UNIVERSITY OF CALIFORNIA
RIVERSIDE

Engineering Human Paraoxonase 2 for Quorum Quenching

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Biochemistry and Molecular Biology

by

Xin Li

September 2017

Dissertation Committee:

Dr. Xin Ge, Chairperson
Dr. Ashok Mulchandani
Dr. Manuela Martins-Green

The Dissertation of Xin Li is approved:

Committee Chairperson

University of California, Riverside

Acknowledgement

After an intensive period, today is the day: writing this note of thanks is the finishing touch on my dissertation. It has been five years of intense learning for me, not only in the scientific arena, but also on a personal level. Writing this dissertation has had a big impact on me. I would like to reflect on the people who have supported and helped me so much throughout this time.

I would express my deepest gratitude to my parents for their continuous love and non-stop support. They are my dearest friends, great listeners, talented cook, and strong allies. At the most depressed time, with their endless love and giving, I did not give up myself. They always wish their baby girl happy, respect her decisions even though sometimes they are ridiculous, and most importantly, they have faith in her. I cannot thank you enough.

My sincere thanks to my advisor Dr. Xin Ge. Thank you for giving me the opportunity to be one of your graduate students. During the past four years working with you, I have learnt quite a few from you, such as scientific thinking, efficient planning, abilities to solve research problems. I would like to thank you for your patience. This training makes me become stronger, smarter, and wiser (at least in my opinion, haha!).

I also want to give my sincere thanks to Dr. Ashok Mulchandani and Dr. Manuela Martins-Green. You gave me enlightening suggestions for my projects when I had difficulty solving the puzzles. Also, your support and understanding

game me a lot of strength when I had concerns about my abilities. I am very grateful for your unselfish assistance.

I would like to thank Dr. Richard Debus, Dr. Dr. Meng Chen, lab safety manager Joshua Halford, TA coworkers Kyu Lee, Carlos Rodriguez, Matthew Young. During many of these busy quarters, you all like great friends to me. Your wisdom, your stories and your experience were warm, funny, and impressive. You were all brightened and smoothed my days.

I would like to thank my lab members, Long Chen, Tyler Lopez, Dr. Chuan Chen, Kibaek Lee, Matthew Chow, Evan Kruchowy; and my undergraduates, Christopher Wang, Guan-Ju Lai, Ran Ye, Jaspreet Wadhwa, Anson Tsang. Thank you all for your daily support, suggestions and discussions, all the scientific sparkling during this time. I am lucky to have you all as my colleagues and closest friends.

I want to take this opportunity to thank my friends, Fei Bu, Yang Yu, Xuye Lang, Chuanxiao Chen, Chen Geng, Qi Ai, Chunyu Chen, and my roommates Jie Shi, Chenwei Tian, Honghong Wu who have helped me and had great life experience with me.

Last but not the least, I would like to express my heart-felt gratitude to Jan Schlemmer. Your love and support, your humor and kindness, made my life colorful. I cannot walk this far without you.

ABSTRACT OF THE DISSERTATION

Engineering Human Paraoxonase 2 for Quorum Quenching

by

Xin Li

Doctor of Philosophy, Graduate Program in Biochemistry and Molecular Biology
University of California, Riverside, September 2017
Dr. Xin Ge, Chairperson

The rapid emergence of antibiotic-resistant bacteria results in a global health crisis. Among approaches to fighting against antibiotic resistance, this study focuses on quorum sensing (QS) quenching. The QS system regulates the expression of non-essential functions – virulence factors – by sensing their own population density. QS inhibitors interfere with QS communication, thus disrupting biofilm formation and inhibiting the expression of virulence factors. Different from antibiotics, manipulating QS system may halt the development of resistance.

Another approach to disrupting QS is the use of quorum quenching (QQ) enzymes to abolish the biological activity of autoinducers (AIs). The first report of degradation AIs was lactonase aiiA isolated from *Bacillus* sp. 240B. We used aiiA for our studies as a positive QQ enzyme control because of its broad-spectrum

AHL-degrading ability. For therapeutic purposes, prokaryotic enzymes are very likely to generate adverse immune responses. Subsequently, it would be desirable to have a therapeutic quality enzyme that is as close as possible to the native human protein. Currently, no such enzyme meets all these requirements.

Human paraoxonases (huPONs) have been discovered to have AHL-lactonase-like activity, hydrolyzing lactones of various modifications, and carbon chain lengths. HuPONs in particular modulates the stress response of endothelial cells to oxidized phospholipids as well as a bacterial quorum-sensing molecule. However, huPONs are difficult to express in soluble forms, and when heterogeneously expressed, the yield is considerably low.

Thus, we intend to enhance the performance of huPON2 by improving its solubility, yield, and activity. By replacing the hydrophobic helices of huPON2 with degenerate short peptide linkers, we isolated human PON2 variants exhibiting high levels of soluble expression. Engineered huPON2s are biologically functional in *P. aeruginosa* swimming, swarming motility and biofilm formation tests and have lactone hydrolysis activities toward a spectrum of QS molecules. Finally, I introduced random mutations to the soluble expressed huPON2s to improve its catalytic activity. This engineered huPON2 as a quorum quenching enzyme shed light on a novel selection pressure-free selection approach to fight against pathogenic infections.

Table of Contents

List of Figures

List of Tables

1 Chapter 1 Introduction

1.1 Threat of Antibiotic Resistance

Sir Alexander Fleming discovered penicillin in 1928. Since then, antibiotics have saved countless lives around the world. Battlefield casualties got the first non-experimental doses of penicillin in 1943, quickly saving soldiers who had been close to death. Yet two years later, when Fleming accepting the 1945 Nobel Prize in Medicine, he mentioned that "It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them…There is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant."

The consequences of the appearance and spread of antibiotic resistance have become a serious worldwide threat to public health and have generated a massive economic burden (**Figure 1-1**). Each year in the United States, over 2 million people obtain serious bacterial and fungal infections, most of which are resistant to one or more than one antibiotic designed to be a treatment. According to John H. Stroger Jr. Hospital of Cook County's 2009 statistical data, for the U.S. alone, more than 2,000,000 people acquire serious infections from these antibiotic resistant pathogens, and 23,000 people die every year as a direct result. The estimated costs have reached as high as $20 billion in excess of direct healthcare costs[1].

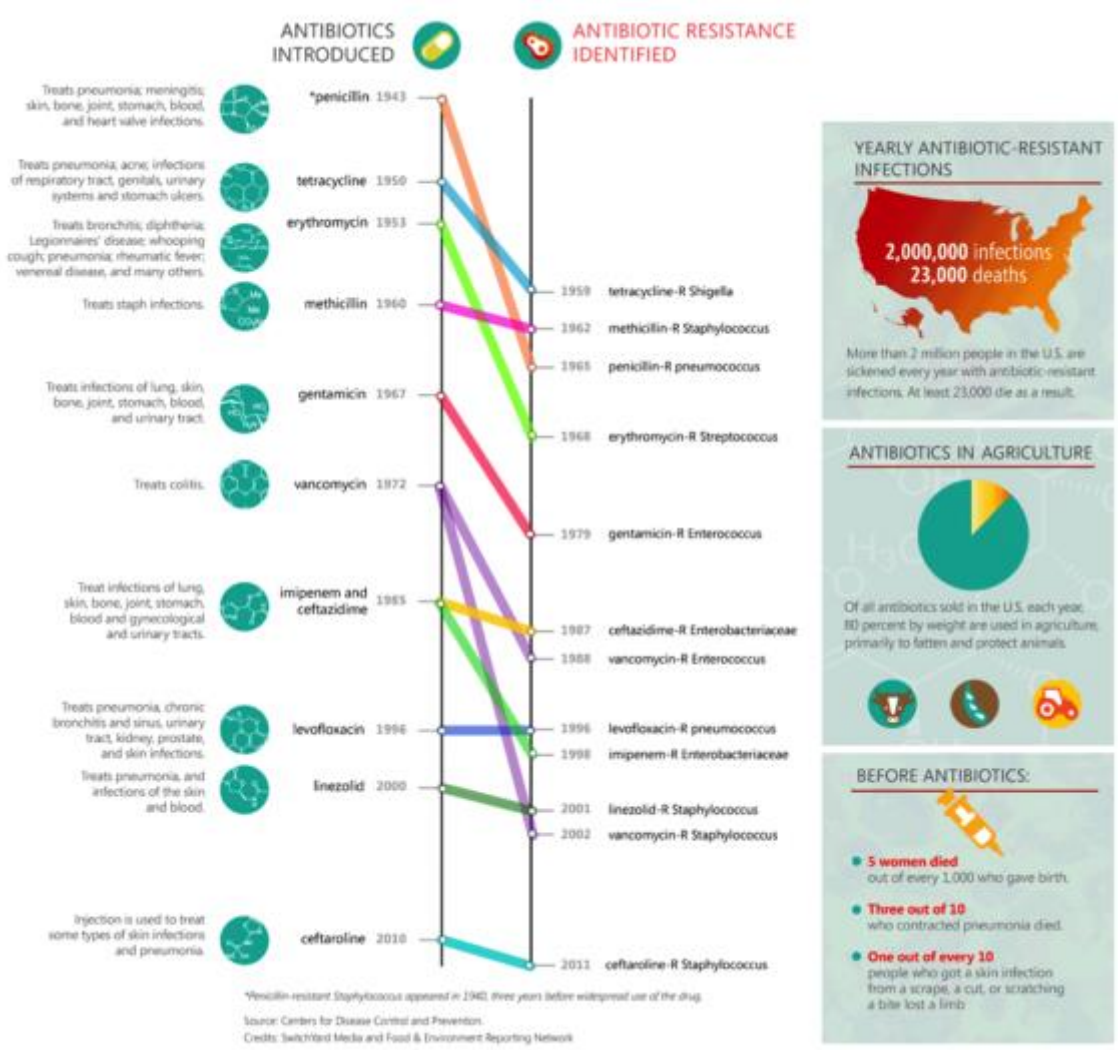

Figure 1-1. Timeline of antibiotic resistance. Many antibiotics have been developed since 1943. However, antibiotics quickly lose their usefulness. As of 2013, every year more than 2 million people in the U.S. develop antibiotic-resistant infections. And at least 23,000 die as a result. 80% of antibiotics sold in U.S. each year are used in agriculture[1].

The crisis is global and the evolutionary pressure for the emergence of antibiotic resistance is great. Thus, the study of how bacteria have been evolving to resist antibacterial products must become a mandatory requirement in the early stages of drug development. The information of bacterial antibiotic resistance mechanisms can provide greater understanding, aid in the discovery of or neutralize existing resistance mechanisms, and provide new targets for discovery.

1.2 Molecular Mechanisms of Antibiotics Resistance

Antibiotic resistance is an adaptation of bacteria interacting with other organisms and/or the environment. Bacteria can be intrinsically resistant to certain antibiotics and can also acquire resistance to antibiotics via mutations in chromosomal genes and by horizontal gene transfer. The intrinsic resistance of a bacterial species to a particular antibiotic is the ability to resist the action of that antibiotic as a result of inherent structural or functional characteristics. For example, the lipopeptide daptomycin is active against Gram-negative bacteria due to an intrinsic difference in the composition of the cytoplasmic membrane; Gram-negative bacteria have a lower proportion of anionic phospholipids in the cytoplasmic membrane than do Gram-positive bacteria. This difference in cytoplasmic membrane composition in turn reduces the efficiency of the Ca^{2+}-mediated insertion of daptomycin into the cytoplasmic membrane that is required

for its antibacterial activity[2]. Many genes are responsible for intrinsic resistance to antibiotics of different classes, including β-lactams and aminoglycosides. Understanding the genetic basis of intrinsic bacterial resistance, and hence the spectrum of activity of an antibiotic, can therefore guide the development of new combinations of agents with improved or expanded activity against target species. Intrinsic resistance can be mediated by several mechanisms, which fall into three main groups: minimization of the intracellular concentrations of antibiotics as a result of poor penetration into the bacterium or of antibiotic efflux; genetic mutations or post-translational modification of the target; and inactivation of the antibiotic by hydrolysis or modification.

1.2.1 Prevention of Access to Target

Through poor penetration into the bacterium or antibiotic efflux, bacteria can minimize the intracellular antibiotic concentrations. Gram-negative bacteria intrinsically tend to be more resistant to lipophilic and amphiphilic antibiotics than Gram-positive bacteria, due to the presence of their outer membrane permeability barrier (**Figure 1-2**). The narrow porin channels on the outer membrane slow down the penetration of small hydrophilic solutes, and the low fluidity of the lipopolysaccharide leaflet decreases the rate of transmembrane diffusion of lipophilic solutes[3-4]. Therefore, reducing the permeability of the outer membrane and limiting antibiotic entry into the bacterial cell can be achieved by the down-regulation of porins or even by the replacement of porins with more

selective channels. For instance, when exposed to carbapenems, *Enterobacter* spp., *Pseudomonas* spp., *Acinetobacter* spp. and *E. coli* gain resistance by their rapid accumulation of porin mutations[5-8].

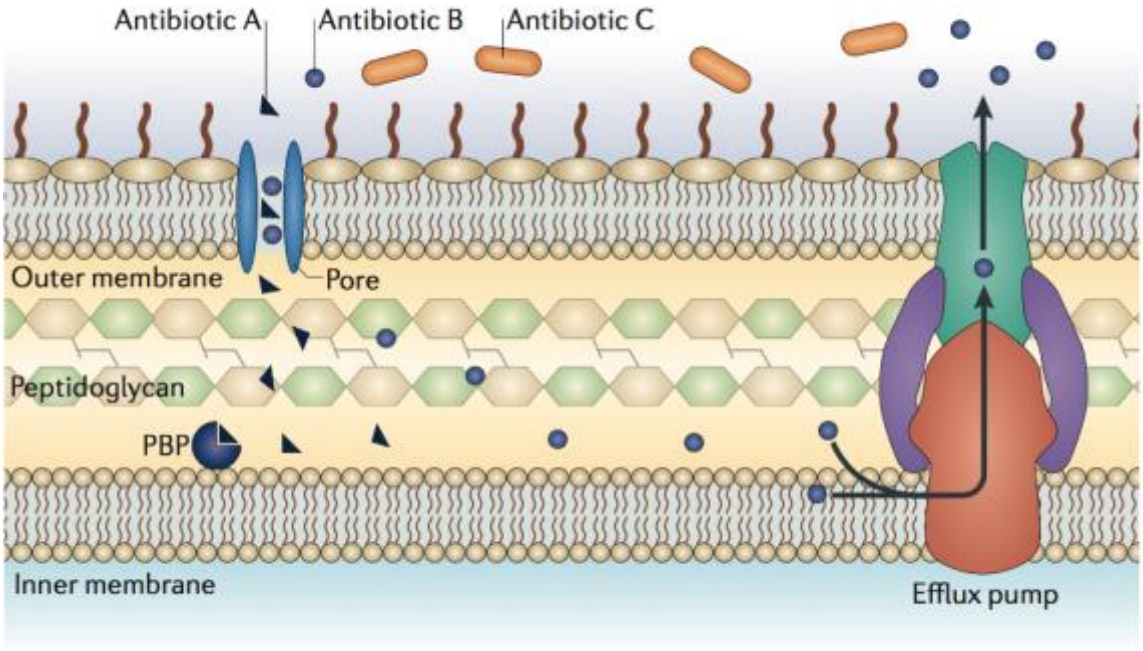

Figure 1-2. Intrinsic mechanisms of resistance. The figure shows an overview of intrinsic resistance mechanisms. The example shown is of β-lactam antibiotics targeting a penicillin-binding protein (PBP). Antibiotic A can enter the cell via a membrane-spanning porin protein, reach its target and inhibit peptidoglycan synthesis. Antibiotic B can also enter the cell via a porin, but unlike Antibiotic A, it is efficiently removed by efflux. Antibiotic C cannot cross the outer membrane and so is unable to access the target PBP[9].

Bacteria possess efflux pumps which can actively transport many antibiotics out of the cell. Efflux pumps are the major contributors to the intrinsic resistance of Gram-negative bacteria. When overexpressed, efflux pumps can

confer high levels of resistance. Some efflux pumps have narrow substrate specificity, but many can transport a wide range of structurally dissimilar substrates and they are known as multidrug resistance (MDR) efflux pumps. MDR efflux pumps are present in all bacteria. Although all bacteria carry multiple genes that encode MDR efflux pumps on their chromosomes, some have been mobilized onto plasmids, that can transfer MDR efflux pumps genes between bacteria. This is a worrying development because the resistance of one pathogen can then be rapidly disseminated to other pathogens[10].

Bacteria can also overexpress efflux pumps to gain resistance. The transcription of genes encoding efflux pumps is controlled by local regulators, which are encoded along the efflux pumps genes, and by global regulators, which have broader biological functions. The high-level expression of efflux genes in multidrug-resistant bacteria is often due to a mutation in the regulatory network controlling expression, within a local repressor, a global transcription factor, or within their regulators[11-13]. Increased expression of efflux pumps can also occur because of induction in response to environmental signals and in conditions in which their function is required. For example, the *acrAB* genes in *E. coli* and *Salmonella* spp. are induced by small molecules that would be encountered during infection, such as indole and bile[14-15]; whereas expression of MtrCDE in *Neisseria gonorrhoeae* is responsive to iron limitation[16].

Understanding the molecular basis of induction of efflux could allow for the development of chemical modulators to prevent efflux de-repression; these

modulators could then be used in conjunction with antibiotics in novel therapeutic strategies.

1.2.2 Targets Protection

Most antibiotics specifically bind to their targets with high affinity, thus preventing the normal activity of the target. During the period of infection, there are often large and diverse populations of pathogens, and if a single site mutation in the gene encoding an antibiotic target can confer resistance to the antibiotic, strains with this mutation can then proliferate. Moreover, uptake of DNA from the environment contributes to resistance through the formation of 'mosaic' genes. A specific example of this is the penicillin resistance in *S. pneumoniae*. The mosaic penicillin-binding protein (*pbp*) genes are believed to be the result of recombination with homologous *pbp* genes from the closely related species *Streptococcus mitis*[17].

Protection by modifications of the target structure can be another effective method of antibiotic resistance that does not involve mutational changes in the target genes. For example, the chloramphenicol-florfenicol resistance (cfr) methyltransferase catalyzes mono- or demethylation of a specific adenine residue A2058, the key nucleotide for the binding of erythromycin in the 23S rRNA. This modification of adenine residue sterically hinders the macrolide, lincosamide, and streptogramin B (MLSB) binding to this pocket and disrupts the hydrogen bonding between the macrolides and the rRNA[18-19].

1.2.3 Direct Modification of Antibiotic Molecule

Apart from preventing antibiotics from entering the cell and altering their targets, bacteria can directly modify antibiotics to resist antibiotics (**Figure 1-3**)[20]. The enzymatic modification of antibiotics has been a major mechanism of antibiotic resistance since the first use of the antibiotic penicillin and the discovery of penicillinase in 1940[21]. Thousands of enzymes have been identified hydrolyzing antibiotics of different classes, including β-lactams, aminoglycosides, phenicols, and macrolides. The carriage of diverse extended-spectrum β-lactamases and carbapenemases – including the IMP (imipenemase), VIM (Verona integrin encoded metallo β-lactamase), KPC (*Klebsiella pneumoniae* carbapenemase), OXA (oxacillinase) and NDM (New Delhi metallo-β-lactamase) in Gram-negative bacteria – has strengthened the emergence of isolates resistant to all β-lactam antibiotics[22-24]. The emergence of these resistant isolates has serious implications for the treatment of severe infections, particularly in hospital patients[25-26].

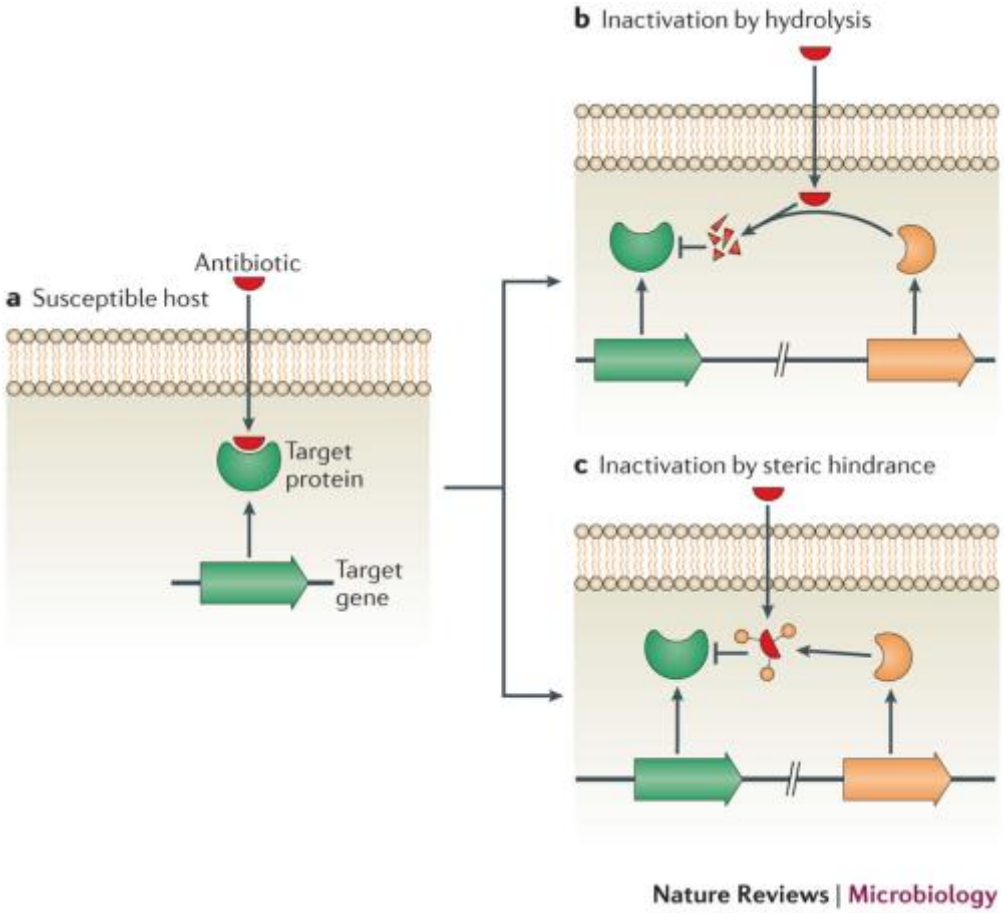

Figure 1-3. Modification of antibiotics with chemical groups. (a) susceptible host with a target that is efficiently inhibited by an antibiotic. (b) Acquisition and production of an enzyme that destroys the antibiotic (for example, β-lactamases) prevents binding to the target and confers resistance. (c) Acquisition and production of an enzyme that modifies the structure of the antibiotic (for example, aminoglycoside-modifying enzymes) can also prevent binding to the target and confer resistance[20].

The addition of chemical groups, including acyl-, phosphate-, nucleotidyl-, and ribitoyl groups[27], to vulnerable sites on the antibiotic molecules by bacterial enzymes can prevent antibiotics from binding to their target protein through steric hindrance (**Figure 1-3**). For example, aminoglycoside antibiotics are particularly susceptible to modification due to their large size and many exposed hydroxyl and amide groups. Three main classes can modify aminoglycosides: acetyltransferases, phosphotransferases and nucleotidyltransferases. These chemical groups can bind to aminoglycosides as their active sites mimic the target environment of the ribosomal binding cleft[28].

As a result of the widespread use of antibiotics in human medicine, as well as in animal treatment, horticulture, beekeeping, anti-fouling paints (used in the marine and oil industries) and laboratories carrying out genetic manipulation, the evolutionary pressure on pathogens for the emergence of antibiotic resistance is considerable[20]. Increasing understanding of resistance aids in the discovery and development of new agents that can circumvent existing resistance mechanisms.

1.3 Development of Novel Antibiotics

With the widespread emergence of antibiotic-resistant bacteria, it is apparent that the success of antibiotics might only have been temporary; we now expect a long-term and perhaps never-ending challenge to find new therapies to combat antibiotic-resistant bacteria. Many non-conventional approaches are

under development. A recent review summarized 19 alternatives to antibiotics[29]. Here, I focused on five of the prospective antibiotic replacements, including peptidomimetic antimicrobials, aminoglycosides and its derivatives, nanoparticles, probiotics, and quorum sensing quenching.

1.3.1 Peptidomimetic antimicrobials

Antimicrobial peptides (AMPs) are termed “host defense peptides” and serve as the first line of defense by disrupting the bacteria membrane[30-33]. The AMPs are small cationic amphiphilic peptides (**Figure 1-4**). They can induce bacterial membrane modifications ranging from minor lipid bending to complete membrane dissolution, depending on the physical structure of the membrane[34]. The structural and sequence diversity of AMPs include amphipathic alpha-helices (e.g. cathelicidins), beta-sheets with 2 to 4 disulfide bridges (e.g. beta defensins and protegrins), extended conformation (indolicidin), and beta-loop peptides (brevinin)[34-37].

Figure 1-4. Three-dimensional structures of human antimicrobial peptides[38].

