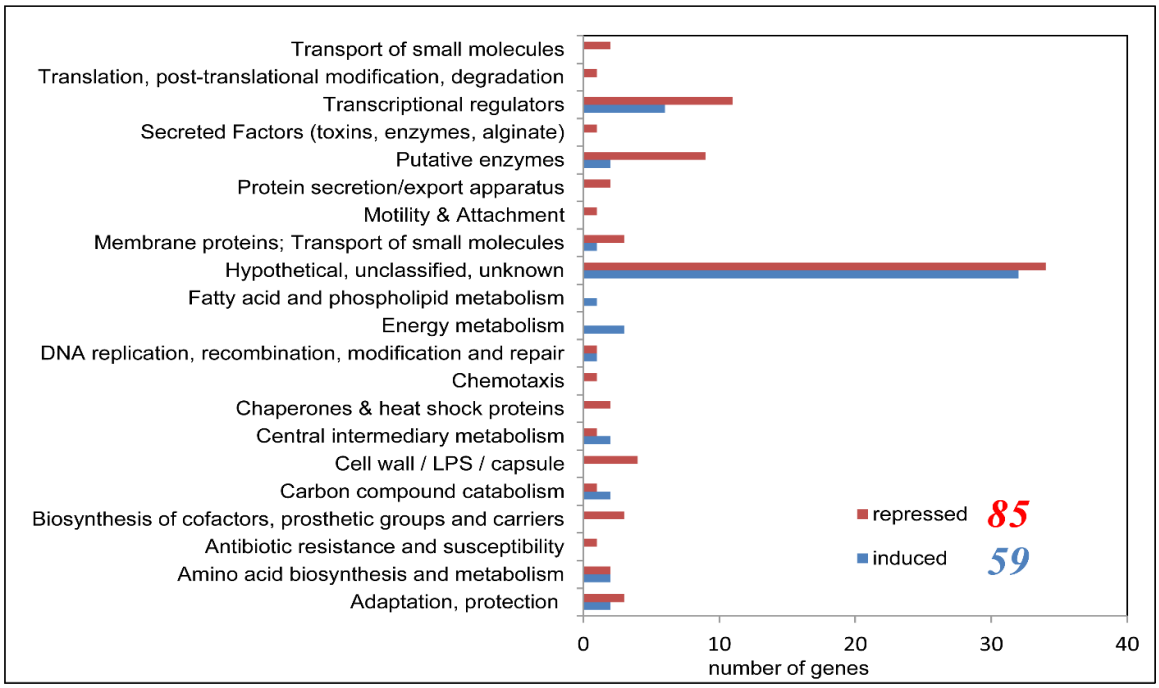

Figure 2-18. Transcriptome profile analysis of *P. aeruginosa* PAO1 persister cells treated with 3-oxo-C_{12}-HSL. The isolated persisters were treated with and without 100 μg/ml 3-oxo-C_{12}-HSL for 1 h.

To confirm the DNA microarray results, we performed the real-time PCR analysis for seven genes including one housekeeping gene (*rpoD*), 3 induced genes (*rsaL*, *mvaT*, and *folD*), and 3 repressed genes (*phoU*, *pilH*, and *hmgA*). As shown in Figure 2-19, the qPCR results of these seven genes are consistent with the DNA microarray data. For example, the *rsaL* gene, which encodes a repressor of *lasI* gene and virulence genes encoding pyocyanin and hydrogen cyanide (*phz1* and *phz2* operons, *phzM*, *phzS*, and *hcnABC*) [32, 33], was induced by about 4 times as shown in our microarray data. Pyocyanin is a toxin produced by *P. aeruginosa* that affects multiple cellular processes such as cell respiration, ciliary function, and cell growth [34]. Thus, it

plays important roles in the pathogenesis mediated by *P. aeruginosa* [34]. The induction of *rsaL* may reduce the production of pyocyanin and hydrogen cyanide and thus repress the pathogenicity of PAO1. Another induced gene *mvaT* is thought to be a global regulator of virulence genes (pyocyanin and elastase) and the *lecA* gene (coding for PA-IL lectin) because inactivation of *mvaT* resulted in increased *lecA* expression and pyocyanin production [35]. The induction of *mvaT* gene may decrease pyocyanin production and result in reduction of PAO1 pathogenicity. For the repressed gene *phoU*, one recent study showed that this gene is involved in the persistence of *E. coli* [12]. Inactivation of PhoU resulted in the high metabolic activity in *E. coli* and thus reduced persister formation [12].

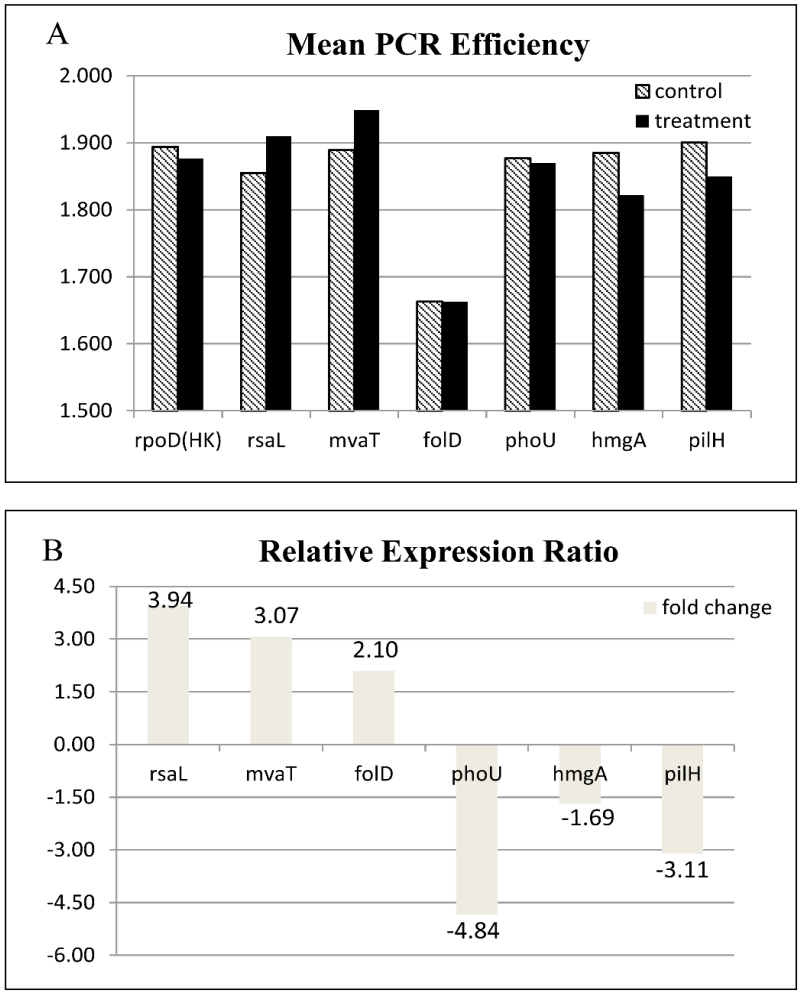

Figure 2-19. qPCR validation of microarray results for selected genes. (A) Shaded and closed bars represent the mean PCR efficiency of seven genes (*rpoD*, *rsaL*, *mvaT*, *folD*, *phoU*, *hmgA*, and *pilH*) in the control and 3-oxo-C_{12}-HSL treated samples, respectively. The *rpoD* gene is the housekeeping gene used in qPCR. (B) Grey bars represent the fold change of six genes including *rsaL*, *mvaT*, *folD*, *phoU*, *hmgA*, and *pilH* shown in qPCR result.

2.5 Discussion

In this study, we found that 3-oxo-C_{12}-HSL can increase the susceptibility of *P. aeruginosa* PDO300 persister cells to Cip. Statistical analysis showed that 3-oxo-C_{12}-HSL didn't affect the

viability of PDO300 persister cells at the range of concentrations tested from 5 to 30 μg/ml. It is interesting that no synergy was observed between 3-oxo-C_{12}-HSL and other classes of antibiotics including Tet, Tob, Gen, and Car. Tet, Tob, Gen target bacterial protein synthesis while Car targets bacterial cell wall synthesis [36, 37]. In addition, our results showed that 3-oxo-C_{12}-HSL can also render *P. aeruginosa* PAO1 persisters susceptible to the antibiotic Cip. However, such synergy was not found between 3-oxo-C_{12}-HSL and the other three antibiotics tested with targets of DNA synthesis (Ofl), protein synthesis (Tob), and cell wall synthesis (Car). We hypothesize that 3-oxo-C_{12}-HSL may interact with Cip or its cellular target; and thus, sensitize PDO300 persister cells to Cip. Further studies of such interactions are helpful for understanding the mechanism.

