8-2013

Controlling Bacterial Persister Cells and Biofilms by Synthetic Brominated Furanones

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ABSTRACT

Bacteria are well known to obtain tolerance to antibiotics by forming multicellular structures, known as biofilms, and by entering dormancy and forming persister cells. Both mechanisms allow bacteria to tolerate antibiotics at concentrations hundreds to thousands of times higher than the lethal dose for regular planktonic cells of the same genotype. Persister formation increases in biofilms; thus, effective control of persister cells, especially those in biofilms is critically important to infection control. Over the past decades, a bacterial signaling system based on cell density, named quorum sensing (QS), has been found to regulate biofilm formation and, in *Pseudomonas aeruginosa*, the level of persistence.

In this study, we characterized the effects of synthetic brominated furanones, a group of QS inhibitors, on the persistence of *P. aeruginosa* and *Escherichia coli*. Our results revealed that (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one (BF8) can reduce persister formation in *P. aeruginosa* PAO1 and *E. coli* RP437 and restore the antibiotic susceptibility of isolated persister cells at growth non-inhibitory concentrations. In addition to planktonic persister cells, BF8 was also found to reduce persister formation in the biofilms of *P. aeruginosa* PAO1 and *E. coli* RP437.

Study at the genetic level using DNA microarrays demonstrated that BF8 induced the gene *mdaB* in both strains and 7 genes encoding oxidoreductases in *P. aeruginosa*. In *E. coli* RP437 persister cells, BF8 was also found to repress the genes for synthesizing indole, a signaling molecule reported to induce persister formation in *E. coli*. Interestingly, although BF8 is a QS inhibitor, the QS signal 3-oxo-C_{12}- acyl homoserine-lactone also rendered the persister cells of *P.*

aeruginosa PAO1 and its mucoid mutant PDO300 more sensitive to antibiotics. Furthermore, BF8 was found to have cidal effects on PDO300.

Besides BF8, some other BFs were also found to restore the susceptibility of *P. aeruginosa* PAO1 persister cells to ciprofloxacin. Collectively, these results indicate that this group of QS inhibitors has promising activities to control multidrug tolerant persister cells; and there might be other bacterial targets of BFs in addition to QS inhibition.

Key words: quorum sensing, biofilm, persister, antibiotic tolerance, brominated furanones.

CONTROLLING BACTERIAL PERSISTER CELLS AND BIOFILMS BY SYNTHETIC BROMINATED FURANONES

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DISSERTATION

Submitted in partial fulfillment of the requirements for the

degree of Doctor of Philosophy in Chemical Engineering

in the Graduate School of Syracuse University

AUGUST 2013

ACKNOWLEDGEMENTS

First of all, I sincerely appreciate the guidance of Dr. Dacheng Ren for leading me to this fascinating research and supporting my work throughout my Ph. D study. I appreciate his instruction with patience, knowledge and talent, which made me able to accomplish this work.

I also thank our excellent collaborators, Dr. Jing An, Dr. Rebecca Bader and Dr. Yan-Yeung Luk for their kind help and access to facilities.

I am grateful to Dr. Thomas K. Wood at Pennsylvania State University, Dr. Michael Givskov at University of Copenhagen, Dr. Matthew Parsek at University of Washington, Dr. John Parkinson at University of Utah and Dr. Robert Austin at Princeton University for sharing bacterial strains. I also thank Professor Frank Middleton and Ms. Karen Gentle at SUNY Upstate Medical University for helping with DNA microarray hybridization.

I also want to thank the U.S. National Science Foundation (CAREER-1055644 and EFRI-1137186) and Syracuse University for supporting my study.

I wish to express my appreciation to my colleagues in the Ren lab, Dr. Shuyu Hou, Miao Duo, Huiqing Zheng, Xi Chen, Xiangyu Yao and Tagbo Herman Roland Niepa, Geetika Choudhary, Ali Adem Bahar, Fangchao Song, Shuyuan Ma, Li Zhang, Jing Wang, Henry Lars Peterson, Ginger Star Gunnip as well as Fei Cheng, Dr. Sijie Yang, Dr. Debjyoti Bandyopadhyay in Luk lab. It was a great pleasure to work with them.

I wish to express my thankfulness to Jacques Lewalle, Dawn M. Long, Sabina Redington, Lynore de la Rosa, Kristin Lingo, Kathleen M. Joyce, Terrie M Monto, Neil Jasper and Karen Low for their help and their kindness. In my struggling days, their lovely and smiling faces are really encouraging.

I also want to thank my friends: Camil Hodzic, Elizabeth Hughes, Burrell Jardin, Bill Dossert, John Kotlarz, Dick Chave and John Banas for their kindness to me.

I wish to give my greatest appreciation to my family: Qin Li and Zheng Pan. I didn't find chance to visit them back in China within 5 years, but their understanding is the biggest support during my study.

Dedication: To my beloved late grandmother Shubin Wang and my late great grandfather Ru Zhu.

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CHAPTER 1

LITERATURE REVIEW

A part of this chapter has been published as Jiachuan Pan and Dacheng Ren. Quorum sensing inhibitors: a patent overview. Expert Opin Ther Pat 2009, 19: 1581-1601.

1.1 Antibiotic resistance on the rise

Although antibiotics have saved millions of lives, more and more antibiotic resistant bacteria strains are emerging and cause increasing attention. The first methicillin-resistant *Staphylococcus aureus* (MRSA) strain was reported in Britain in 1961[1]. Nowadays, "superbugs" that are resistant to essentially all antibiotics are more and more commonly found in hospitals, prisons, schools, and nursing homes.

Recently a study published by Infectious Disease Society of America reported that antibiotic-resistant infections cost the U.S. Healthcare System more than $20 billion annually[2]. In addition, antibiotic-resistant infections also results more than $35 billion in societal costs and more than $8 million due to additional days that patients spend in the hospital. It was estimated that back in 2000, there were 900,000 antibiotic resistant infection cases in the U.S., and this study on 1,391 patients hospitalized shows that 13.5% of patients had antibiotic resistant infections[2]. For each case of antibiotic resistant infection, $18,588 to $29,069 more was cost by medicine; 6.4 - 12.7 days more was spent on hospital stay; and the patients had a risk of death two times higher than the patient without antibiotic resistant infections.

Although antibiotic resistance is naturally occurring based on resistant genes in microbes, misuse and overuse of antibiotics are considered as important factors that promoted the development and spread of antibiotic resistance over the years. Poor compliance of patients with the treatment guidance of antibiotics is also considered as a major factor in increasing rates of bacterial resistance based on Costelloe's study[3]. Clinically, individuals prescribed an antibiotic in primary care for a respiratory or urinary infection were found to develop bacterial resistance to that antibiotic.

Patients with open wounds, implanted medical devices, and weakened immune systems are at greater risks of infection than the general public. The number of species that cause drug resistant infections has also been increasing. MRSA was reported to cause 37% of fatal cases of sepsis in the UK in 1999 while only 4% in 1991[4]. In the US, 50% of all *S. aureus* infections are resistant to penicillin, methicillin, tetracycline and erythromycin[5]. Drug resistant infections are not limited to Gram positive strains. For example, *Pseudomonas aeruginosa*, a highly prevalent opportunistic pathogen was also reported to resist multiple classes of antibiotics[6]. In fact, *P. aeruginosa* is intrinsically tolerant to different antibiotics given by its own multidrug efflux pumps encoded by antibiotic tolerance genes[6]. In addition to intrinsic tolerance, by genetic mutations, or by the horizontal gene transfer of antibiotic resistance genes, *P. aeruginosa* could quickly evolve specific resistance. This evolvement of resistance is facilitated by developing multicellular structures attached to surfaces, named biofilms. Thus, the intrinsic tolerance of *P. aeruginosa* is considered as a major reason for developing chronic infections in humans, such as the patients with cystic fibrosis[7].

An alarming fact is that, on one hand, more and more superbugs are emerging by misuse or overuse of antibiotics; and on the other hand, less and less antibiotics are successfully developed to obtain the FDA approval[8]. For example, in the past 50 years, only one new antibiotic class, daptomycin, was discovered and used clinically[8,9]. Given this challenge, it is important to develop new control method to combat the infections that are increasingly difficult to treat.

1.2 Antibiotic resistance vs. antibiotic tolerance

1.2.1 Acquired resistance to antibiotics

Acquired resistance occurs when bacterial strains obtain the ability to resist the activity of certain antimicrobial agent to which it was previously susceptible[10,11]. Different from intrinsic resistance

that is based on general elements (e,g. efflux pumps) is abroad spectrum of strains of each species, acquired resistance is developed only when a particular bacterial strain acquires resistance by vertical mutations or horizontal gene transfer via transformation, transduction or conjugation[12]. For example, methicillin-resistant *S. aureus* (MRSA) is any strain of *S. aureus* resistant to β-lactam antibiotics (methicillin, penicillin, dicloxacillin, nafcillin, oxacillin, cephalosporins)[12]. Strains sensitive to β-lactams are classified as methicillin-sensitive *S. aureus* (MSSA). When *S. aureus* infection arises, laboratory methods are applied to find out if it is caused by MRSA or MSSA[12].

MRSA strains represent the most studied class of antibiotic resistant strains.[13]. The *mecA* gene is considered as major factor of methicillin resistance in *S. aureus*; e.g., Ubukata[14] reported that a MSSA strain could be converted to MRSA by the introduction of a plasmid carrying a 4.3-kb chromosomal DNA fragment encoding the *mecA* gene from a MRSA strain. With this gene, *S. aureus* strain can produce a penicillin-binding protein (PBP) that has relatively low affinity for most β-lactam antibiotics and it is also reported that absence of PBP in MRSA led to increased sensitivity to β-lactams. It was also found that additional insertion of a plasmid encoding an inducible penicillinase can also trigger the expression of MRSA-PBP. It is possible that penicillinase plasmid contains certain gene(s) required in the mechanism of induction of the *mecA* gene but not contained in the 4.3-kb chromosomal DNA fragment[14]. Thus, production of PBP may cooperate with other functions such as that of penicillinase to achieve this strong resistance.

1.2.2 Intrinsic tolerance in individual cells

1.2.2.1 Porin Proteins

Outer membrane antibiotic uptake/exclulsion mechanisms are considered as a major function for antibiotic tolerance since the outer membrane acts as a semi-permeable barrier to uptake antibiotics or substrate molecules[6]. For example, in *P. aeruginosa* outer membrane, porin proteins are required to uptake small hydrophilic molecules such as ampicillin. Hence, the outer membrane could modulate the movement of hydrophilic antibiotics by changing the activity of those channels. It has been reported that in *P. aeruginosa*, the overall outer-membrane permeability is ranged as 12 to 100 fold lower than other species such as *Escherichia coli*, which is consistent with the fact that *P. aeruginosa* is more tolerant to ampicillin treatment[15]. Among all porin proteins known to date, OprF is responsible for about 65-75% of exclusion capacity of *P. aeruginosa* outer membrane[6].

1.2.2.2 Self promoted uptake

Besides porin protein functions, penetration of outer membrane to antibiotics could also be achieved by an uptake system named self-promoted uptake which could pump polycationic antibiotics into the cells[6,16]. In the self-promoted system, the divalent cation binding sites on cell surface lipopolysaccharide could be bound by polycationic antibiotics which are much larger than native divalent cations. And this binding permeabilizes the membrane and pumped more polycationic antibiotic inside the cells. A shortage of cationic binding site can lead to the low permeability of polycationic antibiotics. For example, it has been reported that *B. cepacia* has no self-promoted uptake system and is tolerant to all polycationic antibiotics[17].

1.2.2.3 Efflux pumps

Another mechanism of antibiotic tolerance is through efflux pumps which require proton motive forces or ATP as energy to exchange the antibiotics through the bacterial membrane. Five classes

of efflux pumps are classified based on their features[18]: small multidrug resistance family (SMR), resistance-nodulation-division (RND) family, major facilitator superfamily (MFS), ATP-binding cassette family and multidrug (ABC) and multi antimicrobial extrusion protein family (MATE). MFS is mostly found in Gram positive bacteria while RND is mostly found in Gram negative bacteria[19]. For example, there is no RND found in *Mycobacterium tuberculosis*; only one RND is found in *Bacillus subtilis*; but 4 found in *E. coli* and 12 in *P. aeruginosa*. In *P. aeruginosa*, RND family attracts more attention for its clinical significance[20]. Its 12 efflux forms 7 functional couplings: MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexGHI-OpmD, MexJK, MexXY and CzrAB-OpmN[21,22], which are responsible for pumping out different classes of antibiotics such as β-lactams, fluoroquinolones, tetracyclines and also small molecules such as acylated homoserine lactones[23], *Pseudomonas* quinolone signals[24] or metal ions[25].

1.2.2.4 Enzymes

In addition to efflux pumps, bacteria also have the strategy to degrade antibiotics for survival. Production of enzymes to degrade antibiotics is one of the methods. The best-known antibiotic-degrading enzymes are β-lactamases, which are responsible for bacterial resistance to beta-lactam antibiotics like penicillins, cephamycins, and carbapenems[26]. These antibiotics share a common four-atom ring which could be broken down by β-lactamase[26]. In *P. aeruginosa*, β-lactamase AmpC enzyme challenges the usage of expanded-spectrum cephalosporins (β-lactam antibiotic) which now is a well-spread gene in different species such as *K. pneumoniae*, *Salmonella spp*. and *P. mirabilis*. Besides β-lactamase, kanamycin phosphotransferase has also been discovered in *P. aeruginosa* chromosomal DNA.

1.2.2.5 Persister cells with unknown mechanisms

Besides the above mechanisms, bacteria can also survive from antibiotic treatment by forming metabolically inactive cells, known as persister cells[7]. Such intrinsic tolerance also facilitates the development of multi-drug resistance through acquired mechanisms, presenting a great challenge to infection control [27]. Since this project is mostly focused on persister cells, more detailed information about its formation and control will be described in a separated section.

1.3 Intrinsic tolerance based on multicellular behaviors

Besides the tolerance through the above mechanisms in individual cells, multicellular bacterial communities are also important to chronic infections and the fertilities of acquired antibiotic resistance[28].

Biofilms are complex microbial communities that function as a cooperative consortium, offer efficient nutrients supply and protect the cells from adverse environment factors[28]. Inside biofilms, there are genotypic and phenotypic variants, dispersal cells and dead cells, as well as extracellular DNA (eDNA), polysaccharide, fatty acid, amyloid fibre, filamentous and phage. All of these components could help to maintain the structure of biofilms (mushroom-like, tower-like or wrinkle-like structure) and facilitated the development of biofilms[28]. Biofilms develop through several stages: first, free swimming bacteria reversibly attach to a surface and subsequently form small cell clusters. Then polysaccharides are produced to form mature biofilms with three-dimensional structures. Biofilm can also disperse from the surface by certain signal or enzymes and other factors[28]. Instead of a single cell or a group of identical individual cells, biofilms are the most common mode of bacterial life in natural, medical and engineered systems.

In disease conditions, biofilms offer bacteria protection from antibiotics and toxins or immune factors of the host. Thus, biofilms are considered a major cause for chronic bacterial infection.

Both Gram-positive (e.g. *Staphylococcus epidermis*) and Gram-negative (e.g. *P. aeruginosa, Vibrio cholerae, and E. coli*) bacteria are well known to form biofilms[29]. Biofilm formation is estimated to be involved in 80% of human infections[30] with up to 1000 times higher tolerance to antibiotics than planktonic counterparts. The persister cells embedded in biofilms also play an important role in chronic infections with high mortality and morbidity[31], e.g., chronic lung infections in cystic fibrosis patients [32].

1.3.1 Bacterial quorum sensing systems

Since the discovery in 1960s [33], a cell-cell signaling system known as quorum sensing (QS) has been identified in numerous microbial species [34]. During cell growth of those species, small signaling molecules are synthesized and secreted into the surrounding environment. As a result, the local QS signal concentration increases with cell density. When the cell density is above a certain threshold, a significant amount of signaling molecules [35] bind to intracellular or membrane receptors and trigger the expression of a series of genes to control a wide spectrum of phenotypes. The ability to synchronize cellular activities including pathogenesis by QS is beneficial, and in some cases crucial, for microbes to survive in challenging environments and establish successful infections. The important roles that QS plays in bacterial pathogenesis also provides an opportunity to control infections with alternative mechanisms rather than using antibiotics alone to inhibit growth, which has been seriously compromised by the rapid development and spread of multidrug resistant microbes [36].

Conceivably, for a QS system to work the cells must be able to synthesize the signaling molecules, detect the signals through specific molecular interactions, and activate or inactivate the target genes by responding to the specific signal-receptor binding events. Interruption of any of these steps could potentially result in repression of virulence and failure for the microbes to

infect a host (Figure 1-1). During the last two decades, a number of natural and synthetic agents have been demonstrated to control QS, which can be categorized as nonpeptide small molecules, peptides and enzymes including antibodies. Most of these agents inhibit QS and associated pathogenesis at concentrations non-inhibitory to microbial growth, providing an alternative approach to controlling microbial infections.

In Gram-negative bacteria, QS systems based on acyl-homoserine lactones (acyl-HSLs or AHLs) are commonly found. In general, each AHL signal is generated by a synthase (called I protein) and detected by a specific autoinducer receptor (called R protein). For example, *P. aeruginosa* produces and responds to two AHL signals including *N*-(3-oxododecanoyl)-HSL (OdDHL) (synthesized by LasI and detected by LasR [37,38]) and *N*-butyryl-HSL (BHL) (synthesized by RhlI and detected by RhlR [39]). In *Vibrio fischeri,* three QS signals have been identified including 3-oxo-hexanoyl-HSL (produced by LuxI and detected by LuxR [40]), *N*-hexanoyl-L-HSL (LuxI is required for its synthesis [41]), and *N*-octanoyl-HSL (produced by AinS and responded by LuxR [42,43]). The specific binding of a QS signal to its receptor is critical for the regulation of gene expression. For example, the binding of *N*-3-oxo-C_8-HSL to the TraR of *A. tumefaciens* has been shown to induce the dimerization of TraR [44], which then enhances the stability of TraR and promotes its binding to the promoters of QS-controlled genes [45,46].

All AHLs have a HSL ring, with the length of the acyl side chain varying from 4 to 18 carbon atoms (Table 1). As demonstrated for *P. aeruginosa* [47] and *V. fischeri* [48], short-chain signals such as BHL could diffuse freely across the cell membrane. In contrast, signals with a long side chain such as OdDHL of *P. aeruginosa* [49] need to be exported by efflux pumps [50].

In addition to AHLs, there is another class of autoinducers in Gram-negative bacteria known as 4-quinolones [51,52]. The best studied one is 2-heptyl-3-hydroxy-4-quinolone in *Pseudomonas* spp., and is therefore named *Pseudomonas* quinolone signal (PQS) [53] PQS is a hydrophobic compound and requires membrane vesicles to transfer it out of the cell after synthesis [54]. In *P. aeruginosa*, PQS controls the expression of virulence determinants. The synthesis and activity of PQS are subject to the regulation of Las and Rhl QS systems [55-57]. Analogues of PQS have also been found in other bacteria; e.g., Diggle *et al* [58] reported that *Burkholderia pseudomallei*, *B. thailandensis*, *B. cenocepacia*, and *P. putida* produce 2-heptyl-4(1H)-quinolone (HHQ, Table 1) as a QS signal.

Besides AHLs, there are also other systems developed for both intra- and inter-species QS. AI-2, derived from S-4,5-dihydroxy-2,3-pentanedione (DPD) with the activity of LuxS, is probably the most conserved QS signal in bacteria [59,60]. Since the discovery of this system in *V. harveyi* [61], homologs of *luxS* gene have been found in more than 250 bacterial genomes [62]. *LuxS* plays a critical role in AI-2-mediated QS in *E. coli*, *Salmonella typhimurium*, and *V. harveyi* [63,64]. It catalyzes the formation of DPD, which is then converted to (2*S*,4*S*)-2,4-dihydroxy-2-methyldihydrofuran-3-one (S-DHMF) and eventually AI-2 molecules [59].

AI-2 has been demonstrated as an interspecies signal for communication among both Gram-negative and Gram-positive species [65]. For example, Xavier et al. [66] reported that the wild-type *E. coli* is able to uptake AI-2 generated by *V. harveyi*, and thereby reduce the QS-controlled bioluminescence in *V. harveyi.*

In addition to AI-2, some other signals were also found to function in multiple species. For example, *cis*-11-methyl-2-dodecenoic acid (DSF, Table 1, [67]), was found to control flagellum

synthesis, aerobic respiration and resistance to toxins and oxidative stress in *Xanthomonas campestris* [68], as well as the factors involved in virulence and antibiotic resistance in *Stenotrophomonas maltophilia* [69].

1.3.2 Quorum sensing and biofilm formation

Because of the high cell density and genotypic and phenotypic diversity in biofilms, it is conceivable that a certain signaling system is required for the biofilm to be maintained chronologically and structurally. Indeed, QS controls biofilm formation in many bacterial species. QS could take place in these confined bacterial communities and has been an important focus in biofilm study for at least two decades. For example, it was found that grown in a flow chamber, *P. aeruginosa* biofilm contains significantly higher OdDHL concentration (632 μM) than in the effluent (14nM) [70]. Consistently, it is reported that in *P. aeruginosa*, the *lasI* mutant, which is incapable of synthesizing the QS signal AHL, lost the mushroom-like biofilm structure and formed a flat and undifferentiated biofilm. In addition, this flat biofilm couldn't protect the cells from sodium dodecyl sulphate (SDS) treatment as compared to the wild-type strain[71]. Although QS was shown to be a promising target for biofilm control, there is also evidence showing that the AHLs can function in biofilm life-cycle to disperse mature biofilm. For example, in a study by Schooling et al[72], rhamnolipid, a biosurfactant regulated by QS, was detected in the culture of *P. aeruginosa* PAO1 (wild-type) but not in its isogenic homoserine lactone (HSL) mutant *P. aeruginosa* PAO-JP2. It was also demonstrated that addition of rhamnolipid into a subculture of PAO1 reduced its biofilm formation[72]. Further, the addition of HSL into both PAO1 and PAO-JP2 cultures elevated the rhamnolipid level. Therefore, AHL was considered as a potential factor to disperse biofilm of *P. aeruginosa*. For Gram positive bacteria, *S. aureus* biofilm was found to be dispersed by Agr, a QS signal. For example, higher *agr* activities were observed during

biofilm dispersion and *agr* mutants showed a high propensity to form biofilms. It was observed that the level of serine protease increased during the dispersion; and the dispersed cells were found to be more sensitive to the antibiotic rifampicin.[73]

Like AHLs, AI-2 also plays a role in biofilm formation. Deletion of the *luxS* gene has been found to influence the biofilm formation of *Streptococcus gordonii* [74] and *S. mutans* [75]; and direct addition of AI-2 has been shown to induce biofilm formation of *E. coli* [76].

1.3.3 Biofilm control by QS inhibitors

As discussed above, QS relies on a sequence of events including signal production, detection and gene activation/inactivation. Interruption of any of these steps could render the QS to fail and potentially cause detrimental consequences to the survival and pathogenesis of bacteria and fungi. It is conceivable that eukaryotic organisms may have developed effective strategies to control QS during evolution. Understanding and improving such strategies with natural or synthetic molecules could potentially lead to better approaches to controlling microbial pathogens and associated infections.

1.3.3.1 Brominated Furanones

A group of potent QSIs, brominated furanones, are produced by the marine alga *Delisea pulchra* to protect it from adhesion and fouling by marine bacteria. This class of compounds has been studied extensively both for their activities and the mechanism of inhibition. The best studied furanone to date is the natural compound (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone (furanone 1, Figure 1-2).

For Gram-negative bacteria, it is reported that furanone 1 can inhibit the swarming and biofilm formation of *E. coli* at concentrations that are non-inhibitory to planktonic growth. The biofilm formed in the presence of furanone 1 was found to lack the appropriate space between cell clusters, which was thought to cause the cells to aggregate and die from starvation and accumulation of metabolic wastes [77].

Several lines of evidence suggest that furanone 1 is an inhibitor of QS based on both AHL and AI-2. For example, furanone 1 at 10 μg/mL inhibits QS via *V. harveyi* AHL by 3300-fold, and QS via *E. coli* AI-2 by 26,600-fold [77]. Recent studies at the molecular level have shown that furanone 1 can directly interact with the regulator R protein in AHL QS system [78] and the LuxS protein in AI-2 QS system[79]. Such interactions were found to increase the turnover of LuxR in *V. fischeri* [80], render the LuxR of *V. harveyi* incapable of binding to its target DNAs [78], and cause LuxS of *E. coli* to loose its activities in AI-2 synthesis[79]. The inhibition of AI-2 QS by furanone 1 is also evidenced by a DNA microarray study, in which furanone 1 was found to repress 56 genes of *E. coli* and 44 (79%) of these genes were also induced by AI-2 [81]. An autoinducer bioassay indicated that 100 μg/mL furanone 1 repressed AI-2 synthesis in *E. coli* by 2-fold. However, the expression of *luxS* was not affected by furanone 1, suggesting that furanone 1 interrupts AI-2 QS at the post-transcriptional level [81].

To improve the activities of brominated furanones and identify the important structural components, a number of synthetic furanones have also been studied and some representative furanones are shown in Figure 1-2. Among them, furanones 3, 4, 6 have been shown to inhibit QS by increasing the turnover of LuxR [80]. Consistent with the important roles of QS in biofilm formation, brominated furanones were also found to be biofilm inhibitors [82-84]. Furanone 4 can cause removal of mature *P. aeruginosa* biofilms when treating biofilms together with 0.1% SDS

[85]. Although furanone 4 has no effect on *P. aeruginosa* growth, it can increase the susceptibility of *P. aeruginosa* biofilm cells to tobramycin. This antibiotic was found to kill 85-90% of furanone-treated biofilm cells, while tobramycin alone only kill the top layers of biofilm cells. In a mouse pulmonary model, furanone 4 was also found to improve the clearance of *P. aeruginosa* infection by the mouse immune system [85].

In a recent study [84], several new regioisomers of brominated furanones (e.g., furanones 8, 9 and 10) were synthesized to compare their activities of biofilm inhibition. It was found that the conjugated exocyclic vinyl bromide on the furanone ring is the most important structural element for non-growth inhibitory reduction of *E. coli* biofilm formation. Furanone 8 has also been described in a patent for its strong inhibition of AHL-mediated QS (demonstrated using a GFP-based reporter), a 90% reduction of AI-2-mediated QS, and a 21% repression of *P. aeruginosa* biofilm formation [86].

In addition to the inhibition of AHL- and AI-2-based QS in Gram-negative bacteria without the inhibition of bacterial growth, some furanones were also shown to inhibit the growth of Gram-positive bacteria [86] and fungi [87,88]. These findings suggest that some furanones may have multiple targets of inhibition. Furanone 1 at 40 μg/ml was found to inhibit biofilm formation of *B. subtilis* by 25% and reduce the percentage of live cells by 63% [89]. Furanones were also found to inhibit the growth of *B. anthracis* (e.g., furanones 1, 2, 4 and 5 [90,91]), *S. aureus* (furanones 8 and 11 [86]) and fungal pathogen *C. albicans* (e.g., furanone 1, 8, 9 and 10 [87,88]). Surface modification with furanone 7 was found to inhibit the infection by *S. epidermidis* in a sheep model [92].

In summary, a relatively large number of brominated furanones have been described. With activities in QS inhibition [93-95], antimicrobial activities [88,96,97], biofilm inhibition [98] and

antifouling applications of brominated furanones [99,100]. Methods for synthesizing new furanone compounds [101,102] has also been developed.

1.3.3.2 HSL analogs

Specific signal-receptor binding is critical for gene regulation by QS; thus, it is possible to develop agonists and antagonists to either stimulate or repress QS by altering the structures of QS signals. Some analogs of N-(3-oxododecanoyl) L-HSL and butyryl L-HSL were described as biofilm inhibitors in an early patent by Davis et al. [103]. The structures of these compounds are shown in Figure 1-3, wherein R_1-R_{21} = CH_3, H, OH, NH_2, SH or C_1-C_4 alkyl group, R_{22}-R_{23} = S or O; R_{24}-R_{28} = H or a halogen. These inhibitors compete with OdDHL and BHL for binding to their receptors, and consequently block the QS and cause inhibition of biofilm formation by Gram-negative bacteria, e.g. *P. aeruginosa*.

A group of AHL analogs with a general structure represented in Figure 1-4A was also developed as QSIs, in which X= O, S, NH or CH_2; R_1, R_2, R_3 = H, C_1-C_{12} acyclic aliphatic group, etc [104]. For example, three of such compounds shown in Figure 1-4 (B, C and D) were identified by screening AHL libraries using three QS reporter strains constructed with TraR of *A. tumefaciens*, LasR of *P. aeruginosa*, and LuxR of *V. fischeri*, respectively. These compounds can significantly inhibit TraR of *A. tumefaciens* and LasR of *P. aeruginosa*, while compound 4E also inhibits LuxR of *V. fischeri*. In addition to QS, compounds 4B and 4D also exhibited strong inhibitory effects on *P. aeruginosa* biofilm formation [105,106].

Another library of HSL analogs was constructed and screened for AHL inhibitors using GFP-based reporters by Suga and coworkers [107,108]. Structures of representative inhibitors are as shown in Figure 1-5A-H and all of them can reduce QS-mediated GFP expression by at least

50%. It was found that the position of H bond acceptors, such as hydroxyl, carboxyamide or pyridyl group, is important for the inhibitory effects.

1.3.3.3 Sulfide AHL analogues

The synthetic compound *N*-(heptylsulfanylacetyl)-L-HSL (Figure 1-6) has been shown as a potential QSI[109]. It is hypothesized that when *N*-(heptylsulfanylacetyl)-HSL binds to TraR, it causes conformational changes so that the β-sheet of TraR is forced to move away from the functional position and renders the TraR unable to bind to its target DNA. As a result, activation of the target genes by QS will be blocked by *N*-(heptylsulfanylacetyl)-HSL [109].

1.3.3.4 Garlic extract

Using a *lasB-gfp* reporter, garlic extract was found to repress QS-controlled expression of *lasB* in *P. aeruginosa* in a dose-dependent manner [110]. For example, at the concentration of 2% (vol/vol), garlic extract reduced the synthesis of GFP by 2-fold without inhibiting the growth of the reporter strain, indicating that repression of *lasB* was by disruption of QS. Interestingly, it was also found that nearly all of *P. aeruginosa* biofilm cells were killed when treated with 1% garlic extract and 340 μg/ml tobramycin simultaneously, while 340 μg/ml tobramycin alone only killed the cells in a thin top layer. Consistent with this finding, the garlic extract was found to cause changes in the architecture of *P. aeruginosa* biofilms. Compared to the heterogeneous biofilms with typical mushroom-like structures, *P. aeruginosa* formed flat and undifferentiated biofilms in the presence of 1% garlic extract. Such structural changes may help explain the enhanced susceptibility of biofilm cells to tobramycin [110]. Some QSIs from garlic extract are shown in Figure 1-7. [109]. These compounds were demonstrated to interrupt LuxR-based QS at the concentrations non-inhibitory to bacterial growth.

1.3.3.5 Other AI-1 inhibitors

In addition to the classes of AHL inhibitors described above, butyrolactone and acetyl-butyrolactone (Figure 1-8) have also been found to repress AHL-mediated QS with no inhibition of *P. aeruginosa* growth [111].

1.3.3.6 Inhibitors of PQS

A recent patent [112] described the activities of some analogs of the autoinducer 2-heptyl-3-hydroxy-4-quinolone, which belongs to the family of 4-quinolone signals. These analogs are hypothesized to inhibit the PQS QS circuit and the binding of the autoinducer molecules to LasR and/or RhlR in *P. aeruginosa*.

1.3.3.7 Analogs of AI-2

A number of AI-2 analogs have been described (Figure 1-9A-I) [113-116]. Consistent with the roles of AI-2 in QS and bacterial pathogenesis, these AI-2 inhibitors have potential applications in control of bacterial infections and biofilm formation. Tedder et al [114] reported that some purine and deazapurine-based AI-2 inhibitors can interact with the MTA nucleosidase, an important enzyme in AI-2 synthesis. These inhibitors, e.g., compounds B and C in Figure 1-9, can bind to the MTA nucleosidase tightly and potentially inhibit AI-2 synthesis.

Some AI-2 analogs with structures shown in Figure 1-9D-I were described in a recent patent [115]. These analogs have been found to interrupt QS based on AI-2 using a *V. harveyi* luminescence assay. The C_2 and C_3 groups of these compounds appeared to play an important role in the activities of QS inhibition. In addition to the above analogs, some boronic acids [117] and DPD analogs [118-121] have also been shown as AI-2 antagonists.

1.4 Persister cells and control.

Bacteria are well known to obtain intrinsic tolerance to antibiotics by forming metabolically inactive cells, known as persister cells[7]. Such intrinsic tolerance also facilitates the development of multi-drug resistance through acquired mechanisms, presenting a great challenge to infection control [27]. Persister formation increases when a culture enters stationary phase. However, the effects of QS on persister formation have been controversial[122]. A piece of evidence suggests that persister formation is influenced by QS was reported by Moker et al [122]. In their study, *P. aeruginosa* cultures contained larger numbers of persister cells in presence of the QS signal 3-oxo-C_{12}-HSL and the pigment PYO (secreted in stationary phase). These signals increased persister formation significantly in logarithmic *P. aeruginosa* PAO1 cultures but not in *E. coli* or *S. aureus* cultures. However, there is also evidence supporting the opposite. Kim Lewis[7] found that the persister level remains the same with or without addition of spent medium to an early exponential culture of *E. coli* [7]. Dörr et al[123] suggested that TisA/TisB, a toxin/antitoxin (TA) module, plays a critical role in persister formation in *E.coli*. In this SOS-TisB response system, reactive oxygen species (ROS) triggers SOS response which induces over expression of the TisB protein. The TisB disrupts the protein motive force, leading to decreased ATP level and increased persister formation. Although adding spent medium not induce persister formation in *E. coli*, there is evidence that QS may at least indirectly affect persister formation.