Peptidomimetic antimicrobials are a new class of AMPs that are stable to enzymatic degradation. To date, peptidomimetics have been designed through the cyclization of linear peptides or the coupling of stable unnatural amino acids. AApeptides are one of the newly developed classes. They mimic natural linear AMPs, adopting globally amphipathic conformations upon initial contact with bacterial membranes (**Figure 1-5**). Some of them are generally compatible, or even superior to the AMP magainin as well as a previous linear α-AApeptide α1 against several bacterial strains[39-41], such as γ-4 containing enhanced bactericidal activity against Gram-positive strains[40]. They are quite toxic to blood cells as well as to other mammalian cells. In fact, the antimicrobial activity of γ-AApeptides is likely to be enhanced if the overall hydrophobicity increases, which at the same time also leads to increased hemolytic activity and cytotoxicity.

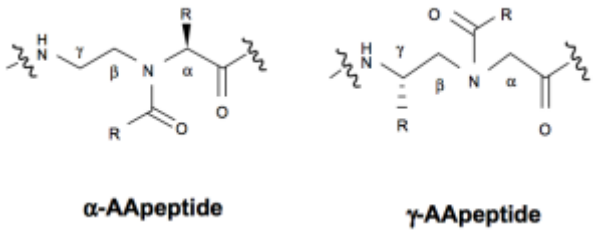

Figure 1-5. Representative structures of α-AA and γ-AApeptides[38].

Peptidomimetics represent an important field in pharmacology as they circumvent the limitations of AMPs used in therapy. The pharmaceutical

company Lytix Biopharma AS has recently commenced Phase I/IIa clinical trials with LytixarTM for nasal decolonization of MRSA. This peptidomimetic, containing a modified tryptophan derivate as lipophilic bulk, displayed a combination of high antibacterial activity against methicillin-resistant *Staphylococci* and *staphylococcal* biofilms[42].

1.3.2 Aminoglycosides and Derivatives

Aminoglycosides (AGs) are bactericidal antibiotics, possessing several amino and hydroxyl functionalities. Because of their polycationic character, AGs show a binding affinity for nucleic acids. Specifically, AGs possess high affinities for certain portions of RNA, especially the prokaryotic rRNA[43-45]. Aminoglycosides interact with 16S RNA and cause a conformational change in the decoding site A, yielding a site that resembles the closed state induced by the interaction between cognate tRNA and mRNA. This change to the decoding sites leads to mistranslation in protein synthesis for Gram-negative and some Gram-positive bacteria.

Enzymatic inactivation by acetylation, adenylation, or phosphorylation at different locations on the aminoglycoside molecules is employed by bacteria to overcome these antibiotics[46-47]. Thus, the development of new AAC-tolerant aminoglycosides, so-called peptidomimetics, is the best way to circumvent this emerging problem. For instance, Plazonicin is currently in clinical trials, and has been shown to have enhanced activity against multidrug-resistant Gram-

negatives[48]. It is not affected by any of the currently-known aminoglycoside-modifying enzymes except AAC (2')-Ia, -Ib, and -Ic, which are only found in the *Providencia* species. Human phase I and II studies have shown no nephrotoxicity or ototoxicity. Plazomicin has completed phase II clinical trials in complicated urinary tract infections[49].

1.3.3 Nanoparticles

Nanoparticles (NPs) have received mounting attention in several fields due to their unique physical, chemical, and affective biological properties. Examples include the utilization of NPs in antibacterial coatings for implantable devices and medicinal materials to prevent infection and promote wound healing, in antibiotic delivery systems to treat disease, in bacterial detection systems to generate microbial diagnostics and in antibacterial vaccines to control bacterial infections. The multiple simultaneous mechanisms of action against NPs would require multiple simultaneous gene mutations in the same bacterial cell for antibacterial resistance to develop. Hence, it is difficult for bacterial cells to become resistant to NPs[50].

NPs need to be in contact with bacterial cells to achieve their antibacterial function. The accepted forms of contact include electrostatic attraction, van der Waals forces, receptor-ligand interactions, and hydrophobic interactions. NPs then cross the bacterial membrane, influencing the shape and function of the cell membrane[51-54]. Thereafter, NPs interact with the bacterial cells' basic

components, such as DNA, lysosomes, ribosomes, and enzymes, leading to oxidative stress, heterogeneous alterations, changes in cell membrane permeability, electrolyte balance disorders, enzyme inhibition, protein deactivation, and changes in gene expression[55-57].

ROS-induced oxidative stress is an important antibacterial mechanism of NPs. ROS is a genetic term for molecules and reactive intermediates that have strong positive redox potential. Different types of NPs produce different types of ROS by reducing oxygen molecules. Under normal circumstances, the production and clearance of ROS in bacterial cells are balanced. In contrast, with excessive production of ROS, the redox balance of the cell favors oxidation[58-59]. This unbalanced state produces oxidative stress, which damages the individual components of bacterial cells. Al_2O_3 NPs cross the cell membrane, and the interaction of Al_2O_3 NPs with the cell membrane eventually triggers the loss of membrane integrity due to oxidative stress[60].

1.3.4 Probiotics approaches

Probiotics were first defined in 1989 as “a live microbial feed supplement which beneficially affects the host by improving its microbial balance”[61]. Four mechanisms control the action of probiotics in the host: the modulation of the content of gut microbiota; the maintenance of the integrity of the gut barrier; the prevention of bacterial translocation; and the modulation of the local immune response by the gut-associated immune system. Most probiotic bacteria belong

to the genera *Lactobacillus* and *Bifidobacterium*. These genera are Gram-positive, lactic acid-producing bacteria that constitute a major part of the normal intestinal microflora in animals and humans. Gut flora resist colonization by pathogenic bacteria in a physiologically restrictive environment with respect to pH, redox potential, and hydrogen sulphide production[62].

Probiotics can adhere to the epithelial gut mucosa by binding to surface-layer proteins so they do not allow free space for pathogenic microorganisms to adhere. The anti-adhesive properties of the probiotics may result from competition for the same receptor, increased production of mucin and biosurfactants, or degradation of carbohydrate receptors. When co-cultured with *Lactobacillus plantarum* 299v or *Lactobacillus rhamnosus* GG with HT20-MTX cells, the induction of MUC3 mucin subsequently inhibited the adhesion of enteropathogenic *E. coli* strain E2348/69[63].

Not limited to the prevention of pathogen binding, probiotics consume micronutrients that would otherwise be utilized by potentially pathogens, consequently inhibiting their growth[64]. An important example of a limited substance in the host is iron. For almost all bacteria, iron is an essential element. The exception is lactonacilli, which do not need iron in their natural habitat[65]. Nevertheless *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* are able to bind ferric hydroxide at their cell surface, rendering it unavailable to pathogenic microorganisms[66].

Certain probiotics in diarrhea are primarily effective due to their ability to protect the host against toxins. Shiga toxin expressed in *E. coli* (STEC) O157:H7 strains can be inhibited in vitro as well as in mice by the anti-infectious activity of probiotic *Bifidobacterium breve* Yakult and *Bifidobacterium pseudocatenulatum* DSM20439. In one study, all animals treated with *B. breve* strain Yakult survived, whereas 90% of the mice in the control group died after challenge with STEC. *In vitro* studies thus imply a high concentration of acetic acid produced by strain Yakult to be responsible for the inhibition of Shiga toxin expression[67].

1.4 Quorum Sensing Quenching

In addition to the four methods mentioned above, quorum sensing quenching intrigues researchers due to its regulation of pathogenic virulence factors without the effect on cell growth, which can highly reduce the selection pressure.

1.4.1 Quorum Sensing System

Pathogens are very clever. When invading a host, they delay the production of virulence factors until sufficient bacteria have amassed and are ready to overwhelm the host immune system. This behavior – when bacteria reach a critical concentration and can sense information from other cells in the population – is called quorum sensing (QS). Bacteria can produce a basal level of small, diffusible signal molecules, which reflect the cell density. These signal molecules are called autoinducers (AIs). AIs can be detected by the receptors

located on either the cytoplasm or the membrane. The QS system can alter gene expressions including bioluminescence signal, antibiotic and toxin production, sporulation, biofilm formation, and virulent factors secretion[68-69]. Moreover, AIs can activate their own production by up-regulating synthase transcription[70], forming a positive feedback loop.

Quorum Sensing signals

AIs are structurally diverse. The most common AIs that Gram-negative bacteria use are *N*-acyl-homoserine-lactones (AHLs). AHLs share a core homoserine-lactone ring and differ in acyl chains of four to eighteen carbons with or without modifications, including carbonyl and hydroxyl moieties at the C3 position (**Figure 1-6a**)[71-72]. Gram-positive bacteria utilize oligopeptide as autoinducing peptides (AIPs). AIPs are varied in both sequence and structure (**Figure 1-6b**)[73-74]. The size of AIPs ranges from 5 to 17 amino acids. They can be further post-translationally modified, linearized, or cyclized [75-77].

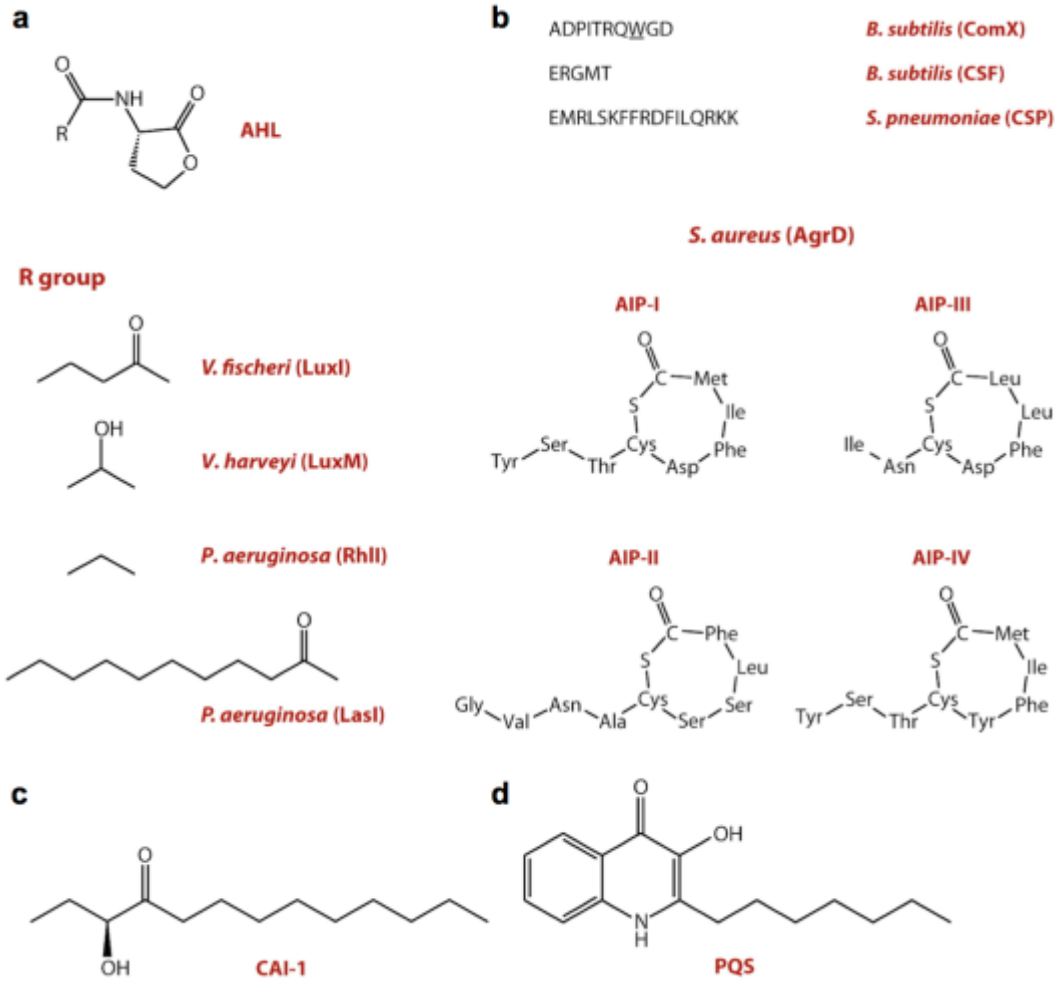

Figure 1-6. Gram-negative and gram-positive bacteria quorum sensing signal structures. (a) For Gram-negative bacteria, acyl-homoserine lactones (AHLs) are the signal molecules. AHLs share a homoserine lactone (HSL) ring and differ in their length of acyl chain as well as the substituents at the 3-position of the acyl chain as R group for different species. (b) For Gram-positive bacteria, oligopeptide autoinducers (AIPs) serve as their communication signals. AIPs typically consist of 5–17 amino acids, sometimes containing unusual side chain modifications. The underline below the tryptophan (W) of ComX indicates a posttranslational isoprenylation of the peptide. (c) CAI-1 is V. cholera AI. (d) P. aeruginosa has a specific AI called PQS[78].

Many Gram-negative plant-associated bacteria can synthesize unusual homoserine lactones (**Figure 1-6c, d**). The photosynthetic bacterium *Rhodopseudomonas palustris* has a 4-coumaroyl-homoserine lactone synthase called RpaI. RpaI can use the host's metabolite *p*-coumarate for its AI[79]. These signaling molecules enable *R. palustris* to associate its quorum sensing response with both the bacterial population density and the accessibility of plant consumables. Another atypical AI, *cis*-11-methyl-2-dodecenoic acid, is produced by *Xanthomonas campestris*. The *cis*-11-methyl-2-dodecenoic acid can modulate *X. campestris'* transitions between its planktonic and biofilm-associated states[80].

Regulation of QS systems

A considerable number of bacteria use more than one type of AI. For example, *P. aeruginosa* has a complicated QS system regulating bacteria virulence and biofilm formation. Over the past two decades, it has been gradually revealed that *P. aeruginosa* has four QS systems (**Figure 1-7**), which are all highly adaptable and capable of responding to external biostress cues. LasR was firstly identified as a key regulator in the expression of lasB, aprA, lasA, and toxA genes[81-84]. It was thought to be a global regulator of the virulence genes. Shortly after, a second factor – the rhl system – was found. It can restore the production of several exoproducts such as elastase, pyocyanin, hemolysin, and rhamnolipids[85]. As the upstream regulator, the las system can activate the rhl system[86]. Both las and rhl systems can produce AHLs. Yet PQS, the third QS signal, which was purified and characterized in 1999, is chemically unique from

the AHLs (**Figure 1-6d**)[87]. PQS is identified as 2-heptyl-3-hydroxy-4-quinolone. The PQS-controlled system, also regulated by lasR, can regulate pyocyanin, elastase, rhamnolipid, and PA-IL lectin production. Recently the fourth inter-cellular communication signal has been found capable of integrating environmental stress cues with the QS network[88]. It was named IQS, and structurally established to be 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde. The genes involved in IQS synthesis are a non-ribosomal peptide synthase gene cluster ambBCDE. The disruption of IQS synthesis results in a reduction in the production of PQS and BHL signals, as well as in the production of virulence factors, such as pyocyanin, rhamnolipids, and elastase. Upon the addition of 10 nmol/L IQS to the mutants, these phenotypes could be fully restored, indicating that IQS is a potent inter-cellular communication signal[88].

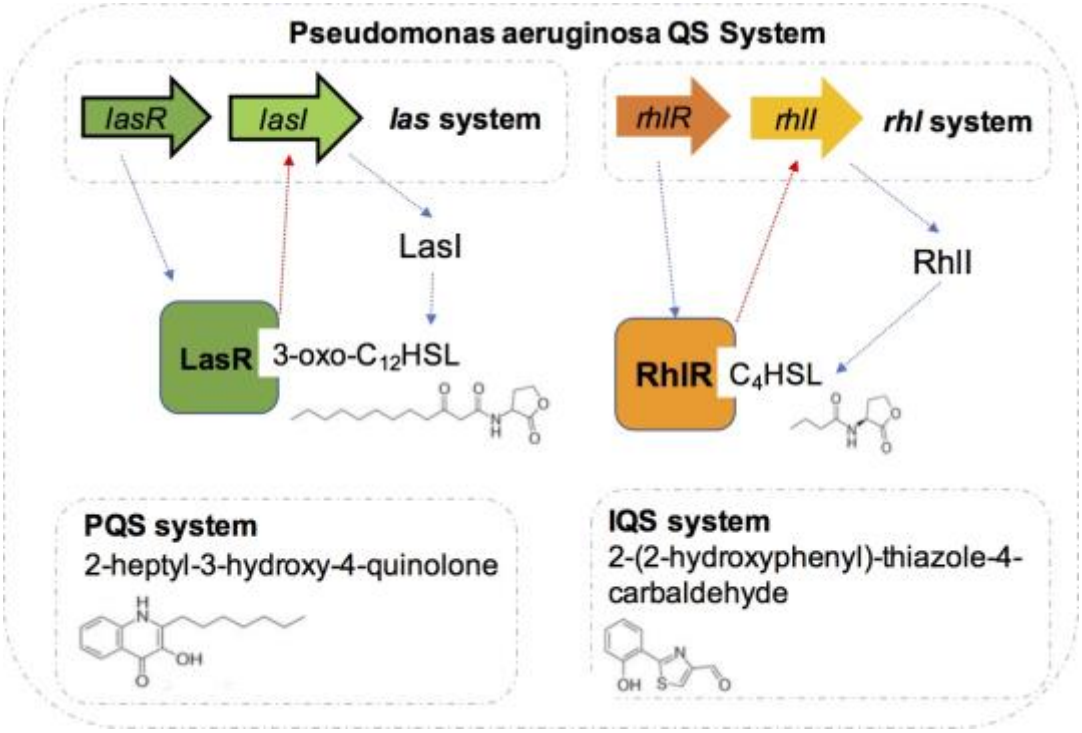

Figure 1-7. *P. aeruginosa* four QS systems: Las, Rhl, PQS, and IQS. The produced autoinducers are 3-oxo-C_{12}-homoserine lactone, C_4-HSL, 2-heptyl-3-hydroxy-4-quinolone, and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde, respectively.

All these QS circuits in *P. aeruginos*a are organized in a hierarchical manner. At the top of the pathway is the las system. The las system can activate the transcription of *rhlR*, *rhl*, *lasI* and other virulence genes that are part of its regulon. The RhlR-BHL complex also dimerizes and similarly activates the expression of its own regulon and rhlI, forming the second positive feedback loop. The las system also positively regulates the PQS transcriptional regulator. In turn, PQS was found to be able to enhance the transcription of rhlI. IQS was also

found to be tightly controlled by LasRI under rich medium conditions. Disruption of either lasR or lasI completely abrogates the expression of ambBCDE and the production of IQS.

As one of the Gram-positive bacterial pathogens, *S. aureus* utilizes P2 promoter regulating the expression of RNAII transcript which encodes its four-component QS system, as described in **Figure 1-8**. Pro-AIPs are encoded by *AgrD*. Pro-AIPs can be transformed to the final AIP and secreted by the transmembrane –transporter protein AgrB[89-90]. Progressing involves truncating the 45 – 47 pro-AIP peptides to a 7 – 9 peptides. This truncating is coupled with the cyclization of a five-membered peptide ring through a thiolactone bond between the central cysteine residue and the carboxyl terminus. When the AIP accumulates, it binds to the membrane-bound histidine kinase AgrC, which autophosphorylates at a conserved histidine and transfers the phosphate group to a conserved aspartate on the response of the regulator AgrA. Then the phosphorylated AgrA binds to the upstream of the P2 promoter sequence, activating the *agr* operon and P3 promoter. The P3 promoter controls the expression of RNAIII[91]. The 5' region of RNAIII harbors the *hld* gene, which encodes the virulence factor δ-hemolysin[92].

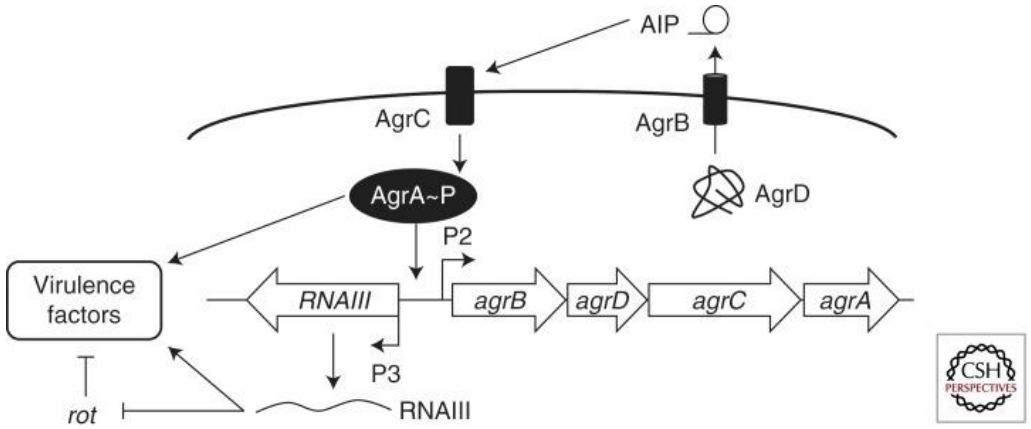

Figure 1-8. *S. aureus* Agr QS circuit. The autoinducing peptide (AIP) is synthesized as a precursor from agrD. The AIP transporter AgrB processes the precursor to the mature AIP and transports it out of the cell. AIPs are detected by a two-component signal transduction pathway. AgrC is the membrane-bound histidine kinase and AgrA is the response regulator. Phosphorylated AgrA activates the P2 and P3 promoters encoding the agr operon (called RNAII) and the RNAIII regulatory RNA, respectively. RNAIII posttranscriptionally activates virulence factor production and represses expression of rot, the repressor of toxins, leading to further de-repression of virulence factors[68].

Signaling molecule diversification also exists within the *S. aureus* community. The *S. aureus* QS system has cross-competition among AIP specificity types. *AgrD* sequences exhibit great sequence diversity throughout their lengths, including the region containing the AIP sequence. For instance, at the amino acid level, only 10% of the residues are identical and 26% of the sequences are replaced with conservative substitutions. Similarly, the alignment of amino acids sequences indicates a wide divergence of *agrC* sequences, particularly within the N-terminal transmembrane region. The C-terminal portion of the *agrB* gene also indicates substantial divergence[93]. This variability leads to

the production of one of four different types of AIPs depending on the strain[94]. Each specific AIP is detected by a corresponding AgrC sensor, and the presence of a noncognate AIP inhibits QS, halting cell-cell communication[95].

In addition to intraspecies signaling, diffusible signals have been involved in interspecies and interkingdom signaling, modulating the behavior of other microorganisms that do not produce the signals. Bacteria talk with their host system, regulating the level of RNA transcription through chemical signals. Numerous effects of bacterial AHLs on eukaryotic cells have been previously described. For example, 3-oxo-C_{12}HSL produced by *P. aeruginosa* differently affects many types of mammalian cells, including macrophages, neutrophils, endothelial cells, epithelial cells, breast carcinoma cells, and T cells. These effects, which were almost exclusively proposed to be detrimental to host cells, include the induction of apoptosis, the stimulation of excessive inflammation, and the modulation of innate immunity. Microarray analysis of lung epithelial cells exposed to 3-oxo-C_{12}HSL demonstrated that approximately 11% of the mammalian transcriptome was significantly altered, which was similar to the magnitude of genes controlled by QS in *P. aeruginosa* itself[96].

1.4.2 Quorum Quenching

Bacterial quorum sensing-mediated signaling can be disrupted by a wide variety of phenomena known as quorum quenching (QQ). All processes of the QS, including synthesis, diffusion, accumulation, and perception of the QS

signals, also the actions and QS targets may be affected. QQ molecules, including chemical compounds and enzymes, are diverse in nature. Usually chemical compounds (QS inhibitors, QSIs) act like competitive inhibitors, and enzymes inactivate QS signals.

QS Inhibitors

The molecules responsible for the inhibition of AI-induced QS systems or the AI-regulated phenotype are called quorum-sensing inhibitors (QSIs). QSIs can be produced by a wide range of organisms, such as bacteria, fungi, plants, and animals from terrestrial, marine, or freshwater ecosystems. The biochemical nature of QSIs is highly diverse, including structural analogs of signal molecules, furanones and their related structural analogs[97-99], bismuth porphyrin complexes[100], glycosylation reagents of glycosylated flavonoids[101], glycomonoterpenols[102], heavy metals[103], and nanomaterials[104-105]. Beside their biochemical diversity, there is no direct correlation between the molecular structure or chemical functional groups of the QSIs and the targeted actor in the QS pathway.

The first marine QSIs were isolated from *Delisea pulchra*, which appears to have developed natural defense mechanisms to prevent microbial biofouling of its surfaces[106]. Secondary metabolites of the halogenated furanones class, which are found at the surface of this alga, exhibit the antifouling activity[107]. Most studies on natural *D. pulchra* furanones involved the (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone. These molecules strongly inhibit AHL

signaling[108]. The inhibitory effect of furanones is primarily due to their structural similarity to AHLs, but some case studies also showed that furanones may function through degrading the luxR-type protein[109] or decreasing the DNA-binding activity of the transcriptional regulator protein luxR[110]. In addition to AHLs, furanones also disrupt the AI-2 biosynthetic pathway by covalently modifying and inactivating luxS[111].