Unlike 3-oxo-C_{12}-HSL, C_4-HSL did not exhibit any synergistic effect with Cip in killing PDO300 persister cells. This phenomenon was observed in PAO1 persister cells as well. Two QS signals, 3-oxo-C_{12}-HSL and C_4-HSL, have the same homoserine lactone ring but differ in the number of carbon atoms in the side-chain. This may be responsible for the difference in interaction with Cip and consequently their synergistic effects in persister control with Cip.

Recently, Pan et al. [27] reported that (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-25(*H*)-one (BF8), as a synthetic QS inhibitor, can restore the antibiotic susceptibility of *P. aeruginosa* PAO1 persister cell.

In *P. aeruginosa*, RsaL protein encoded by the *rsaL* gene was well characterized as a repressor of *lasI* gene encoding the 3-oxo-C_{12}-HSL signal synthase [32, 33]. In addition, RsaL can bind simultaneously with LasR to the *rsaL-lasI* bidirectional promoter thereby to the LasR-dependent activation of both genes. Besides, RsaL also has direct or indirect controls of some genes via

auxiliary regulators [32]. Therefore, as the global regulatory factor, the expression of *rsaL* is essential to modulate of the homeostasis of 3-oxo-C_{12}-HSL and QS circuits involved metabolic status change [38]. The persister specific induction of *rsaL* upon 3-oxo-C_{12}-HSL, as observed in our study, suggests that excess 3-oxo-C_{12}-HSL may perturb the *rsal-lasI*-involved QS network and impact the transition between planktonic and sessile status to sensitize persister cells.

Of interest, the *phoU* gene was repressed three times by 3-oxo-C_{12}-HSL in PAO1 persister cells as shown from our microarray analysis. The *phoU* gene encodes the phosphate uptake regulatory protein PhoU; it is expressed together with other four genes for the ABC-type phosphate-specific transport (PstSABC) within the *pstSCAB–phoU* operon [39, 40]. The Pst-PhoU complex was known to play roles in phosphate sensing and eventual modulate QS network through MvfR and PQS [41]. In a previous study by Li et al. [12], the *phoU* gene has been shown to control persister formation and multidrug tolerance in *E. coli*. Compared to the wild-type strain *E. coli* K-12 W3110, *phoU* mutant becomes more susceptible to different antibiotics, such as gentamicin and norfloxacin, and stresses including starvation, heat, and acid pH [12]. *P. aeruginosa* has a *phoU* homolog in its genome [42, 43]. Therefore, inhibition of *phoU* by 3-oxo-C_{12}-HSL in PAO1 persister cells may initiate the *phoU* regulon through phoR/B two component system and ultimately alter metabolism like in *E. coli* [44], or QS signaling and switch between sessile/virulent life cycle [40, 45].

To date, only a few persistence genes have been described in *P. aeruginosa* including *spoT*, *relA*, *dksA*, *rpoS*, *dinG*, *spuC*, *pilH*, and PA5002 [46]. Earlier studies have shown that PA5002 is part of a larger gene cluster that span PA5012-PA4995 of PAO1 genome, which is associated with the production of *P. aeruginosa* core oligosaccharide biosynthesis genes [47]. The gene adjacent to

PA5002, was characterized as a Mig-14-like protein required for the formation of antimicrobial peptide-tolerant subpopulations [48]. In a recent study by Liebens et al. [49], the gene locus PA5002 was identified as a de-*N*-acetylase (*dnpA*) and was involved in the fluoroquinolone tolerance in *P. aeruginosa*. This was evidenced by the decrease of fluoroquinolone tolerance in PA5002 mutant (both in planktonic culture and biofilm model) while increase of persister fraction in *dnpA*-overexpressing strain. Concurrent with our transcriptome analysis result that 3-oxo-C_{12}-HSL reduced *dnpA* expression by 3-fold, it supports the hypothesis that the reduced expression of PA5002 (*dnpA*) by 3-oxo-C_{12}-HSL diminished the ciprofloxacin persistence through mediating the genes for surface associated processes [49].

In addition, we found that the genes *parC* and *parE*, which encode topoisomerase IV that is the target of the antibiotic Cip [31], were induced in our microarray data. We hypothesize that induction of one Cip target by 3-oxo-C_{12}-HSL may also help increase the susceptibility of PAO1 persister cells to Cip.

In conclusion, the results indicate that 3-oxo-C_{12}-HSL can render *P. aeruginosa* PAO1 persister cells sensitive to the antibiotic Cip. This effect is not directly through *lasR* and *rhlR*. Further studies are needed to understand the underlying mechanism.

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Table

Table 2-2. Induced and repressed genes by 3-oxo-C_{12}-HSL in *P. aeruginosa* PAO1 persister cells. Microarray data were analyzed from two biological replicates.

Gene name	Accession number	Expression ratio	Function
rsaL	PA1431	4.07	Adaptation, protection; Transcriptional regulators; Secreted Factors (toxins, enzymes, alginate)
mvaT	PA4315	2.07	Transcriptional regulators
folD	PA1976	3.03	Nucleotide biosynthesis and metabolism; Biosynthesis of cofactors, prosthetic groups and carriers; Translation, post-translational modification, degradation
ppx	PA5241	2.46	Adaptation, protection; Nucleotide biosynthesis and metabolism
Probable transcriptional regulator	PA3973	2.76	Transcriptional regulators
Probable transcriptional regulator	PA0279	10.25	Transcriptional regulators
coaD	PA0363	2.50	Central intermediary metabolism
metH	PA1843	3.15	Amino acid biosynthesis and metabolism
etfA	PA2951	2.13	Energy metabolism
masA	PA1685	1.84	Amino acid biosynthesis and

			metabolism
azu	PA4922	1.84	Energy metabolism
pssA	PA4693	1.92	Fatty acid and phospholipid metabolism
parC	PA4964	1.50	topoisomerase IV subunit A
parE	PA4967	1.98	topoisomerase IV subunit B
Probable cytochrome c	PA1555	2.63	Energy metabolism
Hypothetical protein	PA2433	2.95	Hypothetical, unclassified, unknown
Hypothetical protein	PA0737	2.79	Hypothetical, unclassified, unknown
Hypothetical protein	PA2243	2.54	Hypothetical, unclassified, unknown
phoU	PA5365	0.34	Membrane proteins; Transcriptional regulators
pilH	PA0409	0.45	Chemotaxis; Two-component regulatory systems; Motility & Attachment
hmgA	PA2009	0.30	Carbon compound catabolism
gcdH	PA0447	0.31	Amino acid biosynthesis and metabolism; Carbon compound catabolism; Fatty acid and phospholipid metabolism
bglX	PA1726	0.58	Carbon compound catabolism
pvdE	PA2397	0.64	Adaptation, protection; Membrane proteins; Transport of small molecules

CHAPTER 3

BIOFILM FORMATION OF *Pseudomonas aeruginosa* PAO1 ON PDMS SURFACE CONTAINING (*Z*)-4-BROMO-5-(BROMOMETHYLENE)-3-METHYLFURAN-2(5*H*)-ONE

3.1 Abstract

Biofilms are highly-structured microbial communities attached to surfaces and enclosed in a matrix of extracellular polymeric substance (EPS). Bacteria in biofilms are up to 1,000 times more resistant to antimicrobial agents than planktonic cells. Due to high-level antibiotic resistance, biofilms cause chronic infections and failure of implanted medical devices. Previously, (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-25(*H*)-one (BF8) has been shown to inhibit quorum sensing and biofilm formation of *P*. *aeruginosa*. Here we engineered polydimethylsiloxane (PDMS) surfaces by including BF8 during polymerization to inhibit *P. aeruginosa* PAO1 biofilm formation. Our results showed that biofilm formation and biofilm-associated persister cells can be significantly reduced on the PDMS surfaces containing BF8 compared to the regular PDMS surfaces.