1.4.1 Persister control

A number of different factors have to be found to promote persister formation, such as toxic metal ions, oxidants, poor nutrition, high temperature, low pH, and membrane acting agents[7,124]. Thus, persister formation may be a strategy for cells to survive in adverse environments and

ensure the survival of the population from being eradicated by severe environmental conditions. In a study by Collins and colleges[125-127]_ENREF_126, it was demonstrated that addition of antibiotics induced cell death by a common mechanism of oxidative stress that relies on the production of ROS by activating cellular respiration and releasing iron (Fenton reaction). Challenged by ROS, bacteria induce the synthesis of antioxidant enzymes such as superoxide dismutase and catalase or small molecules such as ascorbic acid and glutathione or nitric oxide to reduce ROS[128]. Besides, H_2S was found to enhance the tolerance to ROS by suppressing the Fenton reaction and elevating SOD and catalase production[129]. In addition to oxidative compounds, ROS could be also generated from the nutrient stress in bacteria to form persister cells[128]. According to Collins, persister formation is a result of the balance of ROS and antioxidants. Thus, the study of ROS may offer new strategies for persister control.

1.4.2 Potential methods to reduce persisters

Increasing number of antibiotic resistant strains has been reported, indicating that antibiotics may not be an ideal approach for infection control[8]. Since persister cells are highly tolerant to antibiotics, new methods to enhance the antibiotic or to reduce the persistence may offer good opportunity to combat bacterial infections. The current factors reported to reduce the persister level are resuscitation-promoting factor[130], sugar[131], O_2[132].

1.4.2.1 Resuscitation promoting factor

The discovery[130] of resuscitation promoting factor (RPF) was considered as a major breakthrough in the study of dormancy state in Gram positive bacteria. RPF, a 16–17 kDa protein, was reported to promote the growth of dormant cells of *Micrococcus luteus* by 100 times and stimulate the growth of several other high CG Gram-positive strains such as *Mycobacterium*

avium, *Mycobacterium bovis* (BCG), *Mycobacterium kansasii*, *Mycobacterium smegmatis*, and *Mycobacterium tuberculosis*. Mycobacteria were also found to have multiple proteins which contain a highly conserved RPF domain and this conserved domain has high-level structural similarity to lysozyme and soluble lytic transglycosylases. It is consistent with the fact that the RPF cleaves peptidoglycan. In 2008, it is reported[133] that a eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments and the peptidoglycan fragments are potential germinants in *Bacillus subtilis* to wake up the dormant spores. Besides Mycobacteria, the homologues of RPF were also found or predicted to exist in other Gram positive species, for example, there are clear homologues of the Rpf domain in *Streptomyces* and *Corynebacteria*. Deletion of the two *rpf* homologues from *Corynebacteria* did not change the phenotypical properties. However, the lag phase in mutant was prolonged[134].

1.4.2.2 Sugar

Sugar is considered as an important carbon resource for growth. Recently reported, glucose, mannitol, fructose and pyruvate enabled the killing of *E. coli* and *S. aureus* persisters with aminoglycosides in both aerobic and anaerobic conditions[131]. For example, survival of persisters was reduced by 99.9% after 2-h treatment with gentamicin and fructose. It was shown that above sugars or pyruvate could generate a proton-motive force (PMF) facilitating uptake of aminoglycoside; however, they couldn't enhance the killing of persister cells by other classes of antibiotics, such as fluoroquinolone or β-lactam. For example, the persister level after 4 h treatment with mannitol and ampicillin or ofloxacin didn't change compared with the none-sugar treatment, however, persister level after treatment with mannitol and gentamicin were reduced by more than 99.9% after 4 h treatment. Interestingly, it was reported in our study that glucose and mannitol at 10 mM couldn't reduce the persister level during *P. aeruginosa* PAO1 growth, and it

is possible that LB medium offered enough metabolic stimuli to persister cells compared with addition of glucose or mannitol (10mM)[135]. However, tested in other conditions, for example, shown by an in vivo study, sugars enhanced the treatment of chronic infections in a mouse urinary tract infection model. Allison's study[131] on metabolic stimuli establishes a strategy to eradicate bacterial persisters based on metabolism pathway, and highlights the importance of PMF in antibiotic tolerance study which may link to different factors with influence on persister level, such as pH value.

1.4.2.3 Oxygen

The study of persister control by synergy between sugar and aminogluocse indicates a connection between NADH and persister level[131]. Consistently, in a study on dissolved oxygen and persister levels, NADH was found to be involved[132]. In the study by Grant et al[132], it is found that reduction in dissolved oxygen (DO) allows persister cells to survive from antibiotic treatment; while with high levels of DO, all cells can be eradicated by antibiotic treatment. The effects of DO on persister level is consistent with a study by Collins et al[127] that bactericidal antibiotics induce cell death through the production of ROS. In another study by Grant et al, it was demonstrated that high DO levels enhance the killing of persister cells due to high concentrations of ROS; while low DO facilitates persister survival. It was also found that in *M. smegmatis* cultures, persister cells treated with high concentrations of DO could be rescued from antibiotic treatment by adding thiourea, a common reducing agent for peroxides. Consistently, clofazimine, an antibiotic that can increase ROS level via an NADH-dependent redox cycling pathway, can also reduce the persister level in *M. tuberculosis*. The above study[132] showed the promise of ROS in persister control. However, ROS at high level could also damage mammalian cells. In 1950's, certain "prodrugs"[136] were discovered to treat bacterial infection by ROS. These

compounds were harmless outside bacterial cells; however, when they entered the bacterial cells, an enzyme converted them into a toxic form with the similar properties as ROS[8]. In 1960's, due to the lack of study on the target of those prodrugs[8], they were not approved to treat bacterial infection in vivo. Given the increase in biofilm-persister-related chronic infections, prodrugs or the concept of prodrugs might worth further studies[8].

References

(1) Oliveira, D. C.; Tomasz, A.; de Lencastre, H. *The Lancet infectious diseases* **2002**, *2*, 180.

(2) BioMerieux In *PR Newswire* 2012 PR Newswire Association LLC: 2012.

(3) Costelloe, C.; Metcalfe, C.; Lovering, A.; Mant, D.; Hay, A. D. *BMJ* **2010**, *340*.

(4) Das, S. C.; Bhadra, S.; Roy, S.; Saha, S. K.; Islam, M. S.; Bachar, S. C. *Biological* **2012**, 131.

(5) Antibiotic resistance: Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. [Online Early Access]. Published Online: 2012.

(6) Hancock, R. E. W. *Clinical Infectious Diseases* **1998**, *27*, S93.

(7) Lewis, K. *Annu Rev Microbiol* **2010**, *64*, 357.

(8) Lewis, K. *Nature* **2012**, *485*, 439.

(9) Woodworth, J. R.; Nyhart, E.; Brier, G. L.; Wolny, J. D.; Black, H. *Antimicrobial agents and chemotherapy* **1992**, *36*, 318.

(10) MSU. Antimicrobial Resistance learning site for veterinary students. [Online Early Access]. Published Online: 2011.

(11) CDC. Estimating health care-associated infections and deaths in U.S. hospitals. [Online Early Access]. Published Online: 2002.

(12) Enright, M. C.; Robinson, D. A.; Randle, G.; Feil, E. J.; Grundmann, H.; Spratt, B. G. *Proceedings of the National Academy of Sciences* **2002**, *99*, 7687.

(13) Salgado, C. D.; Farr, B. M.; Calfee, D. P. *Clinical Infectious Diseases* **2003**, *36*, 131.

(14) Ubukata, K.; Nonoguchi, R.; Matsuhashi, M.; Konno, M. *Journal of bacteriology* **1989**, *171*, 2882.

(15) Nikaido, H.; Hancock, R. *THE BACTERIA: A TREATISE ON STRUCTURE AND FUNCTION. 1986.* **1986**.

(16) Hancock, R. E. W.; Bell, A. *European Journal of Clinical Microbiology & Infectious Diseases* **1988**, *7*, 713.

(17) Moore, R. A.; Hancock, R. *Antimicrobial agents and chemotherapy* **1986**, *30*, 923.

(18) Lynch, A. S. *Biochemical pharmacology* **2006**, *71*, 949.

(19) Blair, J.; Piddock, L. J. V. *Current opinion in microbiology* **2009**, *12*, 512.

(20) Schweizer, H. P. *Genet Mol Res* **2003**, *2*, 48.

(21) Lomovskaya, O.; Warren, M. S.; Lee, A.; Galazzo, J.; Fronko, R.; Lee, M.; Blais, J.; Cho, D.; Chamberland, S.; Renau, T. *Antimicrobial agents and chemotherapy* **2001**, *45*, 105.

(22) Poole, K. *Journal of Antimicrobial Chemotherapy* **2005**, *56*, 20.

(23) Minagawa, S.; Inami, H.; Kato, T.; Sawada, S.; Yasuki, T.; Miyairi, S.; Horikawa, M.; Okuda, J.; Gotoh, N. *BMC microbiology* **2012**, *12*, 70.

(24) Köhler, T.; Van Delden, C.; Curty, L. K.; Hamzehpour, M. M.; Pechere, J. C. *Journal of bacteriology* **2001**, *183*, 5213.

(25) Stähler, F. N.; Odenbreit, S.; Haas, R.; Wilrich, J.; Van Vliet, A. H. M.; Kusters, J. G.; Kist, M.; Bereswill, S. *Infection and immunity* **2006**, *74*, 3845.

(26) Jalalpour, S.; Ebadi, A. G. *Afr. J. Pharm. Pharmacol* **2012**, *6*, 113.

(27) Lewis, K. *Nat Rev Microbiol* **2007**, *5*, 48.

(28) McDougald, D.; Rice, S. A.; Barraud, N.; Steinberg, P. D.; Kjelleberg, S. *Nature Reviews Microbiology* **2011**.

(29) Stoodley, P.; Sauer, K.; Davies, D. G.; Costerton, J. W. *Annu Rev Microbiol* **2002**, *56*, 187.

(30) Romero, R.; Schaudinn, C.; Kusanovic, J. P.; Gorur, A.; Gotsch, F.; Webster, P.; Nhan-Chang, C. L.; Erez, O.; Kim, C. J.; Espinoza, J. *Am J Obstet Gynecol* **2008**, *198*, 135.

(31) Olson, M. E.; Ceri, H.; Morck, D. W.; Buret, A. G.; Read, R. R. *Can J Veter Res* **2002**, *66*, 86.

(32) Drenkard, E.; Ausubel, F. M. *Nature* **2002**, *416*, 740.

(33) Tomasz, A. *Nature* **1965**, *208*, 155.

(34) Bassler, B. L.; Losick, R. *Cell* **2006**, *125*, 237.

(35) Miller, M. B.; Bassler, B. L. *Annu Rev Microbiol* **2001**, *55*, 165.

(36) Alekshun, M. N.; Levy, S. B. *Cell* **2007**, *128*, 1037.

(37) Pearson, J. P.; Gray, K. M.; Passador, L.; Tucker, K. D.; Eberhard, A.; Iglewski, B. H.; Greenberg, E. P. *Proc Natl Acad Sci USA* **1994**, *91*, 197.

(38) Gambello, M. J.; Iglewski, B. H. *J Bacteriol* **1991**, *173*, 3000.

(39) Pearson, J. P.; Pesci, E. C.; Iglewski, B. H. *J Bacteriol* **1997**, *179*, 5756.

(40) Dunlap, P. V. *J Mol Microbiol Biotechnol* **1999**, *1*, 5.

(41) Kuo, A.; Blough, N. V.; Dunlap, P. V. *J Bacteriol* **1994**, *176*, 7558.

(42) Lupp, C.; Ruby, E. G. *J Bacteriol* **2004**, *186*, 3873.

(43) Lupp, C.; Urbanowski, M.; Greenberg, E. P.; Ruby, E. G. *Mol Microbiol* **2003**, *50*, 319.

(44) Qin, Y.; Luo, Z. Q.; Smyth, A. J.; Gao, P.; von Bodman, S. B.; Farrand, S. K. *EMBO J* **2000**, *19*, 5212.

(45) Zhu, J.; Winans, S. C. *Proc Natl Acad Sci USA* **2001**, *98*, 1507.

(46) Vannini, A.; Volpari, C.; Gargioli, C.; Muraglia, E.; Cortese, R.; De Francesco, R.; Neddermann, P.; Di Marco, S. *EMBO J* **2002**, *21*, 4393.

(47) Pearson, J. P.; van Delden, C.; Iglewski, B. H. *J Bacteriol* **1999**, *181*, 1203.

(48) Kaplan, H. B.; Greenberg, E. P. *J Bacteriol* **1985**, *163*, 1210.

(49) Thomas, G. L.; Böhner, C. M.; Williams, H. E.; Walsh, C. M.; Ladlow, M.; Welch, M.; Bryant, C. E.; Spring, D. R. *Mol BioSys* **2006**, *2*, 132.

(50) Evans, K.; Passador, L.; Srikumar, R.; Tsang, E.; Nezezon, J.; Poole, K. *J Bacteriol* **1998**, *180*, 5443.

(51) Déziel, E.; Lépine, F.; Milot, S.; He, J.; Mindrinos, M. N.; Tompkins, R. G.; Rahme, L. G. *Proc Natl Acad Sci USA* **2004**, *101*, 1339.

(52) Diggle, S. P.; Matthijs, S.; Wright, V. J.; Fletcher, M. P.; Chhabra, S. R.; Lamont, I. L.; Kong, X.; Hider, R. C.; Cornelis, P.; Cámara, M. *Chem Bio* **2007**, *14*, 87.

(53) Pesci, E. C.; Milbank, J. B. J.; Pearson, J. P.; McKnight, S.; Kende, A. S.; Greenberg, E. P.; Iglewski, B. H. *Biochemistry* **1999**, *96*, 11229.

(54) Mashburn, L. M.; Whiteley, M. *Nature* **2005**, *437*, 422.

(55) Diggle, S. P.; Winzer, K.; Chhabra, S. R.; Worrall, K. E.; Camara, M.; Williams, P. *Mol Microbiol* **2003**, *50*, 29.

(56) McKnight, S. L.; Iglewski, B. H.; Pesci, E. C. *J Bacteriol* **2000**, *182*, 2702.

(57) Wade, D. S.; Calfee, M. W.; Rocha, E. R.; Ling, E. A.; Engstrom, E.; Coleman, J. P.; Pesci, E. C. *J Bacteriol* **2005**, *187*, 4372.

(58) Diggle, S. P.; Lumjiaktase, P.; Dipilato, F.; Winzer, K.; Kunakorn, M.; Barrett, D. A.; Chhabra, S. R.; Cámara, M.; Williams, P. *Chem Bio* **2006**, *13*, 701.

(59) Tavender, T. J.; Halliday, N. M.; Hardie, K. R.; Winzer, K. *BMC Microbiol* **2008**, *8*, 98.

(60) Rader, B. A.; Campagna, S. R.; Semmelhack, M. F.; Bassler, B. L.; Guillemin, K. *J Bacteriol* **2007**, *189*, 6109.

(61) Bassler, B. L.; Wright, M.; Showalter, R. E.; Silverman, M. R. *Mol Microbiol* **1993**, *9*, 773.

(62) Vendeville, A.; Winzer, K.; Heurlier, K.; Tang, C. M.; Hardie, K. R. *Nat. Rev. Microbiol.* **2005**, *3*, 383.

(63) Surette, M. G.; Miller, M. B.; Bassler, B. L. *Proc Natl Acad Sci USA* **1999**, *96*, 1639.

(64) Taga, M. E.; Semmelhack, J. L.; Bassler, B. L. *Mol Microbiol* **2001**, *42*, 777.

(65) Schauder, S.; Shokat, K.; Surette, M. G.; Bassler, B. L. *Mol Microbiol* **2001**, *41*, 463.

(66) Xavier, K. B.; Bassler, B. L. *Nature* **2005**, *437*, 750.

(67) Wang, L. H.; He, Y.; Gao, Y.; Wu, J. E.; Dong, Y. H.; He, C.; Wang, S. X.; Weng, L. X.; Xu, J. L.; Tay, L. *Mol Microbiol* **2004**, *51*, 903.

(68) He, Y. W.; Xu, M.; Lin, K.; Ng, Y. J. A.; Wen, C. M.; Wang, L. H.; Liu, Z. D.; Zhang, H. B.; Dong, Y. H.; Dow, J. M. *Mol Microbiol* **2006**, *59*, 610.

(69) Fouhy, Y.; Scanlon, K.; Schouest, K.; Spillane, C.; Crossman, L.; Avison, M. B.; Ryan, R. P.; Dow, J. M. *J Bacteriol* **2007**, *189*, 4964.

(70) Charlton, T. S.; de Nys, R.; Netting, A.; Kumar, N.; Hentzer, M.; Givskov, M.; Kjelleberg, S. *Environ Microbiol* **2000**, *2*, 530.

(71) Davies, D. G.; Parsek, M. R.; Pearson, J. P.; Iglewski, B. H.; Costerton, J. W.; Greenberg, E. P. *Science* **1998**, *280*, 295.

(72) Schooling, S.; Charaf, U.; Allison, D.; Gilbert, P. *Biofilms* **2004**, *1*, 91.

(73) Boles, B. R.; Horswill, A. R. *PLoS pathogens* **2008**, *4*, e1000052.

(74) Blehert, D. S.; Palmer, R. J.; Xavier, J. B.; Almeida, J. S.; Kolenbrander, P. E. *J Bacteriol* **2003**, *185*, 4851.

(75) Merritt, J.; Qi, F.; Goodman, S. D.; Anderson, M. H.; Shi, W. *Infect Imm* **2003**, *71*, 1972.

(76) González Barrios, A. F.; Zuo, R.; Hashimoto, Y.; Yang, L.; Bentley, W. E.; Wood, T. K. *J Bacteriol* **2006**, *188*, 305.

(77) Ren, D.; Sims, J. J.; Wood, T. K. *Environ Microbiol* **2001**, *3*, 731.

(78) Defoirdt, T.; Miyamoto, C. M.; Wood, T. K.; Meighen, E. A.; Sorgeloos, P.; Verstraete, W.; Bossier, P. *Environ Microbiol* **2007**, *9*, 2486.

(79) Zang, T.; Lee, B. W.; Cannon, L. M.; Ritter, K. A.; Dai, S.; Ren, D.; Wood, T. K.; Zhou, Z. S. *Bioorganic & medicinal chemistry letters* **2009**, *19*, 6200.

(80) Manefield, M.; Rasmussen, T. B.; Henzter, M.; Andersen, J. B.; Steinberg, P.; Kjelleberg, S.; Givskov, M. *Microbiol* **2002**, *148*, 1119.

(81) Ren, D.; Bedzyk, L. A.; Ye, R. W.; Thomas, S. M.; Wood, T. K. *Biotech Bioeng* **2004**, *88*, 630.

(82) Janssens, J. C. A.; Steenackers, H.; Robijns, S.; Gellens, E.; Levin, J.; Zhao, H.; Hermans, K.; De Coster, D.; Verhoeven, T. L.; Marchal, K. *Appl Environ Microbiol* **2008**, *74*, 6639.

(83) Rice, S. A.; McDougald, D.; Kumar, N.; Kjelleberg, S. *Curr Opin Investig Drugs* **2005**, *6*, 178.

(84) Han, Y.; Hou, S.; Simon, K. A.; Ren, D.; Luk, Y. Y. *Bioorg Med Chem Lett* **2008**, *18*, 1006.

(85) Hentzer, M.; Wu, H.; Andersen, J. B.; Riedel, K.; Rasmussen, T. B.; Bagge, N.; Kumar, N.; Schembri, M. A.; Song, Z.; Kristoffersen, P. *EMBO J* **2003**, *22*, 3803.

(86) Read, R. W.; Kumar, N. 2001; Vol. AU2001267155B2.

(87) Hölmstrom, G., Pia, Carola; Kjelleberg, S. 2001; Vol. WO 01/68091 A1.

(88) Ren, D.; Luk, Y.-Y. 2008; Vol. 61/114,265.

(89) Ren, D.; Sins, J. J.; Wood, T. K. *Lett Appl Microbiol* **2002**, *34*, 293.

(90) Jones, M. B.; Jani, R.; Ren, D.; Wood, T. K.; Blaser, M. J. *J Infect Dis* **2005**, *191*, 1881.

(91) Jones, M. B.; Blaser, M. J.; Wood, T.; Ren, D. 2004; Vol. US7365184.

(92) Hume, E. B. H.; Baveja, J.; Muir, B.; Schubert, T. L.; Kumar, N.; Kjelleberg, S.; Griesser, H. J.; Thissen, H.; Read, R.; Poole-Warren, L. A. *Biomaterials* **2004**, *25*, 5023.

(93) Kjelleberg, S.; Steinberg, P. D.; De Nys, P. C.; Maximilien, R.; Manefield, M.; Givskov, M.; Gram, L. 1998; Vol. US6555356.

(94) Kjelleberg, S.; Steinberg, P.; Nys, P. C. d.; Maximilien, R.; Manefield, M.; Givskov, M.; Gram, L. 1996; Vol. WO96/29392 A1.

(95) Kjellberg, S.; Rice, S.; McDougald, D. 2001; Vol. WO 2002/047681 A1.

(96) Kjelleberg, S.; Steinberg, P., David; Holmstrom, C.; Back, A. 1999; Vol. WO 99/53915 A1.

(97) Hölmstrom, G., Pia, Carola ; Kjelleberg, S. 2001; Vol. WO 2001/068090 A1.

(98) England, D.; Kjelleberg, S. 2000; Vol. WO 2001/043739 A1.

(99) De Nys, P., Canisius; Steinberg, P.; Charlton, T., Stuart; Christov, V. 1998; Vol. WO 1999/005227 A1.

(100) STEINBERG, P., David; DE NYS, P., Canisius 1995; Vol. WO 1996/001294 A1.

(101) Luk, Y.-Y.; Simon, K. A.; Ren, D. 2008; Vol. 12/233,337, p 12/233.

(102) Read, R.; Kumar, N. 1999; Vol. WO 1999/054323 A1.

(103) Davies, D. G.; Costerton, J. W.; Parsek, M. R.; Greenberg, E. P.; Pearson, J. P.; Iglewski, B. H. 1998; Vol. AU 753329 B2.

(104) Blackwell, H. E.; Geske, G. D.; O'neill, J. C. 2008; Vol. US20080312319 A1.

(105) Blackwell, H. E.; Geske, G. D.; Wezeman, R. W. 2006; Vol. US 20060178430.

(106) Geske, G. D.; O'Neill, J. C.; Miller, D. M.; Mattmann, M. E.; Blackwell, H. E. *J Am Chem Soc* **2007**, *129*, 13613.

(107) Suga, H.; BU., Y. 2007; Vol. US20080027115.

(108) Suga, H.; Smith, K. M. *Curr Opin Chem Biol* **2003**, *7*, 586.

(109) Persson, T.; Hansen, T. H.; Rasmussen, T. B.; Skindersø, M. E.; Givskov, M.; Nielsen, J. *Org Biomol Chem* **2005**, *3*, 253.

(110) Rasmussen, T. B.; Bjarnsholt, T.; Skindersoe, M. E.; Hentzer, M.; Kristoffersen, P.; Kote, M.; Nielsen, J.; Eberl, L.; Givskov, M. *J Bacteriol* **2005**, *187*, 1799.

(111) Whiteley, M.; Lee, K. M.; Greenberg, P. E.; Muh, U. 2000; Vol. US6855513 B1.

(112) Pesci, E. C.; Iglewski, B. H.; Milbank, J. B. J.; Pearson, J. P.; Kende, A. S.; Greenberg, E. P. 2004; Vol. US7442798 B2.

(113) Cooper, S. R.; Yager, K. M. 2002; Vol. US7074776 B2.

(114) Tedder, M. E.; Nie, Z.; Margosiak, S.; Chu, S.; Feher, V. A.; Almassy, R.; Appelt, K.; Yager, K. M. *Bioorg Med Chem Lett* **2004**, *14*, 3165.

(115) Bassler, B. L.; Dammel, C.; Schauder, S.; Shokat, K.; Stein, J.; Surette, M. G. 2001; Vol. US6559176 B1.

(116) Semmelhack, M. F.; Campagna, S. R.; Hwa, C.; Federle, M. J.; Bassler, B. L. *Org Lett* **2004**, *6*, 2635.

(117) Ni, N.; Li, M.; Wang, J.; Wang, B. *Med Res Rev* **2009**, *29*, 65.

(118) Peng, H.; Cheng, Y.; Ni, N.; Li, M.; Choudhary, G.; Chou, H. T.; Lu, C.-D.; Tai, P. C.; Wang, B. *ChemMedChem* **2009**, *0000*, 1.

(119) Li, M.; Ni, N.; Chou, H. T.; Lu, C. D.; Tai, P. C.; Wang, B. *ChemMedChem* **2008**, *3*, 1242.

(120) Lowery, C. A.; Park, J.; Kaufmann, G. F.; Janda, K. D. *J. Am. Chem. Soc.* **2008**, *13*, 9200.

(121) Ganin, H.; Tang, X.; Meijler, M. M. M. *Bioorg Med Chem Lett* **2009**, *19*, 3941.

(122) Moker, N.; Dean, C. R.; Tao, J. *J Bacteriol* **2010**, *192*, 1946.

(123) Dörr, T.; Vulić, M.; Lewis, K. *PLoS Biol* **2010**, *8*, e1000317.

(124) Harrison, J. J.; Ceri, H.; Turner, R. J. *Nat Rev Microbiol* **2007**, *5*, 928.

(125) Kohanski, M. A.; Dwyer, D. J.; Hayete, B.; Lawrence, C. A.; Collins, J. J. *Cell* **2007**, *130*, 797.

(126) Lee, H. H.; Collins, J. J. *Nature Chemical Biology* **2011**, *8*, 6.

(127) Belenky, P.; Collins, J. J. *Science Signalling* **2011**, *334*, 915.

(128) Nguyen, D.; Joshi-Datar, A.; Lepine, F.; Bauerle, E.; Olakanmi, O.; Beer, K.; McKay, G.; Siehnel, R.; Schafhauser, J.; Wang, Y. *Science Signalling* **2011**, *334*, 982.

(129) Shatalin, K.; Shatalina, E.; Mironov, A.; Nudler, E. *Science Signalling* **2011**, *334*, 986.

(130) Mukamolova, G. V.; Kaprelyants, A. S.; Young, D. I.; Young, M.; Kell, D. B. *P Natl Acad Sci USA* **1998**, *95*, 8916.

(131) Allison, K. R.; Brynildsen, M. P.; Collins, J. J. *Nature* **2011**, *473*, 216.

(132) Grant, S. S.; Kaufmann, B. B.; Chand, N. S.; Haseley, N.; Hung, D. T. *Proceedings of the National Academy of Sciences* **2012**, *109*, 12147.

(133) Shah, I. M.; Laaberki, M. H.; Popham, D. L.; Dworkin, J. *Cell* **2008**, *135*, 486.

(134) Ravagnani, A.; Finan, C. L.; Young, M. *BMC genomics* **2005**, *6*, 39.

(135) Pan, J.; Bahar, A. A.; Syed, H.; Ren, D. *PLoS One* **2012**, *7*, e45778.

(136) Stella, V. J. *Prodrugs: Challenges and Rewards, Part 1*; Springer, 2007.

(137) Smith, R. S.; Harris, S. G.; Phipps, R.; Iglewski, B. *J Bacteriol* **2002**, *184*, 1132.

(138) Williams, P. *Microbiol* **2007**, *153*, 3923.

(139) Schaefer, A. L.; Greenberg, E. P.; Oliver, C. M.; Oda, Y.; Huang, J. J.; Bittan-Banin, G.; Peres, C. M.; Schmidt, S.; Juhaszova, K.; Sufrin, J. R.; Harwood, C. S. *Nature* **2008**, *454*, 595.

(140) Chen, X.; Schauder, S.; Potier, N.; Van Dorsselaer, A.; Pelczer, I.; Bassler, B. L.; Hughson, F. M. *Nature a-z index* **2002**, *415*, 545.

(141) Bassler, B. L. *Cell* **2002**, *109*, 421.

(142) Waters, C. M.; Bassler, B. L. *Annu. Rev. Cell Dev. Biol.* **2005**, *21*, 319.

(143) Hogan, D. A. *Eukaryotic Cell* **2006**, *5*, 613.

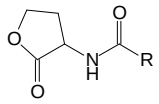
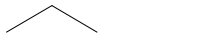
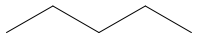
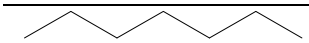
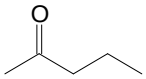
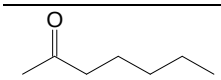
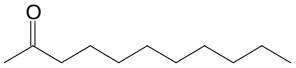
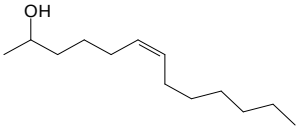
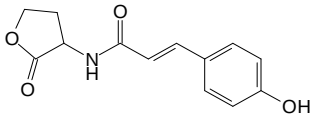
(144) Boon, C.; Deng, Y.; Wang, L. H.; He, Y.; Xu, J. L.; Fan, Y.; Pan, S. Q.; Zhang, L. H. *ISME J* **2008**, *2*, 27.

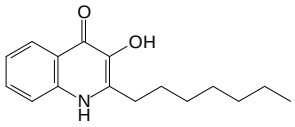
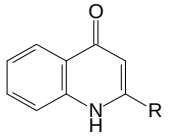
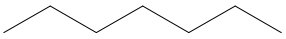
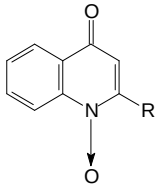
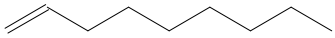
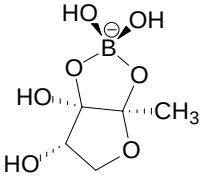
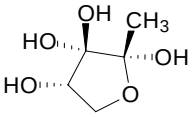
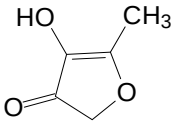
(145) Martinelli, D.; Grossmann, G.; Séquin, U.; Brandl, H.; Bachofen, R. *BMC Microbiol* **2004**, *4*, 1471.

(146) Geske, G. D.; Mattmann, M. E.; Blackwell, H. E. *Bioorg Med Chem Lett* **2008**, *18*, 5978.

(147) Suga, H.; Igarashi, J. 2007; Vol. US 2009/0068120 A1.

Table 1. Representative QS signals.

Category	Structure	Representative microbial species
AHL	 R=  N-butyryl-HSL	*P. aeruginosa* [137]
	 N-hexanoyl-HSL	*Aeromonas salmonicida* [138]
	 N-octanoyl-HSL	*Burkholderia cepacia* [138]
	 N-3-oxo-hexanoyl-HSL	*Vibrio fischeri* [40]
	 N-3-oxo-octanoyl-HSL	*A. tumefaciens* [138]
	 N-3-oxo-dodecanoyl-HSL	*P. aeruginosa* [47]
	 N-3-oxo-5-hydroxyl-C_{14}-HSL	*R. leguminosarum* [108]
p-coumaroyl-HSL	 p-coumaroyl-HSL	*R. palustris* [139]

4-quinolone	 2-heptyl-3-hydroxy-4-quinolone (PQS)	*P. aeruginosa* [53]
	 R=  2-Heptyl-4-hydroxyquinoline N-oxide(HHQ)	*Burkholderia pseudomallei* [58]
	 R=  2-(1-nonenyl)-4(1H)-quinolone	*Burkholderia pseudomallei* [58]
AI-2	 furanosyl borate diester	*V. harveyi, V. cholerae, E. coli* [140 141]
	 R-THMF	*Salmonella enterica* [59] *Salmonella typhimurium* [142]
	 MHF	*V. harveyi* [59]

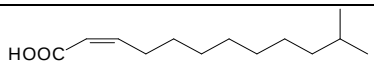
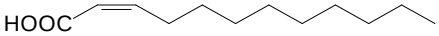
Other signals	 *cis*-11-methyl-2-dodecanoic acid (DSF)	*X. campestris* [143]
	 *cis*-2-dodecenoic acid (BDSF)	*B. cenocepacia* [144]

Figure captions

Figure 1-1. Schematic representation of the general mechanism of QS (A) and the approaches of QS inhibition (B).

Figure 1-2 Structures of representative brominated furanones [77,78,80,81,84-86,89,92,145]. (Note: different numbering systems have been used in literature, attention should be paid to the structures when referring to other publications).

Figure 1-3. General structures of the analogs of N-Butyryl-L-HSL (A) and N-(3-oxododecanoyl)-L-HSL (B).

Figure 1-4. Structures of AHL analogs including the general structure (A) and three representative compounds [105,106,146]

Figure 1-5. Representative inhibitors of AHL (A-H) [107,108] and biofilm formation (I-M) [147]identified from a library of AHL analogs.

Figure 1-6. Structure of *N*-(heptylsulfanylacetyl)-L-HSL.

Figure 1-7. Structures of quorum sensing inhibitors isolated from garlic extract.

Figure 1-8. Structures of butyrolactone (A) and acetyl-butyrolactone (B).