The main source of natural QSIs remains higher plants from very diverse species. These plate species include many medicinal plants, vegetables, and edible fruits. QSIs may be extracted from all types of plant tissues: roots and rhizomes, flowers, bark, leaves, stems, seeds, and fruits[112-115]. The majority of studies have dealt with plant extracts from which the QSI molecules have rarely been isolated. Among identified QSIs, most are cyclic compounds, such as phenolic derivatives or nitrogen cyclics.

QQ enzymes

In addition to QSIs, enzymatic degradation is an alternative approach to inhibiting bacterial signaling. The first reports on enzymatic QQ used enzymes from *Variovorax* and *Bacillus* [116-117]. Since then, numerous enzymes involved in AHL degradation or modification have been reported. They represent four catalytic classes: lactonases that open the homoserine lactone ring, acylases that cleave AHLs at the amide bond and release fatty acid and homoserine lactone, reductases that convert 3-oxo-substituted AHL to their cognate 3-hydroxyl-substituted AHL, and cytochrome oxidases that catalyze oxidation of the

acyl chain[118-123]. These enzymes occur in bacteria, archaea, and eukaryotes (**Figure 1-9**).

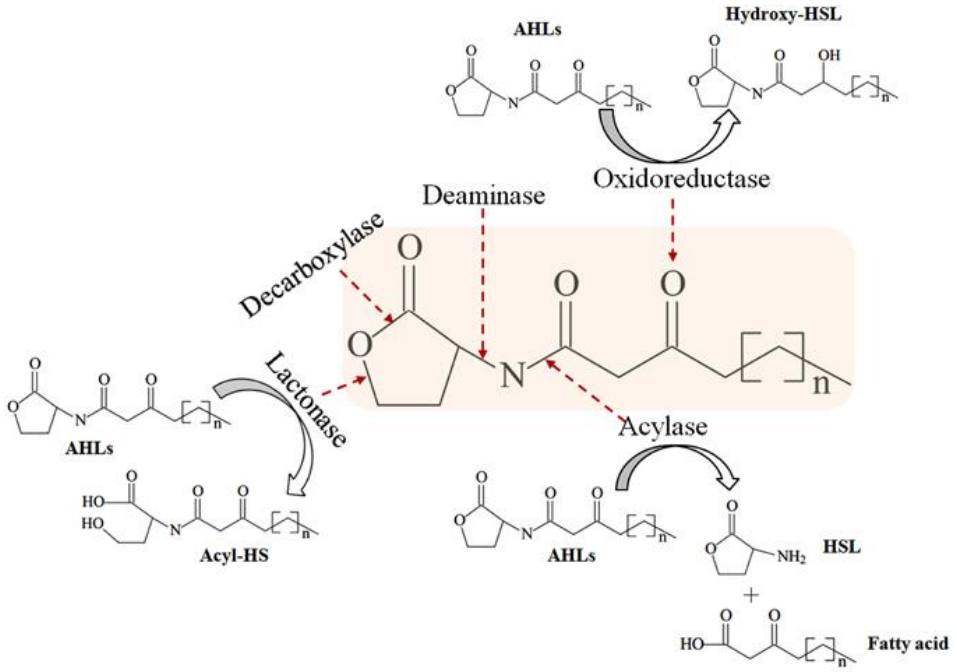

Figure 1-9. Modes of AHL-degrading enzymes and their catalytic sites. They represent four catalytic classes: the lactonases that open the homoserine lactone ring, the acylases that cleave AHLs at the amide bond and release fatty acid and homoserine lactone, the reductases that convert 3-oxo-substituted AHL to their cognate 3-hydroxyl-substituted AHL, and the cytochrome oxidases that catalyze oxidation of the acyl chain[124-125].

Amino acid sequence and architecture of the AHL-degrading enzymes are diverse, especially for the lactonases. Four lactonase families are known: the metallo-β-lactamase-like lactonases, the phosphotriesterase-like lactonases, the paraoxonases, and the α/β-hydrolase fold lactonases. (1) The first enzyme identified as a QQ lactonases, aiiA from *Bacillus* sp. strain[117], belongs to the metallo-β-lactamase superfamily, which has a characteristic Zn^{2+}-binding HXHXDH motif. They usually have a broad substrate specificity and can hydrolyze AHLs with or without c3-substitution and with a preference for medium- to long-chain AHLs[126]. The expression of these genes can prevent the accumulation of AHLs; reduce *Pseudomonas* swarming, twitching, and biofilm formation; and attenuate the virulence factors[122, 127-129]. (2) Phosphotriesterase-like lactonases (PLLs) are members of the amidohydrolase superfamily, possessing a binuclear metal center within a $(\beta/\alpha)_8$-barrel structural scaffold. They generally have a broad substrate spectrum with a preference for hydrophobic lactones, such as SisLac from *Sulfolobus islandicus* and SsoPox from *Sulfolobus solfataricus*[130-131]. (3) The paraoxonases adapt a six-bladed β-propeller, such as PON1. In spite of their different folds, the three families above share a similar catalytic mechanism that uses metal ions and key active site architectures[132]. The lactone substrate binds to the metal cation by its carbonyl oxygen, making the carbonyl carbon more electrophilic. A water molecule is deprotonated by either one active-site metal or by a residue acting as a base. The resulting tetrahedral intermediate is subsequently broken to give the

hydrolyzed product. (4) The structure of α/β-hydrolase fold lactonase named AidH from *Ochrobactrum* is special. It contains no metal-binding motif HXHXDH.

1.4.3 Human Paraoxonases

The paraoxonase family contains three members: PON1, PON2, and PON3. The name derives from the ability of mammalian PON1 to hydrolyze paraoxon, the active metabolite of the organophosphate pesticide parathion. PON1 is a calcium-dependent esterase which is mainly synthesized by the liver and circulated with high-density lipoprotein (HDL) particles[133]. It has been intensively studied for its capacity to protect low-density lipoproteins (LDL) against oxidative stress, reduce macrophage foam cell formation and prevent atherosclerosis development[134]. PON3 is similar to PON1 in terms of expression, function, and location. Both PON1 and PON3 show the capacity to delay LDL oxidation *in vitro*, with PON1 being more effective than PON3[135]. PON2 associates with plasma membranes instead of with HDL particles. PON2 can be expressed in nearly all human tissues, including lungs, liver, heart, kidney, and intestine[136].

HuPON2 structural and functional characterization studies were largely hindered due to the lack of an ample source of recombinant proteins. A rabbit-human hybrid recombinant PON1 was expressed in a soluble and active form in *E. coli*. It exhibited enzymatic properties almost identical to PONs purified from sera[137]. A crystal structure of rePON1 G2E6 was solved, providing the first

structure of a PON family member[138]. According to the crystal structure, PON1 has a six-bladed β-propeller fold structure with two Ca^{2+} ion in its central tunnel[138](**Figure 1-10**). One calcium atom lies at the bottom of the active site and is postulated to play a role in catalysis, while the inner calcium is largely buried and appears to have a structural function. The structure indicated a general-base mechanism reminiscent of secreted phospholipase A2: an activation of a water molecule by a histidine side chain, followed by a nucleophilic attack at the phosphoryl/carbonyl center of the substrates. The negative charge of the resulting intermediates is probably stabilized by the catalytic calcium.

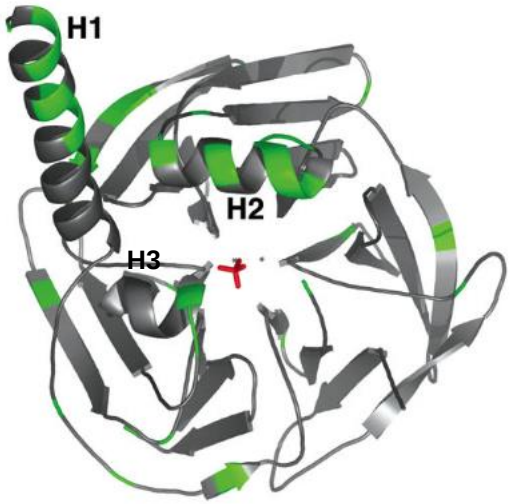

Figure 1-10. Rabbit-human HuPON1 structure (PDB:1V04). Three surface hydrophobic helices are labeled as H1, H2, and H3. Hydrophobic residues are in green[139].

PONs play an important role in innate immune response. This is especially true for PON2. The noteworthy feature of PON2 is its ability to effectively degrade various harmful factors, including acyl-homoserine lactones, that allow bacteria to communicate and coordinate their infection. As early as 2004, it was reported that human airway epithelial cells can inactivate 3-oxo-C_{12}HSL. Later studies revealed that human keratinocytes' PON2 inactivates 3-oxo-C_{12}HSL, and the use of PON2-deficient mice highlighted the involvement of this lactonase in *P. aeruginosa* clearance in the liver, lungs, and spleen, as well as the AHL degradation capability of macrophages[140-141]. Recent work further revealed that the application of 3-oxo-C_{12}HSL on lung epithelial carcinoma cells (human A549 cells) downregulates PON2 mRNA levels, protein and hydrolytic activity of 3-oxo-C_{12}HSL[142]. In addition, a polymorphism found in the PON2 coding sequence, with a substitution of a cysteine for a serine at position 311, alters glycosylation of the enzyme and decreases its lactonase activity, which could be of major importance for innate immunity in the lungs[143].

1.5 Outlines

Our long-term goal is to develop a therapeutic enzyme that can disrupt the pathogenic QS system. This study aims to overcome the aforementioned technical hurdles and develop qualified QQ human-based enzymes. This dissertation is structured into 3 central chapters as follows:

1. In the second chapter, degenerate short peptide linkers based on structure modeling were applied for the removal of hydrophobic helices of

huPON2 without disrupting its folding structure. Modified huPON2s can be solubly expressed in high yields while retaining the enzymatic function and QQ bioactivities. In addition, modified huPON2s inhibit *P. aeruginosa* swimming and swarming motilities.

2. In the third chapter, the soluble expressed huPON2s were further modified by introducing random mutations to enhance the lactonase activity. Five mutants showed strong bioluminescent quenching ability, and two of them, D3H7 (from first round mutagenesis) and 1A3 (from the second roundmutagenesis), elicited the most enhancement of AHL-hydrolysis abilities.
3. In the fourth chapter, QQ agents, C-30 and NPO (chemical molecules) as well as aiiA and huPON2 (QQ enzymes), were applied to both *P. aeruginosa* and chronic wound swabs cultures. C-30, NPO and aiiA exhibited a significant reduction of biofilm for *P. aeruginosa* culture; the engineered huPON2 displays a high potency on *P. aeruginosa* biofilm development. While the influence of aiiA on chronic wound swabs biofilm formation was significant, the potent of engineered huPON2 to the chronic wound swabs biofilm construction needs to be further analyzed.

1.6 References

Uncategorized References

1. Roberts, R. R.; Hota, B.; Ahmad, I.; Scott, R. D., 2nd; Foster, S. D.; Abbasi, F.; Schabowski, S.; Kampe, L. M.; Ciavarella, G. G.; Supino, M.; Naples, J.; Cordell, R.; Levy, S. B.; Weinstein, R. A., Hospital and societal costs of antimicrobial-resistant infections in a Chicago teaching hospital: implications for antibiotic stewardship. *Clin Infect Dis* **2009,** *49* (8), 1175-84.

2. Randall, C. P.; Mariner, K. R.; Chopra, I.; O'Neill, A. J., The Target of Daptomycin Is Absent from Escherichia coli and Other Gram-Negative Pathogens. *Antimicrob Agents Ch* **2013,** *57* (1), 637-639.

3. Nikaido, H.; Vaara, M., Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* **1985,** *49* (1), 1-32.

4. Plesiat, P.; Nikaido, H., Outer membranes of gram-negative bacteria are permeable to steroid probes. *Mol Microbiol* **1992,** *6* (10), 1323-33.

5. Lavigne, J. P.; Sotto, A.; Nicolas-Chanoine, M. H.; Bouziges, N.; Pages, J. M.; Davin-Regli, A., An adaptive response of Enterobacter aerogenes to imipenem: regulation of porin balance in clinical isolates. *International journal of antimicrobial agents* **2013,** *41* (2), 130-6.

6. Tangden, T.; Adler, M.; Cars, O.; Sandegren, L.; Lowdin, E., Frequent emergence of porin-deficient subpopulations with reduced carbapenem susceptibility in ESBL-producing Escherichia coli during exposure to ertapenem in an in vitro pharmacokinetic model. *J Antimicrob Chemother* **2013,** *68* (6), 1319-26.

7. Tamber, S.; Hancock, R. E., On the mechanism of solute uptake in Pseudomonas. *Front Biosci* **2003,** *8*, s472-83.

8. Poulou, A.; Voulgari, E.; Vrioni, G.; Koumaki, V.; Xidopoulos, G.; Chatzipantazi, V.; Markou, F.; Tsakris, A., Outbreak caused by an ertapenem-resistant, CTX-M-15-producing Klebsiella pneumoniae sequence type 101 clone carrying an OmpK36 porin variant. *J Clin Microbiol* **2013,** *51* (10), 3176-82.

9. Munita, J. M.; Arias, C. A., Mechanisms of Antibiotic Resistance. *Microbiol Spectr* **2016,** *4* (2).

10. Dolejska, M.; Villa, L.; Poirel, L.; Nordmann, P.; Carattoli, A., Complete sequencing of an IncHI1 plasmid encoding the carbapenemase NDM-1, the ArmA 16S RNA methylase and a resistance-nodulation-cell division/multidrug efflux pump. *J Antimicrob Chemother* **2013,** *68* (1), 34-9.

11. Kaczmarek, F. S.; Gootz, T. D.; Dib-Hajj, F.; Shang, W.; Hallowell, S.; Cronan, M., Genetic and molecular characterization of beta-lactamase-negative ampicillin-resistant Haemophilus influenzae with unusually high resistance to ampicillin. *Antimicrob Agents Chemother* **2004,** *48* (5), 1630-9.

12. van der Straaten, T.; Janssen, R.; Mevius, D. J.; van Dissel, J. T., Salmonella gene rma (ramA) and multiple-drug-resistant Salmonella enterica serovar typhimurium. *Antimicrob Agents Chemother* **2004,** *48* (6), 2292-4.

13. Webber, M. A.; Piddock, L. J., Absence of mutations in marRAB or soxRS in acrB-overexpressing fluoroquinolone-resistant clinical and veterinary isolates of Escherichia coli. *Antimicrob Agents Chemother* **2001,** *45* (5), 1550-2.

14. Baucheron, S.; Nishino, K.; Monchaux, I.; Canepa, S.; Maurel, M. C.; Coste, F.; Roussel, A.; Cloeckaert, A.; Giraud, E., Bile-mediated activation of the acrAB and tolC multidrug efflux genes occurs mainly through transcriptional derepression of ramA in Salmonella enterica serovar Typhimurium. *J Antimicrob Chemoth* **2014,** *69* (9), 2400-2406.

15. Hirakawa, H.; Inazumi, Y.; Masaki, T.; Hirata, T.; Yamaguchi, A., Indole induces the expression of multidrug exporter genes in Escherichia coli. *Molecular Microbiology* **2005,** *55* (4), 1113-1126.

16. Mercante, A. D.; Jackson, L.; Johnson, P. J. T.; Stringer, V. A.; Dyer, D. W.; Shafer, W. M., MpeR Regulates the mtr Efflux Locus in Neisseria gonorrhoeae and Modulates Antimicrobial Resistance by an Iron-Responsive Mechanism. *Antimicrob Agents Ch* **2012,** *56* (3), 1491-1501.

17. Unemo, M.; Golparian, D.; Nicholas, R.; Ohnishi, M.; Gallay, A.; Sednaoui, P., High-Level Cefixime- and Ceftriaxone-Resistant Neisseria gonorrhoeae in France: Novel penA Mosaic Allele in a Successful International Clone Causes Treatment Failure. *Antimicrob Agents Ch* **2012,** *56* (3), 1273-1280.

18. Leclercq, R.; Courvalin, P., Resistance to macrolides and related antibiotics in Streptococcus pneumoniae. *Antimicrob Agents Ch* **2002,** *46* (9), 2727-2734.

19. Leclercq, R., Mechanisms of resistance to macrolides and lincosamides: Nature of the resistance elements and their clinical implications. *Clinical Infectious Diseases* **2002,** *34* (4), 482-492.

20. Blair, J. M. A.; Webber, M. A.; Baylay, A. J.; Ogbolu, D. O.; Piddock, L. J. V., Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology* **2015,** *13* (1), 42-51.

21. Abraham, E. P.; Chain, E., An enzyme from bacteria able to destroy penicillin. *Nature* **1940,** *146*, 837-837.

22. Livermore, D. M., Defining an extended-spectrum beta-lactamase. *Clin Microbiol Infec* **2008,** *14*, 3-10.

23. Nordmann, P.; Poirel, L.; Walsh, T. R.; Livermore, D. M., The emerging NDM carbapenemases. *Trends in Microbiology* **2011,** *19* (12), 588-595.

24. Voulgari, E.; Poulou, A.; Koumaki, V.; Tsakris, A., Carbapenemase-producing Enterobacteriaceae: now that the storm is finally here, how will timely detection help us fight back? *Future Microbiol* **2013,** *8* (1), 27-39.

25. Johnson, A. P.; Woodford, N., Global spread of antibiotic resistance: the example of New Delhi metallo-beta-lactamase (NDM)-mediated carbapenem resistance. *J Med Microbiol* **2013,** *62* (Pt 4), 499-513.

26. Lynch, J. P.; Clark, N. M.; Zhanel, G. G., Evolution of antimicrobial resistance among Enterobacteriaceae (focus on extended spectrum beta-lactamases and carbapenemases). *Expert Opin Pharmaco* **2013,** *14* (2), 199-210.

27. Wright, G. D., Bacterial resistance to antibiotics: enzymatic degradation and modification. *Adv Drug Deliv Rev* **2005,** *57* (10), 1451-70.

28. Romanowska, J.; Reuter, N.; Trylska, J., Comparing aminoglycoside binding sites in bacterial ribosomal RNA and aminoglycoside modifying enzymes. *Proteins* **2013,** *81* (1), 63-80.

29. Czaplewski, L.; Bax, R.; Clokie, M.; Dawson, M.; Fairhead, H.; Fischetti, V. A.; Foster, S.; Gilmore, B. F.; Hancock, R. E. W.; Harper, D.; Henderson, I. R.; Hilpert, K.; Jones, B. V.; Kadioglu, A.; Knowles, D.; Olafsdottir, S.; Payne, D.; Projan, S.; Shaunak, S.; Silverman, J.; Thomas, C. M.; Trust, T. J.; Warn, P.; Rex, J. H., Alternatives to antibiotics-a pipeline portfolio review. *Lancet Infectious Diseases* **2016,** *16* (2), 239-251.

30. Wu, M. H.; Maier, E.; Benz, R.; Hancock, R. E. W., Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of Escherichia coli. *Biochemistry* **1999,** *38* (22), 7235-7242.

31. Zhang, L. J.; Dhillon, P.; Yan, H.; Farmer, S.; Hancock, R. E. W., Interactions of bacterial cationic peptide antibiotics with outer and cytoplasmic membranes of Pseudomonas aeruginosa. *Antimicrob Agents Ch* **2000,** *44* (12), 3317-3321.

32. Hancock, R. E. W.; Sahl, H. G., Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat Biotechnol* **2006,** *24* (12), 1551-1557.

33. Mendez-Samperio, P., Role of antimicrobial peptides in host defense against mycobacterial infections. *Peptides* **2008,** *29* (10), 1836-1841.

34. Shai, Y., Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. *Biochim Biophys Acta* **1999,** *1462* (1-2), 55-70.

35. Shai, Y., Mode of action of membrane active antimicrobial peptides. *Biopolymers* **2002,** *66* (4), 236-48.

36. Huang, Y.; Huang, J.; Chen, Y., Alpha-helical cationic antimicrobial peptides: relationships of structure and function. *Protein Cell* **2010,** *1* (2), 143-52.

37. Lehrer, R. I.; Lu, W., alpha-Defensins in human innate immunity. *Immunol Rev* **2012,** *245* (1), 84-112.

38. Mendez-Samperio, P., Peptidomimetics as a new generation of antimicrobial agents: current progress. *Infect Drug Resist* **2014,** *7*, 229-37.

39. Padhee, S.; Hu, Y.; Niu, Y.; Bai, G.; Wu, H.; Costanza, F.; West, L.; Harrington, L.; Shaw, L. N.; Cao, C.; Cai, J., Non-hemolytic alpha-AApeptides as antimicrobial peptidomimetics. *Chem Commun (Camb)* **2011,** *47* (34), 9729-31.

40. Niu, Y.; Padhee, S.; Wu, H.; Bai, G.; Harrington, L.; Burda, W. N.; Shaw, L. N.; Cao, C.; Cai, J., Identification of gamma-AApeptides with potent and broad-spectrum antimicrobial activity. *Chem Commun (Camb)* **2011,** *47* (44), 12197-9.

41. Niu, Y.; Wu, H.; Li, Y.; Hu, Y.; Padhee, S.; Li, Q.; Cao, C.; Cai, J., AApeptides as a new class of antimicrobial agents. *Org Biomol Chem* **2013,** *11* (26), 4283-90.

42. Haug, B. E.; Stensen, W.; Kalaaji, M.; Rekdal, O.; Svendsen, J. S., Synthetic antimicrobial peptidomimetics with therapeutic potential. *Journal of Medicinal Chemistry* **2008,** *51* (14), 4306-4314.

43. Fourmy, D.; Recht, M. I.; Blanchard, S. C.; Puglisi, J. D., Structure of the A site of Escherichia coli 16S ribosomal RNA complexed with an aminoglycoside antibiotic. *Science* **1996,** *274* (5291), 1367-1371.

44. Fourmy, D.; Yoshizawa, S.; Puglisi, J. D., Paromomycin binding induces a local conformational change in the A-site of 16 S rRNA. *Journal of molecular biology* **1998,** *277* (2), 333-345.

45. Recht, M. I.; Douthwaite, S.; Puglisi, J. D., Basis for prokaryotic specificity of action of aminoglycoside antibiotics. *Embo Journal* **1999,** *18* (11), 3133-3138.

46. Zaher, H. S.; Green, R., Fidelity at the Molecular Level: Lessons from Protein Synthesis. *Cell* **2009,** *136* (4), 746-762.

47. Ramirez, M. S.; Tolmasky, M. E., Aminoglycoside modifying enzymes. *Drug Resist Update* **2010,** *13* (6), 151-171.

48. Livermore, D. M.; Mushtaq, S.; Warner, M.; Zhang, J. C.; Maharjan, S.; Doumith, M.; Woodford, N., Activity of aminoglycosides, including ACHN-490, against carbapenem-resistant Enterobacteriaceae isolates. *J Antimicrob Chemother* **2011,** *66* (1), 48-53.

49. Cass, R. T.; Brooks, C. D.; Havrilla, N. A.; Tack, K. J.; Borin, M. T.; Young, D.; Bruss, J. B., Pharmacokinetics and safety of single and multiple doses of ACHN-490 injection administered intravenously in healthy subjects. *Antimicrob Agents Chemother* **2011,** *55* (12), 5874-80.

50. Wang, L. L.; Hu, C.; Shao, L. Q., The antimicrobial activity of nanoparticles: present situation and prospects for the future. *Int J Nanomed* **2017,** *12*, 1227-1249.

51. Li, H. H.; Chen, Q. S.; Zhao, J. W.; Urmila, K., Enhancing the antimicrobial activity of natural extraction using the synthetic ultrasmall metal nanoparticles. *Sci Rep-Uk* **2015,** *5*.

52. Armentano, I.; Arciola, C. R.; Fortunati, E.; Ferrari, D.; Mattioli, S.; Amoroso, C. F.; Rizzo, J.; Kenny, J. M.; Imbriani, M.; Visai, L., The Interaction of Bacteria with Engineered Nanostructured Polymeric Materials: A Review. *Sci World J* **2014**.

53. Gao, W. W.; Thamphiwatana, S.; Angsantikul, P.; Zhang, L. F., Nanoparticle approaches against bacterial infections. *Wires Nanomed Nanobi* **2014,** *6* (6), 532-547.

54. Luan, B. Q.; Huynh, T.; Zhou, R. H., Complete wetting of graphene by biological lipids. *Nanoscale* **2016,** *8* (10), 5750-5754.

55. Xu, Y.; Wei, M. T.; Ou-Yang, H. D.; Walker, S. G.; Wang, H. Z.; Gordon, C. R.; Guterman, S.; Zawacki, E.; Applebaum, E.; Brink, P. R.; Rafailovich, M.; Mironava, T., Exposure to TiO2 nanoparticles increases Staphylococcus aureus infection of HeLa cells. *J Nanobiotechnol* **2016,** *14*.

56. Shrivastava, S.; Bera, T.; Roy, A.; Singh, G.; Ramachandrarao, P.; Dash, D., Characterization of enhanced antibacterial effects of novel silver nanoparticles. *Nanotechnology* **2007,** *18* (22).

57. Yang, W.; Shen, C.; Ji, Q.; An, H.; Wang, J.; Liu, Q.; Zhang, Z., Food storage material silver nanoparticles interfere with DNA replication fidelity and bind with DNA. *Nanotechnology* **2009,** *20* (8), 085102.