3.2 Introduction

Bacteria are well known to attach to biotic and abiotic surfaces and form complex multicellular communities known as biofilms [1]. According to the National Institute of Health, biofilms are responsible for more than 60% of all bacterial infections [2, 3]. Bacteria in biofilms are more resistant to antimicrobial agents and host immune defenses, which can be attributed to the barrier of the extracellular matrix, the physiological state of biofilm cells, and the presence of subpopulations with high level drug tolerance known as persister cells [4].

P. aeruginosa is the main cause of lung infections in cystic fibrosis patients and is a well-recognized model organism for biofilm study [1, 5]. The development of *P. aeruginosa* biofilms occurs in a dynamic process including initial attachment, irreversible attachment, maturation and dispersion [1]. In *P. aeruginosa*, three exopolysaccharides, Psl, Pel, and alginate, have been characterized for their roles in formation, structure maintenance and antibiotic resistance of biofilms [5-7].

Recent studies have indicated that quorum sensing (QS) systems play a major role in biofilm formation of different pathogens such as *P. aeruginosa*, *Burkholderia cepacia*, and *Streptococcus intermedius* [8-11]. Thus, interfering with bacterial QS systems may be a promising strategy to treat chronic infections caused by biofilms. Halogenated furanones produced by the marine red alga *Delisea pulchra* were the first example of natural QS inhibitors. These natural furanones have similar structures to acylated homoserine lactones (AHLs) and can disrupt QS by completing with AHLs for binding to the cognate AHL receptors [12, 13]. In order to enhance the QS inhibitory activities, a number of halogenated furanones have been synthesized and studied for the inhibition of biofilm formation by several bacterial species, such as *P. aeruginosa*,

Staphylococcus aureus, *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus epidermidis* [14-18]. For instance, Hentzer et al. [14] developed a novel synthetic furanone compound named furanone 56 [19], which could penetrate the *P. aeruginosa* biofilm matrix and interfere with AHL-mediated QS systems. In addition, this furanone can inhibit the production of QS-regulated virulence factors and biofilm development [14].

Thus, furanones have the potential to be used for antibacterial coatings for the prevention of bacterial adhesion and biofilm formation [20, 21]. Bajeva et al. [22] reported the production of *S. epidermidis* biofilms were significantly inhibited on the polymer biomaterials (for example, polyethylene, poly(vinyl chloride), and polyether polyurethane) with physically absorbed furanone (3-(1'-bromohexyl)-5-dibromomethylene-2(5H)-furanone). Meanwhile, in a separate study by Hume et al. [23], this furanone was covalently linked to polystyrene disks and catheters using two different strategies. Polystyrene-furanone disks were made through the co-polymerization of styrene and furanone compound. For furanone-coated catheters, the catheter surface was coated with a plasma polymer layer and then modified with poly(acrylic acid) and finally grafted by furanone compound [21, 23]. Polystyrene-furanone disks and furanone-coated catheters showed an 89% and 78% reduction in *S. epidermidis* biofilm formation, respectively, compared to control disks and catheters [23]. In addition, some furanone-coated catheters were implanted into a sheep model and were capable of controlling in vivo infection for up to 65 days [23]. In another study, *E. coli* biofilms were inhibited on the polyvinyl chloride material coated with one brominated furanone (3,4-dibromo-5,5-dimethoxypheny-2(5H)-furanone) [24].

Recently, Han et al. [25] reported that (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-25(*H*)-one (BF8) can inhibit *E. coli* biofilm formation. Subsequently, Pan et al. reported that BF8 is

capable of reducing the number of persister cells in established biofilms of *P. aeruginosa* PAO1 [15], PDO300 [16], and *E. coli* [26]. In addition, BF8 appeared to be safe to the normal 3T3-L1 preadipocytes and HS-5 bone marrow stromal cells in vitro and did not show obvious cytotoxicity to mice in vivo [26].

In the present study, we investigated the effects of polydimethylsiloxane (PDMS) surfaces with BF8 on *P. aeruginosa* PAO1 biofilm formation and biofilm-associated persistence. Our results showed that biofilm formation and biofilm-associated persister cells were significantly reduced on the PDMS surfaces containing BF8 compared to the untreated PDMS surfaces.

3.3 Materials and methods

3.3.1 Preparation of PDMS surfaces with and without BF8

PDMS surfaces were prepared using a SYLGARD184 silicone elastomer kit (Dow Corning Co., Midland, MI, USA). As shown in Figure 3-1, the compound BF8 was synthesized as reported previously [25]. Synthetic BF8 was dissolved in ethanol and then stored at -4 ℃ until use. BF8 solution was added before making the PDMS surfaces. The control PDMS surface was added with 300 μl of ethanol to eliminate any solvent effect.

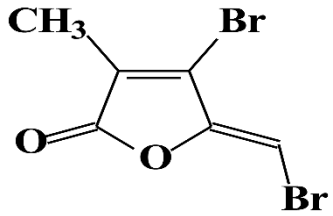


Figure 3-1. Chemical structure of BF8.

3.3.2 Bacterial strain and growth media

P. aeruginosa PAO1 were grown in Luria-Bertani (LB) medium containing 10 g/L NaCl, 10 g/L tryptone, and 5 g/L yeast extract, and cultured with shaking (200 rpm) at 37 ℃ for 18 h.

3.3.3 Effects of PDMS surfaces containing BF8 on PAO1 biofilm formation and biofilm-associated persistence

Overnight culture of *P. aeruginosa* PAO1 was used to inoculate subcultures in LB medium to an optical density at 600 nm (OD600) of 0.05 in petri dishes containing 1 cm × 0.6 cm PDMS surfaces. Petri dishes were incubated at 37 ℃ for certain time of points without shaking. PDMS surfaces without BF8 were used as control. One mL of medium with dispersed cells was washed three times with 0.85% NaCl solution and then plated on LB agar plates to determine the number of CFU of dispersed population. Meanwhile, 1 mL of medium with detached cells was treated with 200 μg/mL ciprofloxacin for 3.5 h at 37 ℃ for persister isolation. Then each sample was washed three times with 0.85% NaCl solution and plated on LB agar plates to determine CFU of surviving persister cells. After certain period of incubation, PDMS surfaces were washed three times by 0.85% NaCl solution. Then each surface was transferred to one 15mL conical tube containing 2mL 0.85% NaCl solution. The biofilm cells were collected by vortexing the surfaces for 1 min and sonicating for 1 min. The drop plate method as described previously [27] was used to determine CFU of collected biofilm cells. To determine the biofilm-associated persisters, the remaining portion of each sample was treated with 200 μg/mL ciprofloxacin for 3.5 h at 37℃. After the treatment, the persister cells were washed three times with 0.85% NaCl solution. This experiment was performed in replicate.

3.3.4 Fluorescence microscopy

To observe the biofilms formed on PDMS surfaces, the dye of acridine orange was used to stain *P. aeruginosa* PAO1 biofilms. This dye would emit green fluorescence when bound to double-stranded DNA and red fluorescence when bound to single-stranded DNA or RNA. After incubation, the PDMS surfaces with PAO1 biofilm cells were gently washed three times with 0.85% NaCl solution. Then the biofilms were stained with 0.5 mg/ml acridine orange for 2 min in the dark. An Axio Imager M1 fluorescence microscope (Carl Zeiss Inc., Berlin, Germany) was used to visualize the biofilms. The biomass of biofilm cells was calculated using COMSTAT software [28].

3.3.5 Statistical analysis

Statistical analysis was conducted using SAS 9.1.3 Windows version (SAS, Cary, NC, USA) to compare the difference between the control and treatment samples. The notes of *, **, and *** indicate that the *p*-value is <0.05, <0.01, and <0.001, respectively.