Figure 1-9. Structures of AI-2 analogs [113-115]. E = B, P or S for compound A [113]. R=$(CH_2)_2$c-C_5H_9 and R^1=CO_2Me or 4-pyridyl for compound B [114]

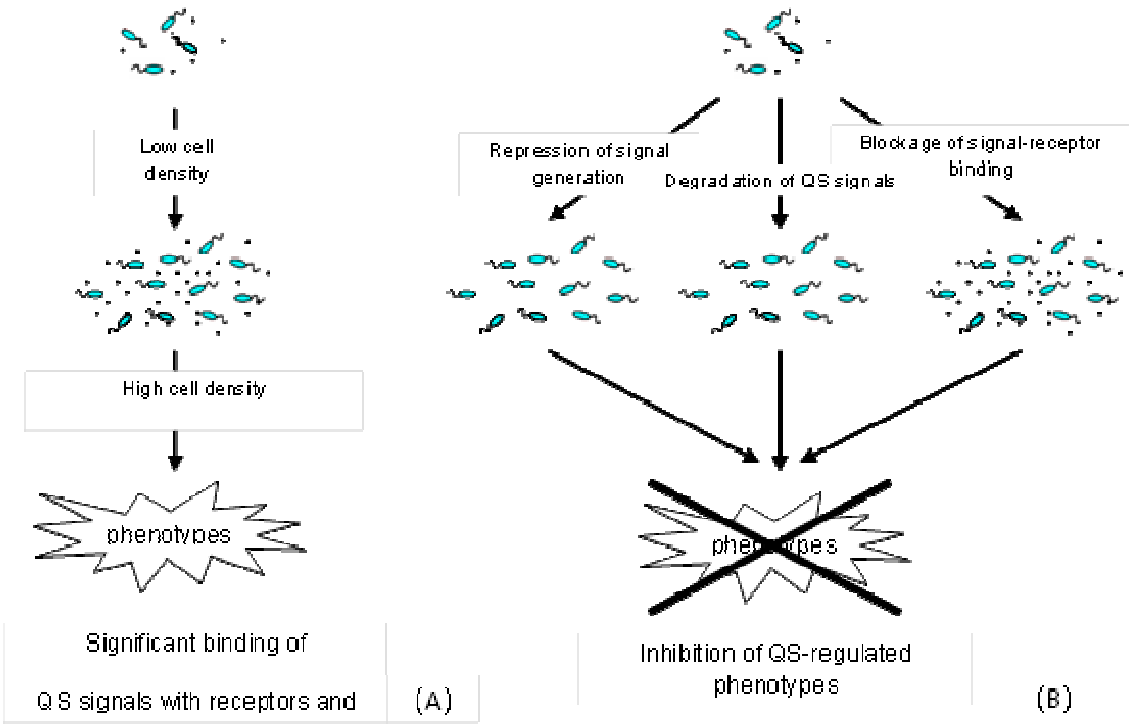
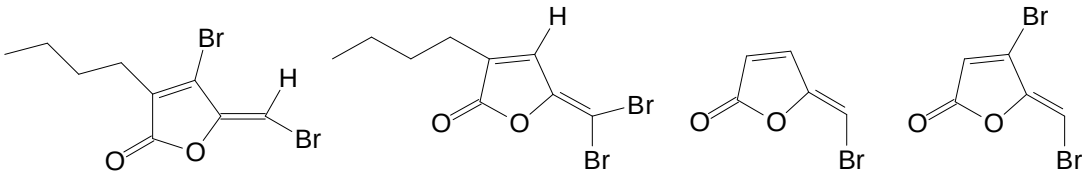
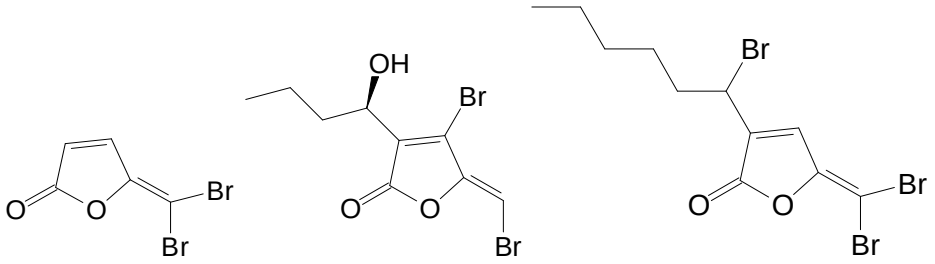

Low cell density
High cell density
phenotypes
Significant binding of QS signals with receptors and
(A)
Repression of signal generation
Degradation of QS signals
Blockage of signal-receptor binding
Inhibition of QS-regulated phenotypes
(B)

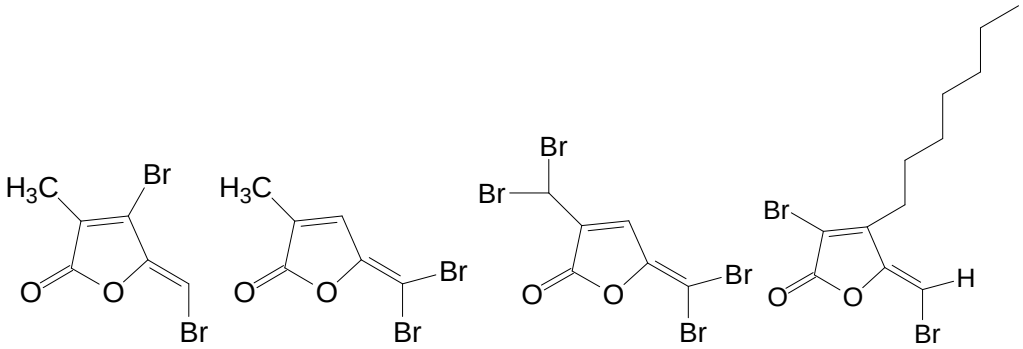
Figure 1-1



1 2 3 4

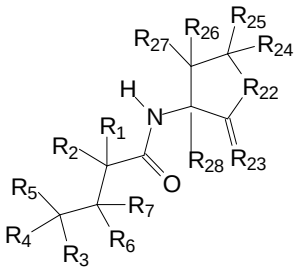


5 6 7

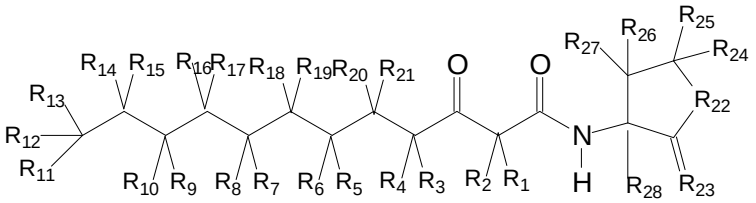


8 9 10 11

Figure 1-2

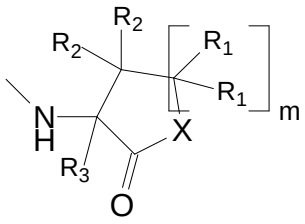

R27
R26
R25
R24
R22
H
N
R1
R2
R28
R23
O
R5
R7
R4
R3
R6

(A)

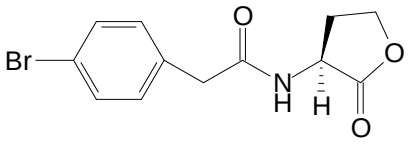

R14
R15
R16
R17
R18
R19
R20
R21
O
O
R27
R26
R25
R24
R13
R22
R12
N
R11
H
R10
R9
R8
R7
R6
R5
R4
R3
R2
R1
R28
R23

(B)

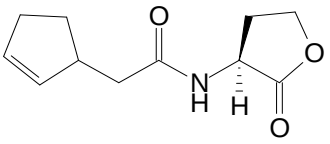
Figure 1-3



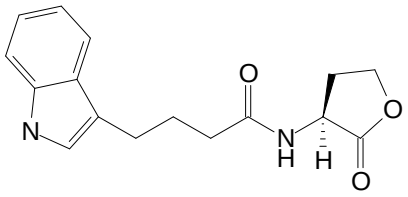
(A)



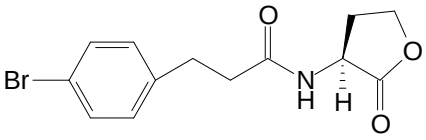
(B)



(C)

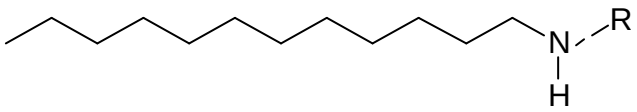


(D)



(E)

Figure 1-4



R=

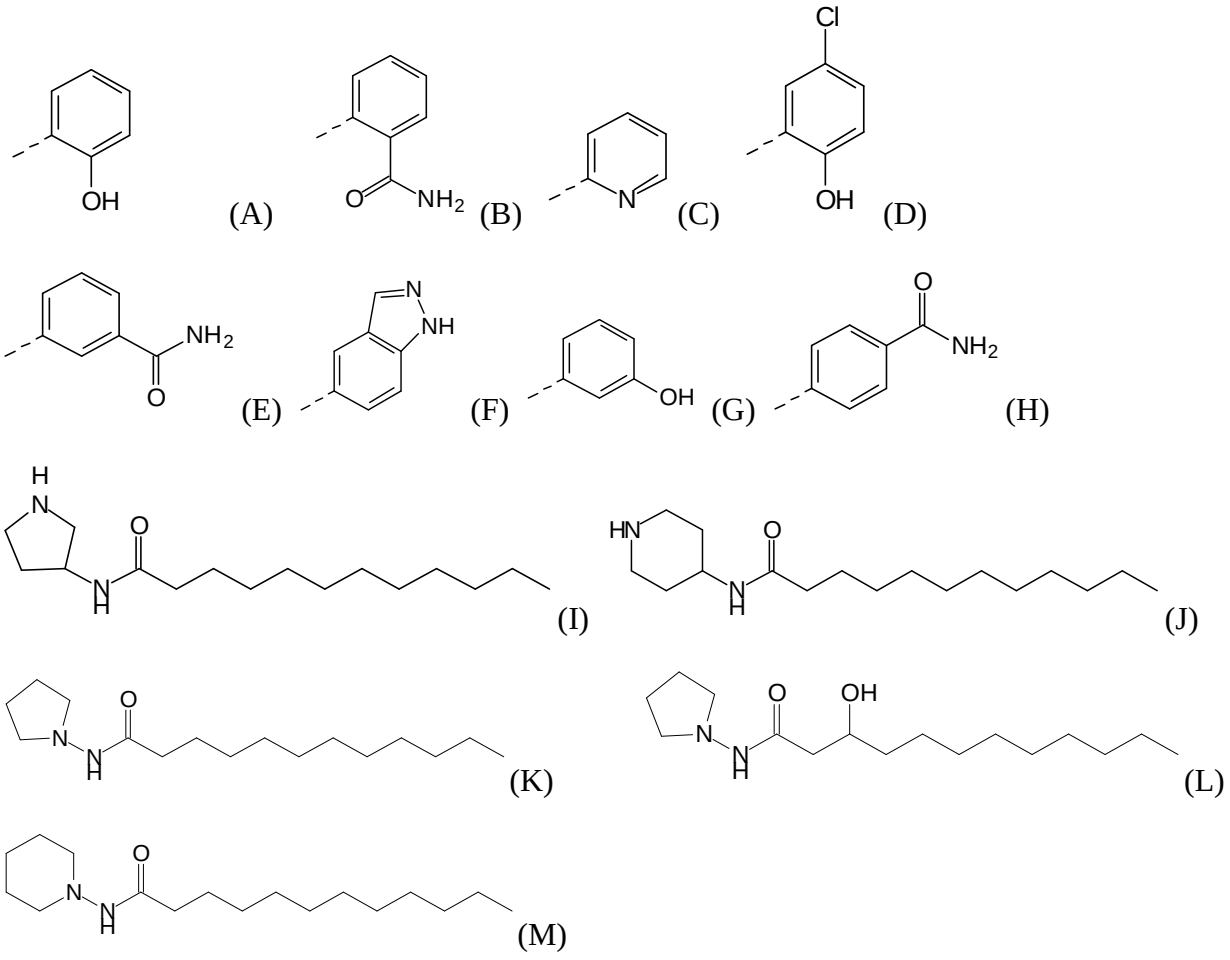


Figure1-5

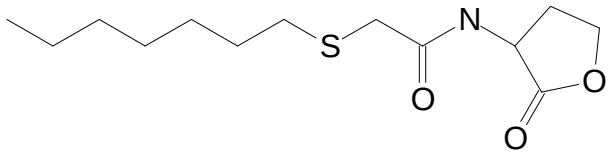
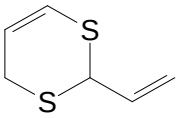
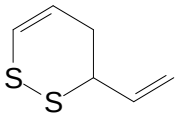


Figure 1-6

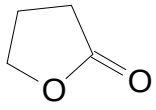


(A)

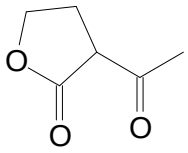


(B)

Figure 1-7

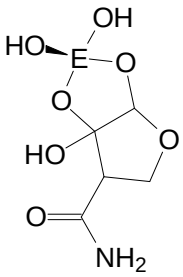


(A)

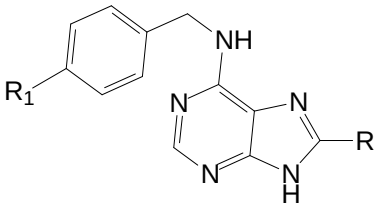


(B)

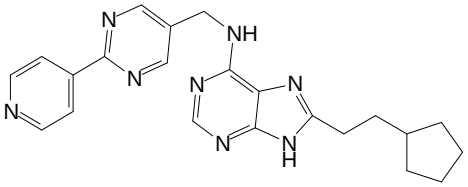
Figure 1-8



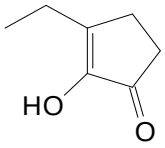
(A)



(B)



(C)



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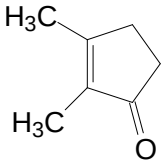
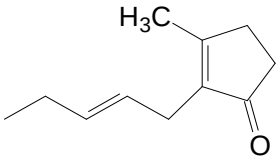
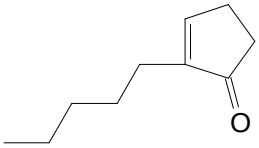


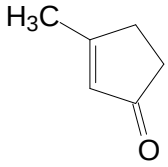
(E)



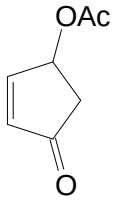
(F)



(G)



(H)



(I)

Figure 1-9

Chapter 2

REVERTING ANTIBIOTIC TOLERANCE OF *Pseudomonas aeruginosa* PAO1 PERSISTER CELLS BY (*Z*)-4-BROMO-5-(BROMOMETHYLENE)-3-METHYLFURAN-2(5*H*)-ONE

This chapter has been published as below with minor modifications. Jiachuan Pan, Ali Adem Bahar, Haseeba Syed and Dacheng Ren. Reverting antibiotic tolerance of bacterial persister cells. PLoS ONE 2012, 7, e45778.

2.1 Abstract

Bacteria are well known to form dormant persister cells that are tolerant to most antibiotics. Such intrinsic tolerance also facilitates the development of multidrug resistance through acquired mechanisms. Thus persister cells are a promising target for developing more effective methods to control chronic infections and help prevent the development of multidrug resistant bacteria. However, control of persister cells is still an unmet challenge. We show in this report that (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one (BF8) can restore the antibiotic susceptibility of *Pseudomonas aeruginosa* PAO1 persister cells at growth non-inhibitory concentrations. Persister control by BF8 was found to be effective against both planktonic and biofilm cells of *P. aeruginosa* PAO1. Interestingly, although BF8 is an inhibitor of quorum sensing (QS) in Gram-negative bacteria, the data in this study suggest that the activities of BF8 to revert antibiotic tolerance of *P. aeruginosa* PAO1 persister cells is not through QS inhibition and may involve other targets. BF8 can sensitize *P. aeruginosa* persister cells to antibiotics.

2.2 Introduction

It is well documented that a small portion of a bacterial population can form metabolically inactive persister cells (1), which are not mutants with drug resistance genes, but rather phenotypic variants of the wild-type strain (2) due to unbalanced production of toxins/anti-toxins (3-6) and other mechanisms related to stress response and translation inhibition (1, 7). This subpopulation can survive the attack of antibiotics at high concentrations, and when the treatment is stopped, they can reestablish the population with a similar percentage of cells as persisters, leading to high levels of antibiotic tolerance (2). Such intrinsic tolerance can cause chronic infections with recurring symptoms after the course of antibiotic therapy and facilitates the development and wide spread of acquired multidrug resistance through genetic mutations and horizontal gene transfer (2). For example, high persistence mutants have been isolated from cystic fibrosis patients with lung infections (8, 9) and from patients with candidiasis (10). Persister phenotypes have also been found in *Mycobacterium tuberculosis*, the bacterium causing chronic tuberculosis (11). Thus, targeting persister cells may help improve infection control and prevent the development of multidrug resistant bacteria (12). However, controlling persister cells is still an unmet challenge.

Conceivably, one approach to eliminating persister cells is to wake up this dormant population and render them to return to a metabolically active stage. These awakened cells are expected to become sensitive to antibiotics. In Gram-positive bacteria, a 17-kDa protein, named resuscitation-promoting factor (Rpf) has been discovered as a potential factor to wake up dormant cells (13). However, a full wakeup call may cause rapid growth of a bacterial pathogen, which can lead to adverse progression of infection if the antibiotics are not admitted during the right window.

Recently, sugars such as mannitol, glucose, fructose and pyruvate have been shown to generate proton-motive force and promote the uptake of aminoglycosides by persister cells of *Escherichia coli* and *Staphylococcus aureus*, which led to enhanced susceptibility of persister cells to this class of antibiotics. The effects were observed within 1 h of incubation, less than what is required for resumption of full growth (14). However, this approach requires relatively high concentrations of sugar (e.g. 10 mM) and is limited to aminoglycosides, but not the β-lactam antibiotic ampicillin and the fluoroquinolone ofloxacin. In addition, sugar molecules can only wake up persister cells, but cannot reduce persistence during growth (see below).

Compared to these approaches, non-metabolites that can potentiate multiple classes of antibiotics and also reduce persistence during bacterial growth may be advantageous. It is well documented that the absolute number of persister cells in a culture increases significantly when the culture enters stationary-phase and when cells form surface-attached highly hydrated structures known as biofilms (15-17). Recent research has demonstrated that quorum sensing (QS), bacterial cell-cell signaling by sensing and responding to cell density, promotes persister formation in *Pseudomonas aeruginosa* PAO1; e.g., acyl-homoserine lactone 3-OC_{12}-HSL and phenazine pyocyanin, QS signals of *P. aeruginosa*, can significantly increase the persister numbers in logarithmic phase cultures of *P. aeruginosa* PAO1 but not *E. coli* or *S. aureus* (18). Thus, we were motivated to test if targeting such pathways may reduce persistence during bacterial growth and/or wake up persister cells and revert their tolerance to antibiotics. We found in this study that the QS inhibitor BF8 has potent activities in persister control, although our data suggest that these activities may not be through QS inhibition and BF8 may have other targets in *P. aeruginosa* (below).

2.3 Materials and Methods

2.3.1 Furanone synthesis.

(*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one (BF8) was synthesized as described previously (20), dissolved in absolute ethanol as 60 mg/mL, and stored at 4°C until use. Briefly, Br_2 (6.22g, 38.9 mmol) in dichloromethane (20 mL) was added dropwise into a flask containing 2.53 g (19.5 nmol) alpha-methyllevulinica acid in 20 mL dichloromethane. The mixture was stirred at 35~40ºC till all the alpha-methyllevulinica acid reacted (based on TLC test); and then the reaction was interrupted by adding ice (~200 mL). The mixture was extracted with dichloromethane three times (80 mL each), washed with $Na_2S_2O_3$ (1 M, 100 mL) to remove residue Br_2, dried with anhydrous sodium sulfate (30 min), filtered with cotton, and then purified by removing solvent using a rotary evaporator. The crude bromo keto acid was added with concentrated H_2SO_4 (98%, 10 mL) and the mixture was heated in an oil bath at 110ºC till all the crude keto reacted (by checking on TLC plates). The raw product was poured into a beaker with 200 mL ice to stop the reaction. The mixture was extracted with dichloromethane three times (50 mL each), washed once with 80 mL H_2O and dried using a rotary evaporator. BF8 was further purified from other impurities using column chromatography (dichloromethane: hexanes = 1 : 4). The structure of BF8 was confirmed using ^{1}H-NMR by comparing with reported data (20).

2.3.2 Bacterial strain and growth media.

Planktonic PAO1 cultures were routinely grown in Luria-Bertani (LB) medium (24) which contains 10 g/L NaCl, 10 g/L tryptone and 5 g/L yeast extract. To minimize the variation in the level of persistence, all overnight cultures of PAO1 were inoculated using single-use glycerol stocks (disposed after use to avoid freeze and thaw) prepared from the same batch of PAO1

overnight culture. The *P. aeruginosa* QS reporter strain PAO1 mini-Tn5-based P*las*B-*gfp*(ASV) (23) was routinely grown in modified LB medium containing 10 g/L trypton, 5 g/L yeast extract, and 4 g/L NaCl. Overnight cultures of *V. harveyi* BB886 were grown in LM medium (38) containing 10 g/L tryptone, 5 g/L yeast extract, and 20 g/L NaCl. PAO1 biofilms were cultured in M63 medium (39) containing 13.6 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, and 0.5 mg/L $FeSO_4 \cdot 7H_2O$, pH 7, supplemented with 0.3% glucose, 1 mM $MgSO_4$ and 0.5% casamino acids.

2.3.3 Persister isolation.

Treatment with Cip up to 50 µg/mL for 3.5 h has been used to isolated PAO1 persister cells previously (18). We confirmed recently that treatment with 50 µg/mL Cip for 3.5 h is also sufficient to kill regular cells of our PAO1 strain since no additional killing was observed with Cip concentration up to 200 µg/mL (the highest concentration tested, Niepa et al., Biomaterials, In press). To further confirm that the treatment time is sufficient, we also tested the killing with 200 µg/mL Cip during 6.5 h of incubation. As shown in Figure 2-6, no additional killing was observed with incubation beyond 1.5 h. Given these results, we chose incubation for 3.5 h with 200 µg/mL Cip to ensure the complete elimination of regular cells. After Cip treatment (200 µg/mL, 3.5 h) of 18-h PAO1 overnight cultures, the surviving persister cells were washed twice with 0.85% NaCl solution to remove residual antibiotics, and then resuspended in 0.85% NaCl solution. The isolated persister cells were then used for different treatments as described below. The cells after each treatment were further treated by supplementing with 200 µg/mL Cip and incubating for 3.5 h. Then the samples were washed three times with 0.85% NaCl solution to quantify the number of cells that remained as persisters. The drop plate method described by Chen et al. (40) was followed to count colony forming units (CFUs).

2.3.4 Effect of BF8 on AHL-mediated QS in the reporter strain *V. harveyi* BB886.

A *V. harveyi* BB886 overnight culture was used to inoculate subcultures in AB medium (22). BF8 was added at different concentrations (0, 0.1, 0.5, 1, 10, 30, 60 µg/mL) after 5.5 h of growth at 37^{o}C with 200 rpm shaking. The incubation continued for another 1.5 h. Then the bioluminescence was measured using a luminometer (20/20n, Turner Design, Sunnyvale, CA, USA). Meanwhile, the CFU of reporter cells was determined using drop method with LM agar plates (22, 41) after washing the cells with 2% NaCl solution. This experiment was performed with two biological replicates and 6 replicates on drop plates were counted for each CFU data point.

2.3.5 Effect of BF8 on QS in PAO1.

A overnight culture of the QS reporter strain PAO1 mini-Tn5-based P*las*B-*gfp*(ASV) (23) was used to inoculate subcultures in modified LB medium (23). When the subcultures reached OD_{600} of 0.8, BF8 was added at different concentrations (0, 5, 10, 15, and 30 µg/mL). Green fluorescence and OD_{450} was measured when OD_{600} reached to around 2.7 by following the previously described protocol (23) to evaluate the effects on QS in PAO1. This experiment was conducted in duplicate.

2.3.6 Effects of BF8 on persistence of PAO1.

A PAO1 overnight culture was used to inoculate subcultures (each contained 5 mL LB medium) to an OD_{600} of 0.05, which were then supplemented with different concentrations of BF8 (0, 5, 10, 30, 50 and 100 µg/mL). The amount of ethanol (solvent of BF8 stock solutions) was adjusted to be the same for each sample to eliminate any solvent effect. Samples were taken after 5 h of

incubation at 37°C with shaking at 200 rpm to count CFU. Meanwhile, the remaining portion of each sample was added with 200 µg/mL Cip and incubated for 3.5 h at 37°C. The samples were then analyzed to quantify the number of persister cells by counting CFU. This experiment was performed with two biological replicates and 6 replicates on drop plates were counted for each CFU data point.

2.3.7 Effects of D-glucose and D-mannitol.

P. aeruginosa PAO1 subcultures were inoculated with an overnight culture to an initial OD_{600} of 0.05 in LB medium. The subcultures were supplemented with 10 mM D-glucose, 10 mM D-mannitol or without sugar (control). The total number of viable cells and the number of persister cells were quantified as described in the experiment of BF8 above. This experiment was conducted with two biological replicates and 5 replicates on drop plates were counted for each CFU data point.

2.3.8 Effects of BF8 on antibiotic susceptibility of isolated persister cells.

Persisters were isolated from overnight cultures as described above. After dilution by 50 times with 0.85% NaCl solution, the persisters were challenged with different concentrations of BF8. Ethanol (the solvent used for making BF8 stock solutions) was adjusted to be the same in all samples to eliminate any solvent effect. After incubation for 2 h at 37°C with shaking at 200 rpm, 1 mL of each sample was taken and washed three times with 0.85% NaCl to quantify the total number of viable cells by counting CFU. The remaining portion of each sample was further tested to quantify the number of cells that remained as persisters as described above. This experiment was conducted with two biological replicates and 5 replicates on drop plates were counted for each CFU data point.

2.3.9 Synergy with other antibiotics.

Persisters were isolated from overnight cultures as described above, and then incubated in 0.85% NaCl for 2 h at 37^{o}C with shaking at 200 rpm in the absence or presence of 5 µg/mL BF8. The amount of ethanol was adjusted to be the same in all samples to eliminate any solvent effect. After incubation, 1 mL of BF8 treated persister samples and BF8-free controls were added with and without different antibiotics [25 µg/mL tetracycline (Tet), 25µg/mL gentamicin (Gen), 25µg/mL tobramycin (Tob), 500 µg/mL carbenicillin (Car), 25 µg/mL ciprofloxacin (Cip)] and incubated for another 3.5 h at 37^{o}C with shaking at 200 rpm. The antibiotic treated persisters were then washed three times with 0.85% NaCl solution to remove antibiotics and plated on LB plates to evaluate the killing by antibiotics by counting CFU. This experiment was conducted with two biological replicates and 5 replicates on drop plates were counted for each CFU data point.

2.3.10 Effects of *N*-(3-Oxododecanoyl)-*L*-homoserine lactone (3-oxo-C_{12}-HSL).

This experiment was conducted by following the same protocol as that of the effects of BF8 on isolated persister cells described above. The QS signal 3-oxo-C_{12}-HSL was tested at 0, 1.5, 3, 6, 15, and 30 µg/mL. This experiment was conducted with three biological replicates and 5 replicates on drop plates were counted for each CFU data point.

2.3.11 Effects of BF8 on persister cells in established biofilms.

P. aeruginosa PAO1 overnight cultures in LB medium were used to inoculate subcultures in M63 medium to an OD_{600} of 0.05 in glass petri dishes containing 2 cm × 1 cm 304L stainless steel coupons. After 18 h of incubation, the coupons with established biofilms were transferred to

a 12 well plate (Becton Dickinson, Franklin Lakes, NJ, USA). Each well contained 4 mL of 0.85% NaCl solution supplemented with different concentrations of BF8 (0, 5, 10, 30, 60 µg/mL). The biofilm samples in 12 well plates were incubated at 37°C for 24 h without shaking. One mL of medium with detached cells was then sampled from each well, washed three times with 0.85% NaCl solution and plated on LB agar plates to determine the viability of PAO1 cells by counting CFU. Meanwhile, 1 mL of medium with detached cells was sampled, added with 200 µg/mL Cip, and incubated for 3.5 h at 37°C to isolate persister cells. Then the samples were washed three times with 0.85% NaCl solution and plated on LB agar plates to determine the number of persister cells by counting CFU. To collect the biofilm cells, the coupons were transferred to 15 mL falcon tubes, each containing 5 mL 0.85% NaCl solution. The biofilm cells were collected by vortexing the coupons for 1 min and sonicating (Ultrasonic cleaner Model No B200, Sinosonic Industrial Co., Ltd, Taipei Hsien, Taiwan) for 1 min (repeat once) (42). Collected biofilm cells were plated on LB plates to count CFU and the rest of the samples were treated with 200 µg/mL Cip for 3.5 h at 37°C for persister isolation. The isolated biofilm-associated persister cells were washed three times and plated on LB agar plates to count CFU. This experiment was conducted with three biological replicates and 5 replicates on drop plates were counted for each CFU data point.

2.3.12 Effects of BF8 on PAO1 biofilm formation.

Biofilms were formed on 2 cm × 1 cm 304L stainless steel coupons in M63 medium. The biofilm cultures with and without 60 µg/mL BF8 (but with the same amount of the solvent ethanol) were inoculated with an overnight culture to an initial OD_{600} of 0.05. After 18 h of incubation at 37^{o}C without shaking, the coupons were gently washed with 0.85% NaCl solution three times to remove unattached planktonic cells. The total number of biofilm cells and the number of

persisters were quantified as described above. This experiment was conducted with three biological replicates and 5 replicates were counted for each CFU sample using drop plate method.

2.3.13 DNA microarray analysis.

Persister cells were harvested from 18-h cultures of PAO1 (100 mL each) using the same methods as described above. The isolated persister cells were resuspended in 0.85% NaCl solution supplemented with 1 µg/mL (3.7 µM) BF8 or with the same amount of ethanol (4.17 µL, to eliminate the solvent effects). After incubation at 37°C for 1 h, treated persister cells were collected by centrifugation at 10,000 rpm for 5 min at 4°C, transferred to 2 mL pre-cooled microcentrifuge tubes and frozen instantly in an ethanol-dry ice bath. The cell pellets were stored at -80°C until RNA isolation.

To isolate the total RNA, the harvested PAO1 cells were lysed by beating at 4,800 oscillations/min using a mini-bead beater (Biospec Products Inc., Bartlesville, OK, USA) after adding 0.5 mm glass beads, 900 µL RLT buffer and 1% 2-Mercaptoethanol. The total RNA was extracted using RNeasy Mini Kit (Qiagen, Austin, TX, USA) with on-column DNase treatment (RNase-Free DNase Set, Qiagen). The RNA samples were sent to the DNA microarray Facilities at SUNY Upstate Medical University for microarray (*P. aeruginosa* Genome Array, Affymetrix, Santa Clara, CA, USA) hybridization. A total of three biological replicates were tested. Using the GeneChip Operating Software (MAS 5.0), genes with a *p*-value of less than 0.0025 or greater than 0.9975 were considered statistically significant based on Wilcoxon signed rank test and Tukey Byweight. To ensure the significance of microarray data, an additional criterion was applied to only select the genes with an expression ratio of 2 or higher from this group as induced and repressed genes. Microarray data has been deposited in Gene Expression Omnibus

(GEO: GSE36753), compliant with Minimum Information About a Microarray Experiment (MIAME) guidelines.

2.3.14 RNA slot blotting.

A total of five genes were tested including PA3523, PA2931, PA0182, PA4167 and PA4943. Primers were designed to include only small inner regions, varying from 368 bp to 448 bp, of these genes. Hybridization probes were labeled with DIG-dUTP (PCR DIG Probe Synthesis Kit, Roche, Mannheim, Germany) in PCR reactions by following the manufacturer's protocol. Total RNA was isolated as described in the DNA microarray section above. The blotting and signal detection were conducted as we described previously (43).

2.3.15 Q-PCR analysis.

To verify if killing of PAO1 cells by Cip led to mRNA degradation in the dead cells, the expression levels of the house-keeping gene *proC* were quantified using Q-PCR. Total RNA was extracted from overnight PAO1 cells before and after 3.5 h of treatment with 200 µg/mL Cip. Then, 200 ng total RNA was taken from each sample to perform cDNA synthesis by using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Two primers were used in Q-PCR including the forward primer CGTGGTCGAGTCCAACGCCG and the reverse primer GCGTCGGTCATGGCCTGCAT. Relative expression ratios were calculated from triplicate reactions.

2.3.16 Minimal inhibitory concentration (MIC) of BF8.

Subcultures of PAO1 were inoculated from an 18-h overnight culture to an OD_{600} of 0.05. BF8 was added at different concentrations (0 - 200 µg/mL) and OD_{600} at this time point was measured.

After 24 h incubation at 37°C, the presence and absence of growth were checked by comparing the OD_{600} before and after incubation. The results indicate that none of the tested concentrations was sufficient to inhibit growth completely. For example, although growth inhibition was observed at 150 µg/mL and 200 µg/mL, significant growth was still present (p = 0.00553 and 0.00395, respectively). Therefore the MIC was found to be higher than 200 µg/mL (shown in Figure 2-5). The experiment was performed with six biological replicates.

2.3.17 Minimal bactericidal concentration (MBC) of BF8.

An 18-h overnight culture of PAO1 was washed and diluted with 0.85% NaCl solution to an OD_{600} of 0.05 supplemented with different concentrations of BF8 (0 - 30 µg/mL). After 2 h of incubation at 37°C in culture tubes, the treated cells were washed and diluted with 0.85% NaCl solution to count CFU using drop plate method. The results indicate that none of the tested concentrations was sufficient to kill more than 99.9% of PAO1 (Figure 2-5). Therefore the MBC (minimum concentration that reduce viability by 99.9%) (26, 27) was found to be higher than 30 µg/mL (Figure 2-5). The experiment was performed with 2 biological replicates.