58. Li, Y.; Zhang, W.; Niu, J. F.; Chen, Y. S., Mechanism of Photogenerated Reactive Oxygen Species and Correlation with the Antibacterial Properties of Engineered Metal-Oxide Nanoparticles. *Acs Nano* **2012,** *6* (6), 5164-5173.

59. Peng, Z. X.; Ni, J. H.; Zheng, K.; Shen, Y. D.; Wang, X. Q.; He, G.; Jin, S. H.; Tang, T. T., Dual effects and mechanism of TiO2 nanotube arrays in reducing bacterial colonization and enhancing C3H10T1/2 cell adhesion. *Int J Nanomed* **2013,** *8*, 3093-3105.

60. Ansari, M. A.; Khan, H. M.; Alzohairy, M. A.; Jalal, M.; Ali, S. G.; Pal, R.; Musarrat, J., Green synthesis of Al2O3 nanoparticles and their bactericidal potential against clinical isolates of multi-drug resistant Pseudomonas aeruginosa. *World J Microb Biot* **2015,** *31* (1), 153-164.

61. Fuller, R., Probiotics in Man and Animals. *J Appl Bacteriol* **1989,** *66* (5), 365-378.

62. Ng, S. C.; Hart, A. L.; Kamm, M. A.; Stagg, A. J.; Knight, S. C., Mechanisms of Action of Probiotics: Recent Advances. *Inflamm Bowel Dis* **2009,** *15* (2), 300-310.

63. Mack, D. R.; Ahrne, S.; Hyde, L.; Wei, S.; Hollingsworth, M. A., Extracellular MUC3 mucin secretion follows adherence of Lactobacillus strains to intestinal epithelial cells in vitro. *Gut* **2003,** *52* (6), 827-33.

64. Grosse, C.; Scherer, J.; Koch, D.; Otto, M.; Taudte, N.; Grass, G., A new ferrous iron-uptake transporter, EfeU (YcdN), from Escherichia coli. *Molecular Microbiology* **2006,** *62* (1), 120-131.

65. Weinberg, E. D., The Lactobacillus anomaly: total iron abstinence. *Perspect Biol Med* **1997,** *40* (4), 578-83.

66. Elli, M.; Zink, R.; Rytz, A.; Reniero, R.; Morelli, L., Iron requirement of Lactobacillus spp. in completely chemically defined growth media. *J Appl Microbiol* **2000,** *88* (4), 695-703.

67. Asahara, T.; Shimizu, K.; Nomoto, K.; Hamabata, T.; Ozawa, A.; Takeda, Y., Probiotic bifidobacteria protect mice from lethal infection with shiga toxin-producing Escherichia coli O157 : H7. *Infection and immunity* **2004,** *72* (4), 2240-2247.

68. Rutherford, S. T.; Bassler, B. L., Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb Perspect Med* **2012,** *2* (11).

69. Li, Y. H.; Tian, X., Quorum sensing and bacterial social interactions in biofilms. *Sensors (Basel)* **2012,** *12* (3), 2519-38.

70. Seed, P. C.; Passador, L.; Iglewski, B. H., Activation of the Pseudomonas aeruginosa lasI gene by LasR and the Pseudomonas autoinducer PAI: an autoinduction regulatory hierarchy. *Journal of bacteriology* **1995,** *177* (3), 654-9.

71. Fuqua, C.; Parsek, M. R.; Greenberg, E. P., Regulation of gene expression by cell-to-cell communication: Acyl-homoserine lactone quorum sensing. *Annual Review of Genetics* **2001,** *35*, 439-468.

72. Ng, W. L.; Bassler, B. L., Bacterial Quorum-Sensing Network Architectures. *Annual Review of Genetics* **2009,** *43*, 197-222.

73. Otto, M.; Sussmuth, R.; Jung, G.; Gotz, F., Structure of the pheromone peptide of the Staphylococcus epidermidis agr system. *Febs Lett* **1998,** *424* (1-2), 89-94.

74. Lazazzera, B. A., The intracellular function of extracellular signaling peptides. *Peptides* **2001,** *22* (10), 1519-27.

75. Magnuson, R.; Solomon, J.; Grossman, A. D., Biochemical and genetic characterization of a competence pheromone from B. subtilis. *Cell* **1994,** *77* (2), 207-16.

76. Mayville, P.; Ji, G.; Beavis, R.; Yang, H.; Goger, M.; Novick, R. P.; Muir, T. W., Structure-activity analysis of synthetic autoinducing thiolactone peptides from Staphylococcus aureus responsible for virulence. *Proceedings of the National Academy of Sciences of the United States of America* **1999,** *96* (4), 1218-23.

77. Bouillaut, L.; Perchat, S.; Arold, S.; Zorrilla, S.; Slamti, L.; Henry, C.; Gohar, M.; Declerck, N.; Lereclus, D., Molecular basis for group-specific activation of the virulence regulator PlcR by PapR heptapeptides. *Nucleic Acids Res* **2008,** *36* (11), 3791-801.

78. Ng, W. L.; Bassler, B. L., Bacterial quorum-sensing network architectures. *Annu Rev Genet* **2009,** *43*, 197-222.

79. 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., A new class of homoserine lactone quorum-sensing signals. *Nature* **2008,** *454* (7204), 595-9.

80. Tao, F.; Swarup, S.; Zhang, L. H., Quorum sensing modulation of a putative glycosyltransferase gene cluster essential for Xanthomonas campestris biofilm formation. *Environ Microbiol* **2010,** *12* (12), 3159-70.

81. Gambello, M. J.; Iglewski, B. H., Cloning and characterization of the Pseudomonas aeruginosa lasR gene, a transcriptional activator of elastase expression. *Journal of bacteriology* **1991,** *173* (9), 3000-9.

82. Gambello, M. J.; Kaye, S.; Iglewski, B. H., Lasr of Pseudomonas-Aeruginosa Is a Transcriptional Activator of the Alkaline Protease Gene (Apr) and an Enhancer of Exotoxin-a Expression. *Infection and immunity* **1993,** *61* (4), 1180-1184.

83. Passador, L.; Cook, J. M.; Gambello, M. J.; Rust, L.; Iglewski, B. H., Expression of Pseudomonas-Aeruginosa Virulence Genes Requires Cell-to-Cell Communication. *Science* **1993,** *260* (5111), 1127-1130.

84. Toder, D. S.; Gambello, M. J.; Iglewski, B. H., Pseudomonas aeruginosa LasA: a second elastase under the transcriptional control of lasR. *Mol Microbiol* **1991,** *5* (8), 2003-10.

85. Brint, J. M.; Ohman, D. E., Synthesis of Multiple Exoproducts in Pseudomonas-Aeruginosa Is under the Control of Rhlr-Rhli, Another Set of Regulators in Strain Pao1 with Homology to the Autoinducer-Responsive Luxr-Luxi Family. *Journal of bacteriology* **1995,** *177* (24), 7155-7163.

86. Pesci, E. C.; Pearson, J. P.; Seed, P. C.; Iglewski, B. H., Regulation of las and rhl quorum sensing in Pseudomonas aeruginosa. *Journal of bacteriology* **1997,** *179* (10), 3127-3132.

87. Pesci, E. C.; Milbank, J. B. J.; Pearson, J. P.; McKnight, S.; Kende, A. S.; Greenberg, E. P.; Iglewski, B. H., Quinolone signaling in the cell-to-cell communication system of Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences of the United States of America* **1999,** *96* (20), 11229-11234.

88. Lee, J.; Wu, J.; Deng, Y. Y.; Wang, J.; Wang, C.; Wang, J. H.; Chang, C. Q.; Dong, Y. H.; Williams, P.; Zhang, L. H., A cell-cell communication signal integrates quorum sensing and stress response (vol 9, pg 339, 2013). *Nature Chemical Biology* **2013,** *9* (6), 406-406.

89. Ji, G.; Beavis, R. C.; Novick, R. P., Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. *Proceedings of the National Academy of Sciences of the United States of America* **1995,** *92* (26), 12055-9.

90. Saenz, H. L.; Augsburger, V.; Vuong, C.; Jack, R. W.; Gotz, F.; Otto, M., Inducible expression and cellular location of AgrB, a protein involved in the maturation of the staphylococcal quorum-sensing pheromone. *Arch Microbiol* **2000,** *174* (6), 452-5.

91. Novick, R. P.; Ross, H. F.; Projan, S. J.; Kornblum, J.; Kreiswirth, B.; Moghazeh, S., Synthesis of Staphylococcal Virulence Factors Is Controlled by a Regulatory Rna Molecule. *Embo Journal* **1993,** *12* (10), 3967-3975.

92. Janzon, L.; Arvidson, S., The role of the delta-lysin gene (hld) in the regulation of virulence genes by the accessory gene regulator (agr) in Staphylococcus aureus. *EMBO J* **1990,** *9* (5), 1391-9.

93. Dufour, P.; Jarraud, S.; Vandenesch, F.; Greenland, T.; Novick, R. P.; Bes, M.; Etienne, J.; Lina, G., High genetic variability of the agr locus in Staphylococcus species. *Journal of bacteriology* **2002,** *184* (4), 1180-6.

94. Ji, G.; Beavis, R.; Novick, R. P., Bacterial interference caused by autoinducing peptide variants. *Science* **1997,** *276* (5321), 2027-30.

95. Geisinger, E.; Muir, T. W.; Novick, R. P., agr receptor mutants reveal distinct modes of inhibition by staphylococcal autoinducing peptides. *Proceedings of the National Academy of Sciences of the United States of America* **2009,** *106* (4), 1216-21.

96. Rumbaugh, K. P.; Kaufmann, G. F., Exploitation of host signaling pathways by microbial quorum sensing signals. *Current Opinion in Microbiology* **2012,** *15* (2), 162-168.

97. Zang, T.; Lee, B. W.; Cannon, L. M.; Ritter, K. A.; Dai, S.; Ren, D.; Wood, T. K.; Zhou, Z. S., A naturally occurring brominated furanone covalently modifies and inactivates LuxS. *Bioorg Med Chem Lett* **2009,** *19* (21), 6200-4.

98. Liu, H. B.; Lee, J. H.; Kim, J. S.; Park, S., Inhibitors of the Pseudomonas aeruginosa quorum-sensing regulator, QscR. *Biotechnol Bioeng* **2010,** *106* (1), 119-26.

99. Steenackers, H. P.; Levin, J.; Janssens, J. C.; De Weerdt, A.; Balzarini, J.; Vanderleyden, J.; De Vos, D. E.; De Keersmaecker, S. C., Structure-activity relationship of brominated 3-alkyl-5-methylene-2(5H)-furanones and alkylmaleic anhydrides as inhibitors of Salmonella biofilm formation and quorum sensing regulated bioluminescence in Vibrio harveyi. *Bioorgan Med Chem* **2010,** *18* (14), 5224-5233.

100. Galkin, M.; Ivanitsia, V.; Ishkov, Y.; Galkin, B.; Filipova, T., Characteristics of the Pseudomonas aeruginosa PA01 Intercellular Signaling Pathway (Quorum Sensing) Functioning in Presence of Porphyrins Bismuth Complexes. *Pol J Microbiol* **2015,** *64* (2), 101-106.

101. Brango-Vanegas, J.; Costa, G. M.; Ortmann, C. F.; Schenkel, E. P.; Reginatto, F. H.; Ramos, F. A.; Arevalo-Ferro, C.; Castellanos, L., Glycosylflavonoids from Cecropia pachystachya Trecul are quorum sensing inhibitors. *Phytomedicine* **2014,** *21* (5), 670-675.

102. Mukherji, R.; Prabhune, A., A new class of bacterial quorum sensing antagonists: glycomonoterpenols synthesized using linalool and alpha terpineol. *World J Microb Biot* **2015,** *31* (6), 841-849.

103. Vega, L. M.; Mathieu, J.; Yang, Y.; Pyle, B. H.; McLean, R. J. C.; Alvarez, P. J., Nickel and cadmium, ions inhibit quorum sensing and biofilm formation without affecting viability in Burkholderia multivorans. *Int Biodeter Biodegr* **2014,** *91*, 82-87.

104. Singh, B. R.; Singh, B. N.; Singh, A.; Khan, W.; Naqvi, A. H.; Singh, H. B., Mycofabricated biosilver nanoparticles interrupt Pseudomonas aeruginosa quorum sensing systems. *Sci Rep-Uk* **2015,** *5*.

105. Miller, K. P.; Wang, L.; Chen, Y. P.; Pellechia, P. J.; Benicewicz, B. C.; Decho, A. W., Engineering nanoparticles to silence bacterial communication. *Front Microbiol* **2015,** *6*.

106. Givskov, M.; DeNys, R.; Manefield, M.; Gram, L.; Maximilien, R.; Eberl, L.; Molin, S.; Steinberg, P. D.; Kjelleberg, S., Eukaryotic interference with homoserine lactone-mediated prokaryotic signaling. *Journal of bacteriology* **1996,** *178* (22), 6618-6622.

107. Dworjanyn, S. A.; De Nys, R.; Steinberg, P. D., Localisation and surface quantification of secondary metabolites in the red alga Delisea pulchra. *Mar Biol* **1999,** *133* (4), 727-736.

108. Hentzer, M.; Wu, H.; Andersen, J. B.; Riedel, K.; Rasmussen, T. B.; Bagge, N.; Kumar, N.; Schembri, M. A.; Song, Z. J.; Kristoffersen, P.; Manefield, M.; Costerton, J. W.; Molin, S.; Eberl, L.; Steinberg, P.; Kjelleberg, S.; Hoiby, N.; Givskov, M., Attenuation of Pseudomonas aeruginosa virulence by quorum sensing inhibitors. *Embo Journal* **2003,** *22* (15), 3803-3815.

109. Manefield, M.; Rasmussen, T. B.; Henzter, M.; Andersen, J. B.; Steinberg, P.; Kjelleberg, S.; Givskov, M., Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. *Microbiol-Sgm* **2002,** *148*, 1119-1127.

110. Defoirdt, T.; Miyamoto, C. M.; Wood, T. K.; Meighen, E. A.; Sorgeloos, P.; Verstraete, W.; Bossier, P., The natural furanone (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone disrupts quorum sensing-regulated gene expression in Vibrio harveyi by decreasing the DNA-binding activity of the transcriptional regulator protein luxR. *Environmental Microbiology* **2007,** *9* (10), 2486-2495.

111. Zang, T. Z.; Lee, B. W. K.; Cannon, L. M.; Ritter, K. A.; Dai, S. J.; Ren, D. C.; Wood, T. K.; Zhou, Z. S., A naturally occurring brominated furanone covalently modifies and inactivates LuxS. *Bioorganic & Medicinal Chemistry Letters* **2009,** *19* (21), 6200-6204.

112. Vandeputte, O. M.; Kiendrebeogo, M.; Rajaonson, S.; Diallo, B.; Mol, A.; El Jaziri, M.; Baucher, M., Identification of Catechin as One of the Flavonoids from Combretum albiflorum Bark Extract That Reduces the Production of Quorum-Sensing-Controlled Virulence Factors in Pseudomonas aeruginosa PAO1. *Applied and Environmental Microbiology* **2010,** *76* (1), 243-253.

113. Chong, Y. M.; Yin, W. F.; Ho, C. Y.; Mustafa, M. R.; Hadi, A. H. A.; Awang, K.; Narrima, P.; Koh, C. L.; Appleton, D. R.; Chan, K. G., Malabaricone C from Myristica cinnamomea Exhibits Anti-Quorum Sensing Activity. *J Nat Prod* **2011,** *74* (10), 2261-2264.

114. Abd-Alla, M. H.; Bashandy, S. R., Production of Quorum Sensing Inhibitors in Growing Onion Bulbs Infected with Pseudomonas aeruginosa E (HQ324110). *ISRN Microbiol* **2012,** *2012*, 161890.

115. Packiavathy, I. A.; Sasikumar, P.; Pandian, S. K.; Veera Ravi, A., Prevention of quorum-sensing-mediated biofilm development and virulence factors production in Vibrio spp. by curcumin. *Appl Microbiol Biotechnol* **2013,** *97* (23), 10177-87.

116. Leadbetter, J. R.; Greenberg, E. P., Metabolism of acyl-homoserine lactone quorum-sensing signals by Variovorax paradoxus. *Journal of bacteriology* **2000,** *182* (24), 6921-6926.

117. Dong, Y. H.; Xu, J. L.; Li, X. Z.; Zhang, L. H., AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of Erwinia carotovora. *Proceedings of the National Academy of Sciences of the United States of America* **2000,** *97* (7), 3526-3531.

118. Zhang, R. G.; Pappas, K. M.; Brace, J. L.; Miller, P. C.; Oulmassov, T.; Molyneaux, J. M.; Anderson, J. C.; Bashkin, J. K.; Winans, S. C.; Joachimiak, A., Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA (vol 417, pg 971, 2002). *Nature* **2011,** *476* (7359).

119. Uroz, S.; Oger, P. M.; Chapelle, E.; Adeline, M. T.; Faure, D.; Dessaux, Y., A Rhodococcus qsdA-encoded enzyme defines a novel class of large-spectrum quorum-quenching lactonases. *Applied and Environmental Microbiology* **2008,** *74* (5), 1357-1366.

120. Lin, Y. H.; Xu, J. L.; Hu, J. Y.; Wang, L. H.; Ong, S. L.; Leadbetter, J. R.; Zhang, L. H., Acyl-homoserine lactone acylase from Ralstonia strain XJ12B represents a novel and potent class of quorum-quenching enzymes. *Molecular Microbiology* **2003,** *47* (3), 849-860.

121. Bijtenhoorn, P.; Mayerhofer, H.; Muller-Dieckmann, J.; Utpatel, C.; Schipper, C.; Hornung, C.; Szesny, M.; Grond, S.; Thurmer, A.; Brzuszkiewicz, E.; Daniel, R.; Dierking, K.; Schulenburg, H.; Streit, W. R., A Novel Metagenomic Short-Chain Dehydrogenase/Reductase Attenuates Pseudomonas aeruginosa Biofilm Formation and Virulence on Caenorhabditis elegans. *Plos One* **2011,** *6* (10).

122. Bijtenhoorn, P.; Schipper, C.; Hornung, C.; Quitschau, M.; Grond, S.; Weiland, N.; Streit, W. R., BpiB05, a novel metagenome-derived hydrolase acting on N-acylhomoserine lactones. *J Biotechnol* **2011,** *155* (1), 86-94.

123. Chowdhary, P. K.; Keshavan, N.; Nguyen, H. Q.; Peterson, J. A.; Gonzalez, J. E.; Haines, D. C., Bacillus megaterium CYP102A1 oxidation of acyl homoserine lactones and acyl homoserines. *Biochemistry* **2007,** *46* (50), 14429-14437.

124. Chen, F.; Gao, Y. X.; Chen, X. Y.; Yu, Z. M.; Li, X. Z., Quorum Quenching Enzymes and Their Application in Degrading Signal Molecules to Block Quorum Sensing-Dependent Infection. *Int J Mol Sci* **2013,** *14* (9), 17477-17500.

125. Zhang, W.; Li, C., Exploiting Quorum Sensing Interfering Strategies in Gram-Negative Bacteria for the Enhancement of Environmental Applications. *Front Microbiol* **2015,** *6*, 1535.

126. Fetzner, S., Quorum quenching enzymes. *J Biotechnol* **2015,** *201*, 2-14.

127. Reimmann, C.; Ginet, N.; Michel, L.; Keel, C.; Michaux, P.; Krishnapillai, V.; Zala, M.; Heurlier, K.; Triandafillu, K.; Harms, H.; Defago, G.; Haas, D., Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in Pseudomonas aeruginosa PAO1. *Microbiol-Sgm* **2002,** *148*, 923-932.

128. Wang, W. Z.; Morohoshi, T.; Ikenoya, M.; Someya, N.; Ikeda, T., AiiM, a Novel Class of N-Acylhomoserine Lactonase from the Leaf-Associated Bacterium Microbacterium testaceum. *Applied and Environmental Microbiology* **2010,** *76* (8), 2524-2530.

129. Morohoshi, T.; Tominaga, Y.; Someya, N.; Ikeda, T., Complete genome sequence and characterization of the N-acylhomoserine lactone-degrading gene of the potato leaf-associated Solibacillus silvestris. *J Biosci Bioeng* **2012,** *113* (1), 20-25.

130. Elias, M.; Dupuy, J.; Merone, L.; Mandrich, L.; Porzio, E.; Moniot, S.; Rochu, D.; Lecomte, C.; Rossi, M.; Masson, P.; Manco, G.; Chabriere, E., Structural basis for natural lactonase and promiscuous phosphotriesterase activities. *Journal of molecular biology* **2008,** *379* (5), 1017-1028.

131. Hiblot, J.; Gotthard, G.; Chabriere, E.; Elias, M., Structural and Enzymatic characterization of the lactonase SisLac from Sulfolobus islandicus. *Plos One* **2012,** *7* (10).

132. Elias, M.; Tawfik, D. S., Divergence and Convergence in Enzyme Evolution: Parallel Evolution of Paraoxonases from Quorum-quenching Lactonases. *Journal of Biological Chemistry* **2012,** *287* (1), 11-20.

133. LaDu, B. N., Structural and functional diversity of paraoxonases. *Nat Med* **1996,** *2* (11), 1186-1187.

134. Aviram, M.; Rosenblat, M., Paraoxonases 1, 2, and 3, oxidative stress, and macrophage foam cell formation during atherosclerosis development. *Free Radical Bio Med* **2004,** *37* (9), 1304-1316.

135. Liu, Y. F.; Mackness, B.; Mackness, M., Comparison of the ability of paraoxonases 1 and 3 to attenuate the in vitro oxidation of low-density lipoprotein and reduce macrophage oxidative stress. *Free Radical Bio Med* **2008,** *45* (6), 743-748.

136. Ng, C. J.; Wadleigh, D. J.; Gangopadhyay, A.; Hama, S.; Grijalva, V. R.; Navab, M.; Fogelman, A. M.; Reddy, S. T., Paraoxonase-2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein. *Journal of Biological Chemistry* **2001,** *276* (48), 44444-44449.

137. Aharoni, A.; Gaidukov, L.; Yagur, S.; Toker, L.; Silman, I.; Tawfik, D. S., Directed evolution of mammalian paraoxonases PON1 and PON3 for bacterial expression and catalytic specialization. *Proceedings of the National Academy of Sciences of the United States of America* **2004,** *101* (2), 482-487.

138. Harel, M.; Aharoni, A.; Gaidukov, L.; Brumshtein, B.; Khersonsky, O.; Meged, R.; Dvir, H.; Ravelli, R. B. G.; McCarthy, A.; Toker, L.; Silman, I.; Sussman, J. L.; Tawfik, D. S., Structure and evolution of the serum

paraoxonase family of detoxifying and anti-atherosclerotic enzymes (vol 11, pg 412, 2004). *Nature structural & molecular biology* **2004,** *11* (12), 1253-1253.

139. Harel, M.; Aharoni, A.; Gaidukov, L.; Brumshtein, B.; Khersonsky, O.; Meged, R.; Dvir, H.; Ravelli, R. B.; McCarthy, A.; Toker, L.; Silman, I.; Sussman, J. L.; Tawfik, D. S., Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. *Nature structural & molecular biology* **2004,** *11* (5), 412-9.

140. Devarajan, A.; Bourquard, N.; Grijalva, V. R.; Gao, F.; Ganapathy, E.; Verma, J.; Reddy, S. T., Role of PON2 in innate immune response in an acute infection model. *Mol Genet Metab* **2013,** *110* (3), 362-70.

141. Simanski, M.; Babucke, S.; Eberl, L.; Harder, J., Paraoxonase 2 Acts as a Quorum Sensing-Quenching Factor in Human Keratinocytes. *J Invest Dermatol* **2012,** *132* (9), 2296-2299.

142. Horke, S.; Witte, I.; Altenhofer, S.; Wilgenbus, P.; Goldeck, M.; Forstermann, U.; Xiao, J. H.; Kramer, G. L.; Haines, D. C.; Chowdhary, P. K.; Haley, R. W.; Teiber, J. F., Paraoxonase 2 is down-regulated by the Pseudomonas aeruginosa quorum-sensing signal N-(3-oxododecanoyl)-L-homoserine lactone and attenuates oxidative stress induced by pyocyanin. *Biochemical Journal* **2010,** *426*, 73-83.

143. Stoltz, D. A.; Ozer, E. A.; Recker, T. J.; Estin, M.; Yang, X.; Shih, D. M.; Lusis, A. J.; Zabner, J., A Common Mutation in Paraoxonase-2 Results in Impaired Lactonase Activity. *Journal of Biological Chemistry* **2009,** *284* (51), 35564-35571.