3.4 Results

It has been reported that BF8, when added in the growth medium, can inhibit biofilm formation of *P. aeruginosa* PAO1 [15]. In the present study, we tested if the PDMS surfaces containing BF8 could affect *P. aeruginosa* PAO1 biofilm formation. As shown in Figure 3-2, at the time point of 5 h, the PDMS surfaces with 1800 μg/mL BF8 reduced the biofilm formation by 37.7% ± 4.3% (one-way ANOVA, $p < 0.0001$) and biofilm-associated persister cells by 67.2% ± 11% (one-way ANOVA, $p < 0.0001$), respectively, compared to the regular PDMS surfaces. The inhibition of biofilms and biofilm-associated persister cells on the PDMS surfaces with BF8 was stronger

after 18 h incubation. For instance, as shown in Figure 3-3, the total number of biofilm cells and biofilm-associated persisters were reduced by 83.8% ±1.1% (one-way ANOVA, $p < 0.0001$) and 88.3% ±4.8% (one-way ANOVA, $p < 0.0001$) respectively, on the PDMS surfaces with 180 μg/mL BF8. At 24 h after inoculation, the PDMS surfaces containing 1800 μg/mL BF8 reduced biofilm formation and biofilm-associated persisters by 98.2% ±0.4% (one-way ANOVA, $p < 0.0001$) and 98.7% ±0.5% (one-way ANOVA, $p < 0.0001$), respectively, compared to the BF8-free PDMS surfaces (Figure 3-4).

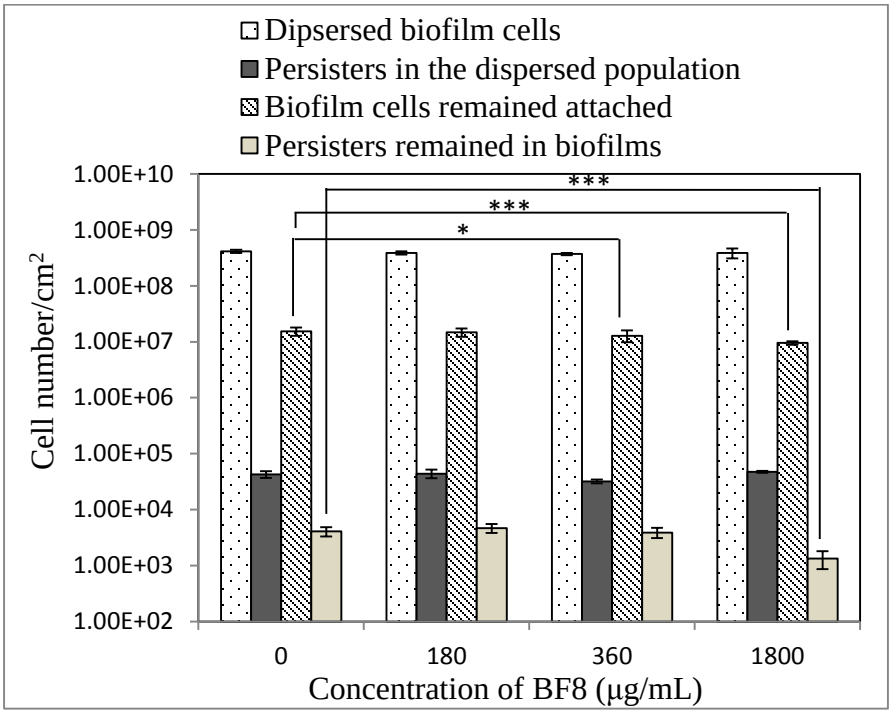


Figure 3-2. Effects of PDMS surfaces containing BF8 on *P. aeruginosa* PAO1 biofilm formation for 5 h. Different concentration of BF8-ethanol solution was added before making the PDMS surfaces. The control PDMS surface was added with 300 μl of ethanol to eliminate any solvent effect.

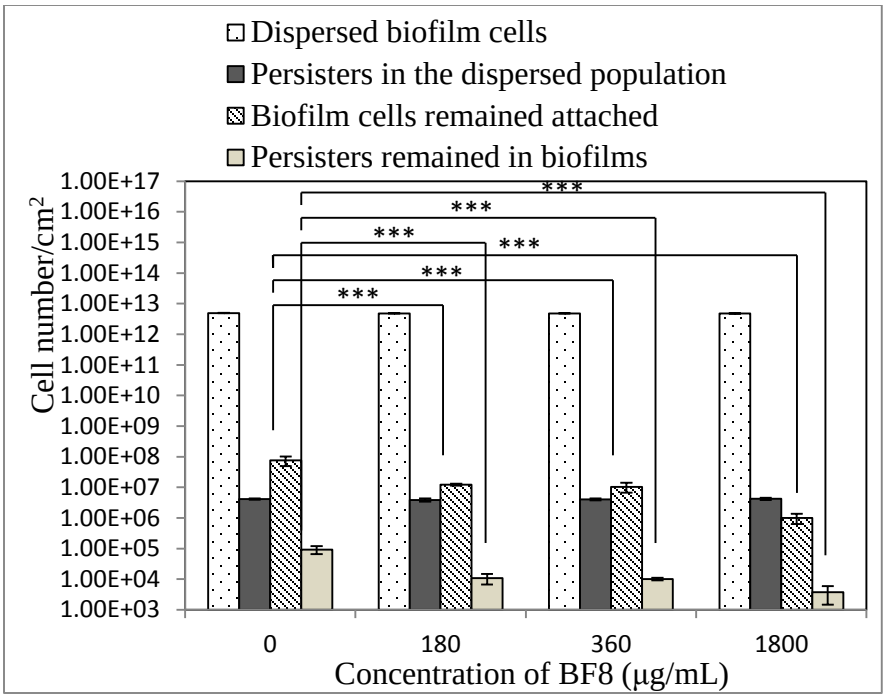


Figure 3-3. Effects of PDMS surfaces containing BF8 on *P. aeruginosa* PAO1 biofilm formation for 18 h.

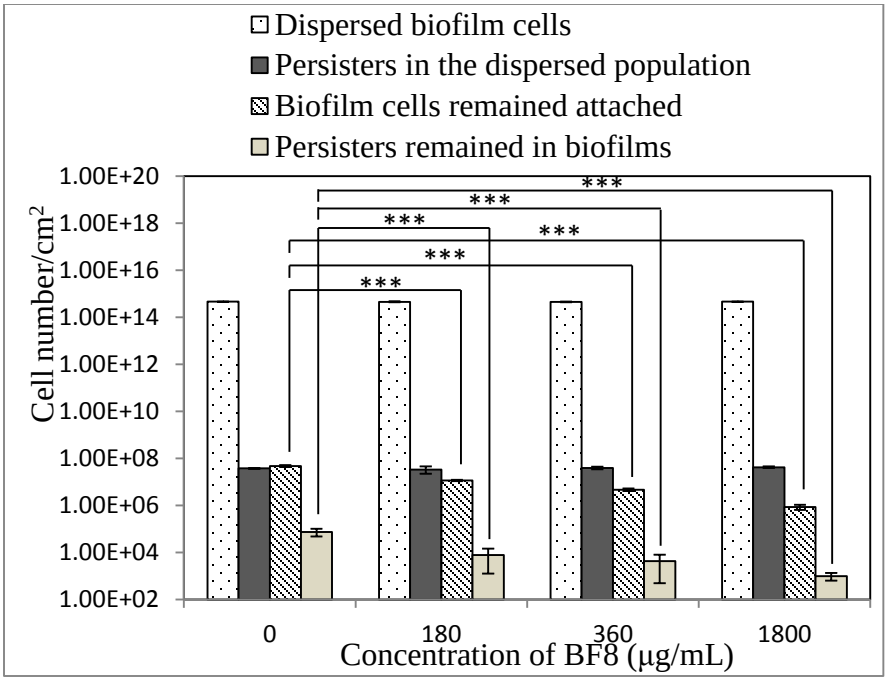


Figure 3-4. Effects of PDMS surfaces with BF8 on *P. aeruginosa* PAO1 biofilm formation for 24 h.

However, the level of biofilm inhibition decreased after 48 h. For example, the number of biofilm cells was reduced by only 50% ±3.2% on the PDMS surface with 1800 μg/mL BF8 (one-way ANOVA, $p < 0.0001$) (Figure 3-5). For 72 h biofilms, we didn't observe the significant inhibition of biofilms on the PDMS surfaces containing 180 μg/mL BF8 (Figure 3-6).