2.3.18 Viability of PAO1 cells challenged with different concentrations of Cip.

An overnight culture of PAO1 was incubated with 200 µg/mL Cip in 37°C on 200 rpm shaker. At different incubation time point (1.5 h-6.5 h), Cip treated cells was sampled, washed by three times, diluted and plated on LB agar plates to determine CFU. The experiment was performed with 2 biological replicates.

2.3.19 Transcription level of the housekeeping gene, *proC*.

Same amount of total RNA extracted from persister cells (after Cip treatment) and total cells before Cip treatment were reverse transcribed and used in Q-PCR reactions to compare *proC* mRNA levels. The persister cell sample was found to have 85.5% less *proC* compared to that of total cells before Cip treatment.

2.4 Results

2.4.1 BF8 is a QS inhibitor.

A wide variety of molecules have been discovered as quorum sensing inhibitors (19). We reported recently that several new synthetic brominated furanones (derivatives of natural brominated furanones) are inhibitors of biofilm formation (20) and quorum sensing (21) in Gram-negative bacteria. Among these compounds, (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one (BF8, Figure 2-1A) is the most effective biofilm inhibitor of *E. coli* and *P. aeruginosa* at growth non-inhibitory concentrations (20). It is also a potent inhibitor of quorum sensing based on AI-2 (21). In this study, the effects of BF8 on AI-1 mediated QS were studied using the reporter strain *Vibrio harveyi* BB886 (ATCC# BAA-1118) (22). By monitoring the bioluminescence and colony forming units (CFU) of the reporter strain, BF8 was found to inhibit QS at concentrations not inhibitory to the viability of the reporter strain. For example, 10 μg/mL BF8 completely inhibited AI-1-mediated QS with no effects on the viability of *V. harveyi* BB886 (Figure 2-1B). To specifically test if BF8 is also an inhibitor of QS in *P. aeruginosa*, the expression of the QS-controlled toxin gene, *lasB*, in the presence of different concentrations of BF8, was characterized using the reporter *P. aeruginosa* PAO1 mini-Tn5-based P*lasB-gfp*(ASV) by following the procedure described previously (23). As shown in Figure 2-1C, expression of

lasB in stationary phase cultures (all around OD_{600} of 2.7) was significantly inhibited by BF8, confirming that BF8 is also an inhibitor of QS in *P. aeruginosa*.

2.4.2 BF8 reduced persistence of PAO1.

To test if BF8 can control persister cells of PAO1, we studied the effects of BF8 (up to 100 µg/mL) on the viability and persistence of *P. aeruginosa* PAO1 (henceforth PAO1) during 5 h of growth in Luria Bertani (LB) medium (24). As shown in Figure 2-2A, the total number of viable cells at the end of incubation was around 3.5×10^9/mL for all the samples (one-way ANOVA followed by Tukey test, $p = 0.122$). Thus, BF8 did not affect the viability of PAO1 directly. Consistently, the MIC (minimum concentration that prevent growth overnight) of BF8 against PAO1 in LB medium was found to be higher than 200 µg/mL (Figure 2-5 A). Interestingly, at the growth non-inhibitory concentrations, the persistence of PAO1 was significantly reduced by BF8 in a dose-dependent manner; e.g., BF8 at 100 µg/mL reduced the number of persister cells by 63 times (98.4% reduction) compared to the untreated control (one-way ANOVA followed by Tukey test, $p = 0.0006$). The reduction of persistence could lead to better efficacy of antibiotics [e.g., ciprofloxacin (Cip) as shown in Figure 2-2A] and help prevent the development of antibiotic resistance. To our best knowledge, this is the first compound known to reduce bacterial persistence during normal growth.

Sugars have been reported to sensitize persisters to antibiotics (14) and Wang et al. (25) reported that relatively high concentrations of fructose and glucose reduced the expression of QS-related gene *pqs*A and the production of extracellular proteases and pyocyanin in *P. aeruginosa*. To test if sugars can also reduce persistence of PAO1 under our experimental condition, we repeated the above experiment using 10 mM D-glucose and D-mannitol instead of BF8. It was found that, unlike BF8, incubation with neither of these sugars affected persistence (Figure 2-2B, one-way

ANOVA followed by Tukey test, $p = 0.43$). These data suggest that persister control by BF8 is through a different mechanism than that by sugars.

2.4.3 BF8 reverted the antibiotic tolerance of PAO1.

In addition to reducing persistence during PAO1 growth, BF8 was also found to revert the antibiotic tolerance of isolated persisters. As shown in Figure 2-3A, treatment with BF8 at all tested concentrations (0.1, 0.5, 1, and 2 µg/mL) increased the susceptibility of persister cells to Cip. For example, although BF8 at 0.5 µg/mL did not affect the viability of persister cells, the antibiotic tolerance of persister cells was reverted since 74.1 ±1.1% of persister cells became sensitive to Cip compared to the untreated control (One-way ANOVA followed by Tukey test, $p = 0.0005$). The effects on persistence reduction increased to 89.8±1.4% when BF8 was added at 2 µg/mL (one-way ANOVA followed by Tukey test, $p = 0.0013$) (Figure 2-3A). At higher concentrations, however, BF8 was found cidal to PAO1 persister cells. For example, treatment with 10 µg/mL BF8 led to significant killing of PAO1 persister cells (data not shown), suggesting that a threshold concentration may exist between growth non-inhibitory reverting of persistence and cidal effects on persister cells. Consistently, BF8 at 2 and 5 µg/mL did not affect the viability of regular PAO1 cells in stationary phase (one-way ANOVA followed by Tukey test, $p = 0.7975$ and $p=0.8572$, respectively, Figure 2-5). It appeared to be cidal to regular cells at 10 µg/mL or higher concentrations (Figure 2-5 B); while the MBC (the minimum concentration that reduces viability by 99.9% (26, 27)) was found to be higher than 30 µg/mL (the highest concentration tested). Overall, the above finding shows that BF8 can revert persistence at concentrations that do not affect the viability of both persister and regular cells of PAO1 (2 and 5 µg/mL under our experimental condition).

We chose 0.85% NaCl solution rather than LB medium to test the effects on isolated persisters because NaCl solution itself does not contain carbon source, allowing the effects on viability to be tested specifically. The concentrations of BF8 that exhibited activities were significantly lower in 0.85% NaCl solution than those in LB medium (to test persistence during growth as described above), presumably because LB medium contains proteins and other large molecules that may bind to BF8 and decrease its activity. It is also worth noticing that the persister numbers are higher in Figure 2-3 (start CFU/mL as $2.1\times10^6 \pm 3.1\times10^5$ in 3A, $2.3\times10^6 \pm 5.7\times10^4$ in 3B, and $2.0\times10^6 \pm 4.0\times10^5$ in 3C) than those in Figure 2-2 ($5.0\times10^5 \pm 1.7\times10^5$/mL for the control) because the persister cells in Figure 2-3 were isolated from overnight cultures (known to have higher persistence (28, 29)) and those in Figure 2-2 were isolated from the growing cultures.

It is also interesting that, unlike sugars which can only potentiate aminoglycosides (14), BF8 was found to restore susceptibility of PAO1 persister cells to both ciprofloxacin and tobramycin (from two different classes of antibiotics). In total, five antibiotics were tested to evaluate the effects on antibiotics with different targets including protein synthesis [tetracycline (Tet), gentamicin (Gen) and tobramycin (Tob)], cell wall synthesis [carbenicillin (Cab)], and functions of DNA gyrase (Cip). In addition to Cip (*t* test, $p = 0.0095$), BF8 at 5 μg/mL was also found to potentiate Tob (*t* test, $p = 0.0271$), while the effects on Tet (*t* test, $p = 0.4096$), Gen (*t* test, $p = 0.0771$), and Car (*t* test, $p = 0.1976$) were not statistically significant (Figure 2-3B).

Since QS is known to stimulate persister formation in PAO1 and BF8 is a QS inhibitor, we further tested if persister controlled by BF8 can be relieved by the QS signal. It was interesting to find that addition of 3-oxo-C_{12}-HSL (Sigma-Aldrich, St. Louis, MO, USA) was not able to

reduce the inhibitory effects of BF8 (Figure 2-3C). Instead, 3-oxo-C_{12}-HSL was also found to sensitize isolated persisters to Cip in a dose dependent manner. For example, after treatment with 30 µg/mL 3-oxo-C_{12}-HSL for 2 h, nearly all the isolated persisters were killed by 200 µg/mL Cip (Figure 2-3C). Interestingly, this AHL was found previously to promote PAO1 persister formation in exponential phase (different experimental condition than described here) (18). Thus, this QS signal may have different effects on PAO1 persisters under different conditions. These findings suggest that, although BF8 is a QS inhibitor, the activities of BF8 to sensitize PAO1 persisters to antibiotics is not through QS inhibition and there are other targets of BF8 in PAO1 persister cells.

2.4.4 Effects of BF8 on PAO1 biofilms and associated persister cells.

Compared to planktonic cells, surface-attached bacterial biofilms are more challenging to microbial control since they are up to 1000 times more tolerant to antibiotics than planktonic cells and are known to harbor a high percentage of persister cells (1, 30). To understand if BF8 can also control persisters in biofilms, we treated 18-h PAO1 biofilms formed on 304L stainless steel coupons with different concentrations of BF8 for 24 h. Both the planktonic (detached cells) and biofilm populations that remained attached were analyzed to evaluate the viability and persistence of PAO1 with and without BF8 treatment. As shown in Figure 2-4A, BF8 dispersed established biofilms and reduced the number of persister cells in both biofilm and detached population. For example, the number of viable cells remained attached after treatment was reduced by 5 µg/mL BF8 from $3.3\times10^{8}\pm1.7\times10^{8}/cm^{2}$ to $7.1\times10^{7}\pm1.4\times10^{7}/cm^{2}$ (one-way ANOVA followed by Tukey test, $p = 0.0025$). Among the cells that remained attached, the number of persisters was reduced from $9.6\times10^{5}\pm9.1\times10^{4}/cm^{2}$ to $7.0\times10^{5}\pm1.1\times10^{5}/cm^{2}$ (one-way

ANOVA followed by Tukey test, $p = 0.002$). At concentrations up to 10 µg/mL, BF8 did not exhibit cidal effects but reduced the percentage of persister cells (0.14±0.01% without BF8 vs. 0.013±0.002% with 10 µg/mL BF8, one-way ANOVA followed by Tukey test, $p = 0.0002$) in the detached population (the total number of cells in suspension increased compared to the control due to detachment); while at high concentrations, BF8 appeared to be cidal to both regular and persister cells. For example, treatment with 60 µg/mL BF8 for 24 h led to 94.2±5.1% reduction of viable persister cells remained on the surface (one-way ANOVA followed by Tukey test, $p = 0.0004$), although the persisters/regular cells ratio in biofilms was not reduced by BF8 (Figure 2-4A). In addition to the effects on established biofilms, BF8 at 60 µg/mL added at inoculation was also found to inhibit PAO1 biofilm formation (incubated for 18 h) by 99.1 ± 0.2% (*t* test, $p = 0.0001$) and reduced the number of biofilm-associated persisters by 99.2 ± 1.3% (*t* test, $p = 0.001$) (Figure 2-4B).

2.4.5 DNA microarray analysis.

It was an interesting finding that BF8 can render persisters sensitive to the antibiotics targeting 30S ribosome RNA (Tob), and topoisomerase (Cip). The capability to sensitize persister cells to antibiotics that target both DNA replication and protein synthesis suggests that BF8 may have made the cells leave the persister stage. To obtain a deeper insight at the genetic level, we investigated the effects of BF8 on sensitization of PAO1 persister cells using DNA microarrays. The gene expression profiles of PAO1 persister cells treated with and without BF8 at 1 µg/mL for 1 h were compared in triplicate. We chose this effective, but relatively low, concentration of BF8 (as shown in Figure 2-3A) so that the most important genes induced by BF8 can be identified. The persister cells were isolated by killing regular cells with 200 µg/mL Cip for 3.5 h. Because average half-life of bacterial mRNA is only a few minutes (31), we expect that the

mRNA in dead cells should be degraded when the cells were harvested. Consistently, we found that 85.5% of the mRNA of the house-keeping gene *proC* was degraded in the persister sample compared to the sample before Cip treatment. Furthermore, since the identical persister cell samples were used for both the control (no BF8) and test (with BF8), only the differentially expressed genes in live cells are expected to be seen in the microarray data.

In total, 28 genes were consistently induced by BF8 by more than 2 fold compared to the control in all three biological replicates (see Table S1 for the full list). In comparison, although a relatively small set of repressed genes was seen in each set, no gene was significantly repressed in all three sets mostly due to low expression ratios in some dataset(s) (test/control < 2.0). This is possibly because persister cells only have low level expression of essential genes due to their dormant nature (32, 33). To validate the DNA microarray results, we conducted RNA slot blotting for five representative genes including one unchanged gene (PA4943) and 4 induced genes (PA3523, PA2931, PA0182 and PA4167). The results of all blots were consistent with the microarray data (Table S2). The consistently induced genes encode oxidoreductases (PA4167, PA1334, PA0182, PA2932, PA2535, PA3223, PA1127), transcriptional factors (PA4878, PA1285, PA3133, PA2196), and hypothetical proteins (PA4173, PA0741, PA1210, PA3240, PA2575, PA0565, PA2580, PA2610, PA2839, PA0422, PA1374, PA2691). Since many reductases are involved in metabolism, our DNA microarray data indicate that some cellular activities or membrane functions of PAO1 persisters can be induced by low concentrations of BF8. In addition, the gene PA2931 was induced by 11 times. This gene encodes a repressor of Cif, a *P. aeruginosa* toxin that causes degradation of the cystic fibrosis transmembrane conductance regulator (CFTR) in mammalian cells (34, 35). The induction of PA2931 indicates

that BF8 can potentially repress the pathogenicity of PAO1. No QS genes were found to be differentially expressed by BF8. This is not surprising since persister cells are relatively dormant and are not expected to have QS activities. This finding further supports that persister control by BF8 involves other pathways and confirms that the mRNAs of differentially expressed genes were indeed from persister cells.

2.5 Discussion

In this study, we show that BF8 can act synergistically with antibiotics to enhance killing of *P. aeruginosa* PAO1 persister cells. Although more work is needed to reveal the exact mechanism, the restoration of antibiotic susceptibility of PAO1 persister cells at growth non-inhibitory concentrations by BF8 is nevertheless interesting. The DNA microarray data suggest that some reductases and proteins for small molecule transfer were induced by BF8. We hypothesize that interaction between BF8 (at growth non-inhibitory concentration) and cell membrane proteins can interrupt specific cellular functions, which led to increase in activities of transport proteins and reductases. Such response should require energy and thus may influence the physiological stage of persister cells and thus restore their susceptibility to antibiotics. Such effects may be mechanistically different from natural wakeup when the persister cells are supplied with new medium. Further study on bacterial membrane potential and metabolism with and without BF8 (at growth non-inhibitory concentrations) can help test this hypothesis. In an earlier work, Shah et al. (33) compared gene expression in regular cells and persisters of *E. coli* and found that around 5% of genes are differentially expressed between these two populations. A number of genes involved in toxin-antitoxin module proteins rather than stationary-phase-specific functions were induced in persisters compared to regular cells. In our PAO1 microarray data, however, only a short list of genes was induced by BF8, which is different from that of regular cells vs.

persister cells (33). These data confirmed that treatment with BF8 was not leading to a full wakeup. Because the cells only activated certain functions, such treatment can be advantageous compared to full wakeup that leads to normal cell growth and potentially higher virulence. Molecules with such activities may have a good opportunity to be applied either before or together with antibiotics to clean infections, without a specific window required for antibiotics to be administered.

To be applied for disease control, it is important to evaluate the safety and efficacy of BF8 *in vivo*. This is part of our ongoing work. Nevertheless, some other brominated furanones have been shown to be safe and effective in animal models such as shrimps (36) and mice (37). For example, furanone C-30 has been shown to reduce the virulence of *P. aeruginosa* and help clear infection from the lungs of mice (37). The activities of persister control found in the present study bring new opportunities to develop more effective therapies based on this class of compounds.

In summary, the results described above indicate that BF8 can reduce persistence during the growth of PAO1 and can also restore the susceptibility of isolated persister cells to antibiotics. This appears to be a promising advantage of BF8 for persister control. The exact targets of BF8 and the chemical nature of such interaction are unknown and are a goal of our ongoing work. It is important to understand if there are a set of specific membrane proteins, activation of which can lead to higher antibiotic susceptibility; and if a subset of such proteins is sufficient for the observed activities. Better understanding of the underlying mechanism will help develop more effective methods to control bacterial persistence and associated chronic infections.

2.6 Acknowledgments

We are grateful to Professor Thomas K. Wood at Pennsylvania State University for sharing the strain of *P. aeruginosa* PAO1, Professor Michael Givskov at University of Copenhagen for sharing the strain *P. aeruginosa* PAO1 mini-Tn5-based P*las*B-*gfp*(ASV), as well as Professor Frank Middleton and Ms. Karen Gentle at SUNY Upstate Medical University for helping with DNA microarray hybridization.

References

1. Lewis K (2010) Persister cells. Annu Rev Microbiol 64: 357-372.
2. Lewis K (2007) Persister cells, dormancy and infectious disease. Nat Rev Microbiol 5: 48-56.
3. Christensen SK, Pedersen K, Hansen FG, Gerdes K (2003) Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. J Mol Biol 332: 809-819.
4. Christensen SK, Gerdes K (2003) RelE toxins from bacteria and Archaea cleave mRNAs on translating ribosomes, which are rescued by tmRNA. Mol Microbiol 48: 1389-1400.
5. Gerdes K, Christensen SK, Løbner-Olesen A (2005) Prokaryotic toxin–antitoxin stress response loci. Nat Rev Microbiol 3: 371-382.
6. Hayes F (2003) Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. Science 301: 1496.
7. Hansen S, Lewis K, Vulić M (2008) Role of global regulators and nucleotide metabolism in antibiotic tolerance in *Escherichia coli.* Antimicrob Agents Chemother 52: 2718-2726.

8. Mulcahy LR, Burns JL, Lory S, Lewis K (2010) Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. J Bacteriol 192: 6191.

9. Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, et al. (2006) Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci 103: 8487.

10. LaFleur MD, Qi Q, Lewis K (2010) Patients with Long-Term Oral Carriage Harbor High-Persister Mutants of *Candida albicans*. Antimicrob Agents Chemother 54: 39.

11. Ojha AK, Baughn AD, Sambandan D, Hsu T, Trivelli X, et al. (2008) Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria Mol Microbiol 69: 164-174.

12. Pearson H (2002) 'Superbug' hurdles key drug barrier. Nature 418: 469.

13. Mukamolova GV, Kaprelyants AS, Young DI, Young M, Kell DB (1998) A bacterial cytokine. Proc Natl Acad Sci 95: 8916.

14. Allison KR, Brynildsen MP, Collins JJ (2011) Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 473: 216-220.

15. Lewis K (2001) Riddle of biofilm resistance. Antimicrob Agents Chemother 45: 999-1007.

16. Lewis K (2005) Persister cells and the riddle of biofilm survival. Biochem (Moscow) 70: 267-274.

17. Spoering AL, Lewis K (2001) Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. J Bacteriol 183: 6746-6751.

18. Moker N, Dean CR, Tao J (2010) *Pseudomonas aeruginosa* increases formation of multidrug-tolerant persister cells in response to quorum-sensing signaling molecules. J Bacteriol 192: 1946-1955.

19. Pan J, Ren D (2009) Quorum sensing inhibitors: a patent overview. Expert Opin Ther Pat 19: 1581-1601.

20. Han Y, Hou S, Simon KA, Ren D, Luk YY (2008) Identifying the important structural elements of brominated furanones for inhibiting biofilm formation by *Escherichia coli*. Bioorg Med Chem Lett 18: 1006-1010.

21. Hou S, Duo M, Han Y, Luk Y-Y, Ren D. Inhibiting microbial biofilm formation by brominated furanones. In: Gilbert JL, editor; 2009; Minneapolis, Minnesota. ASM International. pp. 6-10.

22. Surette MG, Bassler BL (1998) Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. Proc Natl Acad Sci 95: 7046.

23. Hentzer M, Riedel K, Rasmussen TB, Heydorn A, Andersen JB, et al. (2002) Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. Microbiol 148: 87-102.

24. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

25. Wang R, Starkey M, Hazan R, Rahme LG (2012) Honey's ability to counter bacterial infections arises from both bactericidal compounds and QS inhibition. Front Microbiol 3.

26. Taylor P, Schoenknecht F, Sherris J, Linner E (1983) Determination of minimum bactericidal concentrations of oxacillin for *Staphylococcus aureus*: influence and significance of technical factors. Antimicrob Agents Chemother 23: 142-150.

27. Andrews JM (2001) Determination of minimum inhibitory concentrations. J Antimicrob Chemother 48: 5-16.

28. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S (2004) Bacterial persistence as a phenotypic switch. Science 305: 1622.

29. Gefen O, Balaban NQ (2009) The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. FEMS Microbiol Rev 33: 704-717.

30. Mah TF, O'Toole GA (2001) Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 9: 34-39.

31. Goodrich AF, Steege DA (1999) Roles of polyadenylation and nucleolytic cleavage in the filamentous phage mRNA processing and decay pathways in *Escherichia coli.* RNA 5: 972-985.

32. Keren I, Shah D, Spoering A, Kaldalu N, Lewis K (2004) Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. J Bacteriol 186: 8172-8180.

33. Shah D, Zhang Z, Khodursky A, Kaldalu N, Kurg K, et al. (2006) Persisters: a distinct physiological state of *E. coli*. BMC Microbiol 6: 53.

34. Bomberger JM, Ye S, MacEachran DP, Koeppen K, Barnaby RL, et al. (2011) A *Pseudomonas aeruginosa* toxin that hijacks the host ubiquitin proteolytic system. PLoS Pathog 7: e1001325.

35. MacEachran DP, Stanton BA, O'Toole GA (2008) Cif is negatively regulated by the TetR family repressor CifR. Infect Immun 76: 3197.

36. Brazas MD, Hancock REW (2005) Ciprofloxacin induction of a susceptibility determinant in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 49: 3222-3227.

37. Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, et al. (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. EMBO J 22: 3803-3815.

38. Ren D, Sims JJ, Wood TK (2001) Inhibition of biofilm formation and swarming of *Escherichia coli* by (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2 (5*H*)-furanone. Environ Microbiol 3: 731-736.

39. Pabdee AB, Jacob F, Monod J (1959) The genetic control and cytoplasmic expression of "inducibility" in the synthesis of β-galactosidase by *E. Coli*. J Mol Biol 1: 156-178.

40. Chen CY, Nace GW, Irwin PL (2003) A 6 x 6 drop plate method for simultaneous colony counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli.* J Microbiol Meth 55: 475-479.

41. Ren D, Sims JJ, Wood TK (2001) Inhibition of biofilm formation and swarming of *Escherichia coli* by (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2 (5*H*)-furanone. Environ Microbiol 3: 731-736.

42. Chen X, Zhang M, Zhou C, Kallenbach NR, Ren D (2011) Control of Bacterial Persister Cells by Trp/Arg-Containing Antimicrobial Peptides. Appl Environ Microbiol 77: 4878-4885.

43. Duo M, Zhang M, Luk YY, Ren D (2010) Inhibition of *Candida albicans* growth by brominated furanones. Appl Microbiol Biotechnol 85: 1551-1563.

Table

Table 1. List of BF8-inducded genes in PAO1 persister cells. A total of three biological replicates were tested. The genes induced by more than 2 fold in all three data sets are listed below.

Induced gene	**Expression ratio**	**Gene products/Functions**
PA4167	510.6	2,5-diketo-D-gluconate reductase B
PA1334	227.6	oxidoreductase
PA4173	36.7	hypothetical protein
PA0182	97.1	3-ketoacyl-(acyl-carrier-protein) reductase
PA2932(*morB*)	64.9	morphinone reductase
PA0741	16.5	hypothetical protein
PA1210	21	hypothetical protein
PA3240	14.4	hypothetical protein

		Resistance-Nodulation-Cell Division efflux
PA3523	9.6	membrane fusion protein precursor
PA2535	9	oxidoreductase
PA2575	9.1	hypothetical protein
PA2931	11	CifR
PA0565	12.8	hypothetical protein
PA2580	8.3	hypothetical protein
PA2610	7.2	hypothetical protein
PA2839	11	hypothetical protein
PA0422	4.2	hypothetical protein
PA3223(*acpD*)	4.8	AzoR3, azoreductase 3
PA1374	3.4	hypothetical protein
PA3920	3.9	metal transporting P-type ATPase
PA4878	4.2	transcriptional regulator
PA1285	4.4	transcriptional regulator
PA1470	4.1	short chain dehydrogenase
PA3133	3.5	transcriptional regulator
PA2196	4.8	transcriptional regulator
PA2378	3.1	aldehyde dehydrogenase
PA2691	3.7	hypothetical protein
PA1127	3.4	oxidoreductase

Table 2. The primers used in RNA slot blotting and the blotting results. PA4943 was unchanged based on DNA microarray data. All the other 4 genes were induced by BF8 based on microarray results.

Gene	Primers	Expression ratio based on RNA slot blot
PA4943	GAAACGGTGGCATTCGTC GTTTCCAGCTGGGTCTCG	unchanged
PA3523	CCAGCAACTGTTCCTCATCG CAGGTAGGTGCGCTCGTC	2 fold induction
PA2931	CGAGGCGATGGAAATCAG GCATAGAAGGTCGCCAACTC	4 fold induction
PA0182	CGACATCCTGGTCAACAATG GGTGATGTAGGCCGCTTC	2 fold induction

PA4167	GCAGATCTACGGCAACGAG GCAAGTAAGGGCTGAGTTCG	3 fold induction

Figure Captions

Figure 2-1. Dose-dependent inhibition of AHL-mediated QS by BF8. The structure of BF8 (A) and relative QS activities of *V. harveyi* BB886 (B) and PAO1 *lasB* reporter (C) are shown. To study the effects on QS in *V. harveyi* BB886 reporter, an overnight culture of *V. harveyi* BB886 were diluted 1:5000 in AB medium and supplemented with different concentrations of BF8 after 5.5 h of incubation. The QS activity of each sample was characterized by normalizing the bioluminescence of the reporter *V. harveyi* BB886 with its colony forming unit (CFU) after another 1.5 h of incubation. Figure 2-1B shows that QS was inhibited by BF8 in a dose dependent manner. To study the effects on QS in PAO1, the reporter strain PAO1 mini-Tn5-based P*lasB-gfp*(ASV) was cultured till an OD_{600} of 0.8 and then BF8 was added at different concentrations. The green flouresence was measured when the cultures reached stationary phase (OD_{600} around 2.7). The results show that QS in PAO1 was inhibited by BF8.

Figure 2-2. BF8 reduced persistence of PAO1 at growth non-inhibitory concentrations. (A) BF8 reduced persistence of PAO1 cultures during growth. PAO1 was cultured for 5 h in LB medium supplemented with different concentrations of BF8. The total number of viable cells and the number of persister cells after the 5 h incubation were quantified. (B) Sugars did not exhibit the same activities as BF8. The same experimental procedure was followed except that 10 mM glucose or mannitol was added instead of BF8.

Figure 2-3. BF8 and 3-oxo-C_{12}-HSL reverted antibiotic tolerance of isolated PAO1 persister cells. The total number of viable cells and the number of cells that remained as persisters of untreated controls in each graph were normalized as 100% for the convenience of data comparison across the three experiments. (A) BF8 reverted Cip tolerance of isolated PAO1 persister cells. The harvested PAO1 persister cells were treated with different concentrations of BF8 for 2 h in 0.85% NaCl solution and the viability of PAO1 was evaluated by counting CFU. A portion of each sample was then treated with 200 μg/mL Cip to count the number of PAO1 cells that remained as persisters. The start number of persisters was $2.1\times10^6 \pm 3.1\times10^5$/mL. (B) Antibiotic susceptibility of PAO1 persister cells treated with and without 5 μg/mL BF8**.** Persisters were isolated and treated with or without 5 μg/mL BF8 in 0.85% NaCl solution for 2 h. The treated cells were then incubated with different antibiotics for 3.5 h to test antibiotic susceptibility. PAO1 persisters were found to be sensitized to Tob and Cip. The start number of persisters was $2.3\times10^6 \pm 5.7\times10^4$/mL. (C) The QS signal 3-oxo-C_{12}-HSL also sensitized PAO1 persisters to Cip. The same procedure as that in Figure 2-3A was followed except that 3-oxo-C_{12}-HSL was tested instead of BF8. The start number of persisters was $2.0\times10^6 \pm 4.0\times10^5$/mL.

Figure 2-4. BF8 is effective against PAO1 biofilms. (A) BF8 dispersed biofilm and reduced persistence in both biofilm and the detached population. The biofilms were treated with BF8 at different concentrations for 24 h in 0.85% NaCl solution. (B) BF8 inhibited biofilm formation and reduced the number of persister cells in biofilms. BF8 was added at inoculation and the biofilms were cultured for 18 h.

Figure 2-5. Effects of BF8 on growth and viability of *P. aerugionsa* PAO1. (A) Effects on growth. LB medium was inoculated with overnight *P. aeruginosa* PAO1 cultures to an OD_{600} of 0.05. BF8 was added at different concentrations (0 - 200 µg/mL) and the presence and absence of growth were followed after 24 h of incubation at 37°C. The results indicate that none of the tested concentrations was sufficient to inhibit growth completely. Therefore the MIC was found to be higher than 200 µg/mL in LB medium. (B) Effects on viability. An 18-h overnight culture of PAO1 was washed and diluted with 0.85% NaCl solution to an OD_{600} of 0.05 supplemented with different concentrations of BF8 (0 - 30 µg/mL). After 2 h of incubation, the number of viable cells was determined by counting CFU. None of the tested concentrations was sufficient to kill more than 99.9% of PAO1. Therefore the MBC was found to be higher than 30 µg/mL in 0.85% NaCl solution.

Figure 2-6. Effects of Cip treatment time on PAO1 killing. An 18-h overnight culture of PAO1 was treated with 200 µg/mL Cip for different lengths of time to determine the required treatment time for persister isolation.

Figure 2-7. Transcription level of the housekeeping gene, *proC*, from total cells (before Cip treatment) and persister cells quantified with Q-PCR. The persister cells were isolated following the same procedure as described in the manuscript. The cells before and after Cip treatment were used to isolate total RNA and compare the transcription level of *proC*.