2 Chapter 2 Engineering Soluble Human Paraoxonase 2 for Quorum Quenching

This chapter is based on X. Li, C. Wang, A. Mulchandani, X. Ge, *ACS Chem Biol* **2016,** *11* (11), 3122-3131

2.1 Abstract

Many pathogenic bacteria utilize the quorum sensing (QS) system to regulate the expression of their virulence genes and promote the formation of biofilm, which renders pathogens extremely resistant to conventional antibiotic treatments. As a novel approach for attenuating antibiotic resistance and in turn fighting chronic infections, enzymatic inactivation of QS signaling molecules, such as N-acyl homoserine lactones (AHLs), holds great promise. Instead of using bacterial lactonases that can evoke the immune response when administered, we focus on the human paraoxonase 2 (huPON2). However, insolubility when heterologously overexpressed hinders its application as anti-infection therapeutics. In this study, huPON2 was engineered for soluble expression with minimal introduction of foreign sequences. Based on a structure model, degenerate linkers were exploited for the removal of hydrophobic helices of huPON2 without disrupting its folding structure and thus retaining its enzymatic function. High soluble expression levels were achieved with yields of 76 mg of fully human PON2 variants per liter culture media. Particularly, two clones D2 and E3 showed significant quorum quenching (QQ) bioactivities and effectively

impeded *Pseudomonas aeruginosa* swimming and swarming motilities, as signs of early stage of biofilm formation. In addition, by correlating QQ with luminescence signal readouts, quantitative analysis of QQ towards natural or non-natural AHL-regulator combinations was performed. Engineered huPON2 variants D2 and E3 exhibited strong lactone hydrolysis activities towards five AHLs of different side chain lengths and modifications utilized by a variety of biomedically important pathogens.

2.2 Introduction

Besides living as unicellular organisms, bacteria also interact socially by utilizing chemical signaling compounds to communicate within and between species[1-3]. Particularly, to survive in a host and to attack the host defense mechanisms, many bacterial pathogens have evolved multicellular organization, which regulates numerous virulence gene expressions and promotes biofilm development[4-6]. Once biofilm generates, infections such as cystic fibrosis, chronic wounds, and diabetic foot ulcers, become resistant to antibiotics and other conventional antimicrobial agents[5]. Bacteria activate these protective traits only after attaining a particular population density, so that they are able to launch a concerted attack and produce ample virulence factors to overwhelm the host defenses[7]. To sense their population density, bacteria employ quorum sensing (QS), a process in which bacteria secrete and recognize small signaling

molecules called autoinducers or quorum sensing molecules (QSMs)[8]. The concentration of these QSMs correlates with the abundance of secreting microorganisms in the vicinity. Because biofilm development and QS are closely connected, QSMs have been recognized as attractive pharmaceutical targets for fighting infections. In fact, most high-priority Categories A, B and C pathogenic bacteria identified by NIAID involve QS and therefore are potentially targetable by quorum quenching (QQ)[9].

One approach to interfere intercellular communication, thus disrupting biofilm formation and inhibiting the expression of virulence factors, is through the usage of synthetic QSM analogues, e.g. brominated furanone C-30, to act as agonists or antagonists[10-11]. This strategy makes pathogens more sensitive to the host immune system and to some antimicrobials[12]. Its potential for treating infections such as Pseudomonas aeruginosa in cystic fibrosis and Enterobacter cloacae in chronic wounds has been demonstrated[13-14]. In general, disturbing QS communication does not directly cause cell death, therefore applying less selective pressure compared to antibiotics[15]. However, it has also been found that in some cases pathogens tend to evolve resistance such as by increasing efflux of QQ agents[16]. As an alternative approach for disrupting QS, the use of enzymes to abolish the biological activity of QSMs can therefore be an encouraging method of QQ[17].

At least three important criteria are required for the development of an enzymatic anti-biofilm QQ treatment: high catalytic efficiency, minimal immunogenicity, and soluble expression with low production cost. Currently, there is no such enzyme meeting all requirements. Studies of a broad range of bacterial lactonases and acylases suggest they are highly active toward QSMs, i.e., N-acyl homoserine lactones (AHLs), easy to express, and amenable to engineering[18-23]. However, the prokaryotic origin of these enzymes is very likely to evoke adverse immune responses when administrated to human. On the other hand, mammalian paraoxonases (PONs), named after their capability to detoxify organophosphate compounds, can hydrolyze lactones of various modifications and carbon chain lengths[24-25]. Three members of the human PON family (huPON1-3) sharing ~60% sequence homology with highly similar secondary structures have been identified[26]. Among these, huPON2 exhibits the highest AHLs hydrolysis activities, which was suggested as the native function of huPONs[25, 27-28]. Mounting evidence suggests that mammalian PONs play an important role in quenching of bacterial QS[4, 29-30]. For example, paraoxonase-1 transgenic flies were protected from P. aeruginosa lethality[31]; expression of PONs inversely correlated with P. aeruginosa infection in cystic fibrosis[32]; and PON2 deficiency enhanced P. aeruginosa QS in murine tracheal epithelia cells[33].

However, mammalian paraoxonases are cell membrane or high-density lipoproteins (HDL) – associated enzymes, and thus are difficult to be expressed

in soluble forms[34-37]. Active huPON1 has been successfully produced by fermentation in E. coli with considerable yield[38]. Aiming to produce a more soluble version of PON1, directed evolution was applied to shuffle human, rabbit, mouse and rat PON1 genes. Soluble and functional chimeric PON1 recombinants was generated, and one of them was crystalized for structure determination[39-40]. However, sequences of the resulting PON1 variants were more rabbit than human – 91% identity to rabbit PON1 and dozens of amino acids different from human PON1. Further, since the locations of these mutations are scattered throughout the sequence and mainly on the surface of the enzyme, de-immunization of the hybrid PON1 variants, e.g. by B-cell or T-cell epitope removal, would be significantly challenging[41-42]. On the basis of structure analysis, huPON1 has been further engineered by substituting the hydrophobic residues of the HDL binding site with hydrophilic residues derived from the chimeric PON1s. This humanized PON1 variant demonstrated an improved solubility but still differed in a significant number of positions from huPON1[43].

To avoid immunological complications, it would be desirable to have a protein therapeutic that is as close as possible to the human native protein with minimum foreign or immuno-stimulating segments. In this study, we replaced the extruded hydrophobic helices of huPON2 with degenerate short peptide linkers. From the limited-diversity linker library, we isolated fully human PON2 variants exhibiting a high level soluble expression. In addition to lactone hydrolysis

activities toward a spectrum of QSMs found in clinically important pathogens, these engineered huPON2 variants were also biologically functional in P. aeruginosa swimming and swarming motility tests.

2.3 Methods

2.3.1 Design and Construction of the huPON2 Library.

Wild type human paraoxonases 2 (huPON2) sequence (GenBank AAC41995.1) and the crystal structure of a hybrid mammalian PON1 derived from directed evolution (PDB 1V04)[40] were used for the design of the huPON2 library of this study. Twenty-five primers (**Table 2-1**) encoding the huPON2 library were designed using DNAWorks and chemically synthesized. Three segments of huPON2 genes were PCR assembled to construct full-length huPON2 by overlapping PCR. The resulting fragment (904 bp) was gel purified and cloned between NheI and XhoI sites on pET28b to encode N-terminal His-tag fused huPON2 variants. The ligated vectors were transformed into E. coli Jude-1 (DH10B harboring the “F” factor derived from XL1-blue) cells and the quality of the constructed library were verified by DNA sequencing.

Table 2-1. Oligonuceotides used in this chapter

Name	Sequence
PON2a1[1]	CGTACGCTAGCGACCTGCCGCACTGCCATCTGATCAAAGGTA
PON2a2[1,2]	CGTTCGGCAGAATGTCGATGTCTTCAGAACCCGCTTCGATACCTTTGATCAGATGGCAGT
PON2a3[1,2]	ATCGACATTCTGCCGAACGGTCTGGCGTTCTTCTCTGTTGGTCTGAAATTCCCGGGTCTG
PON2a4[1,2]	TCATCAGGATACCACCCGGTTTGTCCGGTGCGAAAGAGTGCAGACCCGGGAATTTCAGAC
PON2a5[1,2]	CCGGGTGGTATCCTGATGATGGACCTGAAGGAAGAAAAACCGCGTGCGCGTGAACTCCGT
PON2a6[1,2]	ATACCGTGAGGATTGAAAGACGCCAGGTCGAAACCACGAGAGATACGGAGTTCACGCGCA
PON2a7[1,2]	CGTCTTTCAATCCTCACGGTATCTCCACCTTCATCGATAACGACGACACCGTTTACCTGT
PON2a8[1,2]	GATTTCAACCGTGTTCTTAAATTCAGGGTGGTTAACAACGAACAGGTAAACGGTGTCGTC
PON2a9[1,2]	TGAATTTAAGAACACGGTTGAAATCTTCAAATTCGAAGAAGCGGAAAACTCTCTGCTGCA
PON2a10[1,2]	CGTTAACAGACGGCAGCAGTTCGTGTTTAACGGTTTTCAGGTGCAGCAGAGAGTTTTCCG
PON2a11[1,2]	TGCTGCCGTCTGTTAACGACATCACCGCAGTTGGTCCGGCGCACTTCTACGCGACCAACG
PON2a12[1]	GGAGAGTAGTAAACAACGTTCGCGCYCGGGCYGTGGTCGTTGGTCGCGTAGAAGTG
PON2b1[1]	GCGAACGTTGTTTACTACTCTCCGAACGAAGTTAAAGTTGTAGCGGAAGGTTTCG
PON2b2[1,2]	TATTTGTCGTCCGGAGAGATGTTGATGCCGTTCGCAGAGTCGAAACCTTCCGCTACAACT
PON2b3[1,2]	CATCTCTCCGGACGACAAATACATCTACGTTGCGGACATCCTGGCGCACGAAATCCACGT
PON2b4[1,2]	CACTTTCAGCTGGGTCAGGTTCATGTTGGTGTGTTTTTCCAGAACGTGGATTTCGTGCGC
PON2b5[1,2]	CCTGACCCAGCTGAAAGTGCTGGAACTGGACACCCTGGTTGACAACCTGTCTATCGACCC
PON2b6[1]	ACCGTTCGGGTGGCAACCAACCCAGATGTCACCAGAAGACGGGTCGATAGACAGGTTGTC
PON2c1[1]	CCGTCTTCTGAAGTTCTGCGTATCCAGAACATCCTGTGCGAAAAACCGA
PON2c2[1,2]	CTGCAGAACAGAACCGTTGTTCGCGTAAACGGTGGTAACGGTCGGTTTTTCGCACAGGAT
PON2c3[1,2]	ACAACGGTTCTGTTCTGCAGGGTTCTTCTGTTGCGTCTGTTTACGACGGTAAACTGCTGA
PON2c4[1,2]	CAGTTCGCAGTACAGCGCACGATGGTACAGGGTACCGATCAGCAGTTTACCGTCGTAAA
PON2bc[1]	GGTTGCCACCCGAACGGTRRCRRCCCGTCTTCTGAAGTTCTGCG
PON2c5[1]	GCGCTGTACTGCGAACTGTAACTCGAGCATGC
PON2c6[1]	GCATGCTCGAGTTACAGTTCGCAGTACAGCGCACG
xl28[3]	GTACCTCGGGGAAAACCTGTATTTTCAGGGAATGACAGTAAAGAAGCTTTATTTCATCCC
xl29[3]	GGACTGAATTCTATATATTCCGGGAACACTCTACAACTC
PON1H1[2]	CTGCCCATGGGTGCGTGGGTTGGTTGTGGCCTGGCTGGTGACCGTGCCGGCTTTTTGGG
PON1H2[2]	GATTACGAAGGGCGAGGAGACGTTCTCCCAAAAAGCCGGCACGG
PON1H3[2]	CTCCTCGCCCTTCGTAATCGTCTTAAAGCGAGCCGCG
PON1H4[2]	GGCAGGTCTACGCTTTCAACTTCGCGGCTCGCTTTAAGAC
PON1H5[2]	GTTGAAAGCGTAGACCTGCCTCACTGCCACCTGATCAAAGGTAT
PON2A12RE[2]	CAGATAGGTACCCAGGTACTTCAGGAACGGATCGCTGAAGTAGTGGTCGTTGGTCGCGTA
PON2H1[2]	GTACCTGGGTACCTATCTGAACTTACACTGGGCGAACGTT
PON2H2[2]	CTTCGTTCGGAGAGTAGTAAACAACGTTCGCCCAGTGTAAG
PON2H3[2]	GTTTACTACTCTCCGAACGAAGTTAAAGTTGTTGCGGAAGGTTT
PON3H1[2]	GTTGCCACCCGAACGGTCAGAAATTATTTGTCTATGACCCGAATAACCCG
PON3H2[2]	CGCAGAACTTCAGAAGACGGCGGGTTATTCGGGTCATAG
PON2CZ[2]	GCGCTGTACTGCGAACTGTAAGGATCCCTAG
PON2CZ_NEW[2]	CTAGGGATCCTTACAGTTCGCAGTACAGCGCACG
xl39[4]	CAGTCCATGGACCTGCCGCACTGCCATC
xl40[4]	CTAGGGATCCTTACAGTTCGCAGTACAGCGCAC
xl65[5]	ACGTCTCGAGGATCCATATGACAGTAAAGAAGCTTTATTTCATCCC
xl66[5]	GGACTTCTAGATTATATATATTCCGGGAACACTCTACAACTC
xl50[6]	GGACCCAACGCTGCCCGAAATTCCTGCGTTTCTACAAACTCTTTCGGTCCGTTG
xl51[6]	GAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAACGGACCGAAAGAGTTTGTAG
xl52[6]	CTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATA
xl63[6]	TTCTTCGATTTTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTG

[1] For gene assembly of huPON2 mutants.

[2] For gene assembly of wild type huPON2.

[3] For gene amplification of lactonase aiiA from genomic DNA of *Bacillus thuringiensis* 4A3.

[4] For gene cloning of D2 and E3 to pMAL-c5x.

[5] For gene cloning of aiiA to pBBR1MCS4.

[6] For gene cloning of D2 and E3 to pBBR1MCS4.

2.3.2 Selection of Soluble Expressed huPON2 Variants.

BL21 (DE3) cells were transformed with huPON2 library plasmids, and 96 colonies were randomly picked and cultured. A total of 200 µL of Terrific Broth supplemented with 50 µg/mL kanamycin was inoculated with 2 µL of overnight seed cultures, and protein expression was induced with 0.2 mM IPTG at RT for 16 h. Cells were harvested by centrifugation at 4000g for 15 min at 4 °C and resuspended in 200 µL of PBS. After sonication and centrifugation, 20 µL cell lysates of all 96 picked clones were analyzed using 12% SDS- PAGE gels to examine soluble huPON2 bands at the expected 34.7 kDa.

2.3.3 Cloning, Expression, and Purification of MBP Fused huPON2 Variants.

Assembled genes of wild type huPON2 and selected mutants D2 and E3 were PCR amplified with primers xl39 and xl40 and cloned between BamHI and NcoI sites on pMAL-c5x (NEB). MBP-PON2 fusion proteins were expressed in E. coli BL21 (DE3) using LB media supplemented with 1 mM $CaCl_2$. Expression was induced with 0.2 mM IPTG at OD_{600} of 0.5−0.6. After culturing at RT for 16 h, cells were harvested by centrifugation, resuspended in column buffer (20 mM Tris-HCl at pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$), and lysed by sonication. Cell lysates were clarified by centrifugation at 10000g for 30 min. MBP-huPON2 fusion proteins were captured by an amylose resin column (NEB) and washed

with column buffer, and MBP-huPON2 proteins were eluted with column buffer supplemented with 10 mM maltose. The eluted fractions containing fusion proteins were dialyzed overnight at 4 °C against 20 mM Tris-HCl at pH 7.4, 5 mM NaCl, and 1 mM $CaCl_2$. Purified protein samples were aliquoted in 20% glycerol and stored at −80 °C. The gene of lactonase aiiA was amplified from genomic DNA of B. thuringiensis 4A3 (Bacillus Genetic Stock Center) with primers xl28 and xl29 and cloned into pMAL-c5x. The MBP-aiiA expression and purification procedure was similar to that of MBP-huPON2s, except 0.5 mM $CoCl_2$ was used in all buffers instead of 1 mM $CaCl_2$.

2.3.4 Lactonase Activity Assays.

Hydrolysis of acyl-homoserine lactones was detected using HPLC by measuring the amount of substrates consumed. Then, 0.4 μM purified MBP-huPON2 variants were preinoculated in 5 mM Tris-HCl (pH 7.4) and 1 mM $CaCl_2$ at 37 °C for 1 min, and reactions were initiated by adding a 1% volume of the 8 mM 3-oxo-C_{12}HSL solution (in methanol) and incubated at 37 °C for various times. Reactions were stopped with an equal volume of acetonitrile, and the mixtures were vortex mixed and centrifuged to pellet the precipitated proteins. Then, 100 μL supernatants were injected to a HPLC system equipped with a ZORBAX Eclipse XDB — C_{18} column (Agilent Technologies, 150 × 4.6 mm, 5 μm particles) and a UV/visible detector set at 205 nm. Samples were eluted

isocratically with water/acetonitrile/formic acid (32:68:0.03 [vol/vol/vol]) at 1 mL/min. The retention time for 3-oxo-C_{12}HSL was around 1.9 min. Assay buffer without enzymes and purified bacterial lactonase aiiA MBP fusion protein (MBP-aiiA) were used as negative and positive controls, respectively, in the enzyme assays. A pH-sensitive colorimetric assay was performed using an Epoch microplate reader. The reactions (200 µL final volume) contained 1 mM HEPES at pH 8.0, 25 mM NaCl, 1 mM $CaCl_2$, 0.002% (w/v) Phenol Red, and 10 µL of purified enzymes. Reactions were initiated with 2 µL of substrates stock solution in methanol, and absorbance decreases at 558 nm were monitored for 3−10 min.

Preparation and Characterizations of Tag-Free E3. MBP-E3 was cleaved by incubating with Factor Xa (1 mg per 50 mg of fusion protein) at RT for 12 h. The cleaved mixture was dialyzed against 20 mM Tris-HCl and 25 mM NaCl at pH 7.4, then loaded to amylose resin. The flow through was saved for SDS-PAGE and following analysis. Size exclusion chromatography experiments were performed on an ÄKTAprime plus system using 20 mM Tris-HCl at pH 7.4, 100 mM NaCl, and 1 mM $CaCl_2$ as the column buffer. Newly digested and purified tag-free E3 was load onto a Superdex 75 10/300 GL column (GE Healthcare) for gel filtration at a flow rate of 0.5 mL/min. The CD spectra of purified E3 were recorded at 25 °C using a Jasco J-815 CD spectrophotometer with a 0.1 mm path length cell. The bandwidth was set at 1 nm, and scans were obtained between 190 and 260 nm with wavelength increments of 1 nm. A total of 1 mg

mL^{-1} of tag-free E3 in 20 mM Tris-HCl at pH 7.4, 25 mM NaCl, and 1 mM $CaCl_2$ was measured. Secondary structure content was analyzed with the online CAPITO server[44]. The data used for graphical presentation and analyses were an average of eight different scans.

2.3.5 Bioluminescence Quorum Sensing Bioassay.

The promoter regions on plasmids pMAL-D2/-E3/-aiiA were replaced with a weak β- lactamase promoter P_{bla} by Gibson assembly. The P_{bla} gene was amplified with primer xl50, xl51, xl52, and xl63. Constructed pMAL- bla - huPONs plasmids were transformed to an sdiA-deficient E. coli. strain JLD271 carrying one of the cognate receptors: rhlR (C_4HSL), lasR (3-oxo-C_{12}HSL), or luxR (3-oxo-C_6HSL)[45]. Single-colony transformants were cultured overnight at 37 °C, in LB media supplemented with 100 μg/mL ampicillin and 20 μg/mL tetracycline. Inoculations were diluted and plated on LB/Amp/Tet Agar plates with 0.1−10 μM C_4HSL, C_6HSL, 3-oxo-C_6HSL, C_{12}HSL, or 3-oxo- C_{12}HSL. After incubation at 37 °C overnight, bioluminescence signals were detected using a ChemiDoc imager (Bio-Rad) with justified exposure times based on signal intensity. Luminescence signals of individual colonies were then quantified using Fiji software[46]. More specifically, each colony region was selected as the region of interest (ROI), and the integrated density values were measured for at least 10 colonies per sample, for calculating mean values of luminescence signals.

2.3.6 P. aeruginosa PAO1 Swarming and Swimming Motility Tests.

Genes of huPON2 mutants D2 and E3 and aiiA were subcloned to a broad-host vector pBBR1MCS4 at XhoI and XbaI sites[47]. After confirmation by DNA sequencing, P. aeruginosa PAO1 competent cells were transformed with the plasmids and cultured at 37 °C overnight in LB media supplemented with 200 μg/mL carbenicillin[48]. Swimming motility was tested on 10 g/L tryptone, 5 g/L NaCl, and 0.3% (w/v) agar plates with 5 μL inoculation of overnight cultures and incubation at 37 °C for 24−48 h. The mean areas of the swimming motility zones were measured from three replicates, and the error bars represent the standard deviations. In swarming motility tests, 0.5% (w/v) agar plates containing 8 g/L nutrient broth (Becton, Dicknson and Company) and 5 g/L D-glucose were dried for 1 h and then inoculated with 2.5 μL overnight cultures on the center of the agar surface. The areas of the swarming motility zones were measured after incubation at 37 °C for 48 h.

2.4 Results and Discussion

2.4.1 Generation of Soluble Expressed huPON2 Variants.

Because of the high similarity in secondary structures among mammalian PONs, huPON2 was modeled based on the crystal structure of rabbit-human hybrid PON1 G2E6 (PDB # 1V04), which has 63.1% amino acid sequence identity to wild type huPON2[26, 49-50]. The modeling demonstrated that huPON2 is

a six-bladed β-propeller with catalytic Ca^{2+} ions at the center and three helices (H1−H3) on the top of its propeller (**Figure 2-1a**). These helices are distanced from the reaction center and highly enriched with hydrophobic residues (shown as sticks in **Figure 2-1a**). Likely, these hydrophobic residues on protruding helices form a putative interface involved with HDL particle and cell membrane anchoring[34, 36]. As a QQ agent to hydrolyze extracellular AHLs, engineered huPON2 will be presumably administered as a topical medication (i.e., for chronic wounds) or lung inhalant (i.e., for cystic fibrosis). Therefore, it is not necessary to localize therapeutic huPON2 on cell membranes or HDLs. Moreover, it is well accepted that extended hydrophobic patches on the protein surface promote aggregation and result in insolubility of recombinant proteins[51]. Notably, dozens of enzymes with propeller topology have been crystallized, and their structures suggest that most of propeller fold proteins do not possess extra helices outside of their main architecture[52]. Also, many of these proteins can be expressed in soluble form at high yields, e.g., 200−300 mg/L of squid phosphotriesterases in *E. coli*[53]. On the basis of these considerations, we hypothesize that H1−H3 of huPON2 are not essential for its catalytic activity, and removal of these hydrophobic helices can substantially improve its solubility, a well-documented protein engineering method for solubility improvement[51, 54].

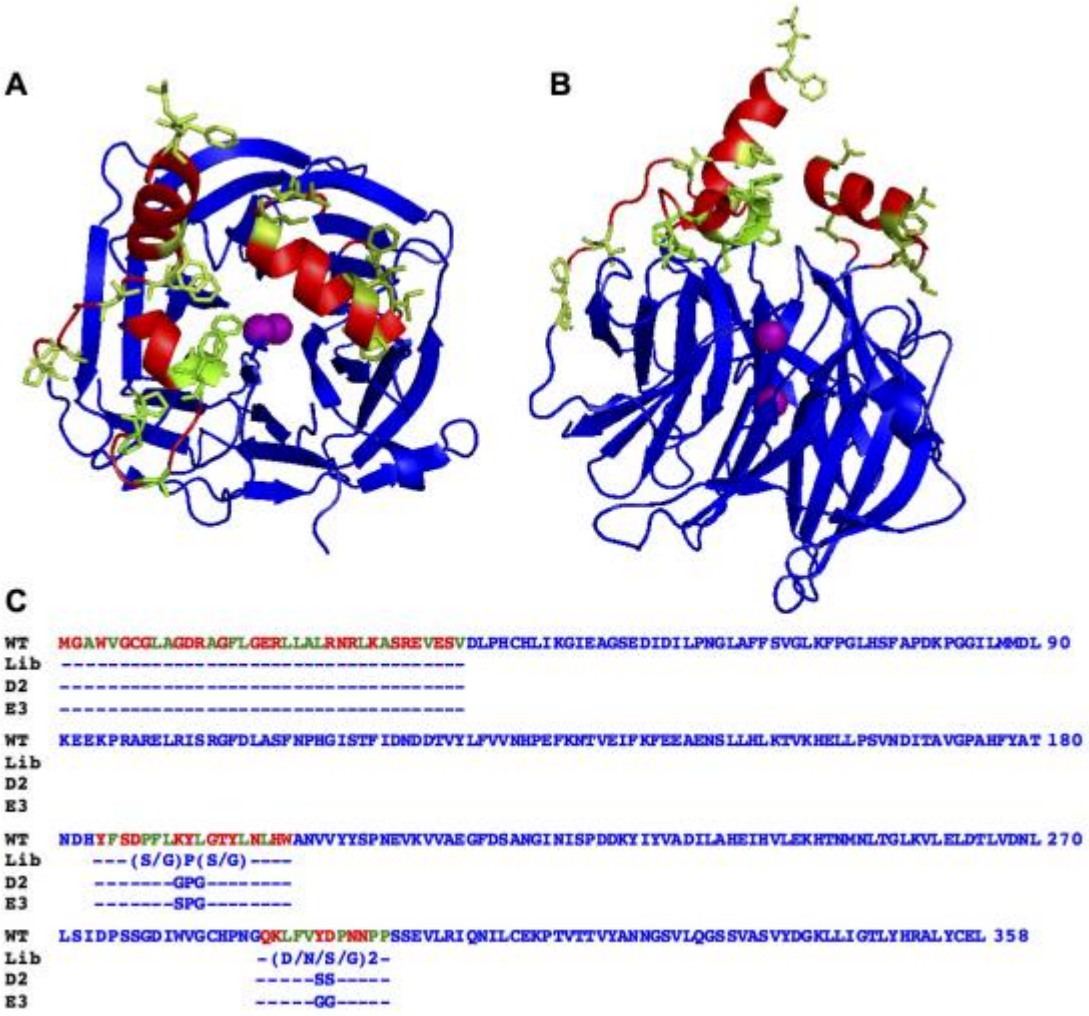

Figure 2-1. Engineering human PON2 for soluble expression by removal of hydrophobic and protruded helixes. (a) Top and side views of huPON2 structure, which is characterized as a six-bladed β propeller fold (blue) with three protruded helices (red, hydrophobic residues within the helices were shown as sticks) and loop regions (green). The reactive Ca^{2+} ions (magenta) are in the center of the propeller fold. Structure mode was generated by Robetta based on a mammalian PON1 (PDB=1V04). (b) Amino acid sequences of wild type huPON2, library design with 64 possibilities, and selected variants D2 and E3. Color matches with the structure in (a). The hydrophobic residues removed or replaced by linkers are underlined.