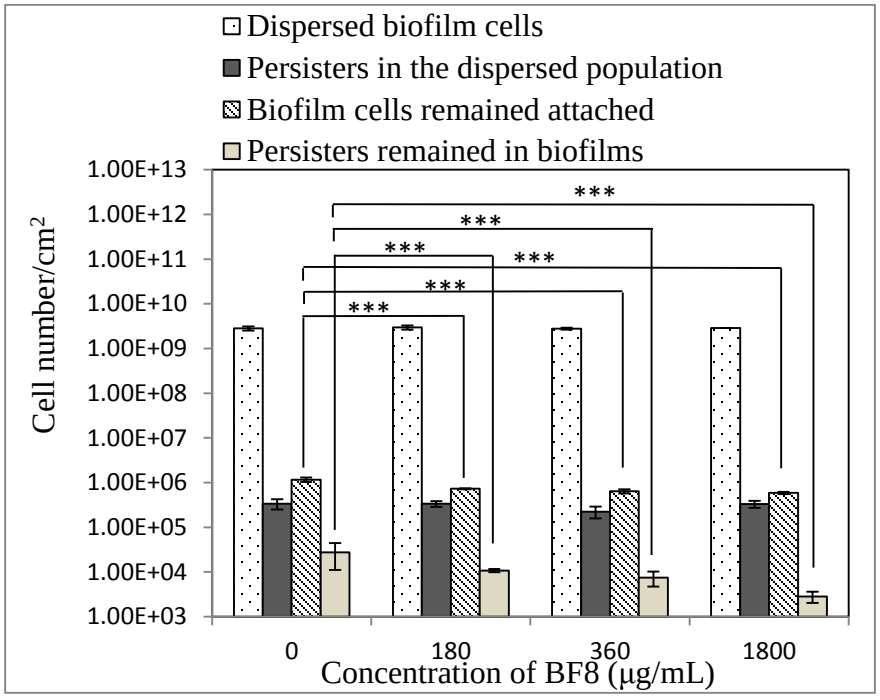


Figure 3-5. Effects of PDMS surfaces with BF8 on *P. aeruginosa* PAO1 biofilm formation for 48 h.

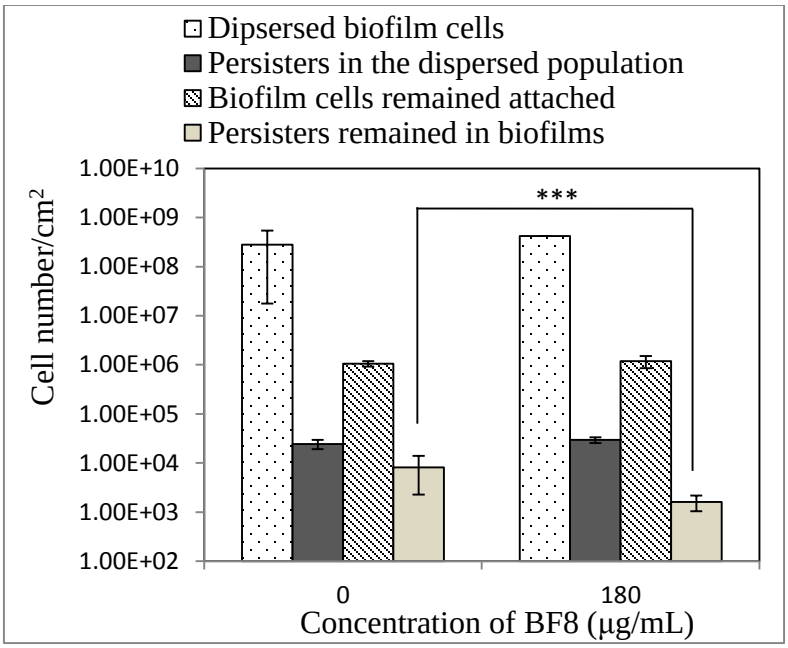
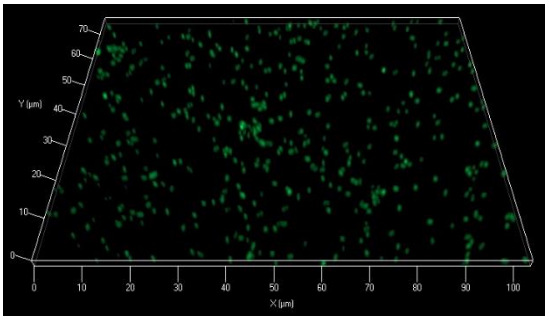


Figure 3-6. Effects of PDMS surfaces with BF8 on *P. aeruginosa* PAO1 biofilm formation for 72 h.

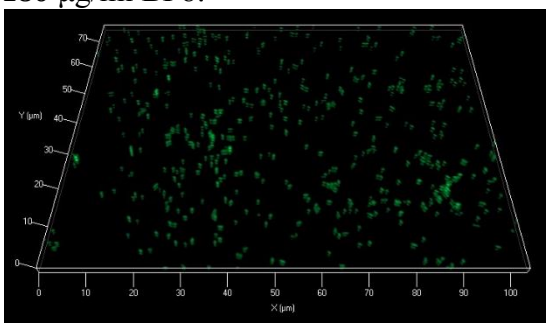
Figure 3-7 shows the representative images of biofilms formed on the PDMS surfaces with and without BF8. Based on the microscopic images of biofilms, the biomass of biofilms on the PDMS surfaces with and without BF8 at different time points was calculated by the COMSTAT software (Figure 3-8). These data are consistent with the above CFU results.

For 5 h biofilms:

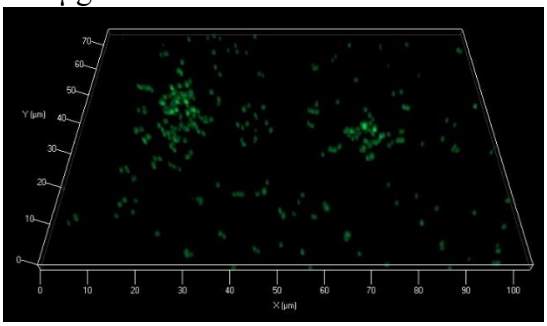
Control:



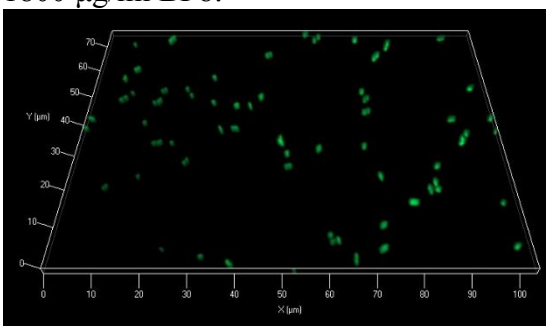
180 μg/ml BF8:



360 μg/ml BF8:

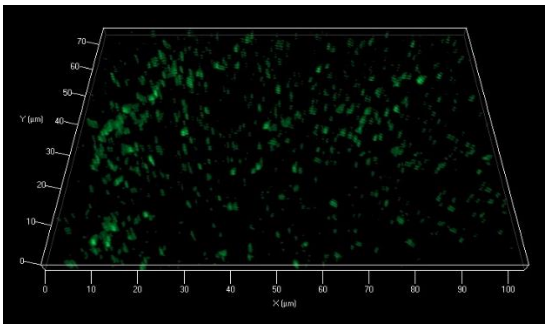


1800 μg/ml BF8:

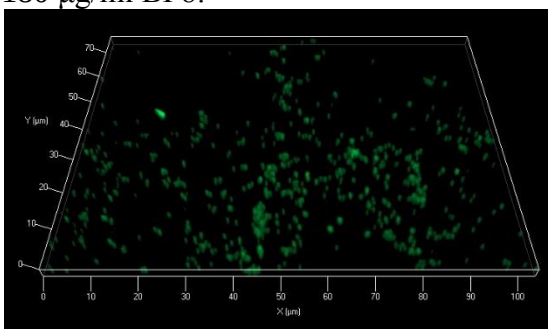


For 18 h biofilms:

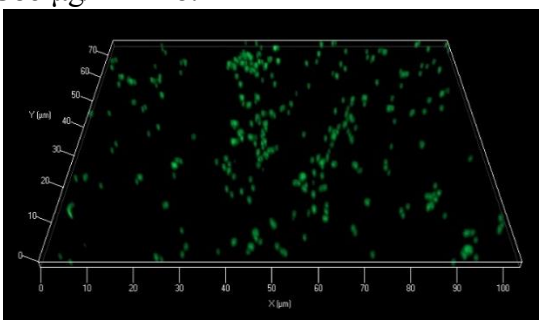
Control:



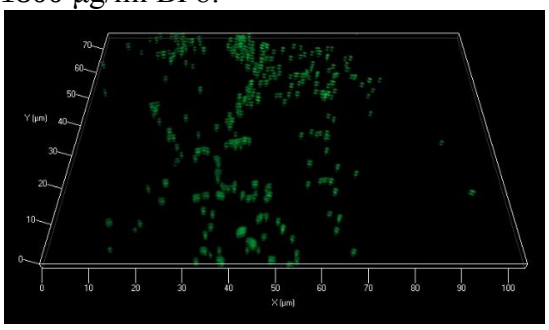
180 μg/ml BF8:



360 μg/ml BF8:

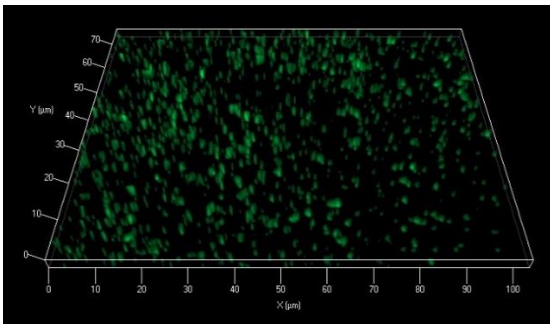


1800 μg/ml BF8:

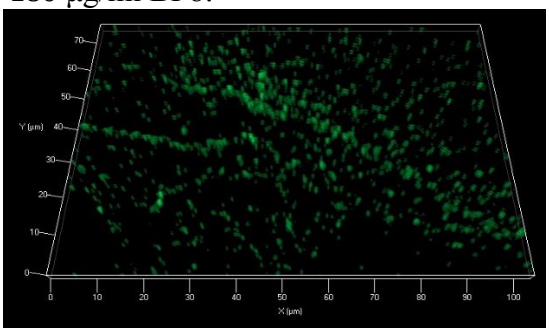


For 24 h biofilms:

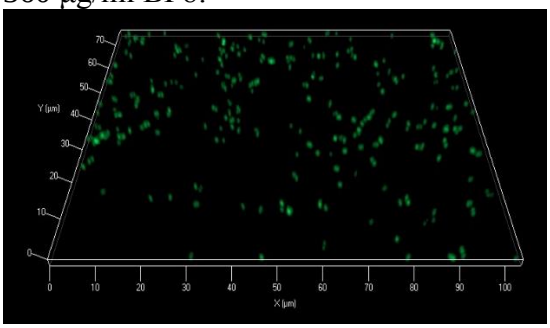
Control:



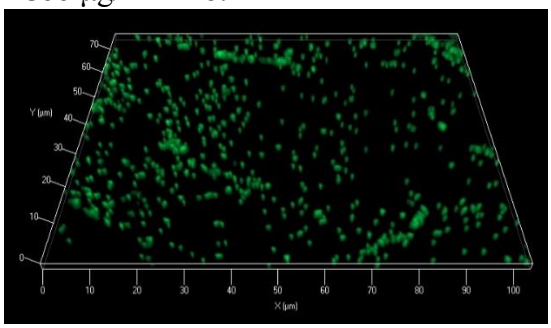
180 μg/ml BF8:



360 μg/ml BF8:

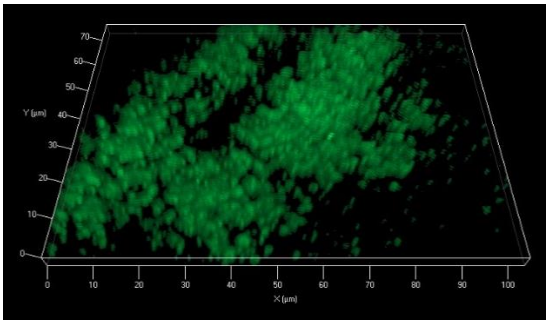


1800 μg/ml BF8:

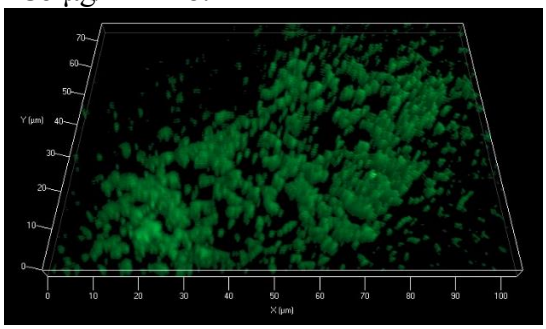


For 48 h biofilms:

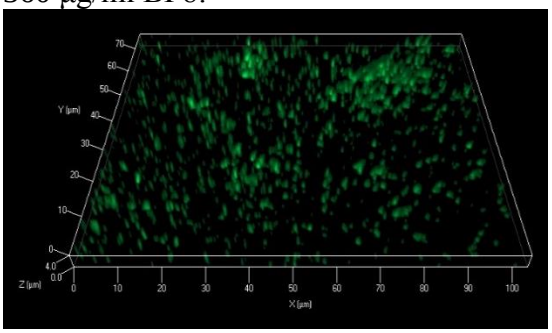
Control:



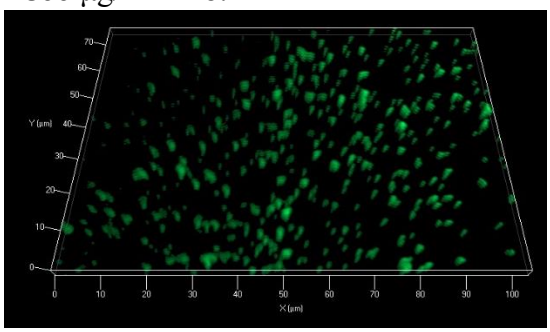
180 μg/ml BF8:



360 μg/ml BF8:

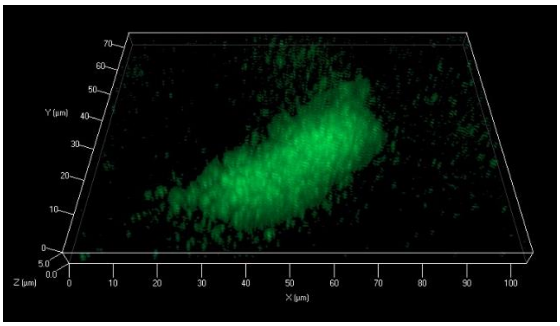


1800 μg/ml BF8:

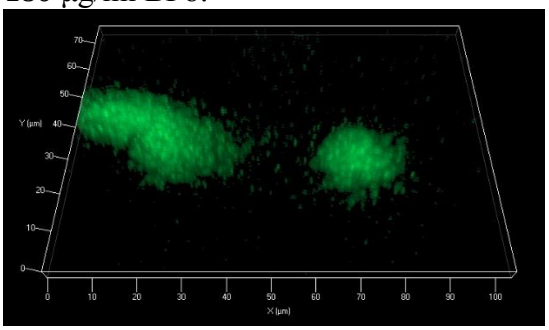


For 96 h biofilms:

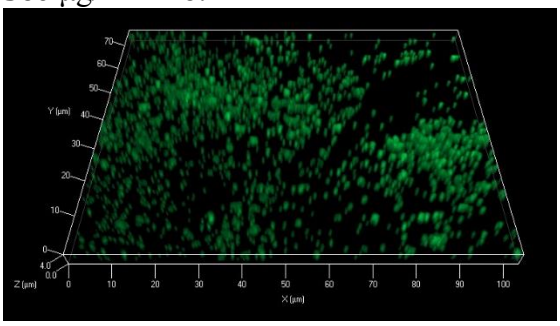
Control:



180 μg/ml BF8:



360 μg/ml BF8:



1800 μg/ml BF8:

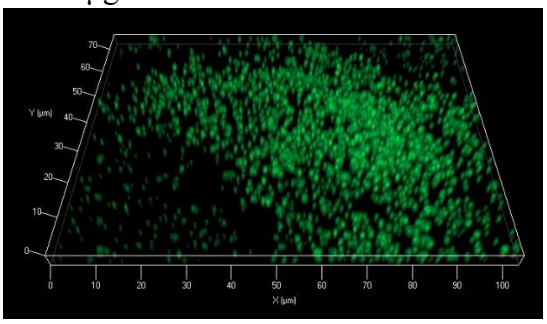


Figure 3-7. Representative microscopic images of *P. aeruginosa* PAO1 biofilm cells on PDMS surfaces with and without BF8. The dye of acridine orange was used to stain *P. aeruginosa* PAO1 biofilms.