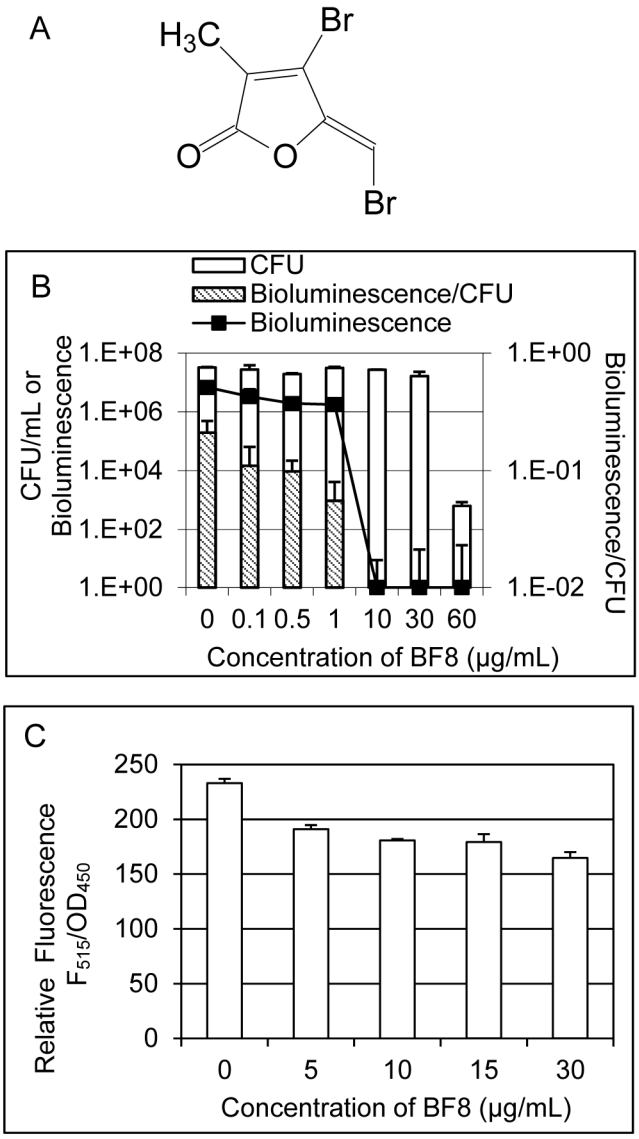

A
H3C
Br
O
O
Br
B
CFU
Bioluminescence/CFU
Bioluminescence
CFU/mL or Bioluminescence
1.E+08
1.E+06
1.E+04
1.E+02
1.E+00
1.E+00
1.E-01
1.E-02
Bioluminescence/CFU
0 0.1 0.5 1 10 30 60
Concentration of BF8 (μg/mL)
C
Relative Fluorescence F515/OD450
250
200
150
100
50
0
0 5 10 15 30
Concentration of BF8 (μg/mL)

Figure 2-1

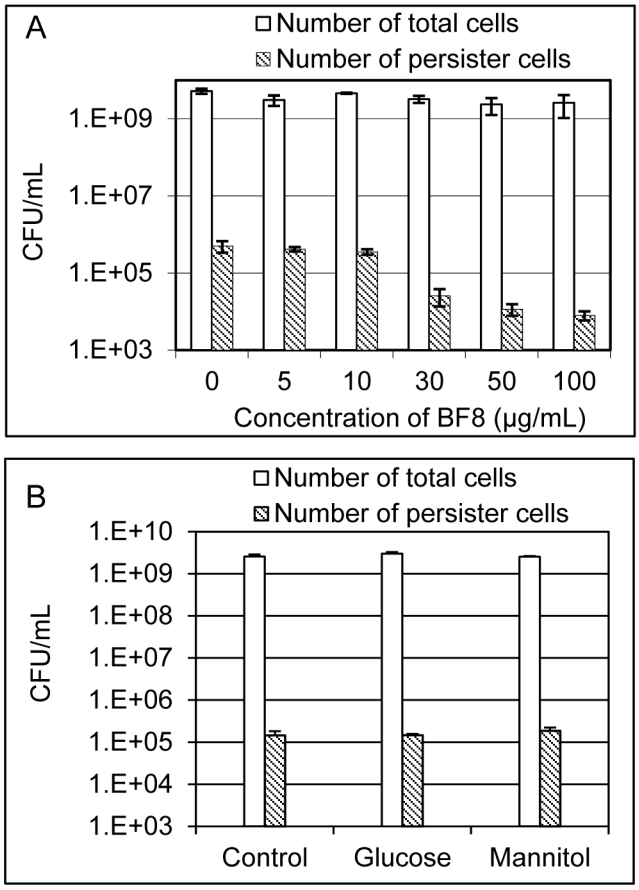

A
Number of total cells
Number of persister cells
CFU/mL
1.E+09
1.E+07
1.E+05
1.E+03
0
5
10
30
50
100
Concentration of BF8 (μg/mL)
B
Number of total cells
Number of persister cells
CFU/mL
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
Control
Glucose
Mannitol

Figure 2-2

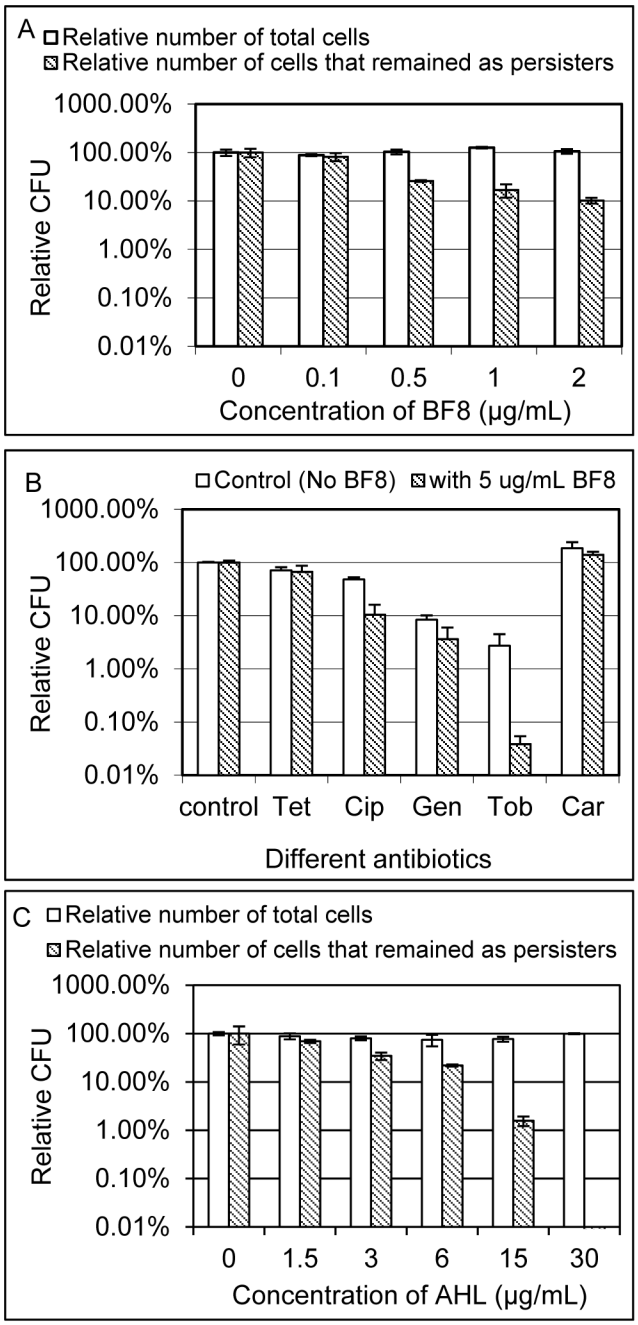

A
Relative number of total cells
Relative number of cells that remained as persisters
1000.00%
100.00%
10.00%
1.00%
0.10%
0.01%
Relative CFU
0
0.1
0.5
1
2
Concentration of BF8 (μg/mL)
B
Control (No BF8)
with 5 ug/mL BF8
1000.00%
100.00%
10.00%
1.00%
0.10%
0.01%
Relative CFU
control
Tet
Cip
Gen
Tob
Car
Different antibiotics
C
Relative number of total cells
Relative number of cells that remained as persisters
1000.00%
100.00%
10.00%
1.00%
0.10%
0.01%
Relative CFU
0
1.5
3
6
15
30
Concentration of AHL (μg/mL)

Figure 2-3

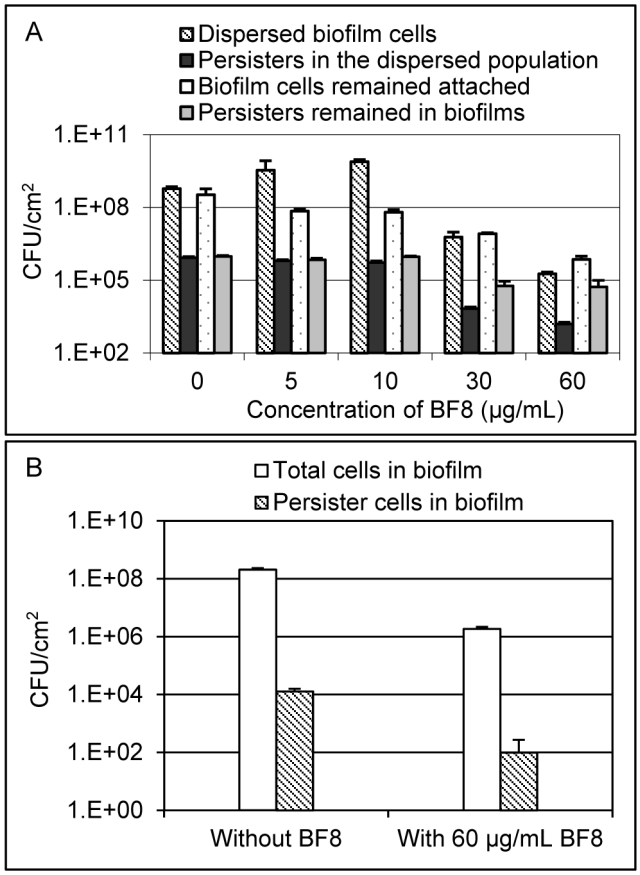

A
Dispersed biofilm cells
Persisters in the dispersed population
Biofilm cells remained attached
Persisters remained in biofilms
1.E+11
1.E+08
1.E+05
1.E+02
CFU/cm2
0
5
10
30
60
Concentration of BF8 (μg/mL)
B
Total cells in biofilm
Persister cells in biofilm
1.E+10
1.E+08
1.E+06
1.E+04
1.E+02
1.E+00
CFU/cm2
Without BF8
With 60 μg/mL BF8

Figure 2-4

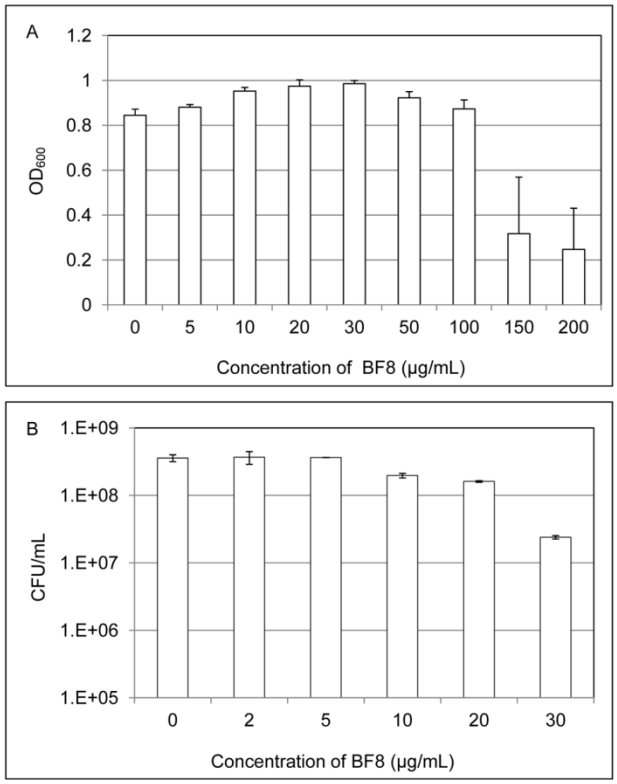

A
1.2
1
0.8
0.6
0.4
0.2
0
OD600
0
5
10
20
30
50
100
150
200
Concentration of BF8 (μg/mL)
B
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
CFU/mL
0
2
5
10
20
30
Concentration of BF8 (μg/mL)

Figure 2-5

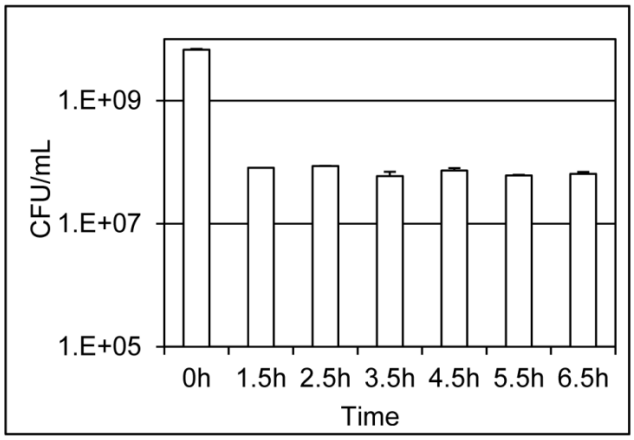


Figure 2-6

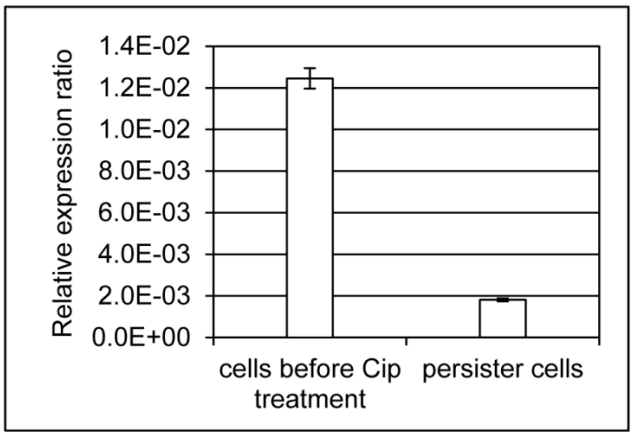

Relative expression ratio
1.4E-02
1.2E-02
1.0E-02
8.0E-03
6.0E-03
4.0E-03
2.0E-03
0.0E+00
cells before Cip treatment
persister cells

Figure 2-7

Chapter 3

(*Z*)-4-BROMO-5-(BROMOMETHYLENE)-3-METHYLFURAN-2(5*H*)-ONE SENSITIZED *Escherichia coli* PERSISTER CELLS TO ANTIBIOTICS

This chapter has been submitted as below with minor modifications. Jiachuan Pan, Ali Adem Bahar, Fangchao Song and Dacheng Ren. (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one sensitizes *Escherichia coli* persister cells to antibiotics. Biotechnology and Bioengineering. 2013.

3.1 Abstract

Persisters are a small subpopulation of bacterial cells that are dormant and extremely tolerant to antibiotics. Such intrinsic tolerance also facilitates the development of multidrug resistance through acquired mechanisms based on drug resistance genes. In this study, we demonstrate that BF8 is effective against persistence in *Escherichia coli*. It was found to reduce persistence during *E. coli* growth and revert the antibiotic tolerance of its persister cells. The effects were more profound when pH was increased from 6 to 8.5. Although BF8 is a quorum sensing (QS) inhibitor, similar effects were observed for the wild type *E. coli* RP437 and *ΔluxS* mutant, suggesting that the effects are not solely through inhibition of AI-2 mediated QS. In addition to planktonic persisters, BF8 was also found to disperse RP437 biofilm and render the cells more sensitive to ofloxacin. These findings broadened the activities of brominated furanones and shed new lights on persister control.

Keywords: antibiotic tolerance, persister, quorum sensing, inhibition, biofilm

3.2 Introduction

It is well documented that bacteria can tolerate antibiotics by entering dormancy and forming so-called persister cells, or by attaching to surfaces and developing multicellular structures, known as biofilms (1). Such high level antibiotic tolerance leads to chronic infections and facilitates the development of multidrug resistance through acquired mechanisms (2); e.g., high persistence mutants of *Pseudomonas aeruginosa* and *Candida albicans* have been isolated from patients with cystic fibrosis and oral thrush biofilm, respectively (3, 4). Persister formation can result from unbalanced toxin/antitoxin production (5), SOS response (5), heavy metal toxicity responses (1, 6) and by cell-cell signaling based on indole (7). Some factors that have been found to stimulate persister formation include toxic metal ions, oxidants, starvation, high temperature, low pH, and membrane acting agents such as TisB (1, 5, 8).

Besides persisters cells, biofilm formation is another important strategy for bacteria to survive in adverse environments. Due to their ubiquitous presence, biofilms are a major form of microbial life in both natural and disease conditions (9). Biofilm matrix contain extracellular polymeric substances (EPS) such as extracellular DNA (eDNA), polysaccharides, proteins and amyloid fibrils which help maintain the biofilm structure and protect the biofilm cells (10). Consistently, biofilm bacteria are up to 1000 times more tolerant to antibiotics than free swimming cells (11)

and are involved in 80% of human bacterial infections (12). Persister formation increases in biofilms and high persister mutants have been isolated from biofilms clinically (3, 13).

Despite the significance, only few studies on persister control have been reported to date. Allison et al (14) demonstrated that sugars, such as mannitol, glucose, fructose and pyruvate could make persister cells more sensitive to aminoglycosides. Their study reported that NADPH generated from sugar is oxidized in the electron transport chain by quinone oxidase, and the proton motive force (PMF) generated in this process facilitates the uptake of aminoglycosides (14). This discovery is consistent with the report of Dörr (5) that TisB, a membrane-acting peptide and a toxin, could decrease the PMF and ATP levels in *E. coli*; and therefore, increase its tolerance to antibiotics (15).

Persister formation of different bacteria species (*E. coli*, *P. aeruginosa*, *Staphylococcus aureus*) has been found to increase when their cultures enter stationary phase (1, 16). However, quorum sensing (QS), a bacterial cell-to-cell signaling system based on cell density, has only been found to promote persister formation in *P. aeruginosa*, but not *E. coli*. For example, adding spend medium (supernatant from stationary phase) to early exponential phase cultures of *P. aeruginosa* PA14 or 3-oxo-C_{12}-homoserine lactone to early exponential phase cultures of *P. aeruginosa* PAO1 led to increased persistence (17). However, spend medium from stationary phase of *E. coli* cultures does not affect its persister formation (1).

Since QS promotes persister formation in *P. aeruginosa*, we recently tested if QS can be a target for persister control. We evaluated the effects of a synthetic QS inhibitor, (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one (BF8), on the persistence of *P. aeruginosa* PAO1 (18). This compound was found to reduce persister formation of *P. aeruginosa* PAO1 and

sensitize the formed persisters to ciprofloxacin and tobramycin. Interestingly, the QS signal *N*-(3-Oxododecanoyl)-*L*-homoserine lactone was also found to sensitize *P. aeruginosa* PAO1 persister cells under our experimental condition. Thus, the effects of BF8 on *P. aeruginosa* PAO1 persisters are, at least, not solely through inhibition of QS via N-Acyl homoserine lactones (AHL).

To further understand this new phenomenon and test if BF8 is also effective against other bacteria. We tested its effects on *E. coli* persister cells. We chose *E. coli* in this study because *E. coli* does not produce AHL (19), allowing us to study the effects of BF8 on persisters in the absence of this class of QS signals at growth non-inhibitory concentrations (Figure 3-6). Also, we have reported that BF8 is an inhibitor of *E. coli* biofilm formation (20) and QS based on AI-2 (21). Thus, it can help to understand general vs. specific effects of BF8 in bacteria that can communicate via AI-2 mediated QS.

3.3 Material and Methods

3.3.1 Furanone Synthesis.

(*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one (BF8) was synthesized as described previously (18, 20), dissolved in absolute ethanol as 60 mg/mL, and stored at 4°C until use.

3.3.2 Bacterial strains and growth media.

E. coli RP437 (henceforth RP437) and *E. coli* KX1485 (RP437 *ΔluxS*, henceforth KX1485) (22) were routinely grown in Luria-Bertani (LB) medium (23) containing 10g/L tryptone, 5g/L yeast extract and 10g/L NaCl. LB medium was also used to form biofilms. Persister cells of *E. coli* were isolated as described previously (14, 18)_ENREF_14: briefly, an overnight culture was

treated with 5 µg/mL ofloxacin (Ofl) for 3.5 h at 37^{o}C with shaking at 200 rpm, washed twice with 0.85% NaCl solution to remove Ofl, and then resuspended in 0.85% NaCl solution. This condition was confirmed to be sufficient to kill regular cells (Figure 3-7). The drop plate method described as described previously (24) was followed to count colony forming units (CFUs) and One-way ANOVA followed by Tukey test was used to test the significance of all results. PBS buffer used in this study was based on 0.85% NaCl solution with NaH_2PO_4 and Na_2HPO_4 added as pH buffering agents. The ionic strength of pH buffering agents was 10 mM. The pH value was adjusted as 6, 6.5, 7, 7.5, 8 or 8.5.

3.3.3 Effects of BF8 on persister formation.

A RP437 overnight culture was used to inoculate sub-cultures in 5 mL LB medium with an initial optical density at 600nm (OD_{600}) of 0.05. The subcultures were then supplemented with different concentrations of BF8 (0, 2, 5 and 10 µg/mL) immediately. The amount of ethanol (solvent of BF8 stock solutions) was adjusted to be the same for all samples to eliminate any solvent effect. Samples were taken after 5 h of incubation at 37^{o}C with shaking at 200 rpm and the total number of viable cells in each sample was determined by counting CFU using drop plate method as described previously (24). Meanwhile, the remaining portion of each sample was added with 5 µg/mL Ofl and incubated for 3.5 h at 37^{o}C with shaking at 200 rpm to count the number of cells that remained as persisters. To compare with the effects of sugars (known to sensitize *E. coli* and *S. aureus* persister cells to aminoglycosides (14)), the same treatment was also tested using 10 mM D-glucose or D-mannitol instead of BF8. To understand if the effect of BF8 on persister formation is through QS inhibition through AI-2, a *luxS* mutant (KX1485) was tested under the same condition.

3.3.4 Effects of BF8 on ofloxacin susceptibility of isolated persister cells.

Persister cells were isolated from an 18 h overnight culture as described above and diluted by 50 times with 0.85% NaCl solution. Then each 3 mL of diluted persister cells was challenged with different concentrations of BF8 (0, 0.1, 0.5, 1, 2 and 5 µg/mL). The concentration of ethanol (the solvent used for making BF8 stock solutions) was adjusted to be the same for all samples to eliminate any solvent effect. After incubation at 37^{o}C for 2 h with shaking at 200 rpm, 1 mL of each sample was taken and the cells were washed three times with 0.85% NaCl solution to evaluate the viability by counting CFU. In addition, the number of cells that remained as persisters were quantified by counting CFU after treatment with 5 µg/mL Ofl for 3.5h at 37^{o}C.

3.3.5 Synergy with other antibiotics.

Persisters were isolated from 18 h overnight cultures, and treated with BF8 at different concentrations. After incubation, 1 mL of BF8 treated persister samples or controls (no BF8) were supplemented with different antibiotics [25 µg/mL tetracycline (Tet), 25µg/mL gentamicin (Gen), 25µg/mL tobramycin (Tob) or 100 µg/mL ampicillin (Amp)] and incubated for another 3.5 h at 37^{o}C with shaking at 200 rpm. The antibiotic-treated persisters were then washed three times with 0.85% NaCl solution to remove antibiotics, diluted and plated on LB agar plates to determine the susceptibility of control and BF8-treated persister cells to different antibiotics.

3.3.6 Synergy with pH change.

Persisters were isolated from 18 h overnight cultures, and then diluted by 50 times with PBS buffer at different pH (6.0, 6.5, 7.0, 7.5, 8.0 or 8.5) or 0.85% NaCl solution. Then 1 µg/mL of BF8 was added to the above PBS or 0.85% NaCl solution with persister cells. After 2 h

incubation at 37°C with shaking at 200 rpm, 1 mL sample was washed, diluted and plated on LB agar plates to count CFU. Meanwhile, a part of BF8-treated persister cells was treated with 5 µg/mL Ofl for 3.5 h again to determine the number of cells that remained as persisters. This experiment was also performed without BF8 as control. To determine if the effects of pH was due to any permanent change in BF8's structure, BF8 was first dissolved in PBS with pH 6, at 10 µg/mL. Then the pH was adjusted to be 6.5, 7, 7.5, 8 or 8.5 with NaOH solution, and incubated at 37°C for 2 h with shaking at 200 rpm. After this treatment, the pH of each sample was adjusted back to 6 using HCl solution. The amount of NaOH and HCl solution was pre-calculated for each sample to ensure the final concentration of BF8 to be 1 µg/mL. These solutions were then tested for their effects on persister cells as described above.

3.3.7 Effects of BF8 on persister formation during biofilm growth.

RP437 overnight cultures were used to inoculate LB medium supplemented with different concentrations of BF8 (0, 5, 10, 30 and 60 µg/mL) to an OD_{600} of 0.05. The amount of ethanol (solvent in BF8 stocks) was adjusted to be the same in all samples to eliminate any solvent effect. Sterile 2 cm × 1 cm 304L stainless steel coupons were transferred to these cultures to form biofilms. After 18 h of biofilm growth at 37°C without shaking, coupons with biofilms were washed gently with 0.85% NaCl solution and soaked in 5 mL 0.85% NaCl solution. The biofilm cells were mechanically detached by vortexing for 1 min and sonicating (Ultrasonic cleaner Model No B200, Sinosonic Industrial Co., Ltd, Taipei Hsien, Taiwan) for 1 min (repeat once) (25). This condition was found to sufficiently detach biofilm cells without affecting cell viability (25, 26). A portion of detached biofilm cells was plated on LB plates to count CFU and the rest of each sample was used for persister isolation as described above. In addition to biofilm cells, 1 mL planktonic cells in each subculture was also washed three times and plated on LB agar plates

to determine the viability by counting CFU. Another 1 mL of planktonic cells in each subculture was sampled to count the number of viable persisters after 3.5 h treatment with Ofl at 5 µg/mL, as described above.

3.3.8 Effects of BF8 on biofilm–associated persister cells.

Biofilms were grown on 304L stainless steel coupons (2cm × 1 cm) in petri dishes as described above but in the absence of BF8. After 18 h of incubation, the coupons with established biofilms were washed gently with 0.85% NaCl solution and transferred to a 12 well plate (Becton Dickinson, Franklin Lakes, NJ,). Each well contained 4 mL of 0.85% NaCl solution supplemented with BF8 at 0, 2, 5, 10 or 30 µg/mL. The biofilm samples in 12 well plates were incubated at 37°C for 12 h without shaking. After treatment, 1 mL of suspension was sampled from each well. The cells were collected by centrifugation, washed three times with 0.85% NaCl solution, and then plated on LB agar plates to determine the viability of RP437 cells by counting CFU. Meanwhile, another 1 mL of planktonic sample was collected to quantify the number of persister cells as described above. To collect the biofilm cells, each coupon was transferred to a 15 mL falcon tube containing 5 mL 0.85% NaCl solution, vortexed for 1 min and sonicated for 1 min (repeat once) (25). The total number of viable cells and number of viable persister cells in the biofilm population were determined using drop plate method as described above.

3.3.9 DNA microarray analysis.

Persister cells were harvested from overnight cultures of RP437 (100 mL each) using the same methods as described above. The isolated persister cells were resuspended in 200 mL 0.85% NaCl solution supplemented with or without 5 µg/mL BF8. The amount of ethanol was adjusted to be the same for both samples to eliminate any solvent effect. After incubation at 37°C for 1 h,

treated persister cells were collected by centrifugation at 10,000 rpm for 5 min at 4^{o}C, transferred to 2 mL pre-cooled centrifuge tubes and frozen instantly in an ethanol-dry ice bath. The cell pellets were stored at -80°C until RNA isolation, using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) as described previously (18). The isolated RNA samples were sent to SUNY Upstate Medical University for hybridization to Affymetrix DNA microarrays. Using GeneChip Operating Software (MAS 5.0), genes with a p value of less than 0.0025 or greater than 0.9975 were considered as statistically significant based on Wilcoxon signed rank test and Tukey Byweight. The genes that are induced/repressed in both data sets are listed.

3.3.10 Quantitative real time PCR analysis.

Six genes were selected to further confirm their transcription level using with Q-PCR. These genes include three induced genes (*mdaB*, *yhhW*, *ybiJ*), two repressed genes (*trpD*, *cspD*), and two genes that were not affected by BF8 (including *hcaD* and housekeeping gene *mdoG*). cDNAs for control and BF8 treated samples were synthesized by using iScript™ cDNA Synthesis Kit (Biorad, Hercules, CA, USA).

Primers for selected genes were designed using OligoPerfect™ Designer (Life Technologies, USA) to keep the melting temperatures between 59.9˚C and 60.1˚C and product size between 152-220 bp. The sequences of primers are listed in Table S1.

Q-PCR reaction was carried with an Eppendorf Mastercycler Realplex thermal cycler (Eppendorf, Hauppauge, NY, USA) and Fast SYBR Green Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). The following conditions were applied in Q-PCR reactions: enzyme activation for 1 min at 95˚C followed by 40 cycles with denaturation for 5 s at 95˚C, and annealing/extension for 45 s at 60˚C.

The DNA microarray data have been deposited in Gene Expression Omnibus (GEO: GSE44273)

3.4 Results

3.4.1 BF8 reduced persister formation during *E. coli* growth in LB medium.

The subcultures of RP437 were treated with BF8 for 5 h in LB medium at growth-non inhibitory concentrations (0, 2, 5 or 10 µg/mL). As shown in Figure 3-1A, at 2 µg/mL, BF8 reduced persistence by 63.9±5.0% compared to BF8-free control (p= 0.0047); while at a higher concentration of 10 µg/mL, BF8 reduced persistence by 83.5±1.2% (p=0.0018). Consistent with what we found for *P. aeruginosa* (18), 10 mM of mannitol and glucose, which were reported to sensitize *E. coli* persister cells to aminoglycosides (14), did not exhibit the same effects as BF8 (p = 0.9273) (Figure 3-1B). Since BF8 is an inhibitor of AI-2 mediated QS (21), we also tested its effects on an AI-2 mutant to understand if the effects of BF8 were specifically through AI-2 inhibition. The persister levels of RP437 and its *ΔluxS* mutant in 5 h cultures were found to be the same (Figure 3-8). As shown in Figure 3-1C, BF8 at growth non-inhibitory concentrations also reduced persistence of the *ΔluxS* mutant (KX1485) during 5 h incubation. For example, at 10 µg/mL, BF8 didn't reduce the growth of KX1485; however, the number of persister cells was reduced by 68.9 ± 2.4% compared to the BF8-free control. The similar activities of BF8 on RP437 and its *ΔluxS* mutant suggest that persister control by BF8 is at least not solely through QS inhibition and BF8 may have other target(s).

3.4.2 BF8 sensitized *E. coli* persisters to antibiotics.

BF8 was also tested for its effects on persisters isolated from an overnight culture. As shown in Figure 3-2A, BF8 didn't exhibit significant effects on the viability of isolated persister cells (p=0.2519); however, the persister cells were rendered sensitive to Ofl dose-dependently by BF8. For example, 91.6± 0.3% persister cells were killed by Ofl treatment after incubation with 5 µg/mL BF8 for 2 h (p=0.0018). To test if BF8 can also revert the tolerance of *E. coli* persister cells to other antibiotics, representative antibiotics from another two classes of antibiotics were tested with targets of protein synthesis (Tet, Gen and Tob) and cell wall synthesis (Amp), respectively. Persister cells isolated from overnight cultures were treated with BF8 at different concentrations for 2 h and then challenged with these antibiotics. As shown in Figure 3-2B-E, after treatment with BF8 at 5µg/mL, RP437 persister cells were rendered more sensitive to Tet, Tob and Gen treatment but not to Amp. For example, at 5 µg/mL, BF8 sensitized isolated persisters to: Tet by 100.0 ± 0.0% (p<0.0001), Tob by 74.7 ± 3.9% (p=0.0020) and Gen by 79.8 ± 6.7% (p=0.0257). We chose 0.85% NaCl solution rather than LB medium for this test because 0.85% NaCl solution does not support growth so that we can study the effects of BF8 on isolated persister cells specifically. It is worth noticing that the concentrations of BF8 that exhibited activities are lower in 0.85% NaCl than in LB, presumably because LB medium contains large molecules that can bind to BF8 and reduce its activities.

3.4.5 Synergy with pH change.

Since change in PMF has been shown to sensitize persister cells to aminoglycosides, we hypothesized that the effects of BF8 can be enhanced by adjusting the pH during treatment. A pH range of 6-8.5 was tested and the results are shown in Figure 3-3A. At pH from 6 to 7.5, BF8 didn't show significant cidal effect on persister cells (p=0.0689); however, BF8 was more effective in sensitizing persister cells to Ofl when pH increased from 6 to 7 and further more at

pH 7.5. For example, tolerance to Ofl was reduced by 85.5±0.5% at pH 7.5 compared to pH 6 (p=0.0144). At pH values higher than 7.5, BF8 exhibited cidal effects on persister cells and further reduced persistence; e.g., at pH of 8, 69.2± 5.4 % persister cells were killed by 1 µg/mL BF8 and 98.5 ±0.7% of persister cells were rendered sensitive to Ofl compared to pH 6. Overall, the potency of BF8 to sensitize RP437 persister cells to Ofl was found to increase with pH in the tested range (pH 6-8.5); and no cidal effect of BF8 was observed for pH values not higher than 7.5. To compare the results, isolated persister cells were also treated with the same buffers with varying pH without BF8. As shown in Figure 3-3B, in the absence of BF8, persister cells remained tolerant to Ofl under all the pH values tested (p=0.0663) and no cidal effects were observed (p=0.9564). Thus, the pH change itself did not affect persistence, but exhibited interesting synergy with BF8. To determine if this is a true synergy or permanent change in BF8's structure in alkaline condition, BF8 was treated at high pH values and then adjusted to pH 6 before it was tested on isolated persister cells. As shown in Figure 3-3C, pH-treated BF8 showed no cidal effect on persister cells (p=0.2674) or the change in the ability to sensitize isolated persister cells (p=0.3589). So the increase in potency of BF8 at higher pH was indeed through synergistic effects, rather than permanent changes in BF8's structure.

3.4.6 BF8 reduced persistence in RP437 biofilms.

We reported previously that BF8 can inhibit biofilm formation of *E. coli* RP437 at growth-non inhibitory concentrations (20). In this study, we were interested in further characterizing its effects on biofilm-associated persister cells. To achieve this goal, RP437 biofilm was grown in LB medium in the absence and presence of different concentrations of BF8. The number of total viable cells and the number of persisters were quantified for both the biofilm and planktonic populations. As shown in Figure 3-4, BF8 reduced biofilm formation of RP437 on stainless steel

304L coupons, consistent with the report of Han et al. (20) For example, BF8 at concentrations up to 60 µg/mL showed no cidal effect (p=0.1107). However, BF8 significantly reduced biofilm formation; e.g., at 30 µg/mL, the total number of biofilm cells was reduced by 93.2 ± 2.3% (p=0.0197), compared with the BF8-free control. In addition to inhibition of biofilm formation, BF8 was found to reduce the number of persister cells in both the planktonic and biofilm populations. For example, at 30 µg/mL, the total number of persisters in planktonic and biofilm population were reduced by 90.3 ± 2.8% (p<0.0001) and 87.4 ± 3.1% (p=0.0218) respectively, compared with the BF8-free control.

3.4.7 BF8 reduced the persistence of pre-formed biofilms.

In addition to the effects on biofilm formation and associated persistence, BF8 was also found to detach RP437 cells from 24-h biofilms and reduce the number of persister cells dose-dependently. For example, shown in Figure 3-5, at 5 µg/mL, the viable cells in planktonic phase were not reduced significantly (p=0.0543); while the number of viable cells in biofilm was reduced dramatically by 94.4 ± 1.0% (p<0.0001). BF8 also reduced the persister cell number in both planktonic and biofilm populations. For example, at 5 µg/mL, the number of persister cells in planktonic population was reduced by 93.1 ± 5.6% (p<0.0001); and the number of viable persister cells in biofilm was reduced by 99.0 ± 0.1% (p<0.0001).

3.4.8 DNA microarray study.

Treatment of RP437 persister cells with 5 µg/mL BF8 for 1 h was found to induce 6 genes and repress 10 genes consistently in duplicated (two biological replicates) DNA microarray tests (Table 1). These results were also confirmed with a Q-PCR study by choosing 6 of these 17 genes, including *trpD*, *cspD*, *mdaB*, *yhhW*, *ybiJ* and *hcaD* (Table 2). It is interesting to note that

mdaB was induced by BF8. This gene has a homolog PA2580 in *P. aeruginosa* which was also induced by BF8 (18).