To test this hypothesis, we modified huPON2 by removing its N-terminal H1 region (residue 17−37, LGERLLALRNRLKASREVESV), replacing the H2 region (residue 184−201, YFSDPFLKYLGTYLNLHW) with a degenerate tripeptide linker carrying a proline turn flanked by one flexible residue at both sides Gly/Ser-Pro-Gly/Ser, and replacing the H3 region (residue 288−298, QKLFVYDPNNP) with a flexible and hydrophilic degenerate dipeptide linker Asp/Asn/Ser/Gly-Asp/ Asn/Ser/Gly (**Figure 2-1b**). The proline turn and the lengths of linkers replacing H2 and H3 were determined according to huPON2 structure modeling (**Figure 2-1a**). The total design diversity of the linker library was 64, which was encoded by associated degenerate codons (**Table 1**).

The helix-free huPON2 genes with degenerate linkers were constructed by DNA assembly, cloned in pET28b vector, and expressed for SDS-PAGE analysis (**Figure 2-2**). Given the small design diversity, 96 clones were randomly picked, and their cell lysates (clarified by centrifugation at 10 000g for 30 min) were analyzed without purification. Soluble expressed helix-free huPON2 variants were identified as bands with an expected MW of 34.7 kDa. Results indicated that 34 of 96 picked clones carried soluble expressed huPON2 variants (**Figure 2-3**). Particularly, clones D2 and E3 displayed strong bands associated with the helix-free huPON2, suggesting a high expression level (**Figure 2-4**). DNA sequencing revealed that the linkers of D2 and E3 were Gly-Pro-Gly and Ser-Pro-Gly for their H2 regions and Ser-Ser and Gly-Gly for their H3 regions,

respectively. As polyhistidine-tag fusion proteins, D2 and E3 were soluble expressed with yields of 6.2 mg and 3.2 mg per liter of culture medium after purification.

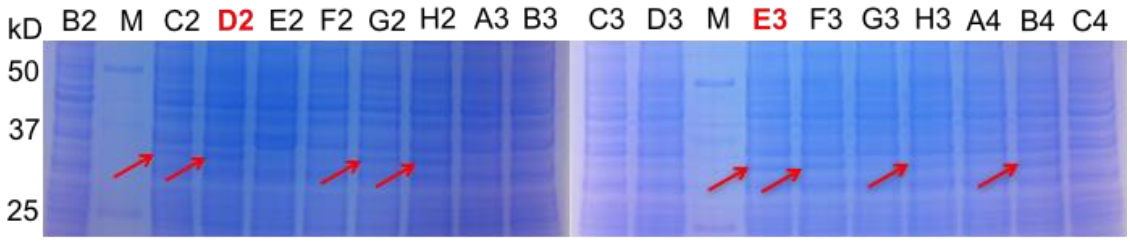

Figure 2-2. Identification of soluble expressed helix-free huPON2 variants. Whole cell lysates without purification were analyzed by SDS-PAGE to identify soluble expressed PON2 variants (arrowed) at the expected MW of 34.7 kD. Totally 96 colonies were tested (analysis of other clones are shown in Figure 2-3).

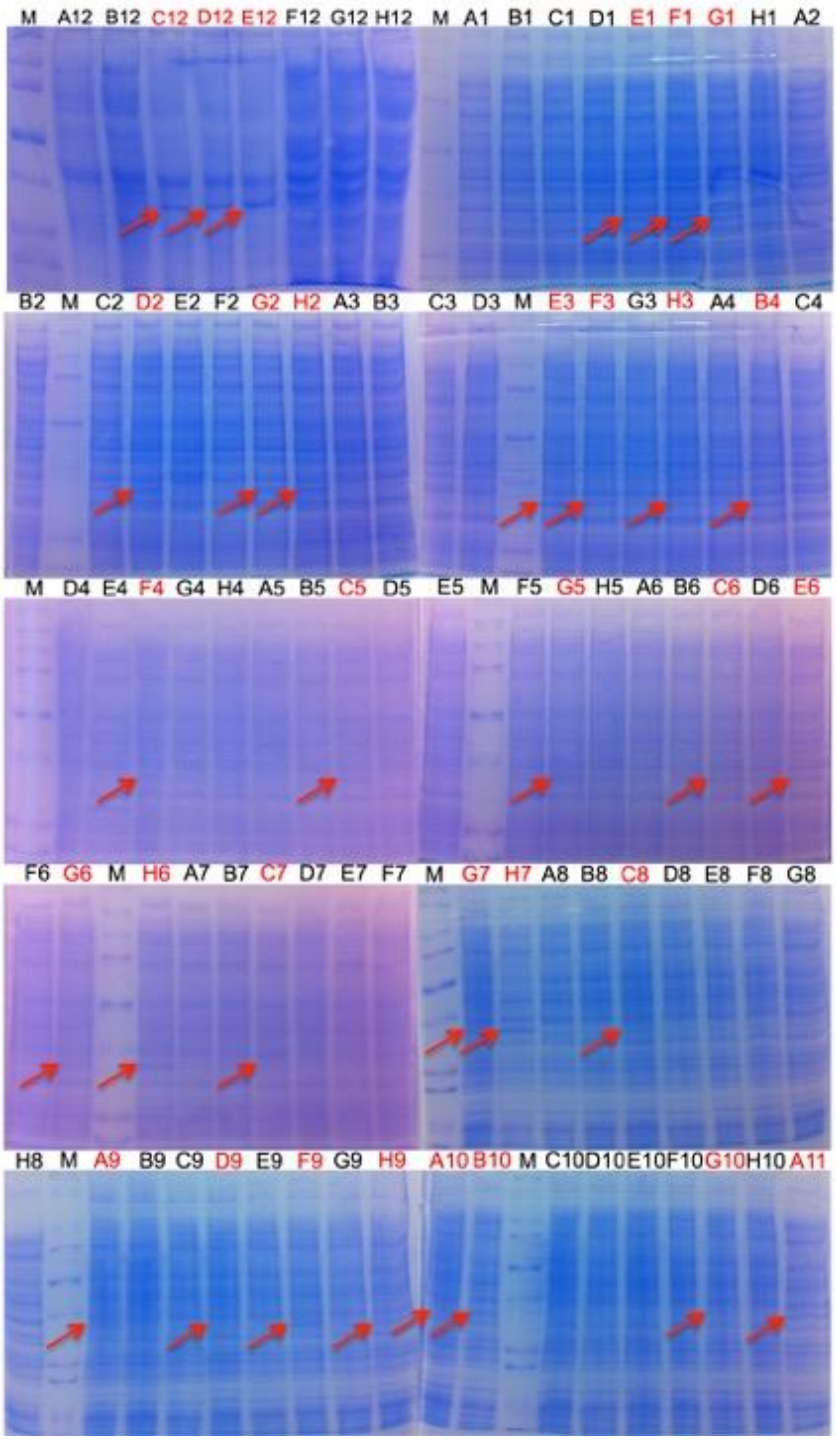

Figure 2-3. Identification of soluble huPON2 variants by SDS-PAGE. Totally 34 out of 96 randomly picked clones carried soluble expressed huPON2 variants.

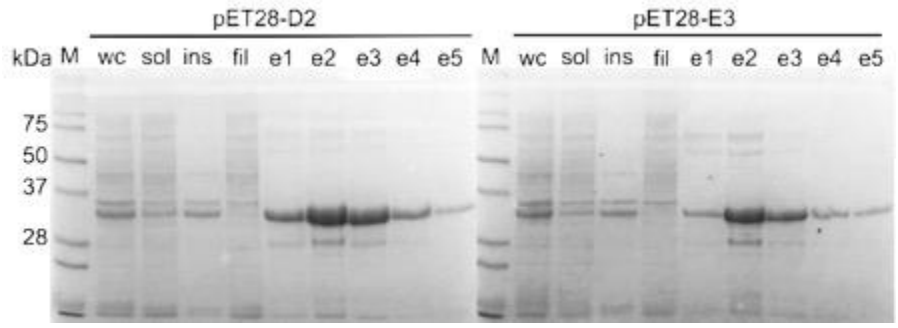

Figure 2-4. Expression and purification of soluble huPON2 variants D2 and E3. Samples included whole cell (wc), soluble fraction (sol), insoluble fraction (ins), filtrate through His-tag resin columns (fil), and elution fractions from the columns (e1-e5).

To increase their soluble expression, D2, E3, and wild-type huPON2 were subcloned into the C-terminal of maltose binding protein (MBP). SDS-PAGE analysis of expression and purification profiles after amylose resin-based chromatography indicated that while the majority (90%) of MBP-wtPON2 was expressed in its insoluble form, almost all helix-free variants MBP-D2 and MBP-E3 were present in soluble fractions (**Figure 2-5**). The typical after purification yields for MBP-D2 and MBP-E3 variants were 320 and 200 mg of protein per liter of culture medium, respectively. The enzymatic activity of purified MBP- E3 was measured using N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C_{12}-HSL) as the substrate. The consumption of substrate and the production of hydroxycarboxylic acid N-(3-oxodecanoyl)-L-homoserine were monitored using

HPLC (**Figure 2-6**). Results indicated that when 80 μM 3- oxo-C_{12}-HSL was used, MBP-E3 exhibited a specific activity of 0.16 μmol/min/mg of protein.

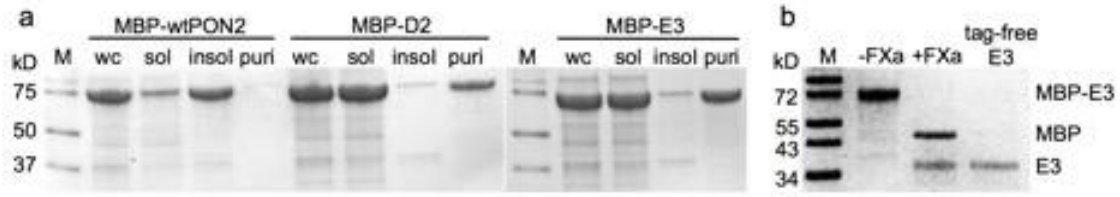

Figure 2-5. Production of soluble huPON2 variants D2 and E3. (a) The MBP fusion proteins were expressed in E. coli and purified by amylose resin chromatography. Samples of whole cell (wc), soluble cell lysate (sol), insoluble pellet (insol), and purified proteins (puri) were normalized based on cell density and loaded to gels. After purification, 320 mg MBP-D2 and 200 mg MBP-E3 were typically yielded from per liter of culture media. (b) Production of tag-free E3. Samples of purified MBP-E3 fusion, cleaved by protease Factor Xa, and separated MBP-free E3 were analyzed by SDS-PAGE. 76 mg of tag-free E3 was produced from per liter of culture media.

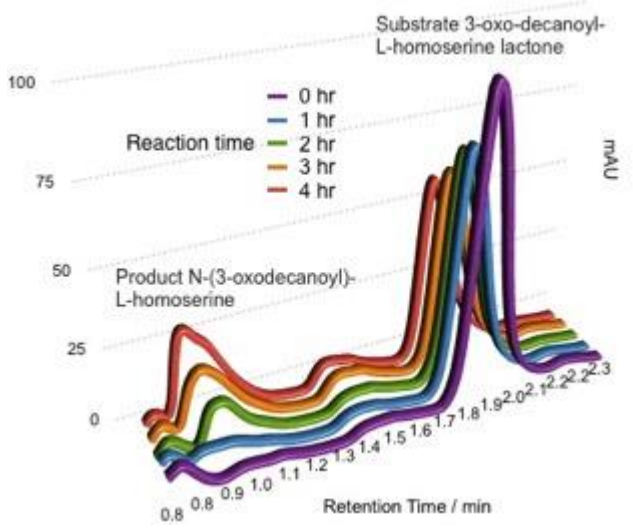

Figure 2-6. AHL hydrolysis activity of MBP-E3 measured by HPLC. 3-oxo-C_{12}-HSL was used as the substrate (with a retention time of 1.9 min) and the associated hydroxycarboxylic acid N-(3-oxodecanoyl)-L-homoserine (with a retention time of 0.9 min) was produced. Background auto- hydrolysis of AHLs without presence of enzymes was subtracted for specific activity calculations.

We further measured the kinetics of MBP-D2 and MBP-E3 on AHL hydrolysis using Phenol Red as the pH indicator. The results showed that MBP-D2/E3 hydrolyzed a panel of AHLs having side chains ranging from C_4 to C_{12} in length with or without 3-oxo substituents, including C_4-, C_6-, 3-oxo-C_6-, and 3-oxo-C_{12}-HSLs (**Table 2**). Among tested AHLs, 3-oxo-C_{12}-HSL was the most efficient substrate for both MBP-D2 and MBP-E3 mainly due to its low K_m values. In addition, turnover rates of MBP-E3 in general were 1 or 2 orders of magnitude higher than these of MBP-D2, but considerably lower than G2E6[28]. To attenuate chronic bacterial infections, repeated administrations are needed, therefore it is expected that a tag-free enzyme without the MBP component would be preferred over the fusion protein to avoid an adverse immune response. In order to produce MBP-free huPON2, purified MBP-E3 fusion was digested with protease Factor Xa, and E3 was separated from MBP by amylose resin chromatography. Per liter of culture medium, 76 mg of tag-free E3 was typically obtained (**Figure 2-5**). Size exclusion chromatography analysis demonstrated that purified tag-free E3 was mainly a soluble monomer with smaller amounts of dimer and trimer (**Figure 2-7**), an observation consistent with the wild type huPONs recombinantly produced in mammalian cells[55]. Furthermore, CD spectra of purified tag-free E3 showed a broad minimum centered around 210 nm with a crossover point of 205 nm (**Figure 2-8**), in a good agreement with known β-fold proteins such as diisopropylfluorophosphatase[53, 56]. And compared with wild type huPON1, E3 has

the negative peak shifted from 217 to 210 nm, indicating less composition of α-helices[57]. The enzymatic kinetics of tag-free E3 was also measured toward C_4-HSL and C_6-HSL. The results (k_{cat} of 1.54 min^{-1} and 1.94 min^{-1} and K_m of 4.7 mM and 0.46 mM, respectively) suggested a similar specific activity to that of MBP-E3 (**Table 2-2**).

Table 2-2. Specific Lactonase Activates of MBP-D2 and MBP-E3

	Substrate	**K_m (mM)**	**k_{cat} (min^{-1})**	**k_{cat}/K_m (mM^{-1}-min^{-1})**
MBP-E3	C_4HSL	1.28	2.45	1.91
	C_6HSL	0.49	2.24	4.57
	3-oxo-C_6HSL	1.29	1.63	1.26
	3-oxo-C_{12}HSL	0.15	1.22	8.13
MBP-D2	C_4HSL	2.53	0.30	0.12
	C_6HSL	2.70	0.22	0.08
	3-oxo-C_6HSL	4.57	0.20	0.04
	3-oxo-C_{12}HSL	0.12	0.23	1.95

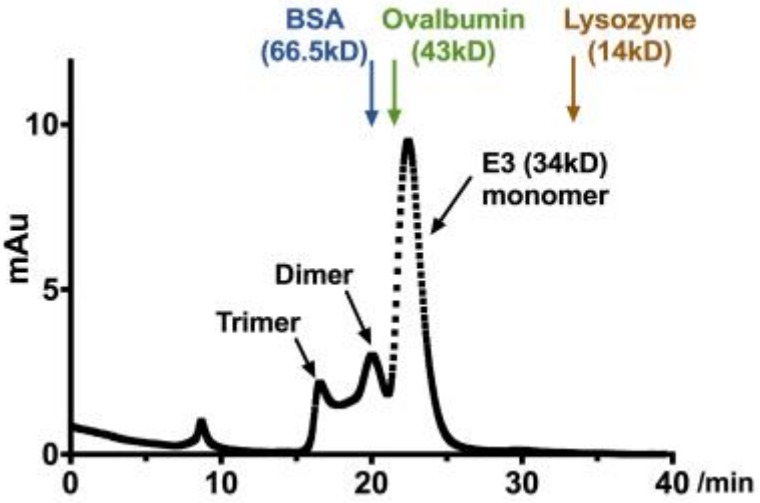

Figure 2-7. Size exclusion chromatography of purified tag-free E3 (MW= 34kDa). 103 µg of E3 in column buffer (20 mM Tris-HCl pH7.4, 200 mM NaCl, 1 mM $CaCl_2$) was loaded to Superdex 75 10/300 GL column at a flow rate of 0.5 mL/min. The retention times at 22.5 min (monomer) and 19.5 min (dimer) indicate tag-free E3 is a soluble protein without aggregation. E3 trimmer was also observed, consistent with the results of wt huPONs produced by recombinant mammalian cells[55].

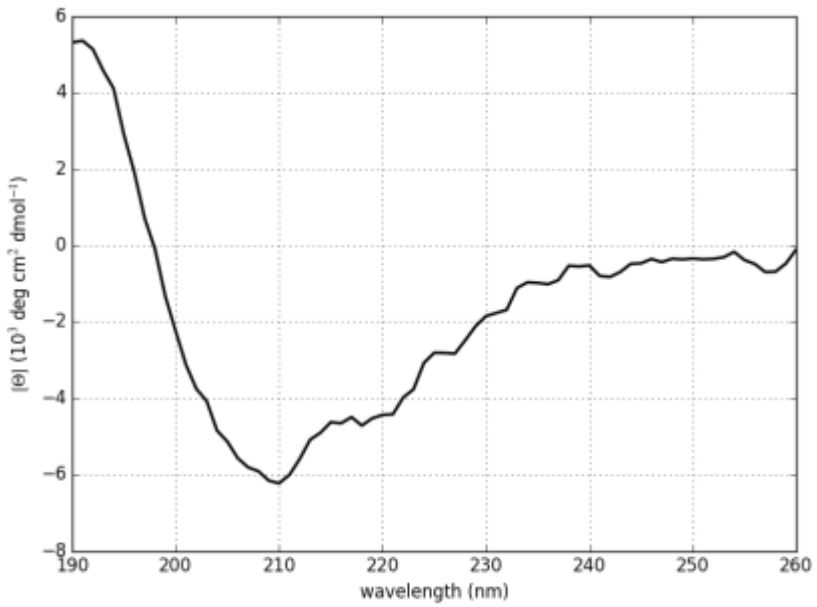

Figure 2-8. CD spectra of purified tag-free E3. A broad negative peak at 210 nm with a crossover point of 205 nm is in a good agreement with known β-fold proteins such as diisopropylfluorophosphatase[53, 56].

2.4.2 Quorum Quenching Activity of Helix-free Soluble huPON 2s.

QQ activities of soluble huPON2 variants D2 and E3 were examined by bioluminescence bioassay using reporter strains, which mimic the luxR-luxI type QS system of natural Gram-negative bacteria (**Figure 2-9**). Particularly, the host contained two plasmids an enzyme plasmid encoding MBP-huPON2 fusion and a reporter plasmid carrying a QS regulator gene LasR/RhlR/LuxR and the complete luciferase operon luxCDABE under the control of the associated QS promoter $P_{lasI}/P_{rhlI}/P_{luxI}$[58]. When exogenously added, AHLs such as 3-oxo-C_{12}-

HSL will freely transport into cells and bind with QS regulators and thus induce the expression of luxCDABE, which generates bioluminescence signals on agar plates. In the presence of active huPON2, AHLs will be hydrolyzed, resulting in no or low bioluminescence signals known as QQ.

As shown in **Figure 2-10**, helix-free huPON2 mutants D2 and E3 exhibited strong lactonase activity toward 3-oxo-C_{12}-HSL in the QQ bioassay. In the absence of AHLs, the background luminescence of all the clones was undetectable. Increasing the concentration (0.1−10 μM) of 3-oxo-C_{12}HSL generated a stronger luminescence signal as a result of the higher expression level of the luxCDABE gene cassette. However, when D2 or E3 was expressed (either with or without IPTG induction), the luminescence signal intensity was quenched to the background level even at 10 μM 3-oxo-C_{12}HSL. QQ activity was also confirmed with the positive control aiiA, a bacteria lactonase possessing a high activity toward a broad range of AHLs[59].

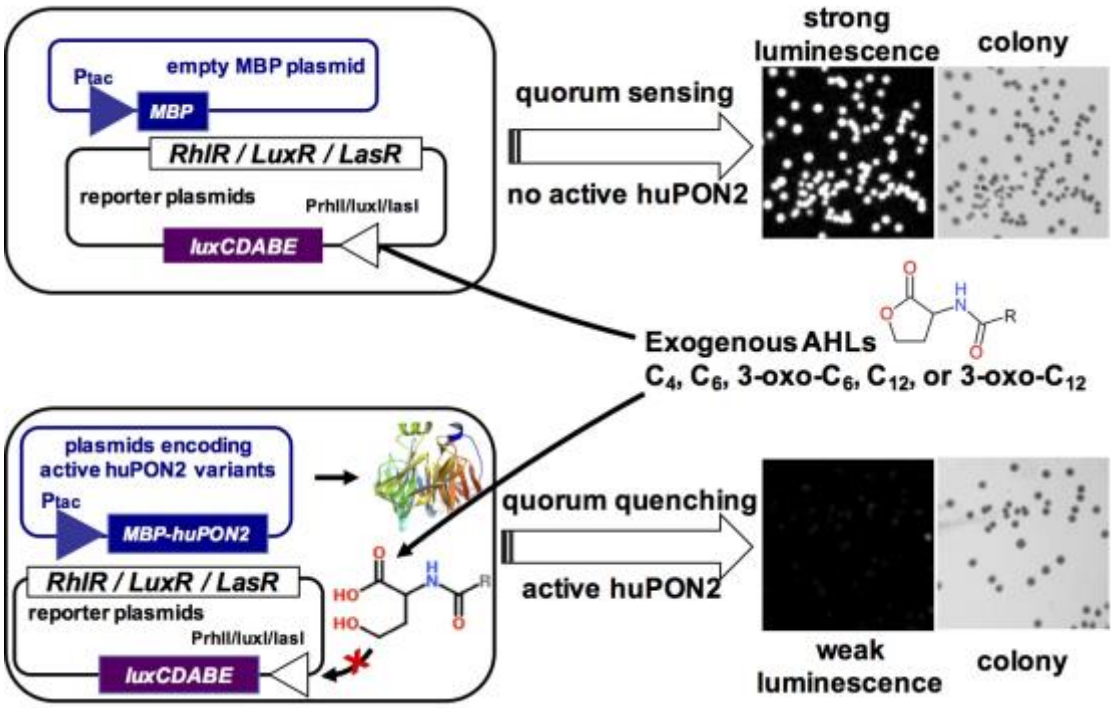

Figure 2-9. Characterization of lactone hydrolysis activity of D2 and E3 by bioluminescence assays[60]. Exogenously added AHLs bind to regulator LasR/RhlR/LuxR and induce the expression of luxCDABE cassette through and promoter $P_{lasI}/P_{rhlI}/P_{luxI}$, resulting in strong bioluminescence signals on agar plates. (Bottom) With expression of active huPON2 variants, hydrolysis of AHLs leads to quorum quenching and low bioluminescence signals. Five lactones N-butyryl-/ N-hexanoyl-/ N-(β-ketocaproyl)-/ N-dodecanoyl- / N-3-oxo-dodecanoyl-L-homoserine were tested in the assays.