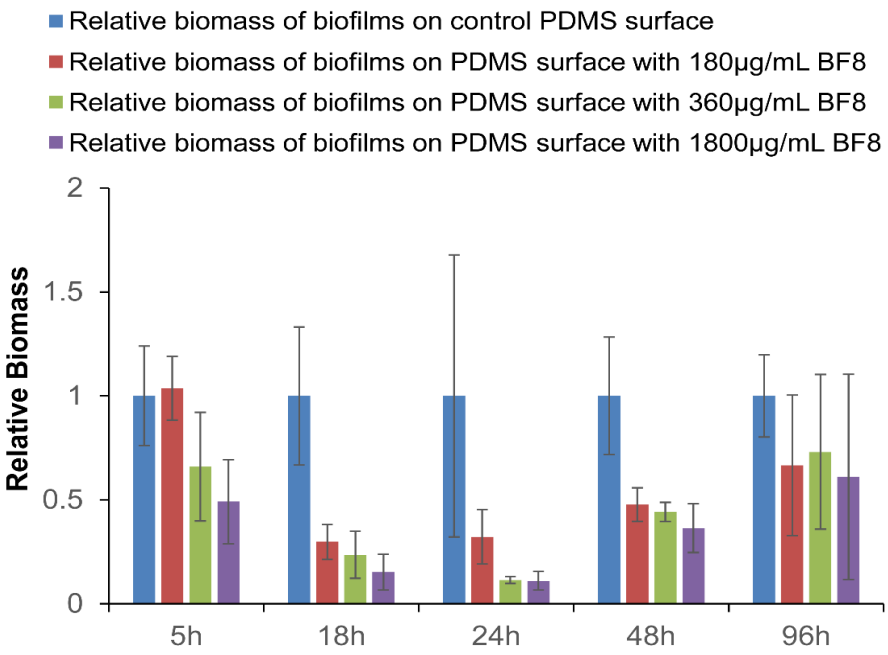


Figure 3-8. Relative biomass of biofilm cells on PDMS surfaces with and without BF8. The biomass of biofilms on the PDMS surfaces with and without BF8 was calculated by the COMSTAT software. The biomass of untreated controls at different time points were normalized to 100%.

The number of dispersed biofilm cells and persister cells in the dispersed population didn't change on the PDMS surfaces with different concentrations of BF8 at the different time points. This suggests that BF8 did not diffuse out of PDMS significantly.

3.5 Discussion

In this chapter, PDMS surfaces containing BF8 were prepared and compared with regular PDMS for *P. aeruginosa* PAO1 biofilm formation. Our results revealed that *P. aeruginosa* PAO1 biofilm formation and biofilm-associated persisters were significantly reduced on the PDMS

surfaces containing BF8 compared to the PDMS surface without BF8 within 48 h. After inoculation for more than 48 h, however, the difference decreased.

One previous study showed that a conjugated exocyclic vinyl bromide of BF8 is the key element for the inhibition of *E. coli* biofilm formation [25]. Pan et al. [15] reported that *P. aeruginosa* PAO1 biofilms and biofilm-associated persisters were significantly inhibited by adding 60 μg/ml BF8 as free molecules in the growth medium at inoculation. It should be noted that direct covalent immobilization of BF8 to a surface may attenuate its inhibition activity of biofilm formation [25]. By adding BF8 in the PDMS directly, we obtained significant biofilm inhibition. However, since most of BF8 molecules are embedded in the PDMS, it may be more efficient to combine this with surface coating strategies.

In summary, the results from this study further support that bacterial cell-to-cell signaling is a possible target for controlling biofilm formation and persistence. Future studies with optimized material design and in vivo experiments will help develop better biomaterials. Additionally, studies on the genetic mechanism will provide a better understanding of the activities of BF8 and better control of biofilm-associated infections caused by *P. aeruginosa*.

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APPENDIX

Experimental protocols

Protocol 1

PAO1 Persister RNA Isolation

Harvesting Cells

1. Grow 60 mL overnight culture of PAO1 for 18 hours.

2. Wash the cells twice by 0.85% NaCl solution at 8000 rpm for 10 min.

3. Add 200 μg/mL Cip and incubate for 3.5 hours to get persisters.

4. Wash persisters thrice by 0.85% NaCl solution at 8000 rpm and 4 ℃ for 10 min.

5. Dilute the persister culture 5 times by 0.85% NaCl solution and divide the diluted culture into 8 falcon tubes. Each falcon tube contains 37.5 mL diluted persisters. (4 tubes for control and 4 tubes for treatment)

6. Treat the diluted persister culture with the specific condition of interest.

7. During this time, pre cool all the tubes and centrifuges.

8. Centrifuge the persister culture at 10,000 rpm and 2 ℃ for 2 min and decant supernatant.

9. Flash-freeze in dry-ice, store at -80^{o}C until use.

Kit: RNeasy Mini Kit (50) Source: Qiagen Cat log. Number: 74104

RNA Isolation

Preparation:

1. Add 200uL Zirconia/Silica beads to bead beater tube & cool on ice

2. RLT Buffer: 10 μL βME per 1 mL RLT (2 mL/sample)

3. RPE Buffer: 8 mL EtOH per 2 mL RPE (4 mL/sample)

4. DNase Mix: 45 μL DNase I stock per 315 μL RDD Buffer (360μL/2 samples)

Procedure:

1. Add 450 uL RLT buffer to all the 8 tubes with pellets. Vortex them. Combine 2 tubes of control and 2 tubes of treatment. In total there will be 4 tubes with 900 uL RLT mixed with cells: 2 for control and 2 for treatment.

2. Transfer 900 μL of RLT with cells in bead beater tubes with zirconia/silica beads.

3. Beat 60s (set timer at 6) at speed 48 (~5000 rpm), although PAO1 is Gram negative.

Centrifuge 15s at 13000rpm and 4 ℃.
(All centrifuge steps at these settings unless noted)

4. Collect supernatant and add 445 μL EtOH.

5. Load 700 μL sample onto RNeasy column (Qiagen), and then centrifuge.

6. Add 350 μL RW1 and centrifuge. Then repeat once.

7. Add 180 μL DNase I incubation mix <u>directly onto membrane.</u>

8. Incubate at RT for 45 min.

9. Add 350 µL RW1 and centrifuge. Then repeat once.

10. Add 500 µL RPE and centrifuge. Then repeat twice.

11. Add 500 µL RPE and centrifuge for 2 min.

12. Replace collection tube and centrifuge for 1 min.

13. Place column in 1.5 collection tube.

14. Add 40 µL RNase-free water and centrifuge for 1 min. Then repeat once.

Clean-up

1. Add 900 µL RLT Buffer to primary RNA product.

2. Add 445 µL EtOH.

3. Load 700 µL sample onto RNeasy column (Qiagen), and then centrifuge.

4. Add 350 µL RW1 and centrifuge. Then repeat once.

5. Add 180 µL DNase I incubation mix directly onto membrane.

6. Incubate at RT for 30 min.

7. Add 350 µL RW1 and centrifuge. Then repeat once.

8. Add 500 µL RPE and centrifuge. Then repeat twice.

9. Add 500 µL RPE and centrifuge for 2 min.

10. Replace collection tube, centrifuge for 1 min.

11. Place column in 1.5 collection tube

12. Add 40 µL RNase-free water and centrifuge for 1 min. Then repeat once.

Save flow-through!