BF8 repressed *ycfR* by 15.3 fold compared to the BF8-free control. Deletion of *ycfR* was reported to induce more biofilm formation and change *E. coli* cells from hydrophilic to hydrophobic (27). Thus, induction of this gene by BF8 is consistent with biofilm inhibition by BF8. Among the repressed genes, *trpABCDE* were repressed by 3-4 fold. This operon is involved in tryptophan synthesis and indole synthesis. It has been reported that deletion mutant of *trpE* produces 10-fold less indole than the wild-type (27, 28) and indole has been shown to be signal to promote antibiotic tolerance in *E. coli* (7). It will be interesting to further study if indole synthesis is affected by BF8.

3.5 Discussion

In this study, the effects of (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one (BF8) on RP437 and KX1485 were tested. The obtained results show that BF8 could reduce the persistence during *E. coli* growth and sensitize the isolated persister cells to Ofl (DNA gyrase inhibitor), Gen, Tet, and Tob (aminoglycosides), but not Car (β-lactamase). The effects of BF8 were found more effective with increase in pH (pH 6-8.5 tested). In addition to planktonic cells, BF8 was found to reduce biofilm formation, reduce the number of viable cells of pre-formed biofilms and reduce the number of associated persister cells.

This study shows that a QS inhibitor BF8 can reduce persister formation during the growth of *E. coli* RP437 in LB medium, at the concentrations that have no cidal effects. It has been reported that one resuscitation-promoting factor can sensitize persister cells of Gram- positive bacteria (29), and 3-[4-(4-methoxyphenyl)piperazin-1-yl]piperidin-4-yl biphenyl-4-carboxylate can

sensitize persister cells of Gram-negative bacteria (30) to norfloxacin. Also sugars have been reported to sensitize both Gram-positive and Gram-negative persister cells to aminoglycoside (14). Compared to these compounds, BF8 is the first QS inhibitor that has been shown to control persisters. Interestingly, both in our recent study on *P. aeruginosa* persisters and in the present study on *E. coli*, we obtained data suggesting the activities of BF8 in persister control are not solely through QS inhibition. The synergy with pH shown in this study indicates that BF8 may interact with cell membrane.

In a previous study, it was found that PMF from oxidization of NADH facilitates aminoglycoside uptake by persister cells (14). In this process, quinones(Q), NADH and quinol oxidase are involved (14). Interestingly, our DNA microarray data show that *mdaB* encoding a modulator of drug activity B, a NADPH quinone oxidoreductase, was induced by BF8. This enzyme has a predicted binding site specific for flavin adenine dinucleotide (FAD) (31), which is an active redox cofactor and transfers two electrons(31). In addition, the depletion of flavin mononucleotide pool was considered to cause persister formation (1, 32). This implies that waking up persister may involve the redox reactions catalyzed by FAD. Besides *P. aeruginosa* and *E. coli*, *mdaB* is conserved in many other species including *Helicobacter hepaticus* (33). Thus, it will be helpful to test if BF8 might show similar effects on other bacterial species. Our microarray data also showed reduction in *cspD*. This result is consistent with Kim's report (34) that toxin cspD can increase persister formation.

DNA microarray study showed no QS genes were directly affected by BF8. This is expected because the microarray study was based on dormant persister cells. However the expression of gene related to indole-production was repressed by BF8; e.g. multiple genes in the *trp* operon (*trpABCDE*) were repressed by 3.0 to 4.7 fold (Table 1).

Deletion of *TrpE* has been shown to reduce indole production (28) and indole is a signal known to stimulate persister formation (35). The results are consistent with the finding in the present study that BF8 can revert the persistence in *E. coli* and repress *trpABCDE* genes. Interestingly, both indole (28) and BF8 (this study) are inhibitors of *E. coli* biofilm formation which warrants further research.

Collectively, the data obtained in this study suggest that BF8 may have multiple targets for persister control. Further studies on its effects on indole signaling and cell membrane functions will shed new lights on this interesting phenomenon.

3.6 Acknowledgements

We thank the U.S. National Science Foundation (CAREER-1055644 and EFRI-1137186) for support.

We are grateful to Dr. Frank Middleton and Ms. Karen Gentle at SUNY Upstate Medical University for helping with DNA microarray hybridization. We also thank Prof. John Parkinson at University of Utah for sharing *E. coli* RP437 and to Prof. Robert Austin at Princeton University for sharing *E. coli* KX1485.

References

1. **Lewis K.** 2010. Persister cells. Annu Rev Microbiol **64:**357-372.
2. **Lewis K.** 2007. Persister cells, dormancy and infectious disease. Nat Rev Microbiol **5:**48-56.

3. **Mulcahy LR, Burns JL, Lory S, Lewis K.** 2010. Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. J Bacteriol **192:**6191-6199.

4. **LaFleur MD, Qi Q, Lewis K.** 2010. Patients with long-term oral carriage harbor high-persister mutants of *Candida albicans*. Antimicrob Agents Ch **54:**39-44.

5. **Dörr T, Vulić M, Lewis K.** 2010. Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. PLoS Biol **8:**e1000317.

6. **Harrison JJ, Ceri H, Roper NJ, Badry EA, Sproule KM, Turner RJ.** 2005. Persister cells mediate tolerance to metal oxyanions in *Escherichia coli*. Microbiology **151:**3181-3195.

7. **Lee HH, Molla MN, Cantor CR, Collins JJ.** 2010. Bacterial charity work leads to population-wide resistance. Nature **467:**82-85.

8. **Harrison JJ, Ceri H, Turner RJ.** 2007. Multimetal resistance and tolerance in microbial biofilms. Nat Rev Microbiol **5:**928-938.

9. **Hall-Stoodley L, Costerton JW, Stoodley P.** 2004. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol **2:**95-108.

10. **McDougald D, Rice SA, Barraud N, Steinberg PD, Kjelleberg S.** 2011. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Nat Rev Microbiol **10**:39-50

11. **Olson ME, Ceri H, Morck DW, Buret AG, Read RR.** 2002. Biofilm bacteria: formation and comparative susceptibility to antibiotics. Can J Vet Res **66:**86.

12. **Romero R, Schaudinn C, Kusanovic JP, Gorur A, Gotsch F, Webster P, Nhan-Chang CL, Erez O, Kim CJ, Espinoza J.** 2008. Detection of a microbial biofilm in intraamniotic infection. Am J Obstet Gynecol **198:**135. e131-135.

13. **LaFleur MD, Kumamoto CA, Lewis K.** 2006. *Candida albicans* biofilms produce antifungal-tolerant persister cells. Antimicrob Agents Ch **50:**3839-3846.

14. **Allison KR, Brynildsen MP, Collins JJ.** 2011. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature **473:**216-220.

15. **Unoson C, Wagner EGH.** 2008. A small SOS-induced toxin is targeted against the inner membrane in *Escherichia coli*. Mol Microbiol **70:**258-270.

16. **Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K.** 2006. Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett **230:**13-18.

17. **Moker N, Dean CR, Tao J.** 2010. *Pseudomonas aeruginosa* increases formation of multidrug-tolerant persister cells in response to quorum-sensing signaling molecules. J Bacteriol **192:**1946-1955.

18. **Pan J, Bahar AA, Syed H, Ren D.** 2012. Reverting antibiotic tolerance of *Pseudomonas aeruginosa* PAO1 persister cells by (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2 (5H)-one. PLoS One **7:**e45778.

19. **Houdt R, Aertsen A, Moons P, Vanoirbeek K, Michiels CW.** 2006. N-acyl-l-homoserine lactone signal interception by *Escherichia coli*. FEMS Microbiol Lett **256:**83-89.

20. **Han Y, Hou S, Simon KA, Ren D, Luk YY.** 2008. Identifying the important structural elements of brominated furanones for inhibiting biofilm formation by *Escherichia coli*. Bioorg Med Chem Lett **18:**1006-1010.

21. **Hou S, Duo M, Han Y, Luk Y-Y, Ren D.** 2009. Inhibiting microbial biofilm formation by brominated furanones, p 6-10. Materials & Processes for Medical Devices Conference, Minneapolis, Minnesota.

22. **Park S, Wolanin PM, Yuzbashyan EA, Lin H, Darnton NC, Stock JB, Silberzan P, Austin R.** 2003. Influence of topology on bacterial social interaction. P Natl Acad Sci USA **100:**13910-13915.

23. **Sambrook J, Russell DW.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

24. **Chen CY, Nace GW, Irwin PL.** 2003. A 6 x 6 drop plate method for simultaneous colony counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes,* and *Escherichia coli.* J Microbiol Meth **55:**475-479.

25. **Chen X, Zhang M, Zhou C, Kallenbach NR, Ren D.** 2011. Control of bacterial persister cells by Trp/Arg-containing antimicrobial peptides. Appl Environ Microb **77:**4878-4885.

26. **Niepa THR, Gilbert JL, Ren D.** 2012. Controlling *Pseudomonas aeruginosa* persister cells by weak electrochemical currents and synergistic effects with tobramycin. Biomaterials. **33**:7356-7365

27. **Zhang XS, García-Contreras R, Wood TK.** 2007. YcfR (BhsA) influences *Escherichia coli* biofilm formation through stress response and surface hydrophobicity. J Bacteriol **189:**3051-3062.

28. **Lee J, Jayaraman A, Wood TK.** 2007. Indole is an inter-species biofilm signal mediated by SdiA. BMC Microbiol **7:**42.

29. **Mukamolova GV, Kaprelyants AS, Young DI, Young M, Kell DB.** 1998. A bacterial cytokine. P Natl Acad Sci USA **95:**8916.

30. **Kim JS, Heo P, Yang TJ, Lee KS, Cho DH, Kim BT, Suh JH, Lim HJ, Shin D, Kim SK.** 2011. Selective killing of bacterial persisters by a single chemical compound without affecting normal antibiotic-sensitive cells. Antimicrob Agents Ch **55:**5380-5383.

31. **Adams MA, Jia Z.** 2006. Modulator of drug activity B from *Escherichia coli*: crystal structure of a prokaryotic homologue of DT-diaphorase. J Mol Biol **359:**455-465.

32. **Hansen S, Lewis K, Vulić M.** 2008. Role of global regulators and nucleotide metabolism in antibiotic tolerance in *Escherichia coli*. Antimicrob Agents Ch **52:**2718-2726.

33. **Hong Y, Wang G, Maier RJ.** 2008. The NADPH quinone reductase MdaB confers oxidative stress resistance to *Helicobacter hepaticus*. Microb Pathogenesis **44:**169-174.

34. **Kim Y, Wood TK.** 2010. Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in *Escherichia coli*. Biochem Bioph Res Co **391:**209-213.

35. **Vega NM, Allison KR, Khalil AS, Collins JJ.** 2012. Signaling-mediated bacterial persister formation. Nat Chem Biol **8:**431-433.

Tables

Table 1

Gene	Expression ratio*	Gene products/Functions
mdaB	4.3	modulator of drug activity B
ybiJ	15.0	hypothetical protein
ycfR	15.3	Inhibition of *ycfR* has been shown to reduce biofilm formation (27)_ENREF_28_E
ygiD	6.5	hypothetical protein
yhaK	3.8	hypothetical protein
yhhW	6.1	hypothetical protein
aldA	-2.9	aldehyde dehydrogenase
cdd	-2.8	cytidine deaminase
cspD	-1.7	DNA replication inhibitor
ryeA	-2.7	non-coding RNA (small RNA that interacts with Hfq, a RNA chaperon)
trpA	-3.0	tryptophan synthase subunit alpha
trpB	-4.7	tryptophan synthase subunit beta

trpC	-3.5	bifunctional indole-3-glycerol phosphate
trpD	-3.9	bifunctional glutamine
trpE	-3.2	anthranilate synthase
udp	-3.8	uridine phosphorylase

* Expression ratio (BF8 vs. control, average of the two biological replicates)

Table 2

Gene	**Expression ratio (BF8 vs. control)**	
	DNA microarray	**Q PCR**
cspD	-1.7	-1.6
trapD	-3.9	-1.9
mdaB	+4.3	+1.4
yhhW	+6.1	+3.2
ybiJ	15.0	+5.0
hcaD	1 (no change)	1 (no change)

Figure Caption

Figure. 3-1 BF8 reduced persistence during RP437 growth. (A) BF8 reduced the number of RP437 persisters during growth. RP437 was cultured for 5 h in LB medium supplemented with BF8 at different concentrations. The total number of cells and the number of persister cells were normalized by the corresponding BF8-free control. (B) D-glucose and D-mannitol did not affect persistence. The same experimental procedure was followed by using 10 mM D-glucose or D-mannitol instead of BF8. The number of total cells and the number of persister cells of untreated controls were normalized as 100%.

Figure. 3-2 BF8 sensitized the persister cells to other antibiotics. BF8 sensitized RP437 persister cells isolated from an overnight culture to different antibiotics. The RP437 persister cells were isolated from an 18 h overnight culture and treated with different concentrations of BF8 (0, 0.5, 1, 2, 5 μg/mL) for 2 h in 0.85% NaCl solution. The viability of RP437 was evaluated by counting CFU and a portion of each sample was then treated with antibiotics to count the number of RP437 cells that remained as persisters. The tested antibiotics include 5

µg/mL Ofl (A), 25 µg/mL Tet (B), 25 µg/mL Tob (C), 25 µg/mL Gen (D) and 100 µg/mL Amp (E). The untreated controls were normalized as 100%.

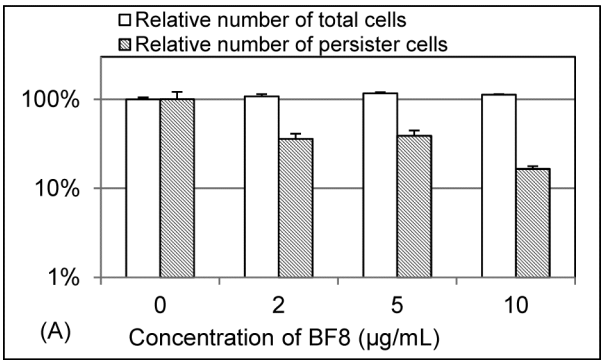
Figure. 3-3 Synergistic effects between BF8 and pH change. (A) BF8 exhibited higher activities with increase in pH. The effects of BF8 increased with pH between 6-8.5. The RP437 persister cells were isolated from an 18 h overnight culture and treated with different concentrations of BF8 for 2 h in PBS with different pH. The viability of RP437 was evaluated by counting CFU and a portion of each sample was then treated with 5 µg/mL Ofl to count the number of cells that remained as persisters. The cell numbers were normalized by those at pH of 6. (B) Changing pH alone did not affect persistence. The same experimental procedure was followed in the absence of BF8. The cell numbers were normalized by those at pH of 6. (C) Increase in the activity of BF8 is through synergy with pH change rather than permanent change in BF8's structure. BF8 was treated with PBS at different pH (6, 6.5, 7, 7.5, 8, 8.5) for 2 h, and then adjust back to pH of 6 before tested for their effects on RP437 persisters.

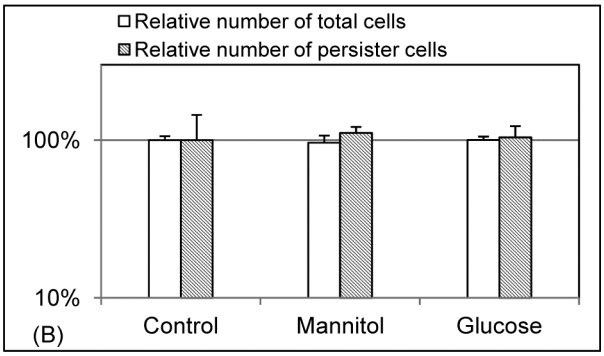
Figure. 3-4 BF8 reduced biofilm formation and associated persistence. (A) BF8 reduced biofilm formation and persister cells inside biofilm during the growth of biofilm. BF8 at different concentrations was added at inoculation and the biofilms were cultured for 18 h. (B) *E. coli* RP437 was cultured with BF8 at different concentrations (0, 5, 10, 30 and 60 µg/mL) for 18 h. Biofilm formation was reduced at growth-non inhibitory concentrations.

Figure. 3-5 BF8 is effective against pre-formed biofilm. BF8 reduced the total cell number and persister number in both planktonic population and biofilm population. The 18-h biofilms were treated with BF8 at different concentrations for 12 h in 0.85% NaCl solution.

Figure 3-6. Comparison of persister levels of RP437 and KX1485 after 5 h of growth. The subcultures of RP437 and KX1485 were grown for 5 h and the total cells and persister cells were quantified. The results show these two strains have the same level of persistence.

Figure 3-7. Concentration and treatment time of ofloxacin (Ofl) for persister isolation. (A) Effects of Ofl concentration on the viability of RP437 cells. RP437 cells from an 18 h culture were challenged with different concentrations of Ofl for 3.5 h. Then the number of cells that remain viable was determinated by counting CFU. (B) Effects of treatment time on the viability of RP437 cells. RP437 cells from an 18 h culture were incubated with 5 µg/mL Ofl for different lengths of time. The number of cells that remained viable was determined by counting CFU. The results indicate that treatment with 5 µg/mL Ofl for 3.5 h is sufficient for isolating persister cells of RP437.





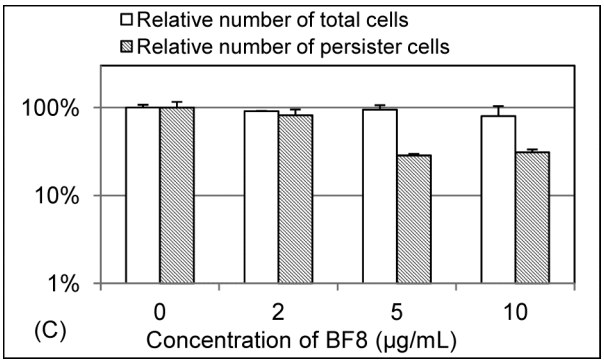
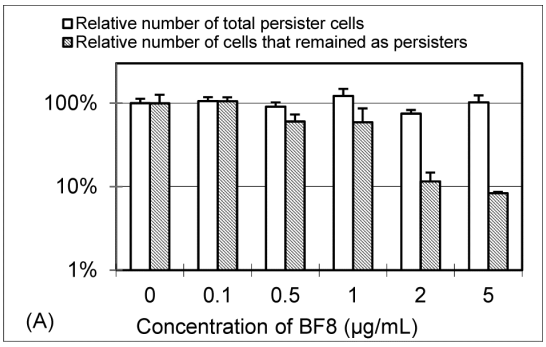
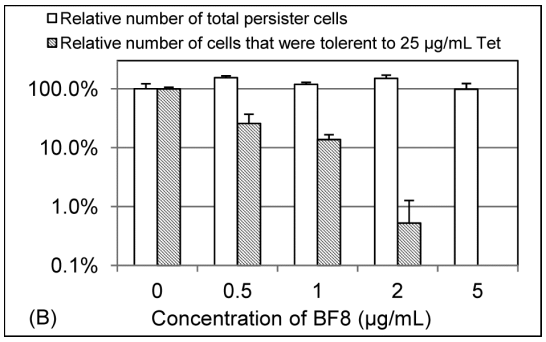
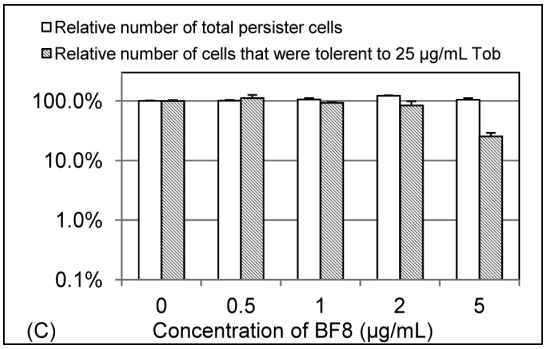
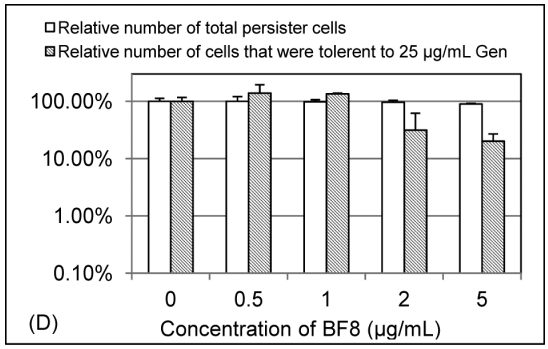

Relative number of total cells
Relative number of persister cells
100%
10%
1%
0
2
5
10
(C)
Concentration of BF8 (μg/mL)

Figure 3-1


Relative number of total persister cells
Relative number of cells that remained as persisters
100%
10%
1%
0
0.1
0.5
1
2
5
(A)
Concentration of BF8 (μg/mL)


Relative number of total persister cells
Relative number of cells that were tolerent to 25 μg/mL Tet
100.0%
10.0%
1.0%
0.1%
0
0.5
1
2
5
(B)
Concentration of BF8 (μg/mL)


Relative number of total persister cells
Relative number of cells that were tolerent to 25 μg/mL Tob
100.0%
10.0%
1.0%
0.1%
0
0.5
1
2
5
(C)
Concentration of BF8 (μg/mL)


Relative number of total persister cells
Relative number of cells that were tolerent to 25 μg/mL Gen
100.00%
10.00%
1.00%
0.10%
0
0.5
1
2
5
(D)
Concentration of BF8 (μg/mL)

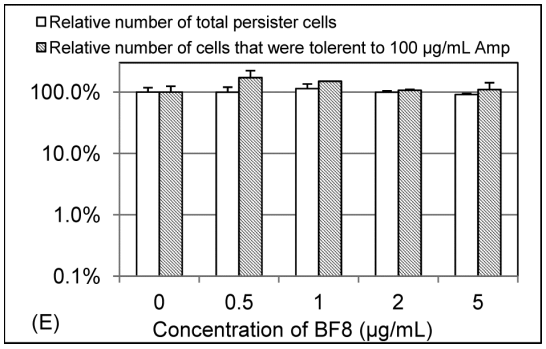
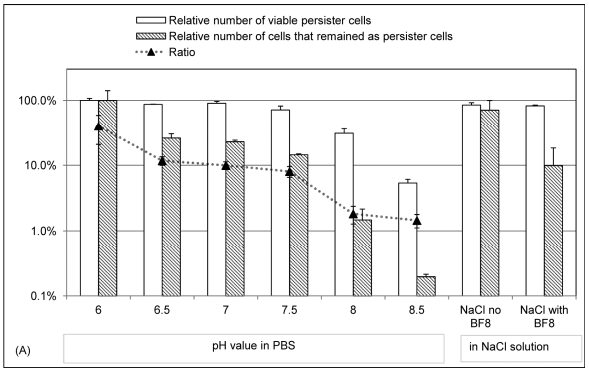
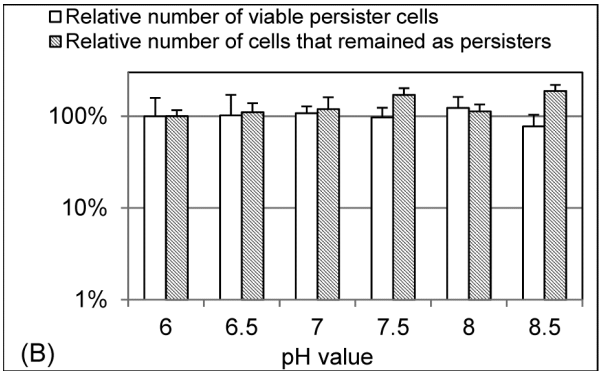

Relative number of total persister cells
Relative number of cells that were tolerent to 100 μg/mL Amp
100.0%
10.0%
1.0%
0.1%
0
0.5
1
2
5
(E)
Concentration of BF8 (μg/mL)

Figure 3-2


Relative number of viable persister cells
Relative number of cells that remained as persister cells
Ratio
100.0%
10.0%
1.0%
0.1%
6
6.5
7
7.5
8
8.5
NaCl no BF8
NaCl with BF8
(A)
pH value in PBS
in NaCl solution


Relative number of viable persister cells
Relative number of cells that remained as persisters
100%
10%
1%
6
6.5
7
7.5
8
8.5
(B)
pH value

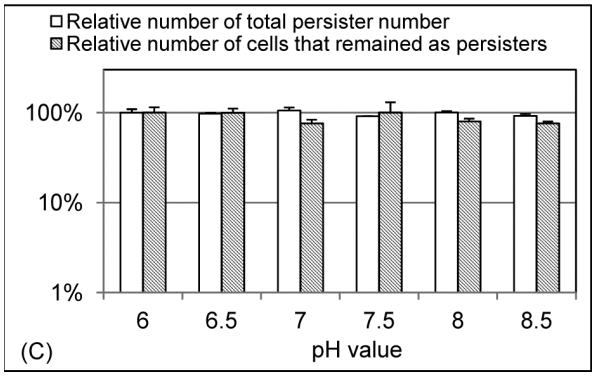
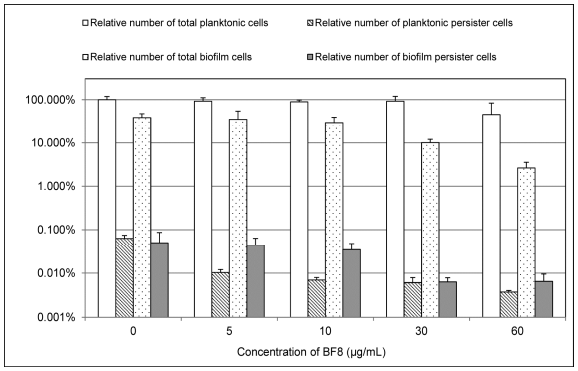

Relative number of total persister number
Relative number of cells that remained as persisters
100%
10%
1%
6
6.5
7
7.5
8
8.5
(C)
pH value

Figure 3-3


Relative number of total planktonic cells
Relative number of planktonic persister cells
Relative number of total biofilm cells
Relative number of biofilm persister cells
100.000%
10.000%
1.000%
0.100%
0.010%
0.001%
0
5
10
30
60
Concentration of BF8 (µg/mL)

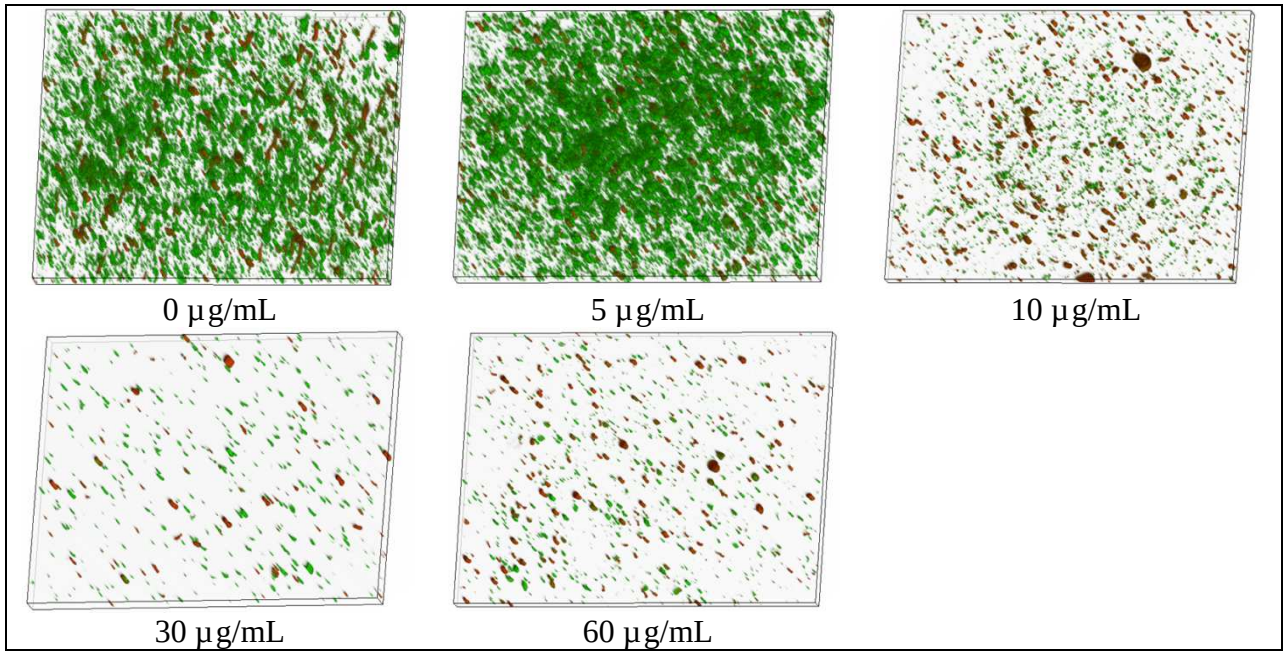

0 µg/mL
5 µg/mL
10 µg/mL
30 µg/mL
60 µg/mL

Figure 3-4

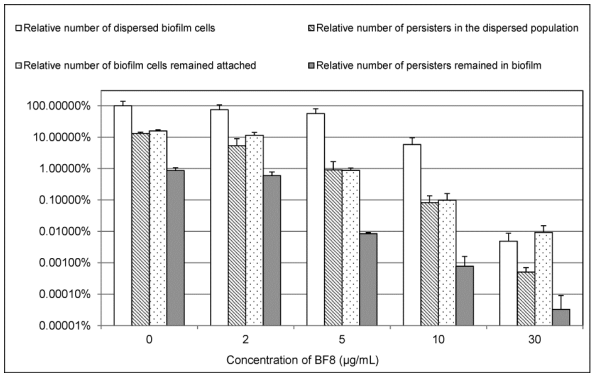

Relative number of dispersed biofilm cells
Relative number of persisters in the dispersed population
Relative number of biofilm cells remained attached
Relative number of persisters remained in biofilm
100.00000%
10.00000%
1.00000%
0.10000%
0.01000%
0.00100%
0.00010%
0.00001%
0
2
5
10
30
Concentration of BF8 (μg/mL)

Figure 3-5

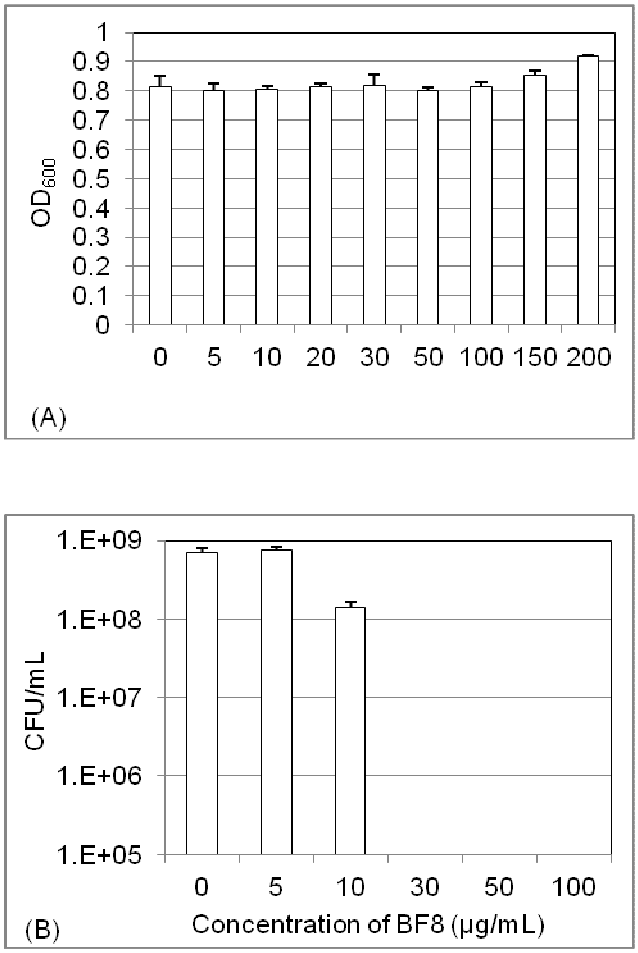

1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD600
0 5 10 20 30 50 100 150 200
(A)
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
CFU/mL
0 5 10 30 50 100
Concentration of BF8 (μg/mL)
(B)

Figure 3-6

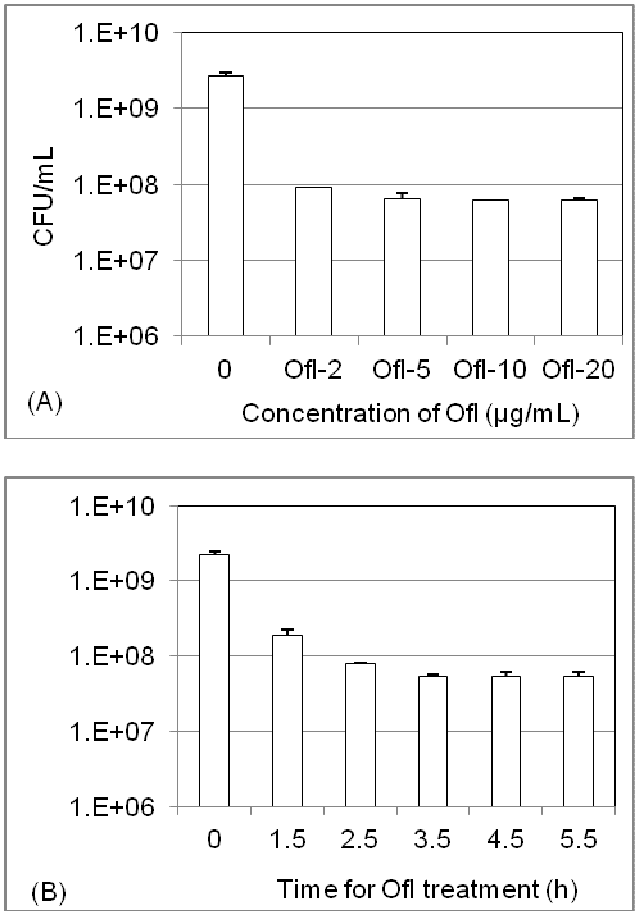


Figure 3-7

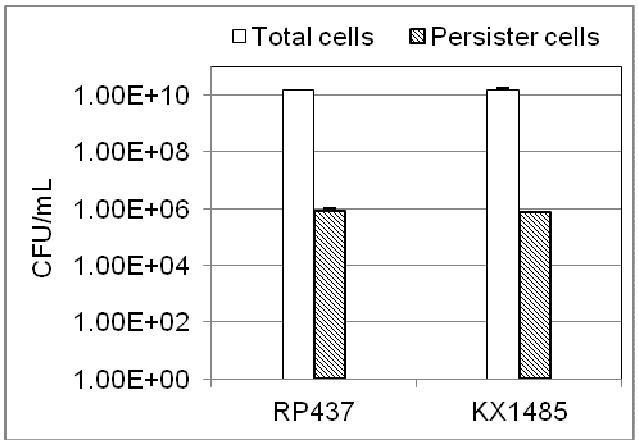

Total cells
Persister cells
1.00E+10
1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
CFU/mL
RP437
KX1485

Figure 3-8

Chapter 4

CONTROLLING PERSISTER CELLS OF *Pseudomonas aeruginosa* PDO300 BY (*Z*)-4-BROMO-5-(BROMOMETHYLENE)-3-METHYLFURAN-2(5*H*)-one

This chapter has been submitted as below with minor modifications. Jiachuan Pan, Fangchao Song and Dacheng Ren. Controlling persister cells of *Pseudomonas aeruginosa* PDO300 by (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran -2(5*H*)-one. Bioorganic & Medicinal Chemistry Letters. 2013.