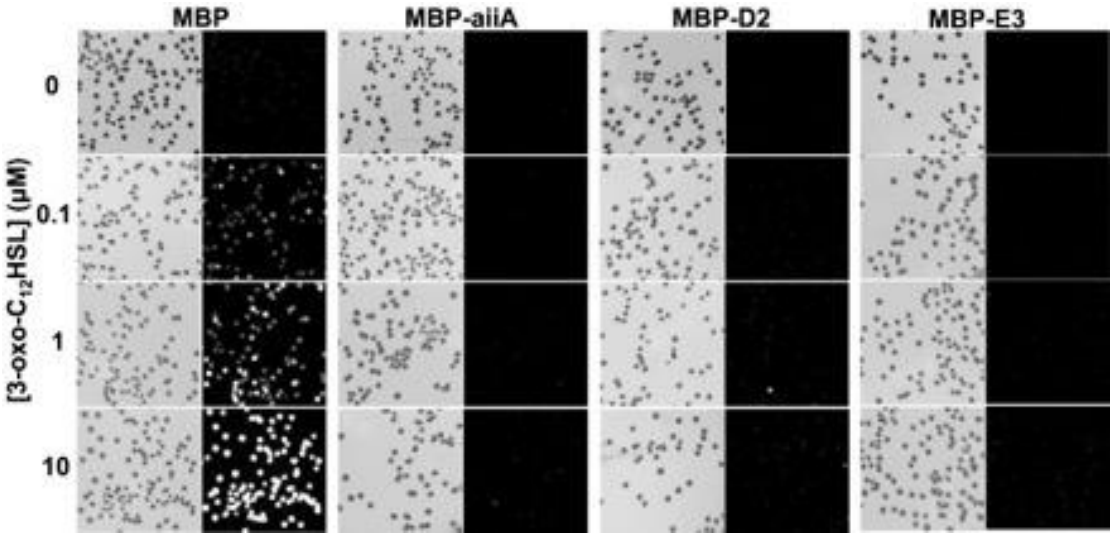

Figure 2-10. Quorum quenching activities of D2 and E3. LasR/P_{lasI} receptor system and its associated QSM 3-oxo-C_{12}HSL (0 to 10 μM) were used for the assays. Without active enzymes, bioluminescence signals were intensified with increased concentrations of AHL. With expression of D2 or E3, QS signals were completely quenched even at 10 μM of AHL. A highly active bacterial lactonase aiiA was included as the positive control. Locations of colonies are shown in the left columns.

2.4.3 Inhibition of *P. aeruginosa* Swimming and Swarming Motilities.

At the early stage of biofilm formation, bacteria use their flagella to sense the environment and swim as a group[61]. It is well-known that syntheses of flagella, polysaccharides, and rhamnolipids are effected by QS, through both the direct regulation by the rhl system and the indirect regulation by the las system[62]. As a model strain for motility study in an aqueous environment, *P. aeruginosa* is commonly used for swimming assays, in which cells migrate away from the point of inoculation and form a concentric chemotactic ring. To test the effects of

soluble huPON2 on swimming motility of *P. aeruginosa*, D2, E3, and aiiA genes were subcloned to a shuttle plasmid, and transformed into *P. aeruginosa* PAO1. After 24 h of culture at 37 °C, P. aeruginosa carrying the empty vector quickly spread out with an area of 14.1 ± 1.8 cm^2 and at 48 h covered the entire plates with a spreading zone area of 41.3 ± 3.3 cm^2 (**Figure 2-11**). However, when huPON2 mutants D2 or E3 were expressed, swimming motility was significantly attenuated with spreading region areas, respectively, of 11.2 ± 4.4 cm^2 and 11.4 ± 2.0 cm^2 at 24h and 28.7 ± 3.6cm^2 and 30.2 ± 4.1cm^2 at 48 h, which account for a ~30% average decrease. As a positive control, *P. aeruginosa* expressing aiiA was tested in parallel and showed a ~70% decrease of the spreading zone area, suggesting a stronger QQ bioactivity compared with engineered huPON2 variants. We further tested swimming motilities when 100 nM C_4-HSL and/or 3-oxo-C_{12}-HSL were present in agar to mimic the social behavior of *P. aeruginosa* colonies. Results showed that with exogenous AHLs, *P. aeruginosa* exhibited increased motility as expected. However, transformants with aiiA or E3 genes significantly reduced swimming motilities compared to the strain carrying the empty vector (**Figure 2-12**).

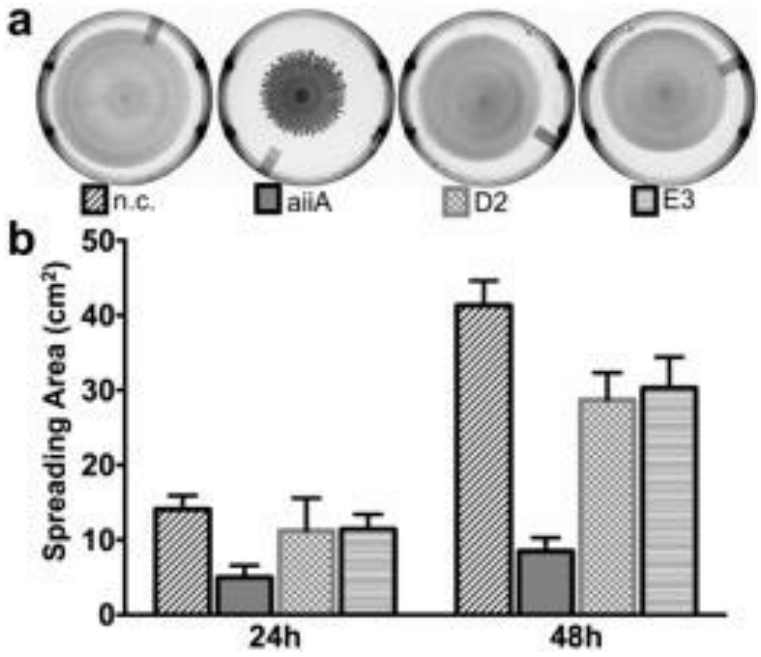

Figure 2-11. Inhibition effects of D2 and E3 on Pseudonomas aeruginosa PAO1 swimming motility. (a) Representative agar plates showing PAO1 spreading zones after 48 hours of culture. PAO1 cells were transformed with D2, E3, aiiA or the vector plasmid (n.c.) (b) Quantitative measurements of spreading zone areas.

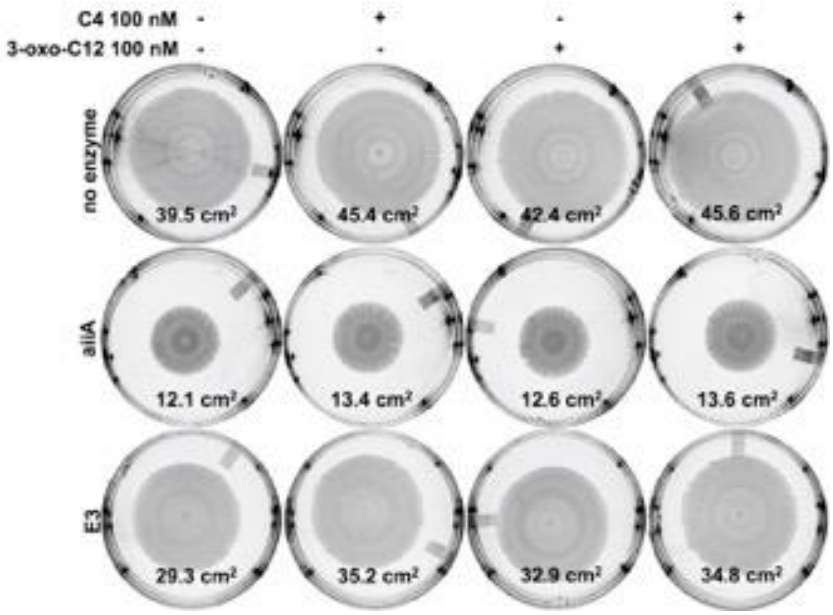

Figure 2-12. E3 mediated swarming motility reduction in the presence of exogenous AHLs. Enzymes were endogenously expressed by transformed P. aeruginosa PAO1. Adding 100 nM C_4 or/and 100 nM 3-oxo-C_{12}HSL in agar plates promoted swimming motilities by 13-20 %. However, transformants with aiiA or E3 genes significantly reduced swimming motilities compared to the strain carrying the empty vector. Colony spreading areas were measured and values were shown for each agar plate.

Bacteria exhibiting biofilm formation also display the feature of swarming, which is a specialized flagella-driven surface motility that bacteria employ on semisolid surface under nitrogen limiting conditions[63]. Swarming cells express two polar flagella, which can facilitate bacteria movement by providing more propulsion, and thus gain a competitive advantage in searching for nutrient-rich environments[64]. In addition, production of surfactant rhamnolipid is fundamental for the swarming motility to facilitate insoluble hydrocarbon biodegradation and reducing surface tension[65]. Because both flagella and rhamnolipid synthesis are regulated by the rhl system, swarming motility is closely related to QS as well[66]. We evaluated the effect of endogenous D2 or E3 expression on *P. aeruginosa* swarming motility. After 48 h of incubation at 37 °C, surface areas covered by swarming were measured. Results indicated that cells transformed with empty plasmid (as a negative control) freely spread across the media surface exhibiting typical dendritic-like patterns (**Figure 2-13a**).

In contrast, the swarming motility was inhibited in *P. aeruginosa* clones carrying huPON2 variants, with 76% and 42% reduction of spreading zones for D2 and E3, respectively. As expected, *P. aeruginosa* transformed with aiiA also reduced *P. aeruginosa* swarming motility by 70%, a similar efficacy compared with D2. In therapeutic applications, the engineered AHL hydrolyzing enzymes will likely be topically administrated. Therefore, it will be more relevant to test motilities when D2 or E3 was exogenously added to culture medium rather than

endogenously expressed. For this test, a PAO1 strain that pronounces significant branch formations was used[67]. Results demonstrated that when 720 µg of MBP-D2 or 520 µg of MBP-E3 were applied on agar plates during inoculation, P. aeruginosa swarming motilities were significantly obstructed or completely abolished (**Figure 2-13b**).

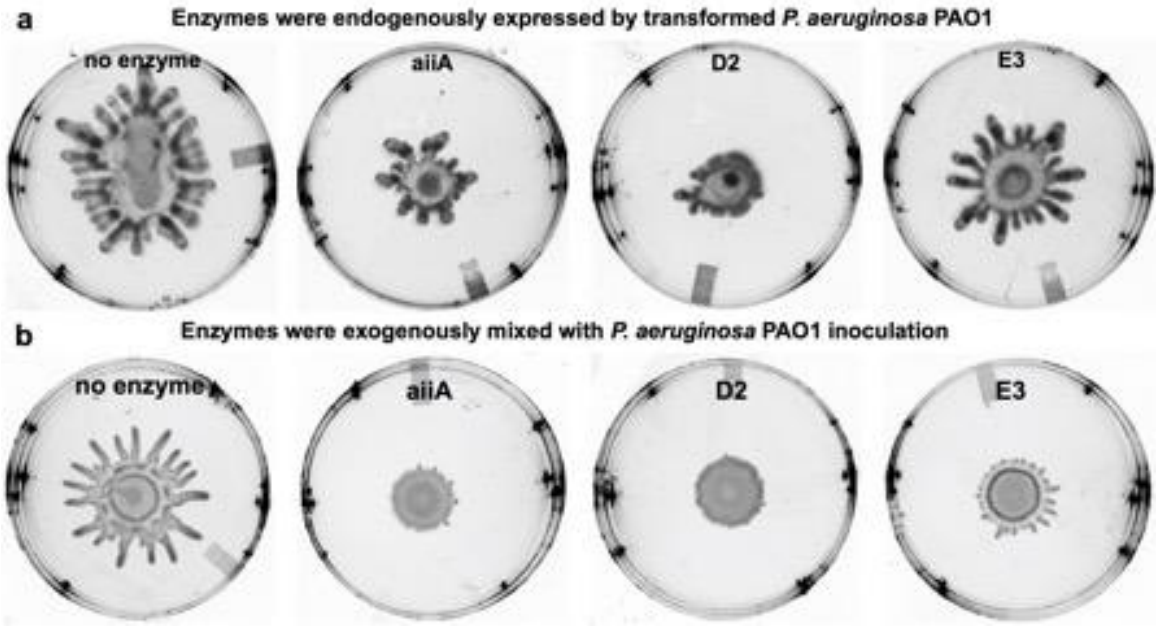

Figure 2-13. Reduction of P. aeruginosa PAO1 swarming motility with D2 or E3. Enzymes were either endogenously expressed by (a) transformed POA1 or (b) exogenously added on agar plates.

2.4.4 Promiscuous Hydrolysis Activities of Soluble huPON2.

Because QS systems play important roles in numerous pathogenic bacteria for their survival from host immune responses, many NIAID category A, B, and C priority pathogens are associated with QS. As one of the major signaling molecules for Gram-negative bacteria, AHLs thus serve as an excellent group of targets to attenuate chronic infections. However, a broad range of AHLs with different 3′ modifications and side chain lengths are utilized in a variety of pathogens. For example, in *Yersinia pestis* (plague) the predominant AHLs are 3-oxo-C_8-HSL and 3-oxo-C_6-HSL synthesized by gene *YspI*[60], and in *Burkholderia pseudomallei* (melioidosis) at least three luxI and five luxR homologues are involved in its pathogenicity, and several signaling molecules including C_8-/C_{10}-/OH-C_8-/OH-C_{10}-/3-oxo-C_{14}-HSLs are generated[68].

To evaluate the effects of engineered soluble huPON2 variants D2 and E3 on different AHLs and the associated QS receptors, we tested five AHLs including C_4-HSL, C_6-HSL, 3- oxo-C_6-HSL, C_{12}-HSL, and 3-oxo-C_{12}-HSL with a combination of three receptors *P. aeruginosa* rhlR (C_4-HSL as its natural QSM), *P. aeruginosa* lasR (3-oxo-C_{12}-HSL as its natural QSM), and *Vibrio fischeri* [45]. E. coli JLD271 carrying one of the reporter plasmids was transformed with an enzyme plasmid encoding MBP-D2, MBP-E3, or MBP-aiiA (included as the positive control). The empty vector encoding the MBP tag alone was also

transformed into the QS reporter cells as the negative controls. These double transformants were cultured on LB-Amp-Tet plates supplemented with different AHLs at variable concentrations. After 16 h of incubation, bioluminescence signal images were taken (**Figure 2-14a**), and 10 colonies from each sample were randomly selected for quantitative analysis. As demonstrated in **Figure 2-14b**, high luminescence signals represent strong QS and low luminescence signals represent QQ and thus high lactone hydrolysis activities. The collected data for all AHL-regulator combinations were then compiled to generate heat maps. Initially, when MBP fusion proteins were cloned following a strong promoter P_{tac}, all the QQ effects of D2, E3, and aiiA were significantly strong, and signals were indistinguishable from each other even at high concentrations of AHLs, *i.e.* 10 μM of 3-oxo-C_{12}-HSL (**Figure 2-15**). To better characterize huPON2 mutants by increasing the response range, P_{tac} was replaced by a weak and constitutive β-lactamase promoter P_{bla}. SDS-PAGE verified an approximately 20-fold reduced expression level for MBP-D2 under P_{bla} (**Figure 2-16**) and thus making it more suitable to evaluate huPON2 mutants.

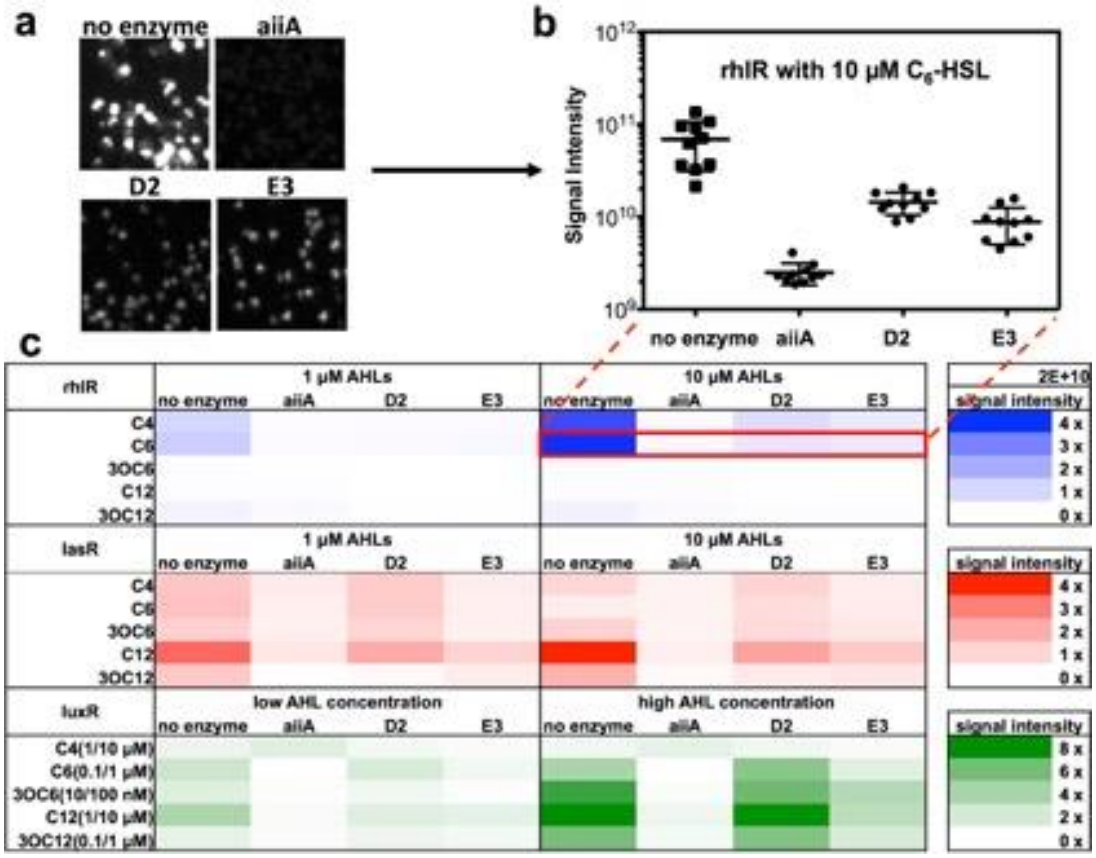

Figure 2-14. Quorum quenching analysis of D2 and E3 on different AHL-regulator combinations. Three quorum sensing signal receptors, rhlR, lasR and luxR, and five AHLs at various concentrations were utilized for the analysis. D2, E3 and aiiA were under the control of a weak β-lactamase promoter to lessen their expression levels. (a) Colony bioluminescence images of agar plates for rhlR with 10 μM C6-HSL as an example. (b) Bioluminescence signal intensities of ten representative colonies for each sample were randomly selected for quantitative analysis. (c) Heat maps of quorum quenching analysis on huPON2 variants with different AHL-regulator combinations.

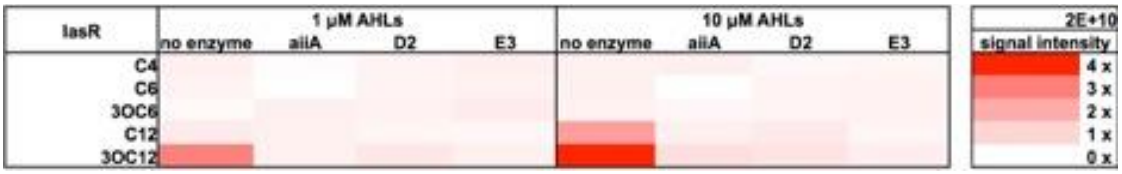

Figure 2-15. Quorum quenching analysis of MBP-huPON2 variants when expressed at the downstream of a strong promoter P_{tac}.

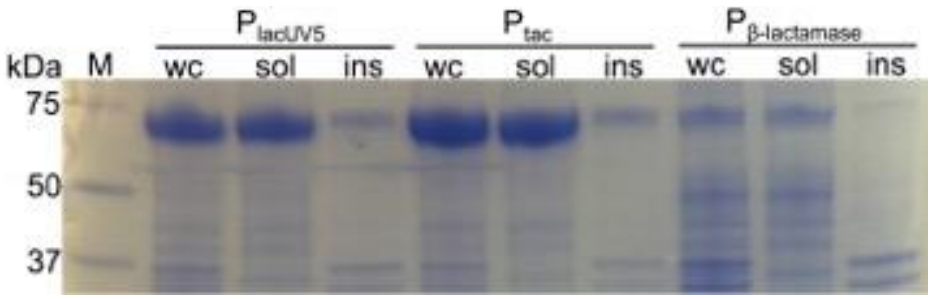

Figure 2-16. SDS-PAGE analysis of MBP-D2 expression levels under the control of promoters P_{lacUV5}, P_{tac} or P_{bla}. P_{lacUV5} and P_{bla} resulted in approximately 3- and 20-folds lower expression than P_{tac}.

The overall results of QQ analysis of D2 and E3 toward different AHL-regulator combinations are shown as heat maps in **Figure 2-14c**. Briefly, in the absence of lactonase, receptor rhlR strongly responded to its natural QSM C_4-HSL and non-natural QSM C_6-HSL at the same level, but C_{12}-HSL and 3-oxo-C_{12}-HSL were not able to stimulate the rhlR-mediated QS and resulted in low luminescence signals. In contrast, lasR and luxR exhibited AHL preferences to C_{12}-HSL with moderate response to 3-oxo-C_6-HSL and 3-oxo-C_{12}-HSL and low response to C_4- HSL and C_6-HSL. As expected, the higher AHL concentrations produced the stronger luminescence signals. In the presence of huPON2 variants D2 or E3, luminescence signals were potently quenched in general. Importantly, D2 and E3 exhibited lactone hydrolysis activities toward all five AHLs tested. Their catalytic promiscuity is not surprising given that wild type huPON1−3s can hydrolyze a broad range of substrates including lactones, organophosphates, aryl esters, etc[28]. Notably, E3 was more effective than D2 on QQ for most AHL-receptor combinations tested. Since D2 was usually expressed ~40% more than E3, these results suggested E3 exhibited a higher specific activity than D2, consistent with kinetics measurements (**Table 2-2**). As a positive control, expression of bacterial lactonase aiiA gave very low luminescence signals and effectively eliminated QS for all five AHLs, in excellent agreement with a previous report[18]. Collectively, the bioluminescence systems demonstrated

here can be used for evaluation of lactonases and pave the way for further selection of huPON2 variants with high AHL hydrolysis activities targeting QSM-regulator combinations of biomedical importance.

2.5 Conclusion

In this study, huPON2 was engineered for soluble expression with minimal introduction of foreign sequences. Both a rational approach based on structure modeling and a combinatory approach of degenerate linker design are exploited for removing the hydrophobic helices without disruption of the folding structure and thus retaining the function of huPON2. High soluble expression levels were achieved with yields of 76 mg of fully human PON2 variants per liter of culture media. Particularly, two clones, D2 and E3 were characterized for their quorum quenching bioactivities. Results demonstrated that D2 and E3 effectively inhibited *P. aeruginosa* swimming and swarming motilities. In addition, using the reporter strains to correlate QQ with luminescence signal readouts, quantitative analysis of QQ toward natural or unnatural AHL-regulator combinations was performed, which allowed us to better evaluate huPON2 variants for the desired lactonase activities toward pathogens of interest. Compared with bacterial lactonase aiiA, the engineered soluble huPON2 mutants D2 and E3 exhibited relatively moderate catalytic activities of lactone hydrolysis. For the generation of therapeutic QQ human enzymes with required high turnover rates, directed

evolution by random and site-directed mutagenesis followed by bioluminescence-based screening is currently undertaken. Moreover, the technique of engineering highly soluble huPON2 described in this paper can be readily applied for huPON1, which exhibits organophosphate hydrolysis activities, for use in environmental bioremediation of pesticides and detoxication of nerve agents.

2.6 Reference

Uncategorized References

1. Atkinson, S.; Williams, P., Quorum sensing and social networking in the microbial world. *J R Soc Interface* **2009,** *6* (40), 959-78.

2. Raina, S.; De Vizio, D.; Odell, M.; Clements, M.; Vanhulle, S.; Keshavarz, T., Microbial quorum sensing: a tool or a target for antimicrobial therapy? *Biotechnol Appl Biochem* **2009,** *54* (2), 65-84.

3. Galloway, W. R.; Hodgkinson, J. T.; Bowden, S. D.; Welch, M.; Spring, D. R., Quorum sensing in Gram-negative bacteria: small-molecule modulation of AHL and AI-2 quorum sensing pathways. *Chem Rev* **2011,** *111* (1), 28-67.

4. Antunes, L. C.; Ferreira, R. B.; Buckner, M. M.; Finlay, B. B., Quorum sensing in bacterial virulence. *Microbiology* **2010,** *156* (Pt 8), 2271-82.

5. Dickschat, J. S., Quorum sensing and bacterial biofilms. *Nat Prod Rep* **2010,** *27* (3), 343-69.

6. Rasko, D. A.; Sperandio, V., Anti-virulence strategies to combat bacteria-mediated disease. *Nat Rev Drug Discov* **2010,** *9* (2), 117-28.

7. de Kievit, T. R.; Iglewski, B. H., Bacterial quorum sensing in pathogenic relationships. *Infection and immunity* **2000,** *68* (9), 4839-49.

8. Lowery, C. A.; Dickerson, T. J.; Janda, K. D., Interspecies and interkingdom communication mediated by bacterial quorum sensing. *Chem Soc Rev* **2008,** *37* (7), 1337-46.

9. Asad, S.; Opal, S. M., Bench-to-bedside review: Quorum sensing and the role of cell-to-cell communication during invasive bacterial infection. *Crit Care* **2008,** *12* (6), 236.