Quantification

1. Measure OD at 260 nm and 280 nm (using TE as the background)

- Yield: OD_{260} of 1.0 = 40 µg/mL
- Calculation: RNA concentration = 40 µg/mL × OD_{260} × dilution factor
- Ratio: OD_{260}/OD_{280} should be >2.0

2. Run 1.4% agarose gel (0.42g agarose, 30 mL 1xTAE)

- Should have two clear bands (23S at 3.1kb, 16S at 1.5kb)
- Smearing patterns at low molecular range indicate RNase contamination

Protocol 2

qPCR Analysis

cDNA Synthesis

1. Thaw the components in the iScript cDNA synthesis kit and then mix them as shown below.

Components	Volume per Reaction
5x iScript reaction mix	4 µl
iScript reverst transcriptase	1 µl
Nuclease-free water	12.5 µl
RNA template	2.5 µl
Total volume	20 µl

2. Incubate the above reaction mix in the thermo cycler and run the program shown as: 5 minutes at 25 ℃, 30 minutes at 42 ℃, 5 minutes at 85 ℃, hold at 4 ℃.

Reaction mix preparation and thermal cycling

1. Thaw cDNA samples and dilute them to the concentration of 40ng/µl.

2. Thaw forward and reverse primers, iTaq Universal SYBR Green supermix and nuclease-free water. Then mix them as shown below:

Components	Volume per 20 µl Reaction
iTaq Universal SYBR Green supermix	10 µl
Primer mix (forward and reverse)	1 µl
Nuclease-free water	4 µl
cDNA	5 µl
Total volume	20 µl

3. Add the reaction mix into the wells of a qPCR plate. Spin the qPCR plate by using the plate centrifuge at 3000rpm for 5min.

4. Set the program of qPCR instrument and then run the PCR.

Protocol 3

Monocyte cell culture (THP-1)

Subculturing procedures for frozen cells

1. Add 5 ml complete culture medium (RPMI 1640 + 5% FBS + 1% Pen/Strep) into a 25 cm^2 tissue culture flask. Put the flask into the incubator for at least 15 minutes to make sure that the pH value of the medium is in the range of 7.0-7.6.

2. Thaw the vial of macrophage cells in the water bath under 37℃ for about 2 minutes.

3. Add 9.0mL complete culture medium into a 15ml centrifuge tube.

4. Remove the vial from the water bath immediately after the vial contents are thawed. Withdraw the vial contents into the above 15ml centrifuge tube.

5. Centrifuge them at approximately 125xg for 5 to 7 minutes. Remove the supernatant from the centrifuge tube.

6. Take out the 25cm^2 tissue culture flask containing complete culture medium from the incubator. Add certain medium into the above centrifuge tube. Withdraw the cells into the 25cm^2 tissue culture flask.

7. Use the microscope to check macrophage cells. Then put the 25cm^2 tissue culture flask into the incubator.

Subculturing procedures for the suspension cells

1. After put 25cm^2 tissue culture flask containing macrophage cells into the incubator, use the microscope to check macrophage cells.

2. Add 4ml complete culture medium into a 25cm^2 tissue culture flask. Put the flask into the incubator for at least 15 minutes to make sure that the pH value of the medium is in the range of 7.0-7.6.

3. When the macrophage cell concentration reaches 8×10^5 cells/ml, use the pipette aid to withdraw 1ml cells suspension into the above 25cm^2 tissue culture flask containing 4.0ml fresh medium (RPMI 1640 + 5% FBS + 1% Pen/Strep).

4. Put the flask into the 37℃ incubator.

Freezing cells grown in suspension culture

1. Transfer cell solution to a 15ml conical tube and then centrifuge it at approximately 125 xg for 5 min.

2. Remove supernatant and resuspend pellet in the above medium (RPMI 1640 + 5% FBS + 1% Pen/Strep + 5% DMSO) at a density of 10^6 to 10^7 cells/ml.

3. Transfer 1ml aliquots of cell suspension into cryovials and then put cryovials into the cryopreservation container.

4. Put the cryopreservation container in the -80 freezer for overnight and then put the cryovials into the liquid nitrogen.

Phagocytosis assay

Day 1

1. Culture the THP-1 cells as mentioned above and transfer 5 ml of THP-1 cell culture into a new culture dish.

2. Add 100 ng/ml PMA into the culture of THP-1 cells for differentiation and put the culture dish into the incubator (in Room 437) for 2 days.

Day 3

1. After 2 days, add 5 ml complete culture medium (RPMI 1640 + 5% FBS) into a tissue culture dish. Put the culture dish into the incubator for at least 15 minutes to make sure that the pH value of the medium is in the range of 7.0-7.6.

2. Take out the culture dish containing differentiated THP-1 cells from the incubator. Then replace the old medium in the culture dish with the preheated culture medium from tissue culture flask.

3. Add 100 ng/ml E.coli LPS into the culture dish containing differentiated THP-1 cells for 1 day.

4. Make the overnight culture of *E. coli* RP 437 (or *acrA* mutant, *acrB* mutant).

Day 4

1. After 1 day, make the 0.1mg/ml FITC dye (dissolved in 0.1M $NaHCO_3$) solution before use. Then stain the *E. coli* RP 437 cells using 0.1mg/ml FITC dye for 1 hr at room temperature and then washed bacteria once using 1×PBS.

2. Add the FITC-labeled bacteria into the culture dish containing THP-1 cells for 2 hrs.

3. After 2h, add the 50 μg/ml ethidium bromide and then observe using inverted microscope.

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TECHNICAL SKILLS

- **Biomedical analytical techniques:** Mammalian cell culture, DNA/RNA and plasmid extraction, cloning, PCR, real time PCR, gel electrophoresis, Western blot, SDS-PAGE, fluorescence microscopy
- **Chemical analysis:** HPLC, GC, Karl Fischer, UV-VIS spectrophotometer
- **Material characterization:** DSC, TGA, FTIR, XRD, NMR, SEM, column chromatography
- **Software:** MS office, SAS, Origin, AutoCAD, Matlab, Photoshop

RESEARCH EXPERIENCE

Research Assistant Syracuse University 2012 – 2014

- Designed a method for controlling the chronic infections caused by bacteria biofilms and persister cells by manipulating cell-cell signaling
- Researched the effects of cell-cell signals on bacteria cells and their genetic mechanisms
- Performed microbiological experiments including growth, maintenance, and storage of bacterial cultures.
- Performed molecular biology techniques including DNA/RNA extraction, cloning, gel electrophoresis, SDS-PAGE and Western blot.
- Designed primers for specific genes and performed classic PCR and real time PCR assays
- Performed mammalian cell culture techniques including growth, expansion, and differentiation of murine/human macrophage cells
- Did the phagocytosis assay of normal bacteria by macrophage cells
- Developed a novel PDMS surface with synthetic brominated furanones for biofilm inhibition
- Synthesized, purified and characterized a group of brominated furanones
- Trained/mentored 2 undergraduate/graduate students

Research Assistant Tianjin University 2010 – 2012

- Designed and optimized the reactive crystallization of ceftazidime using DSC, TGA, HPLC, and GC
- Assisted in determining the reactive crystallization kinetics
- Assisted in designing and optimizing the cooling crystallization using DSC, Karl Fischer, and HPLC

Teaching Assistant Syracuse University 2012 – 2013

- Lead a chemical engineering lab section by presenting pre-lab lectures, executing lab set up and lab procedures, and grading lab reports

EDUCATION

M.S. Chemical Engineering **GPA: 3.67** Syracuse University, NY 2012 – 2014

- Thesis: Controlling *Pseudomonas aeruginosa* biofilms and persister cells by manipulating

cell-cell signaling
- Graduate Coursework: Biochemical Engineering; Biomaterials & Medical Devices; Green Engineering; Kinetics; Fluid Dynamics

M.S. Chemical Engineering **GPA: 3.8** Tianjin University, China 2009 – 2012

- Thesis: Study on isoelectric crystallization process of ceftazidime
- Graduate Coursework: Life Sciences and Biotechnology; New Chemical Experiment Technology; Chemical Engineering Thermodynamics; Chemical Reaction Engineering

PUBLICATIONS

- Wang YL, Ma SY, Lv XD. Determination of reactive crystallization kinetics of 5-difluoromethoxy-2-mercapto-1H-benzimidazole in a batch crystallizer. Crystal Research and Technology 2012
- Ma SY, Ren D. Effects of *N*-(3-oxododecanoyl)-L-homoserine lactone on *Pseudomonas aeruginosa* persister cells. PLOS ONE 2014. In preparation
- Ma SY, Ren D. Biofilm formation of *Pseudomonas aeruginosa* PAO1 on PDMS surface containing (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one. FEMS Microbiol Lett 2014. In preparation