4.1 Abstract

Pseudomonas aeruginosa is a major pathogen causing chronic pulmonary infections; e.g., 80% of cystic fibrosis patients get infected by this bacterium as the disease progresses. Such chronic infections are challenging because *P. aerugionsa* exhibits high-level tolerance to antibiotics by forming biofilms (multicellular structures attached to surfaces), by entering dormancy and forming persister cells, and by conversion to the mucoid phenotype. Recently, we reported that a synthetic quorum sensing inhibitor, (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one (BF8), can sensitize the persister cells of *P. aeruginosa* PAO1 to antibiotics at the concentrations non-inhibitory to its growth. In this study, we report that BF8 has cidal effects on the mucoid strain *P. aeruginosa* PDO300, a *mucA22* derivative of PAO1, and has synergistic effects in killing PDO300 with antibiotics. These results broaden the activities of this class of compounds and indicate that BF8 also has other targets in *P. aerugionsa* in addition to quorum sensing.

Keywords: antibiotic tolerance, persister, quorum sensing, biofilm, PDO300

4.2 Introduction

Persister cells are commonly found as a small subpopulation in a bacterial culture, which is metabolically inactive and highly tolerant to antibiotics[1]. Such intrinsic tolerance is not acquired through drug resistance genes, but can also lead to chronic infections with reoccurring symptoms after the course of antibiotic therapy[1], which facilitate the development and wide spread of acquired multidrug resistance based on genetic mutations and horizontal gene transfer.

High persistence mutants of *P. aeruginosa* have isolated from sputa of cystic fibrosis patients[2]. In this autosomal recessive genetic disorder, the loss of function of transmembrane conductance regulator (CFTR) leads to overproduction and accumulation of mucus in the breathing passages of patients' lungs [3]. This condition is favorable to the adherence of bacteria, such as *P. aeruginosa*, and formation of multicellular structures with cells embedded in a polysaccharide matrix, known as biofilms. Both biofilm cells and persister cells are highly tolerant to antibiotics[4,5].

Another important factor of pathogenesis is the conversion of *P. aeruginosa* to the mucoid phenotype, which is characterized by overproduction of the polysaccharide alginate and enhanced antibiotic tolerance[6,7]. Conversion to mucoid strain occurs due to mutations in *P. aeruginosa* genes, such as *algU*[8], *mucA*[7], *and mucB*[9], and plays an important role in the establishment of chronic infection of this pathogen with high mortality and morbidity[6].

Recently, we reported that (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one (BF8), an inhibitor of bacterial quorum sensing (a system that regulates bacterial gene expression in response to their cell density), can sensitize the persister cells of *P. aeruginosa* PAO1 to antibiotics at growth non-inhibitory concentrations. To further understand persister control by BF8, we tested its effects on the persister cells of a mucoid strain *P. aeruginosa* PDO300. *P.*

aeruginosa PDO300 is a *mucA22* derivative of PAO1 induced by reactive oxygen species (ROS)[7]. To our best knowledge, this is the first effort to study the effects of a quorum sensing inhibitor on the persistence of a mucoid strain.

4.3 Material and Method

4.3.1 Furanone Synthesis.

(*E*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one (BF8) was synthesized as described previously[10,11], dissolved in absolute ethanol as 60 mg/mL, and stored at 4°C until use.

4.3.2 Bacterial strain and growth media.

P. aeruginosa PDO300 (henceforth PDO300) and PAO1 (henceforth PAO1) were routinely grown in Luria-Bertani (LB) medium[12]. The LB medium was also used to form biofilms. To isolate persister cells, 18-h overnight cultures were treated with 200 µg/mL ciprofloxacin (Cip) for 3.5 h at 37^{o}C with shaking at 200 rpm (unless otherwise specified), washed twice with 0.85% NaCl solution to remove carryover of antibiotics, and then resuspended in 0.85% NaCl solution. The cell-washing steps mentioned above were performed by centrifugation at 13,200 rpm for 3 min at room temperature and then resuspended in 0.85% NaCl solution. The drop plate method described by Chen et al.[13]_ENREF_15_ENREF_14_ENREF_14 was followed to count colony forming units (CFUs). One-way ANOVA followed by Tukey test was applied for statistical analysis to test the significance.

4.3.3 Effects of BF8 on persister formation.

A PDO300 overnight culture was used to inoculate subcultures in 5mL fresh LB medium to an initial optical density at 600 nm (OD_{600}) of 0.05. Then the subcultures were supplemented with

different concentrations of BF8 (0, 5, 10 and 30 µg/mL) immediately. The amount of ethanol (solvent of BF8 stock solutions) was adjusted to be the same in each sample to eliminate any solvent effect. Samples were taken after 5 h or 12 h of incubation and the total cell number in each sample was quantified by counting CFU. Meanwhile, persisters were isolated from the remaining portion of each sample. The number of persisters formed after 5 h or 12 h growth was quantified by counting CFU.

4.3.4 Effects of BF8 and AHLs on the susceptibility of PDO300 persister cells to Cip.

PDO300 persister cells were isolated from 18-h overnight cultures as described above. Isolated persister cells were diluted by 50 times with 0.85% NaCl solution and then each 3 mL of diluted persister cells was challenged with varying concentrations of BF8 (0, 5, 10, 25 and 50 µg/mL). Ethanol (the solvent used for making BF8) was adjusted to be the same in all the samples to eliminate any solvent effect. After incubation for 2 h at 37^{o}C with shaking at 200 rpm, 1 mL of each sample was taken and washed three times with 0.85% NaCl solution to quantify the total number of viable cells by counting CFU. Meanwhile, the persister cells in the remaining portion were isolated after additional treatment with 200 µg/mL Cip, diluted and plated on LB agar plates to quantify the number of cells that remained as persisters.

4.3.5 Effects of BF8 on persister formation in biofilms.

PDO300 overnight cultures were used to inoculate subcultures in LB medium supplemented with different concentrations of BF8 (0, 20, 40 and 60 µg/mL) to an OD_{600} of 0.05. The amount of ethanol was adjusted to be the same in all the samples to eliminate any solvent effect. In each subculture, 2 cm × 1 cm 304L stainless steel coupons were put at the bottom of the petri dish to form biofilms. After 18 h of biofilm growth at 37^{o}C without shaking, stainless steel coupons with

biofilm were rinsed gently with 0.85% NaCl solution and soaked into 5 mL 0.85% NaCl solution. Then then biofilm cells were mechanically detached by vortexing for 1 min and sonicating (Ultrasonic cleaner Model No B200, Sinosonic Industrial Co., Ltd, Taipei Hsien, Taiwan) for 1 min (repeat once)[14,15]. Detached biofilm cells were plated on LB plates to count CFU and the rest of the samples were used for persister isolation as described above. Meanwhile, 1 mL planktonic cells from each subculture was washed three times and plated on LB agar plates to determine the viability of total planktonic cells by counting CFU. Another 1 mL from planktonic cells in each subculture was sampled for persister isolation. The persister samples from the above treatment were diluted and plated on LB agar plates to determine the number of persister cells in each planktonic or biofilm sample.

4.3.6 Effects of BF8 on persister cells in established biofilms.

PDO300 overnight cultures in LB medium were used to inoculate subcultures in LB medium to an OD_{600} of 0.05. Coupons of 304L stainless steel (2 cm × 1 cm) were placed in petri dishes containing above PDO300 culture. After 18 h of incubation, the coupons with established biofilms were transferred to a 12 well plate (Becton Dickinson, Franklin Lakes, NJ,). Each well contained 4 mL of 0.85% NaCl solution supplemented with different concentrations of BF8 (0, 2, 5, 10 and 30 μg/mL). The biofilm samples in 12 well plates were incubated at 37°C for 24 h without shaking. After 24 h of treatment, 1 mL of medium in each well was sampled, washed three times with 0.85% NaCl solution and plated on LB agar plates to determine the viability of RP437 cells by counting CFU. At the same time, another 1 mL of medium was sampled and persister cells were isolated as described above. To collect the biofilm cells, the coupons were transferred to 15 mL falcon tubes, each containing 5 mL 0.85% NaCl solution. The biofilm cells were detached by vortexing for 1 min and sonicating for 1 min (repeat once)[14]. Detached biofilm

cells were diluted and plated on LB plates to count CFU and the persister cells in the rest of the samples were isolated as described above. The isolated planktonic persister cells and biofilm-associated persister cells were diluted and plated on LB agar plates to count CFU.

4.4 Results:

4.4.1 BF8 exhibited different effects on PDO300 and PAO1 persister cells.

Recently (Chapter 2), we demonstrated the BF8 can reduce persister formation during 5 h of PAO1 growth in LB medium[11]. In comparison, BF8 showed no effect on the viability (p=0.1647) and persister formation (p=0.0838) of PDO300 during the 5 h growth; however, with longer treatment time (12 h), BF8 reduced the persister formation without growth inhibition (Figure 4-2). For example, after 12 h of incubation, BF8 did not affect the growth of PDO300 [Figure 4-2(B), p = 0.4079], but reduced the persister level dose-dependently [Figure 4-1B, p=0.0038]. For example, at 30 µg/mL, BF8 reduced number of persisters by 88.5±4.3% (p=0.0014) compared to the BF8-free control (Figure 4-1B).

4.4.2 BF8 is cidal to PDO300 persister cells.

As described in Chapters 2 and 3, BF8 can sensitize isolated persister cells of PAO1 and *E. coli* RP437 to antibiotics without affecting the viability. Interestingly, we found that BF8 can directly killed PDO300 persister cells at the concentrations of 5 to 50 µg/mL (Figure 4-3, p<0.001). For example, at 5 µg/mL, BF8 killed PDO300 persister cells by 80.9 ± 1.9% (p=0.0064) and the remaining viable persister cells were not sensitized to Cip. At higher concentration of BF8, e.g., 50 µg/mL, BF8 killed all the isolated persister cells.

4.4.3 BF8 reduced biofilm formation and the number of persister cells inside biofilm.

BF8 was reported to be an inhibitor of quorum sensing and biofilm formation of *P. aeruginosa* PAO1 [11,16] [5]. Here, we show that BF8 can also dose-dependently reduce the biofilm formation of PDO300 and the number of persister cells in its biofilms (Figure 4-4A). At all the concentrations tested, BF8 didn't reduce the number of viable cells in the planktonic phase (p=0.1526), but reduced biofilms dose dependently (p=0.0002). For example, at 60 μg/mL, biofilm formation was reduced by 91.6 ± 0.5% (p<0.0001). In addition, the number of viable persister cells inside the biofilms was reduced by 98.3 ± 0.7% (p=0.0003). Consistent with the results shown in Figure 4-2B, the number of viable persister cells in planktonic cells were also reduced dose-dependently by BF8 (p<0.0001). For example, at 60 μ/mL, the number of persister cells was reduced by 96.5 ± 1.3% (p<0.0001).

4.4.4 BF8 is also effective against PDO300 in established biofilms.

Treatment of 18-h PDO300 biofilms with BF8 was found to reduce the number of viable biofilm cells dose-dependently (Figure 4-4B, p=0.0031] and kill the persister cells in biofilms [Figure 4-4B, p=0.0288]. For example, after treatment with 30 μg/mL BF8 for 24 h, no viable cells were detected in the planktonic phase; and only 0.01±0.01% biofilm cells remained on the surface were viable (p= 0.0024) with no viable persister cells were detected. Compared with the results about PAO1[11], the results in this chapter indicate that BF8 is more effective against PDO300 biofilms than PAO1 biofilms.

4.5 Discussion

In this study, we should that BF8 can also control persister cells of the mucoid strain *P. aeruginosa* PDO300. It is an interesting finding that a longer treatment time is needed for BF8 to significantly reduce persistence during PDO300 growth. This may be due to the presence of

alginate, which could retard the penetration of BF8. It is also interesting to find that, at the minimum concentration to control persistence, BF8 has cidal effects against PDO300 persister cells in 0.85% NaCl buffer, but not in LB medium. Different from the effects on PAO1 persister cells, BF8 at 5 to 50 μ/mL killed PDO300 persister cells directly without synergic effect with further Cip treatment. The underlying mechanism is unknown; however, since overproduce of alginate is major phenotypic difference between PAO1 and PDO300, alginate may be an important factor [7]. Alginate (Figure 4-1B) can be deprotonated at high pH (>6.5) [17] and become negatively charged. Since alginate is around PDO300 cells[18], it is possible that the change in the charge of alginate and local pH may influence the interaction between BF8 and PDO300 cells and cause cidal effects in 0.85% NaCl solution. In Chapter 4, we described that BF8 is more effective against *E. coli* RP437 persister cells in PBS when pH increased slightly above 7. Thus, it will be important to investigate the effects alginate on the pH around cells, and associated changes in proton motive force, and the mechanism/target of persister control by BF8.

Overall, the finding described in this Chapter present additional activities of this class of quorum sensing inhibitors and indicate the existence of other targets in *P. aerugionsa*. Further study about the underlying mechanism will help understand bacterial persistence and develop effective approaches to control *P. aerugionsa* infections with mucoid strains.

4.6 Acknowledgements

We thank the U.S. National Science Foundation (CAREER-1055644 and EFRI-1137186) for partial support of this work. We are grateful to Prof. Matthew Parsek at University of Washington for sharing *P. aeruginosa* PAO1 and PDO300 strains.

References

(1) Lewis, K. *Biochemistry (Moscow)* 2005, *70*, 267.

(2) Mulcahy, L. R.; Burns, J. L.; Lory, S.; Lewis, K. *J Bacteriol* 2010, *192*, 6191.

(3) Yankaskas, J. R.; Marshall, B. C.; Sufian, B.; Simon, R. H.; Rodman, D. *CHEST Journal* 2004, *125*, 1S.

(4) Olson, M. E.; Ceri, H.; Morck, D. W.; Buret, A. G.; Read, R. R. *Canad. J Vet Res* 2002, *66*, 86.

(5) Lewis, K. *Annu Rev Microbiol* 2010, *64*, 357.

(6) Hassett, D. J.; Cuppoletti, J.; Trapnell, B.; Lymar, S. V.; Rowe, J. J.; Sun Yoon, S.; Hilliard, G. M.; Parvatiyar, K.; Kamani, M. C.; Wozniak, D. J. *Advanced drug delivery reviews* 2002, *54*, 1425.

(7) Mathee, K.; Ciofu, O.; Sternberg, C.; Lindum, P. W.; Campbell, J. I. A.; Jensen, P.; Johnsen, A. H.; Givskov, M.; Ohman, D. E.; Søren, M. *Microbiology* 1999, *145*, 1349.

(8) Schurr, M.; Yu, H.; Martinez-Salazar, J.; Boucher, J.; Deretic, V. *Journal of bacteriology* 1996, *178*, 4997.

(9) Martin, D.; Schurr, M.; Mudd, M.; Deretic, V. *Molecular microbiology* 1993, *9*, 497.

(10) Han, Y.; Hou, S.; Simon, K. A.; Ren, D.; Luk, Y. Y. *Bioorg Med Chem Lett* 2008, *18*, 1006.

(11) Pan, J.; Bahar, A. A.; Syed, H.; Ren, D. *PLoS One* 2012, *7*, e45778.

(12) Sambrook, J.; Russell, D. W. *Molecular cloning: a laboratory manual*; 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001.

(13) Chen, C. Y.; Nace, G. W.; Irwin, P. L. *J Microbiol Meth* 2003, *55*, 475.

(14) Chen, X.; Zhang, M.; Zhou, C.; Kallenbach, N. R.; Ren, D. *Appl Environ Microb* 2011, *77*, 4878.

(15) Niepa, T. H. R.; Gilbert, J. L.; Ren, D. *Biomaterials* 2012, *33*, 7356.

(16) Hentzer, M.; Riedel, K.; Rasmussen, T. B.; Heydorn, A.; Andersen, J. B.; Parsek, M. R.; Rice, S. A.; Eberl, L.; Molin, S.; Høiby, N. *Microbiology* 2002, *148*, 87.

(17) Cheng, Y.; Luo, X.; Betz, J.; Payne, G. F.; Bentley, W. E.; Rubloff, G. W. *Soft Matter* 2011, *7*, 5677.

(18) Hassett, D. J.; Sutton, M. D.; Schurr, M. J.; Herr, A. B.; Caldwell, C. C.; Matu, J. O. *Trends in microbiology* 2009, *17*, 130.

Figure captions

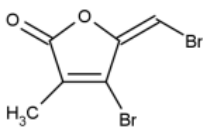
Figure 4-1 Structures of BF8 (A) and alginate deprotonated at pH of 6.5 (B).

Figure 4-2. BF8 reduced persister formation during PDO300 growth, but required a longer time of treatment than PAO1. (A) BF8 did not significantly reduce persistence during 5-h treatment. (B) BF8 reduced the persistence of PDO300 dose-dependently after treatment for 12 h.

Figure 4-3. BF8 exhibited cidal effects against PDO300 persister cells in 0.85% NaCl solution.

Figure 4-4. Effects of BF8 on PDO300 biofilms and associated persister cells. (A) BF8 reduced biofilm formation in LB medium and the number of biofilm-associated persister cells at concentrations that did not affect the viability of planktonic cells. (B) BF8 can kill PDO300 persister cells in established biofilms when treated in 0.85% NaCl solution.

Figure 4-5. Effects of 3-oxo-C_{12}-AHL and C_4-AHL on isolated PDO300 persister cells. (A) 3-oxo-C_{12}-AHL sensitized PDO300 persister cells without compromising viability. (B) C_4-AHL at the same concentration did not sensitize persister cells or reduce the viability of persister cells.



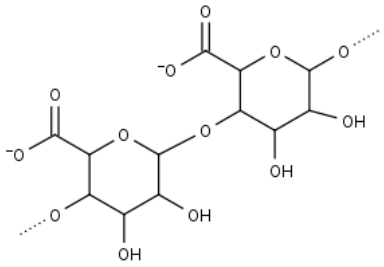
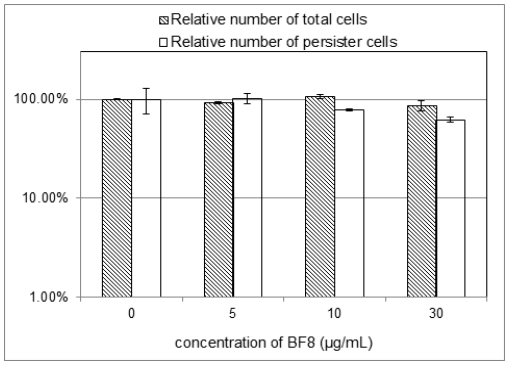


Figure 4-1


Relative number of total cells
Relative number of persister cells
100.00%
10.00%
1.00%
0
5
10
30
concentration of BF8 (µg/mL)

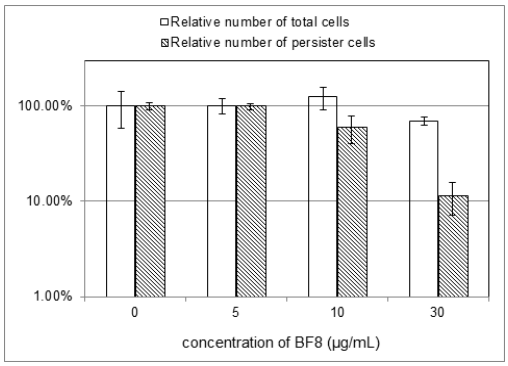

Relative number of total cells
Relative number of persister cells
100.00%
10.00%
1.00%
0
5
10
30
concentration of BF8 (µg/mL)

Figure 4-2

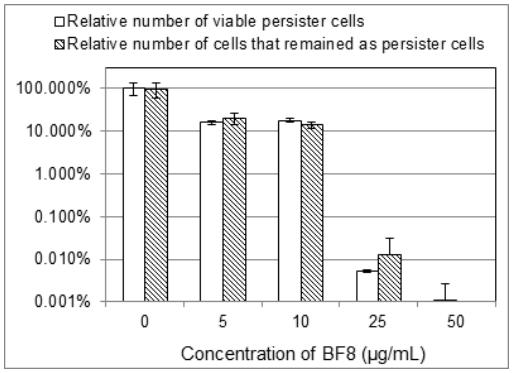
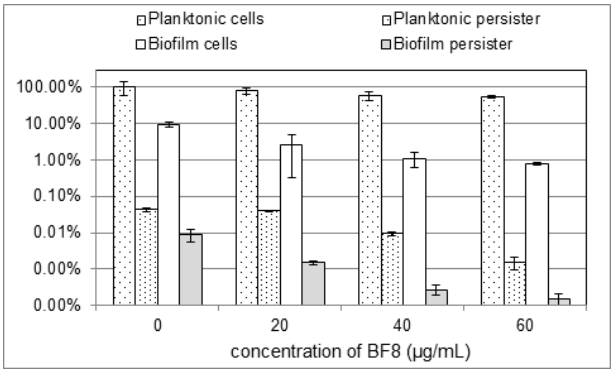

Relative number of viable persister cells
Relative number of cells that remained as persister cells
100.000%
10.000%
1.000%
0.100%
0.010%
0.001%
0
5
10
25
50
Concentration of BF8 (μg/mL)

Figure 4-3


Planktonic cells
Planktonic persister
Biofilm cells
Biofilm persister
100.00%
10.00%
1.00%
0.10%
0.01%
0.00%
0.00%
0
20
40
60
concentration of BF8 (µg/mL)

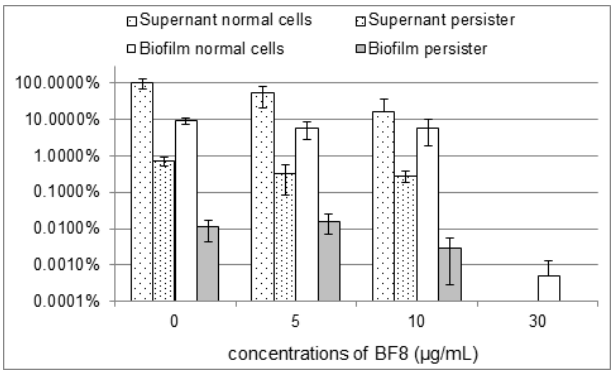
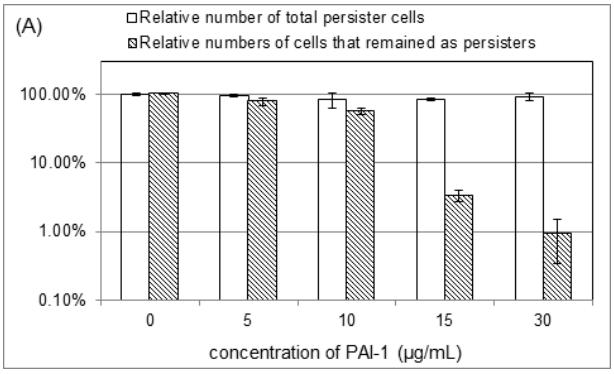

Supernant normal cells
Supernant persister
Biofilm normal cells
Biofilm persister
100.0000%
10.0000%
1.0000%
0.1000%
0.0100%
0.0010%
0.0001%
0
5
10
30
concentrations of BF8 (µg/mL)

Figure 4-4


(A)
Relative number of total persister cells
Relative numbers of cells that remained as persisters
100.00%
10.00%
1.00%
0.10%
0
5
10
15
30
concentration of PAI-1 (μg/mL)

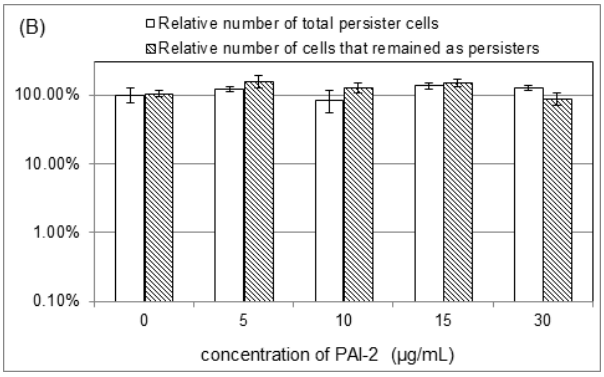

(B)
Relative number of total persister cells
Relative number of cells that remained as persisters
100.00%
10.00%
1.00%
0.10%
0
5
10
15
30
concentration of PAI-2 (μg/mL)

Figure 4-5

Chapter 5

STRUCTURAL EFFECTS ON PERSISTER CONTROL BY BROMINATED FURANONES

This chapter has been submitted as below with minor modifications. Jiachuan Pan, Fangchao Song and Dacheng Ren. Structural effects on persister control by brominated furanones. Bioorganic & Medicinal Chemistry Letters. 2013.

5.1 Abstract

In chapter 2, 3 and 4, we reported that a quorum sensing (QS) inhibitor, (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one (BF8) can restore antibiotic susceptibility of *Pseudomonas aeruginosa* PAO1 persister cells. In this chapter, a group of synthetic brominated furanones with similar structures was tested to identify for potential persister control. The results show that these furanones are QS inhibitors and can also restore the antibiotic susceptibility of *P. aeruginosa* PAO1 persisters.

5.2 Introduction

Persister cells are phenotypic variants that can be found in virtually any bacterial and are tolerant to different antibiotic treatments[1]. Consistently, bacterial strains with high-level persistence have been isolated from patients with cystic fibrosis and tuberculosis [].

Recently, it is reported that (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one (BF8) can revert antibiotic tolerance of *Pseudomonas aeruginosa* persister cells[3]. BF8 is an inhibitor of bacterial quorum sensing (QS)[3, 4], a gene regulation system in response to bacterial population and regulates different phenotypes[5] such as biofilm and bioluminescence. Although this QS inhibition also controls *P. aeruginosa* persister cells, the role of QS in persister formation remains elusive. The QS signal 3-oxo-C_{12}-HSL (AHL) has been shown to promote persister formation in exponential culture of *P. aeruginosa*. However, adding spent media was not found to enhance persistence in *Escherichia. coli*. We also found that can sensitize *P. aeruginosa* persister cells. Thus, comparison test of BFs with similar structures for their effects on persisters and QS will provide useful information for understanding the activities of BFs and designing better control method.

Han and coworkers characterized a group of synthetic BFs with related structures and identified important structural elements for biofilm control. Here we test their effects on QS and persistence of *P. aeruginosa*.

5.3 Material and Method

5.3.1 Effects on AHL- mediated QS.

The QS reporter *V. harveyi* BB886 [ATCC (BAA-1118)] was used to characterize the effects of BFs on QS. This strain produces bioluminescence in response to AHL[8]. The procedure as described by Surette and Bassler was followed with slight modifications. Briefly, *V. harveyi* BB886 was grown in AB medium overnight and then diluted by 1:5000 with the fresh AB medium. After incubation for 5.5 hours, subculture was added with a brominated furanone or non-brominated furanone. Concentrations of 0, 0.1, 0.5, 1, 10, 30 and 60µg/mL were tested for each furanone. After 1.5 h of incubation, the bioluminescence was measured using a luminometer (Turner Biosystem 20/20n); and then the cells from each sample were washed by centrifugation and resuspension in 20g/L NaCl solution to count CFU.

5.3.2 Effects of BFs on persistence.

The BFs tested in our study exhibited different activities in restoring the susceptibility of *P. aeruginosa* PAO1 to ciprofloxacin. The persisters were isolated by adding 200µg/mL ciprofloxacin to an 18 h overnight culture and incubating at 37^{o}C with shaking at 200 rpm. Then the cells were washed three times to remove the antibiotic residue and resuspended in 0.85% NaCl solution with 50 times dilution. The tested BF was added to the cell suspension and incubated for 2h. One mL of BF-treated persisters was washed three times and plated on LB agar plates for counting CFU. Another portion of BF-treated persisters was added with 200 µg/mL

ciprofloxacin and incubated for 3.5 h to determine the number of cells that remained as persisters. The amount of ethanol was adjusted to be the same for all samples to eliminate any solvent effect.

5.4 Results

5.4.1 Effects on AHL- mediated QS.

As shown in Figure 5-2A, addition of BF9 reduced bioluminescence dose-dependently with no effects on the viability of *V. harveyi* BB886 observed when BF9 was added up to 10µg/mL. For example, the bioluminescence was reduced by 99.96±0.69% when BF9 was added as 10µg/mL. At higher concentrations, e.g. 30 µg/mL, the viability of *V. harveyi* BB886 was reduced by BF9 and no bioluminescence was detected. BF11 and BF12 also exhibited similar activities: inhibiting QS without affecting the viability of the reporter strain when the concentration is below a threshold (Figure 5-2). For example, at 0.5µg/mL, BF9, BF11 and BF12 reduced the relative bioluminescence (bioluminescence/CFU of the *V. harveyi* BB886) by 84.7±13.6%, 55.9±0.3% and 91.2±4.3%, respectively.

For comparisons in this test, two non-brominated furanones (purchased from ATCC) were included as controls. Shown in Figure 5-1G and Figure 5-2G, NF1 was found toxic to *V. harveyi* BB886 at 1µg/mL or higher concentrations and NF2 (Figure 5-1H and Figure 5-2H) did not affect the viability and QS of *V. harveyi* BB886 at concentrations up to 60µg/mL. This result indicates that the bromine atoms are important to QS control by BFs.

5.4.2 Effects of BFs on persistence.

As shown in Figure 5-2, BF11 at certain concentrations showed the capability to sensitize persister cells to ciprofloxacin without reducing the viability of persister cells. For example, after treatment with BF11 at 2 μg/mL, 99.99±0.02% PAO1 persisters were killed by ciprofloxacin. But BF11 at 2 μg/mL by itself did not affect the viability.

In comparison, BF10, BF12 and BF13 can kill PAO1 persister cells directly and exhibited synergistic effects with Cip in killing persister cells. BF10, BF13 and BF12 at 2μg/mL killed PAO1 persister cells by 99.9±0.0%, 98.9±0.2% and 93.0±0.1%, respectively.

5.5 Discussion

Recently, Allison et al[10] reported that sugars, such as glucose, mannitol, fructose and pyruvate can potentiate metabolically *E. coli* persisters to aminoglycosides. As shown in Figure S1, glucose at 2μg/mL does not affect the viability or antibiotic tolerance of PAO1 persisters. The non-brominated furanones NF1 and NF2 also did not exhibit notable effects (Figure 5-3G&H).

Although the mechanism of persister control by BFs is still unknown, the differences in QS and persister control by BFs suggest that these compounds also have other targets in *P. aeruginosa*. BF8 could induce a repressor of the *P. aeruginosa* toxin Cif, toxin which interferes with a ubiquitin proteolytic system of its host and degrades the cystic fibrosis transmembrane conductance regulator (CFTR) in mammalian cells, increasing the chance for cystic fibrosis[11, 12]. Thus, these BFs and other similar compounds may have activities to control related chronic infections. Further study with animal cells/ animal models will provide valuable information for infection control by targeting persister cells.

5.6 Acknowledgements

We thank the U.S. National Science Foundation (CAREER-1055644 and EFRI-1137186) for partial supports of this work.

We are grateful to Professor Thomas K. Wood at Pennsylvania State University for sharing the strain of *P. aeruginosa* PAO1, as well as Professor Yan-Yeung Luk at Syracuse University for facilities.

Supplementary data

Supplementary data associated with this article can be found in the online version. At XXX

References

1. Lewis, K. *Annu Rev Microbiol* **2010,** *64*, 357.

2. Lewis, K. *Nat Rev Microbiol* **2007,** *5*, 48.

3. Pan, J.; Bahar, A. A.; Syed, H.; Ren, D. *PLOS ONE* **2012,** *7*, e45778.

4. Hou, S.; Duo, M.; Han, Y.; Luk, Y.-Y.; Ren, D. Inhibiting Microbial Biofilm Formation by Brominated Furanones; ASM International: Minneapolis, Minnesota, 2009;

5. Miller, M. B.; Bassler, B. L. *Annual Reviews in Microbiology* **2001,** *55*, 165.

6. Moker, N.; Dean, C. R.; Tao, J. *J Bacteriol* **2010,** *192*, 1946.

7. Han, Y.; Hou, S.; Simon, K. A.; Ren, D.; Luk, Y. Y. *Bioorganic and medicinal chemistry letters* **2008,** *18*, 1006.

8. Surette, M. G.; Miller, M. B.; Bassler, B. L. *Proceedings of the National Academy of Sciences* **1999,** *96*, 1639.

9. Shah, D.; Zhang, Z.; Khodursky, A.; Kaldalu, N.; Kurg, K.; Lewis, K. *Bmc Microbiology* **2006,** *6*, 53.

10. Allison, K. R.; Brynildsen, M. P.; Collins, J. J. *Nature* **2011,** *473*, 216.

11. MacEachran, D. P.; Stanton, B. A.; O'Toole, G. A. *Infection and immunity* **2008,** *76*, 3197.

12. Bomberger, J. M.; Ye, S.; MacEachran, D. P.; Koeppen, K.; Barnaby, R. L.; O'Toole, G. A.; Stanton, B. A. *PLoS pathogens* **2011,** *7*, e1001325.

Figure caption

Figure 5-1 Structures of brominated furanones and non-brominated furanones used in this study.

Figure 5-2 Effects of BFs on AHL-mediated quorum sensing. *V. harveyi* BB886 was challenged by BFs and NFs at different concentrations. The number of viable *V. harveyi* BB886 cells after BF treatment and the bioluminescence are shown. The relative bioluminescence normalized by CFU is also shown for the convenience of comparison.

Figure 5-3 Effects of BFs and NFs on isolated PAO1 persister cells. After treatment for 2 h, the number of surviving persisters was quantified by CFU. Meanwhile, a portion of each sample was further challenged with 200μg/mL Cip for 3.5 h to determine the number of cells that remained as persister by counting CFU.

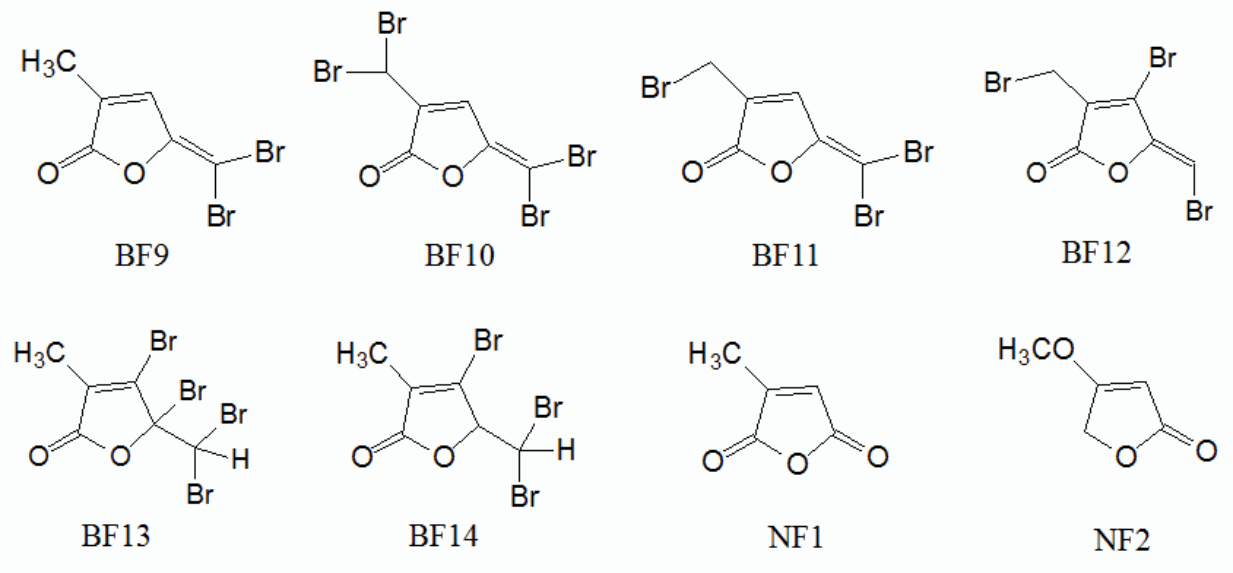

H3C
O
O
Br
Br
BF9
Br
Br
O
O
Br
Br
BF10
Br
O
O
Br
Br
BF11
Br
Br
O
O
Br
BF12
H3C
Br
Br
Br
O
O
H
Br
BF13
H3C
Br
Br
O
O
H
Br
BF14
H3C
O
O
O
NF1
H3CO
O
O
NF2

Figure 5-1

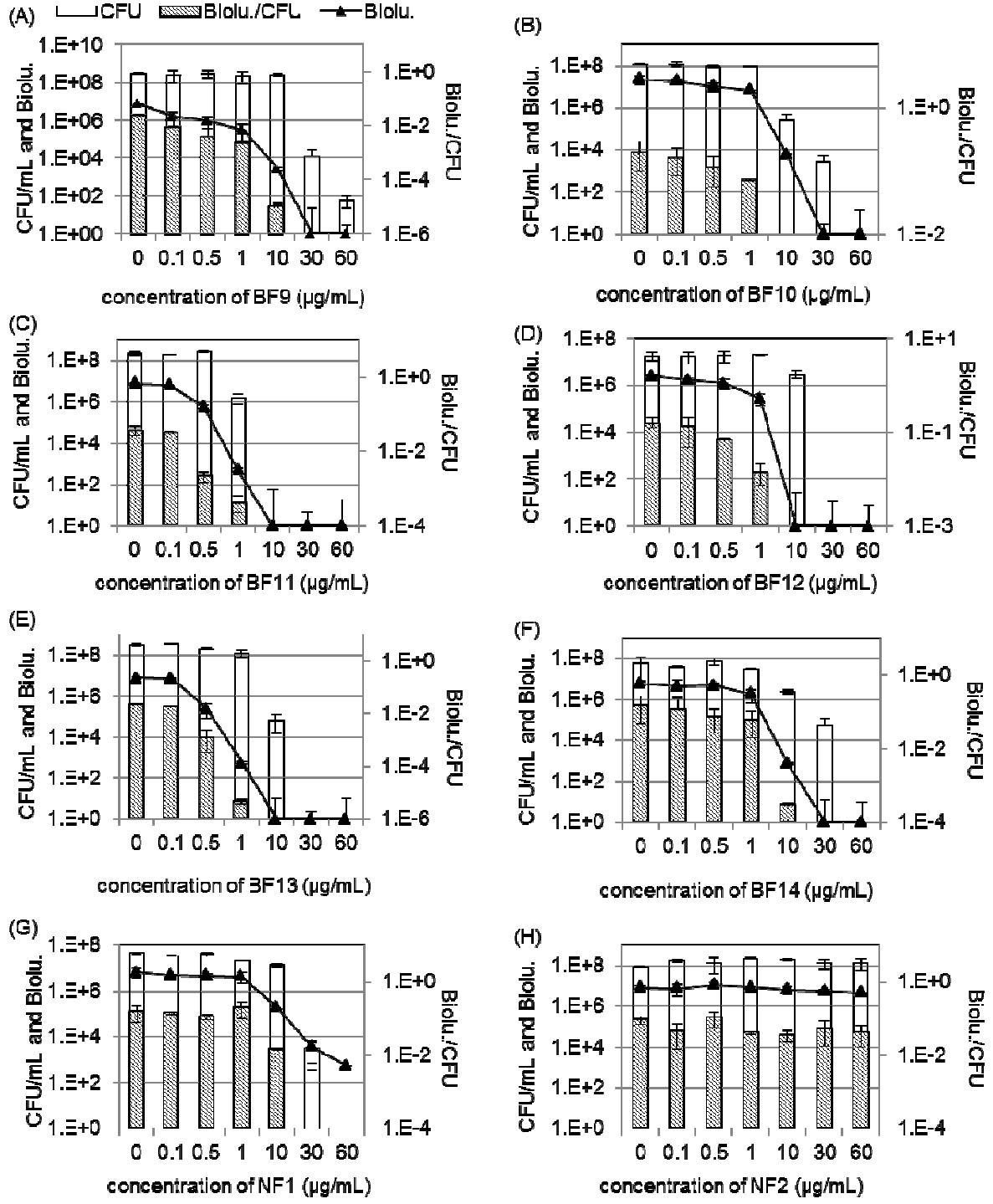

(A)
CFU
Biolu./CFU
Biolu.
CFU/mL and Biolu.
Biolu./CFU
concentration of BF9 (μg/mL)
(B)
concentration of BF10 (μg/mL)
(C)
concentration of BF11 (μg/mL)
(D)
concentration of BF12 (μg/mL)
(E)
concentration of BF13 (μg/mL)
(F)
concentration of BF14 (μg/mL)
(G)
concentration of NF1 (μg/mL)
(H)
concentration of NF2 (μg/mL)
0 0.1 0.5 1 10 30 60

Figure 5-2

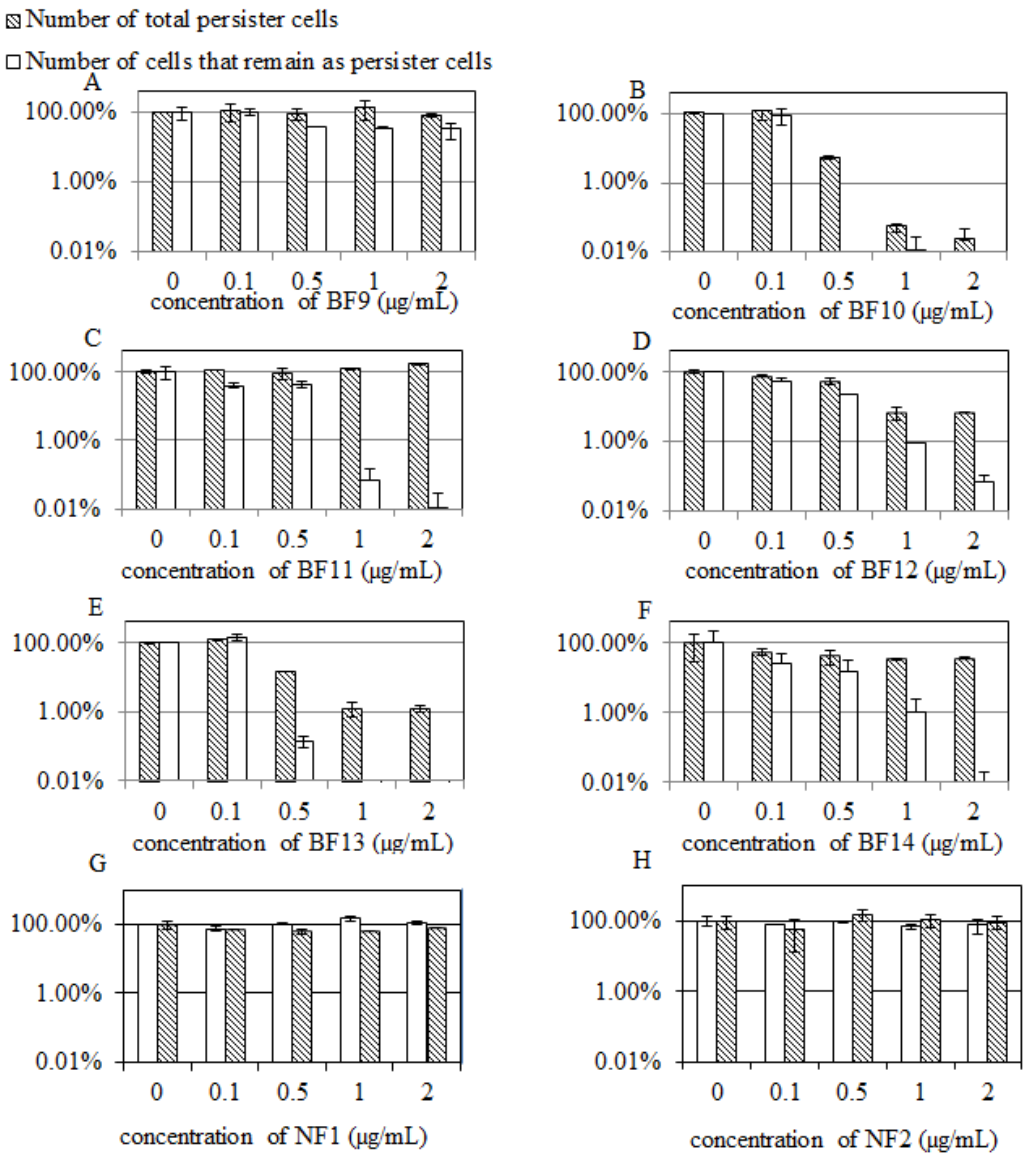

Number of total persister cells
Number of cells that remain as persister cells
A
100.00%
1.00%
0.01%
0 0.1 0.5 1 2
concentration of BF9 (μg/mL)
B
100.00%
1.00%
0.01%
0 0.1 0.5 1 2
concentration of BF10 (μg/mL)
C
100.00%
1.00%
0.01%
0 0.1 0.5 1 2
concentration of BF11 (μg/mL)
D
100.00%
1.00%
0.01%
0 0.1 0.5 1 2
concentration of BF12 (μg/mL)
E
100.00%
1.00%
0.01%
0 0.1 0.5 1 2
concentration of BF13 (μg/mL)
F
100.00%
1.00%
0.01%
0 0.1 0.5 1 2
concentration of BF14 (μg/mL)
G
100.00%
1.00%
0.01%
0 0.1 0.5 1 2
concentration of NF1 (μg/mL)
H
100.00%
1.00%
0.01%
0 0.1 0.5 1 2
concentration of NF2 (μg/mL)

Figure 5-3

APPENDIX

Experimental protocols

Protocol 1

Synthesis of (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one (BF8)

This protocol is developed based on the procedures described by Han et al [1]. The major steps are: Br addition, ring-closing reaction; and purification using chromatography.

Cleaning glassware

All glassware should be thoroughly cleaned, leaving on oil or salt stains (including irons) and dried in an oven before use..

1. Clean the glassware with acetone to move organic chemicals.

2. Clean the glassware with soap and water to move salts.

3. Wash with acetone again.

4. Dry in an oven.

I Br addition

The device is set up as shown in Figure A1. Adjust the **T** (temperature) to be around 35~40ºC. Prepare 200 mL ice.

1. Take alpha-methyllevulinica acid from refrigerator (4^{o}C) and keep it at room temperature for around 30 min before weighting to avoid water condensation.. Add 2.53g alpha-methyllevulinica acid in the round-bottom flask.

3. Add 20mL dichloromethane (DCM) in the round bottom flask.

5. Add 20mL DCM to a funnel (addition, graduated), followed by 1 mL of Br_2 Double gloving is highly recommended for this step due to the corrosiveness of Br_2.

6. Add Br_2 dropwise into the round-bottom flask.

7. After 1 h of relax, check how much of the starting material has reacted using TLC (EA: Hexane=1:2). If the reaction is complete, go to the next step. Otherwise, more time should be given for the reflax.

8. Stop the reaction by pouring all the products onto ice in a beaker around 200mL.

II Extract

1. Extract the residue with DCM (3×80 mL)..

2. Add $Na_2S_2O_3$ (1M, 100 mL, 15.8g) to the extract to remove any Br_2.

3. Washing the extract once with DI water (80mL)

4. Dry the extract with anhydrous sodium sulfate by putting anhydrous sodium sulfate into extract and waiting for 30 min.

5. Filter the extract from the salt by using a funnel with cotton in the middle.

6. Using rotary evaporator to get crude keto.

7. Weigh the product and calculate the yeild (W2)

III Ring-Closing reaction

The device is set up as shown in Figure A1. Adjust the **T** (temperature) to be around 110ºC1. Add concentrated H_2SO_4 (95~98%, 10 mL) to the crude keto at room temperature.

2. The round-bottom flask with mixture in is heated in an oil-bath at 110ºC for 20 min.

3. Before stopping the reaction, check if the material has all reacted by TLC (DCM: Hexane=1:4, twice). If so, go on to the next step.

4. Stop the reaction by pouring all the products into a beaker of ice (around 200mL).

IV Extract

1. Extract the products with DCM (3×50 mL). 2. Wash the extract once with DI water (80mL).

3. Dry the extract with anhydrous sodium sulfate.

4. Filter the extract from the salt by using a funnel with cotton in the middle.

5. Remove the solvent using a rotary evaporator .

6. Weigh the product to calculate the yield.

V Purification using chromatography

Set up the device as shown in Figure A2.

3. Mix silicon powder with hexane, and pour into the column. Make sure that the top surface of silicon is flat.

4. Using compressed air to make the silicon layer tight and without any bubble.

5. Load the sample in the column slowly.

6. Add washed sea sand on the top.

7. Start chromatography with DCM: Hexane = 1:4 as the mobile phase.

8. Collect the the elute and use TLC (DCM: Hexane=1:4) to check the yield ad purity of the product.

Reference

[1] Yongbin Han, Shuyu Hou, Karen A. Simon, Dacheng Ren, and Yan-Yeung Luk "Identifying the important structural elements of brominated furanones for inhibiting biofilm formation by *Escherichia coli"* Bioorganic & Medicinal Chemistry Letters. 2008, 18: 1006-1010.

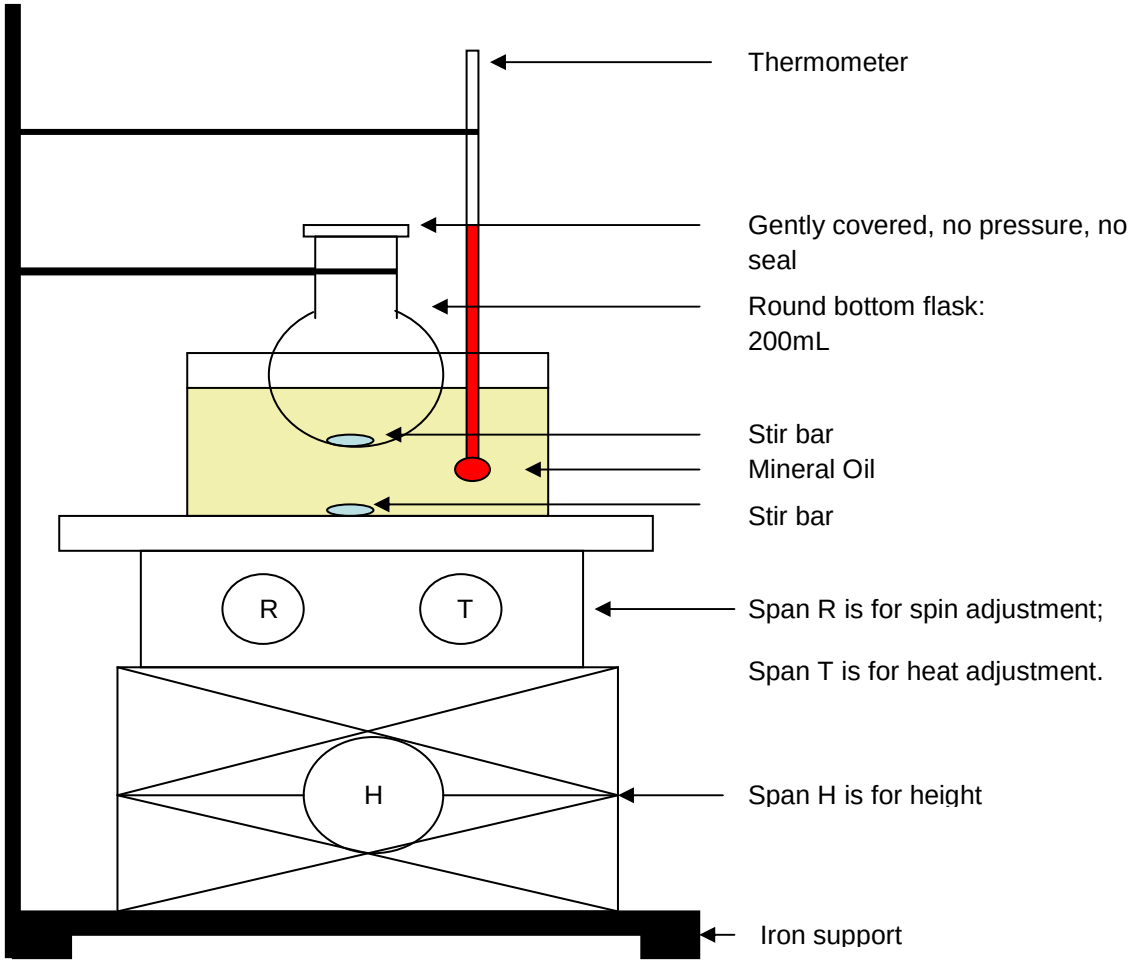
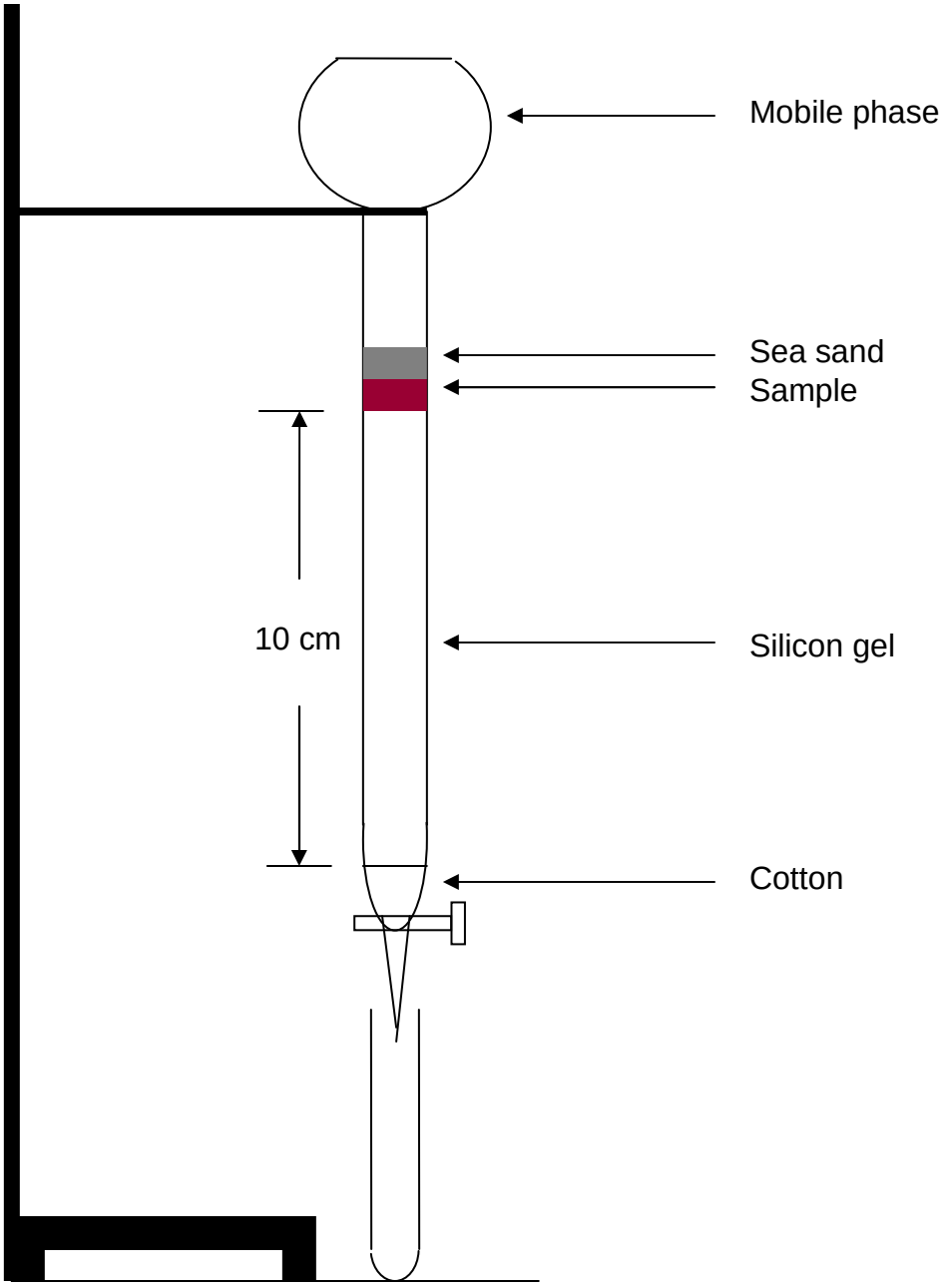


Figure A1 Device for Br addition and Ring-Closing reaction



FigureA2 Device for purification using chromatography

Protocol 2.

RNA Isolation from Persister Cells

Harvesting Cells

1. Grow 100 mL overnight culture of PAO1/RP437 for 18 h.
2. Collect cells using centrifugation and treat the cells with 200 µg/mL Cip. (for *P.aeruginosa* PAO1) or 5 µg/mL Ofl. (for *E.coli* RP437) for 3.5 hto kill regular cells .
3. Wash persister cells twice with 0.85% NaCl solution.
4. Dilute the persister suspension by 5 times with 0.85% NaCl solution and divide the diluted culture into two flasks, and the final volume of 0.85% NaCl solution is adjusted to 250 mL.
5. Treat the persister cells with conditions of interest.
6. During treatment, cool all the tubes and centrifuge rotor to 0-4ºC.
7. Collect the persister cells by centrifugating10,000 rpm for 6 min and decant supernatant.
8. Resuspend the cells with cold ice and transfer the cells to a cold 2 mL bead beater tube.
9. Centrifuge
10. Freeze the cells in a dry-ice/ethanol bath and store the cells at -80^{o}C until RNA isolation.

RNA Isolation using RNeasy Mini Kit (Qiagen:74104)

- Preparation:
    1. Add 200uL Zirconia/Silica beads to each bead beater tube with cells & cool on ice
    2. RLT Buffer: 10 µL 2-mercaptoethanol (βME) per 1 mL RLT buffer (2 mL/sample)
    3. RPE Buffer: 8 mL EtOH per 2 mL RPE buffer (4 mL/sample)

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

- Isolation:
  1. Add 900 µL RLT Buffer to the bead beater tube with cell pellet/beads
  2. Beat for 60s (set timer at 6) at speed setting of 48 (~5000 rpm).
  3. Centrifuge for 15s at 13000rpm at 4C

     (All centrifuge steps were carried under this condition unless noted)
  4. Collect the supernatent, add 445 µL EtOH
  5. Load 700 µL sample onto RNeasy column (Qiagen) and centrifuge
  6. Add 350 µL RW1, centrifuge twice
  7. Add 180 µL DNase I incubation mix directly onto the column
  8. Incubate at RT for 30min
  9. Add 350 µL RW1 and centrifuge(repeat once)
  10. Add 500 µL RPE and centrifuge (repeat twice)
  11. Add 500 µL RPE and centrifuge for two min
  12. Replace collection tube and centrifuge for 1m
  13. Place the column in a 1.5 collection tube
  14. Add 40 uL RNase-free water and centrifuge for 1m; collect the flow-through (repeat once)
- RNA Clean-up
  1. Add 900 µL RLT Buffer to previously collected RNA sample
  2. Add 445 µL EtOH.
  3. Load 700 µL sample onto RNeasy column (Qiagen), centrifuge

4. Add 350 µL RW1, centrifuge (x2)
5. Add 180 µL DNase I incubation mix directly onto membrane
6. Incubate at RT for 30m
7. Add 350 µL RW1, centrifuge(x2)
8. (x3) Add 500 µL RPE, centrifuge
9. Add 500 µL RPE, centrifuge 2m
10. Replace collection tube, centrifuge 1m
11. Place column in 1.5 collection tube
12. (x2) Add 40 uL RNase-free water, centrifuge 1m

    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 ug/mL
   - Ratio: OD_{260}/OD_{280} should be >2.0
2. Check the samples on a 1.4% agarose gel
   - Should have two clear bands (23S at 3.1kb, 16S at 1.5kb)
   - Smear patterns at low molecular range indicate RNase contamination

Jiachuan (Jacqueline) Pan

EDUCATION

Syracuse University, NY **9/2008-6/2013**

Ph.D., Chemical Engineering, GPA: 3.5/4.00

Thesis work: Chemical compounds were synthesized to interrupt bacterial signaling system and demonstrated to reduce biofilm formation and antibiotic tolerance of *P. aeruginosa* and *E. coli*.

Funding resources: U.S. National Science Foundation (CAREER and EFRI)

Wallace H. Coulter Foundation

Syracuse University Fellowship

PATENTS

Ren, D. and **Pan, J**. Syracuse University. System and method for reverting antibiotic tolerance of bacterial persister cells. US2011/047080 **2011**; WO/2012/021514 **2012**.

AWARDS based on my project in Syracuse University:

1. ASM Student Travel Grant, American Society for Microbiology, 6th Conference on Biofilms, Miami, FL, **2012**.

2. First place achievement, Syracuse Biomaterials Institute Offsite Poster Session, **2011**.

3. Department winner in Nunan Research Day Poster Competition, Syracuse, NY, **2013**.

Chinese Academy of Sciences, Beijing and Xiamen, China **9/2005-7/2008**

Master's degree, Environmental Engineering, GPA: 3.5/4.00

Thesis work: Setting up the biofilm reactor for the fermentation of sulphate reducing bacteria.

PATENT

Cao, H.; **Pan, J**.; Zhang Y.; Li Y. A method to isolate anaerobic bacteria. CN101270334; **2008.**

China Agriculture University, Beijing, China **9/2001-7/2005**

Bachelor's degree, Environmental Engineering, GPA: 3.9/4.00 (Rank 4/144)

Thesis work: Identifying and optimizing the conditions for the fermentation of a sulfur-oxidizing bacterium.

AWARDS based on my course study and social work in China Agriculture University:

First Place Achievement for Study (2002), Third Place Achievement for Social Work (2002), First Place Achievement for Study (2003), Second Place Achievement for Study (2004)

TEACHING EXPERIENCE AND PROFESSIONAL MENTORSHIP

Teaching Assistant (Biological Principles for Engineers, Syracuse University): Preparing materials and teaching a laboratory course for 32 undergraduate students. The course content includes plasmid isolation, plasmid transformation, DNA digestion, protein gel and western blotting. **2009-2010**

Teaching Assistant (Chemical Engineering Principles, Syracuse University): Grading home assignments, answering questions and claims on home assignments, and helping the students to review and organize what they had learned. **2009-2010**

Professional Mentorship (Ren Lab, Syracuse University): Teaching an undergraduate student supported by a summer REU fellowship from the Syracuse Biomaterials Institute to design and present his/her project in **2008**; Teaching a master's student to use different devices, deign his/her thesis, perform experiments, analyze the data and report data, which were published in **2012**.

EXPERTISE AND SKILLS

Organic Synthesis, Microbiological Assay Design and Data Analysis, Genetic Engineering Skills, Literature Search and Organizing skills.

Optical Microscope (Phase, DIC, Water Immersion and Oil Immersion), Fluorescence Microscopes, Confocal Microscope, Scanning Electron Microscope, Ion Chromatograph, HPLC, GC, Electrochemical Workstation, Atomic Absorption Spectroscopy (AAS), NMR, Gas Chromatography (GC), Spectrophotometer, Fluorescence Spectrophotometer, pH Meter, Clean Bench Workstation, PCR Device and Gel Image System.

COMPUTER EXPERIENCE:

Endnote, Microsoft Office, ChemWindow, Primer Designer Online, BLAST for bioinformatics, Adobe Photoshop, Statistical Analysis Software (SAS), Auto-CAD

Softwares for ZEISS Microscopes, HPLC, Ion Chromatography, NMR and other analysis equipments.

PUBLICATIONS

1. **Pan, J** and Ren, Dacheng Quorum sensing inhibitors: a patent overview. *Expert Opinion Therapeutic Patents* **2009**, *19*, 1581.

2. **Pan, J**; Bahar Ali.; Syed, Hassiba. and Ren, D. Reverting antibiotic tolerance of *Pseudomonas aeruginosa* PAO1 persister cells by (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran- 2(5*H*)-one. *PLoS One* **2012**,*7(9)*, e45778.

3. **Pan, J**.; Song, F. and Ren, D. Controlling persister cells of *Pseudomonas aeruginosa* PDO300 by (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one (in print, by Bioorganic & Medicinal Chemistry Letters, **2013**)

4. **Pan, J.**; Cao, H.; Shao, Z.; Sheng, Y. and Zhang. Y. Marine SRB community reducing sulfate wastewater in flue gas desulfurization. *Environmental Science* **2009**, *30*, 504.

5. **Pan, J.**; Shao, Z.; Cao, H. and Sheng Y. Review on the isolation and purification methods of sulfate reducing bacteria. *Journal of Microbiology* **2007**, 27, 79.

6. **Pan, J.**; Song, F.; Bahar A. and Ren, D. (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5*H*)-one sensitizes the persister cells of *Escherichia coli* to antibiotics. (under review)

7. **Pan, J.** and Ren, D. Controlling persister cells of *Pseudomonas aeruginosa* PAO1 by a group of brominated furanones. (In preparation)

ABSTRACTS AND PRESENTATIONS

1. **Pan, J**.; Hou, S.; Han, Y.; Luk, Y. and Ren, D. Controlling bacterial biofilms and persister cells with novel brominated furanones. Recent Advances in Microbial Control, Arlington, VA, **2010**.

2. **Pan, J.** and Ren, D. Reverting antibiotic tolerance of bacterial persisters by brominated furanones. BMES 2011 Annual Meeting, Hartford, CT, **2011**.

3. **Pan, J.**; Bahar, A.; Syed, H. and Ren, D. Controlling bacterial persister cells with brominated furanones. ASM 112th General meeting, San Francisco, CA, **2012**.

4. **Pan, J.**; Bahar, A.; Syed, H. and Ren, D. Reverting antibiotic tolerance of bacterial persisters by brominated furanones. ASM 6th Conference on Biofilms, Miami, FL, **2012**.

5. **Pan, J**. and Ren, D. Reverting antibiotic tolerance of *Pseudomonas aeruginosa* persister cells by synthetic brominated furanones. 14th International Conference on *Pseudomonas*, Switzerland, **2013**