10. Hentzer, M.; Givskov, M., Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *J Clin Invest* **2003,** *112* (9), 1300-7.

11. Camps, J.; Pujol, I.; Ballester, F.; Joven, J.; Simo, J. M., Paraoxonases as Potential Antibiofilm Agents: Their Relationship with Quorum-

Sensing Signals in Gram-Negative Bacteria. *Antimicrob Agents Ch* **2011,** *55* (4), 1325-1331.

12. Hentzer, M.; Givskov, M., Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. *Journal of Clinical Investigation* **2003,** *112* (9), 1300-1307.

13. Wu, H.; Song, Z. J.; Hentzer, M.; Andersen, J. B.; Heydorn, A.; Mathee, K.; Moser, C.; Eberl, L.; Molin, S.; Hoiby, N.; Givskov, M., Detection of N-acylhomoserine lactones in lung tissues of mice infected with Pseudomonas aeruginosa. *Microbiol-Sgm* **2000,** *146*, 2481-2493.

14. Percival, S. L.; Hill, K. E.; Williams, D. W.; Hooper, S. J.; Thomas, D. W.; Costerton, J. W., A review of the scientific evidence for biofilms in wounds. *Wound Repair and Regeneration* **2012,** *20* (5), 647-657.

15. Gerdt, J. P.; Blackwell, H. E., Competition Studies Confirm Two Major Barriers That Can Preclude the Spread of Resistance to Quorum-Sensing Inhibitors in Bacteria. *Acs Chemical Biology* **2014,** *9* (10), 2291-2299.

16. Maeda, T.; Garcia-Contreras, R.; Pu, M. M.; Sheng, L. L.; Garcia, L. R.; Tomas, M.; Wood, T. K., Quorum quenching quandary: resistance to antivirulence compounds. *Isme Journal* **2012,** *6* (3), 493-501.

17. Amara, N.; Krom, B. P.; Kaufmann, G. F.; Meijler, M. M., Macromolecular Inhibition of Quorum Sensing: Enzymes, Antibodies, and Beyond. *Chem Rev* **2011,** *111* (1), 195-208.

18. Wang, L. H.; Weng, L. X.; Dong, Y. H.; Zhang, L. H., Specificity and enzyme kinetics of the quorum-quenching N-acyl homoserine lactone lactonase (AHL-lactonase). *Journal of Biological Chemistry* **2004,** *279* (14), 13645-13651.

19. Liu, D. L.; Thomas, P. W.; Momb, J.; Hoang, Q. Y. Q.; Petsko, G. A.; Ringe, D.; Fast, W., Structure and specificity of a quorum-quenching lactonase (AiiB) from Agrobacterium tumefaciens. *Biochemistry* **2007,** *46* (42), 11789-11799.

20. Bokhove, M.; Jimenez, P. N.; Quax, W. J.; Dijkstra, B. W., The quorum-quenching N-acyl homoserine lactone acylase PvdQ is an Ntn-hydrolase with an unusual substrate-binding pocket. *Proceedings of the National Academy of Sciences of the United States of America* **2010,** *107* (2), 686-691.

21. Leadbetter, J. R.; Greenberg, E. P., Metabolism of acyl-homoserine lactone quorum-sensing signals by Variovorax paradoxus. *Journal of bacteriology* **2000,** *182* (24), 6921-6926.

22. Chow, J. Y.; Wu, L.; Yew, W. S., Directed Evolution of a Quorum-Quenching Lactonase from Mycobacterium avium subsp paratuberculosis K-10 in the Amidohydrolase Superfamily. *Biochemistry* **2009,** *48* (20), 4344-4353.

23. Chow, J. Y.; Xue, B.; Lee, K. H.; Tung, A.; Wu, L.; Robinson, R. C.; Yew, W. S., Directed Evolution of a Thermostable Quorum-quenching Lactonase from the Amidohydrolase Superfamily. *Journal of Biological Chemistry* **2010,** *285* (52), 40911-40920.

24. Costa, L. G.; Richter, R. J.; Li, W. F.; Cole, T.; Guizzetti, M.; Furlong, C. E., Paraoxonase (PON 1) as a biomarker of susceptibility for organophosphate toxicity. *Biomarkers* **2003,** *8* (1), 1-12.

25. Draganov, D. I.; Teiber, J. F.; Speelman, A.; Osawa, Y.; Sunahara, R.; La Du, B. N., Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *Journal of lipid research* **2005,** *46* (6), 1239-47.

26. PrimoParmo, S. L.; Sorenson, R. C.; Teiber, J.; LaDu, B. N., The human serum paraoxonase arylesterase gene (PON1) is one member of a multigene family. *Genomics* **1996,** *33* (3), 498-507.

27. Bar-Rogovsky, H.; Hugenmatter, A.; Tawfik, D. S., The Evolutionary Origins of Detoxifying Enzymes THE MAMMALIAN SERUM PARAOXONASES (PONs) RELATE TO BACTERIAL HOMOSERINE LACTONASES. *Journal of Biological Chemistry* **2013,** *288* (33), 23914-23927.

28. Khersonsky, O.; Tawfik, D. S., Structure-reactivity studies of serum paraoxonase PON1 suggest that its native activity is lactonase. *Biochemistry* **2005,** *44* (16), 6371-6382.

29. Amara, N.; Krom, B. P.; Kaufmann, G. F.; Meijler, M. M., Macromolecular inhibition of quorum sensing: enzymes, antibodies, and beyond. *Chem Rev* **2011,** *111* (1), 195-208.

30. Estin, M. L.; Stoltz, D. A.; Zabner, J., Paraoxonase 1, quorum sensing, and P. aeruginosa infection: a novel model. *Adv Exp Med Biol* **2010,** *660*, 183-93.

31. Stoltz, D. A.; Ozer, E. A.; Taft, P. J.; Barry, M.; Liu, L.; Kiss, P. J.; Moninger, T. O.; Parsek, M. R.; Zabner, J., Drosophila are protected from Pseudomonas aeruginosa lethality by transgenic expression of paraoxonase-1. *J Clin Invest* **2008,** *118* (9), 3123-31.

32. Griffin, P. E.; Roddam, L. F.; Belessis, Y. C.; Strachan, R.; Beggs, S.; Jaffe, A.; Cooley, M. A., Expression of PPARgamma and paraoxonase 2 correlated with Pseudomonas aeruginosa infection in cystic fibrosis. *PLoS One* **2012,** *7* (7), e42241.

33. Stoltz, D. A.; Ozer, E. A.; Ng, C. J.; Yu, J. M.; Reddy, S. T.; Lusis, A. J.; Bourquard, N.; Parsek, M. R.; Zabner, J.; Shih, D. M., Paraoxonase-2 deficiency enhances Pseudomonas aeruginosa quorum sensing in murine tracheal epithelia. *Am J Physiol Lung Cell Mol Physiol* **2007,** *292* (4), L852-60.

34. Deakin, S.; Leviev, I.; Gomaraschi, M.; Calabresi, L.; Franceschini, G.; James, R. W., Enzymatically active paraoxonase-1 is located at the external membrane of producing cells and released by a high affinity, saturable, desorption mechanism. *Journal of Biological Chemistry* **2002,** *277* (6), 4301-4308.

35. Draganov, D. I.; Stetson, P. L.; Watson, C. E.; Billecke, S. S.; La Du, B. N., Rabbit serum paraoxonase 3 (PON3) is a high density lipoprotein-associated lactonase and protects low density lipoprotein against oxidation. *Journal of Biological Chemistry* **2000,** *275* (43), 33435-33442.

36. Gaidukov, L.; Tawfik, D. S., High affinity, stability, and lactonase activity of serum paraoxonase PON1 anchored on HDL with ApoA-I. *Biochemistry* **2005,** *44* (35), 11843-11854.

37. Josse, D.; Ebel, C.; Stroebel, D.; Fontaine, A.; Borges, F.; Echalier, A.; Baud, D.; Renault, F.; le Maire, M.; Chabrieres, E.; Masson, P., Oligomeric states of the detergent-solubilized human serum paraoxonase (PON1). *Journal of Biological Chemistry* **2002,** *277* (36), 33386-33397.

38. Stevens, R. C.; Suzuki, S. M.; Cole, T. B.; Park, S. S.; Richter, R. J.; Furlong, C. E., Engineered recombinant human paraoxonase 1 (rHuPON1) purified from Escherichia coli protects against organophosphate poisoning. *Proceedings of the National Academy of Sciences of the United States of America* **2008,** *105* (35), 12780-12784.

39. Aharoni, A.; Gaidukov, L.; Yagur, S.; Toker, L.; Silman, I.; Tawfik, D. S., Directed evolution of mammalian paraoxonases PON1 and PON3

for bacterial expression and catalytic specialization. *Proceedings of the National Academy of Sciences of the United States of America* **2004,** *101* (2), 482-487.

40. Harel, M.; Aharoni, A.; Gaidukov, L.; Brumshtein, B.; Khersonsky, O.; Meged, R.; Dvir, H.; Ravelli, R. B.; McCarthy, A.; Toker, L.; Silman, I.; Sussman, J. L.; Tawfik, D. S., Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. *Nature structural & molecular biology* **2004,** *11* (5), 412-9.

41. Cantor, J. R.; Yoo, T. H.; Dixit, A.; Iverson, B. L.; Forsthuber, T. G.; Georgiou, G., Therapeutic enzyme deimmunization by combinatorial T-cell epitope removal using neutral drift. *Proceedings of the National Academy of Sciences of the United States of America* **2011,** *108* (4), 1272-1277.

42. Laroche, Y.; Heymans, S.; Capaert, S.; De Cock, F.; Demarsin, E.; Collen, D., Recombinant staphylokinase variants with reduced antigenicity due to elimination of B-lymphocyte epitopes. *Blood* **2000,** *96* (4), 1425-1432.

43. Sarkar, M.; Harsch, C. K.; Matic, G. T.; Hoffman, K.; Norris, J. R., 3rd; Otto, T. C.; Lenz, D. E.; Cerasoli, D. M.; Magliery, T. J., Solubilization and humanization of paraoxonase-1. *Journal of lipids* **2012,** *2012*, 610937.

44. Wiedemann, C.; Bellstedt, P.; Gorlach, M., CAPITO--a web server-based analysis and plotting tool for circular dichroism data. *Bioinformatics* **2013,** *29* (14), 1750-7.

45. Lindsay, A.; Ahmer, B. M., Effect of sdiA on biosensors of N-acylhomoserine lactones. *Journal of bacteriology* **2005,** *187* (14), 5054-8.

46. Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; Tinevez, J. Y.; White, D. J.; Hartenstein, V.; Eliceiri, K.; Tomancak, P.; Cardona, A., Fiji: an open-source platform for biological-image analysis. *Nature methods* **2012,** *9* (7), 676-682.

47. Kovach, M. E.; Elzer, P. H.; Hill, D. S.; Robertson, G. T.; Farris, M. A.; Roop, R. M., 2nd; Peterson, K. M., Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **1995,** *166* (1), 175-6.

48. Choi, K. H.; Kumar, A.; Schweizer, H. P., A 10-min method for preparation of highly electrocompetent Pseudomonas aeruginosa cells:

Application for DNA fragment transfer between chromosomes and plasmid transformation. *J Microbiol Meth* **2006,** *64* (3), 391-397.

49. Kim, D. E.; Chivian, D.; Baker, D., Protein structure prediction and analysis using the Robetta server. *Nucleic Acids Res* **2004,** *32*, W526-W531.

50. Harel, M.; Aharoni, A.; Gaidukov, L.; Brumshtein, B.; Khersonsky, O.; Meged, R.; Dvir, H.; Ravelli, R. B. G.; McCarthy, A.; Toker, L.; Silman, I.; Sussman, J. L.; Tawfik, D. S., Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes (vol 11, pg 412, 2004). *Nature structural & molecular biology* **2004,** *11* (12), 1253-1253.

51. Nieba, L.; Honegger, A.; Krebber, C.; Pluckthun, A., Disrupting the hydrophobic patches at the antibody variable/constant domain interface: Improved in vivo folding and physical characterization of an engineered scFv fragment. *Protein Eng* **1997,** *10* (4), 435-444.

52. Jawad, Z.; Paoli, M., Novel sequences propel familiar folds. *Structure* **2002,** *10* (4), 447-454.

53. Hartleib, J.; Ruterjans, H., High-yield expression, purification, and characterization of the recombinant diisopropylfluorophosphatase from Loligo vulgaris. *Protein Expres Purif* **2001,** *21* (1), 210-219.

54. Mosavi, L. K.; Peng, Z. Y., Structure-based substitutions for increased solubility of a designed protein. *Protein Eng* **2003,** *16* (10), 739-745.

55. Draganov, D. I.; Teiber, J. F.; Speelman, A.; Osawa, Y.; Sunahara, R.; La Du, B. N., Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *Journal of lipid research* **2005,** *46* (6), 1239-1247.

56. Kuznetsov, S. V.; Hilario, J.; Keiderling, T. A.; Ansari, A., Spectroscopic studies of structural changes in two beta-sheet-forming peptides show an ensemble of structures that unfold noncooperatively. *Biochemistry* **2003,** *42* (15), 4321-4332.

57. Yeung, D. T.; Josse, D.; Nicholson, J. D.; Khanal, A.; McAndrew, C. W.; Bahnson, B. J.; Lenz, D. E.; Cerasoli, D. M., Structure/function analyses of human serum paraoxonase (HuPON1) mutants designed from a DFPase-like homology model. *Bba-Proteins Proteom* **2004,** *1702* (1), 67-77.

58. Lindsay, A.; Ahmer, B. M. M., Effect of sdiA on biosensors of N-acylhomoserine lactones. *Journal of bacteriology* **2005,** *187* (14), 5054-5058.

59. Momb, J.; Wang, C.; Liu, D.; Thomas, P. W.; Petsko, G. A.; Guo, H.; Ringe, D.; Fast, W., Mechanism of the quorum-quenching lactonase (AiiA) from Bacillus thuringiensis. 2. Substrate modeling and active site mutations. *Biochemistry* **2008,** *47* (29), 7715-25.

60. Kirwan, J. P.; Gould, T. A.; Schweizer, H. P.; Bearden, S. W.; Murphy, R. C.; Churchill, M. E., Quorum-sensing signal synthesis by the Yersinia pestis acyl-homoserine lactone synthase YspI. *Journal of bacteriology* **2006,** *188* (2), 784-8.

61. Harshey, R. M., Bacterial motility on a surface: many ways to a common goal. *Annu Rev Microbiol* **2003,** *57*, 249-73.

62. Daniels, R.; Vanderleyden, J.; Michiels, J., Quorum sensing and swarming migration in bacteria. *FEMS Microbiol Rev* **2004,** *28* (3), 261-89.

63. Kearns, D. B., A field guide to bacterial swarming motility. *Nat Rev Microbiol* **2010,** *8* (9), 634-44.

64. Tremblay, J.; Deziel, E., Gene expression in Pseudomonas aeruginosa swarming motility. *BMC Genomics* **2010,** *11*, 587.

65. Kohler, T.; Curty, L. K.; Barja, F.; van Delden, C.; Pechere, J. C., Swarming of Pseudomonas aeruginosa is dependent on cell-to-cell signaling and requires flagella and pili. *Journal of bacteriology* **2000,** *182* (21), 5990-5996.

66. Reimmann, C.; Ginet, N.; Michel, L.; Keel, C.; Michaux, P.; Krishnapillai, V.; Zala, M.; Heurlier, K.; Triandafillu, K.; Harms, H.; Defago, G.; Haas, D., Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in Pseudomonas aeruginosa PAO1. *Microbiology* **2002,** *148* (Pt 4), 923-32.

67. Davies, D. G.; Parsek, M. R.; Pearson, J. P.; Iglewski, B. H.; Costerton, J. W.; Greenberg, E. P., The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **1998,** *280* (5361), 295-8.

68. Ulrich, R. L.; Deshazer, D.; Brueggemann, E. E.; Hines, H. B.; Oyston, P. C.; Jeddeloh, J. A., Role of quorum sensing in the pathogenicity of Burkholderia pseudomallei. *J Med Microbiol* **2004,** *53* (Pt 11), 1053-64.

3 Chapter 3 Engineering Human Paraoxonase 2 for Enhanced Quorum-Quenching Activity

3.1 Abstract

Human paraoxonase 2 (huPON2) plays an important role in innate immunity, atherosclerosis, and other associated diseases due to its ability to hydrolyze quorum sensing molecules, such as *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}HSL). In Chapter 2, huPON2 has been engineered for soluble expression while retaining its enzymatic activities. In this Chapter, we aim to improve its lactone hydrolysis activities toward biomedical important *N*-acyl homoserine lactones (AHLs). Random mutagenesis was introduced by error-prong PCR, and the huPON2 library was screened by bioluminescence assays. Five variants with enhanced quorum quenching ability were isolated. These isolated variants exhibited strong quenching ability towards five AHLs with different carbon chain length and substitution. Among these variants, D3H7 from the first round of screening and 1A3 from the second round showed the most improvements in *in vitro* kinetic assays. Whereas, isolated huPON2 mutants did not possess enhanced inhibition toward *Pseudomonas aeruginosa* swimming motility.

3.2 Introduction

Human paraoxonases (huPONs) play an important role in innate immunity, apoptosis in cancer cells, and other human disorders including atherosclerosis, diabetes, cerebrovascular disease, Alzheimer disease, amyotrophic lateral sclerosis, organophosphate susceptibility, and Parkinson disease[1-5]. Particularly, huPON2 has been implicated in oxidative stress, inflammation and quorum sensing regulation, due to the highest activity among three huPONs against N-acyl-homoserine lactones (AHLs)[6]. By hydrolyzing AHLs, which allows Gram negative bacteria to communicate and coordinate in their infection[7], PON2 maintains intestinal homeostasis, degrades carious harmful factors, and favors solid protection for mucosal barrier integrity[8-9]. In addition, huPON2 lessen quorum sensing of pathogenic *P. aeruginosa* in cystic fibrosis sufferers[10-11]. These findings collectively suggest that PON2 represents an interesting pharmaceutical target for the prevention of epithelial infections.

HuPON2 structural and functional characterization studies were largely hindered due to the lack of an ample source of recombinant proteins. HuPONs are rather unstable and tend to aggregate in the absence of detergents[12]. To facilitate their studies and enable their engineering for improving catalytic efficiency and specificity, attempts to express PONs in *E. coli* have been started since 2004. Through family shuffling of four PON1 genes, including human, rabbit, mouse and rat, the recombinant PON1 G2E6 gave stable and well-

diffracting crystals. Likewise, the expression of recombinant PON3 was achieved by DNA shuffling and random mutagenesis[13]. Later, N-terminal hydrophobic patch removing or polar mutations in the putative HDL binding site, PON1 was engineered for improvement of solubility, stability and activities[14-16].

However, most of these engineered PON variants carry non-human sequences, e.g. G2E6 has a 91% identity to rabbit PON1 with dozens of amino acids different from human PON1. These non-human sequences could generate immunogenicity issues when applied as biopharmatheuticals. In Chapter 2, we overcome the issue by removing and replacing the protruding hydrophobic helices with short peptide linkers[17], and achieved high expression level. To further improve their catalytic activities, we applied directed evolution in this current study.

3.3 Materials and Methods

3.3.1 Generation of HuPON2 Random Mutagenesis Library

The plasmids encoding huPON2 clones D2 or E3 were mixed as templates for error-prone PCR with primers xl39 (5'-CAGTCCATGGACCTGCCGCACTGCCATC-3') and xl40 (5'-CTAGGGATCCTTACAGTTCGCAGTACAGCGCAC-3') and annealing temperature at 55 °C. 100 μL reaction mixture contained 1 μL Taq DNA polymerase (NEB), 0.25 μM xl39, 0.25 μM xl40, 20 ng template DNA, 2.4 mM

$MgCl_2$, 0.5 mM $MnCl_2$, 0.12 mM dATP, 0.1 mM dCTP, 0.55 mM dGTP, 3.85 mM dTTP and 0.5 μg BSA. PCR product was cloned between BamHI and NcoI sites on pMAL-*bla*-c5x vector, and ligated DNA was electro-transformed into *E. coli* Jude-1 (DH10B harboring the "F" factor derived from XL1-blue) competent cells.

3.3.2 Bioluminescence Quorum Quenching Screening Assay

Constructed library plasmids were amplified by miniprep and transformed into *E. coli* strain JLD271 for screening. Cells were cultured overnight at 37 °C in LB broth supplemented with 100 μg/mL ampicillin and 20 μg/mL tetracycline, then plated on LB/Amp/Tet agar plates containing 0.1 μM 3-oxo-C_6HSL. Each plate contained 500 - 2000 colonies and 200 plates were screened to cover the entire library. After incubation at 37 °C overnight, bioluminescence signals were detected by the ChemiDoc imager (Bio-Rad) with justified exposure time based on the signal intensity. Low bioluminescence colonies were picked and inoculated with 200 μl LB media in 96-deep-well plates for overnight culture at 37 °C. Colonies with consistent quorum quenching behavior were selected for DNA sequencing.

3.3.3 Expression and Purification of HuPON2 Variants

Genes of isolated huPON2 mutants were amplified with xl39 and xl40, and then subcloned into pMAL-c5x with P_{tac} promoter. HuPON2 variants were

expressed in *E. coli* BL21 (DE3) using LB media supplemented with 1 mM $CaCl_2$. Expression was induced with 0.2 mM IPTG at OD_{600} of 0.5−0.6. After culturing at room temperature for 16 h, cells were harvested by centrifugation, resuspended in column buffer (20 mM Tris-HCl at pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$), and lysed by sonication. Cell lysates were clarified by centrifugation at 10,000×g for 30 min. The proteins were captured by amylose resin columns (NEB) and column was washed thoroughly with column buffer, and then eluted with elution buffer (column buffer supplemented with 10 mM maltose). The eluted fractions containing huPON2 mutant proteins were dialyzed overnight at 4 °C against storage buffer (20 mM HEPES at pH 7.4, 5 mM NaCl, and 1 mM $CaCl_2$). Purified protein samples were aliquoted in 20% glycerol and stored at -80 °C.

3.3.4 SDS-PAGE and Western Blotting

Purified huPON2 variants were analyzed by SDS-PAGE and transferred (1h at 100V) onto PVDF membranes in 0.375 M Tris-glycine buffer (pH8.3). The membranes were blocked by incubation with skim milk 5% (w/v) in 1x Tris-buffered saline (0.2 M Tris pH 7.5, 1.5 M NaCl) containing 0.05% Tween (v/v) and incubated for 1 h with an anti-MBP mouse monoclonal antibody (NEB). After washing, the membranes were incubated for 1 h with anti-mouse IgG-peroxidase conjugate (NEB), and developed with a chemiluminescent substrate.

3.3.5 Lactonase Activity Assays

N-acyl-homoserine lactonase activity of huPON2 variants was measured with phenol red as a spectrophotometric pH indicator. pH change was monitored in 96-well plates using a microplate reader (Biotek). 200 µL reaction contained 1 mM HEPES (pH 8.0) at pH 8.0, 5 mM NaCl, 1 mM $CaCl_2$, 0.002% (w/v) phenol Red, and 2 µL of substrates stock solution in methanol. The absorbance decreases at 558 nm were monitored for 3-10 min. Background substrates hydrolysis was subtracted from enzyme kinetic measurements.

3.3.6 Inhibition of Swimming Motility of *P. aeruginosa* PAO1 by HuPON2 Variants

Genes of huPON2 mutants were subcloned to a broad-host vector pBBR1MCS4 between XhoI and XbaI sites[18]. After confirmation by DNA sequencing, *P. aeruginosa* PAO1 competent cells were transformed with the plasmids and cultured at 37 °C overnight in LB media supplemented with 200 µg/mL carbenicillin[19]. Swimming motility was tested on 10 g/L tryptone, 5 g/L NaCl, and 0.3% (w/v) agar plates with 5 µL inoculation of overnight culture and incubation at 37 °C for 24h.

3.4 Results

3.4.1 Identification of huPON2 Variants with Increased Quorum Quenching Activity.

In Chapter 2, wild type huPON2 was modified by removing and replacing the protruding hydrophobic helices with short peptide linkers[17]. huPON2 D2 and E3, derived from wt huPON2, could be solubly expressed. Meanwhile, D2 and E3 retained their enzymatic functions toward five different AHLs. Additionally, they showed significant quorum quenching bioactivities and effectively impeded *P. aeruginosa* swimming and swarming motilities. Thus, D2 and E3 were used as the templates for huPON2 library construction. Gene libraries with 1×10^6 variants were prepared by error-prone PCR (**Figure 3-1a**). From ten randomly chosen clones, 7 were in correct reading frame, 2 had advanced stop codons, and 1 had reading-frame shift.

The constructed plasmids carrying library DNA were transformed into an *sdiA*-deficient *E. coli* strain JLD271[20], a reporter strain containing pAL103, which has a transcriptional activator luxR (naturally recognizing 3-oxo-C_6HSL) and a bioluminescence gene cassette luxCDABE (**Figure 3-1b**). When screening on agar plates for enhanced quorum quenching activity, the exogenous AHLs, such as 3-oxo-C_{12}HSL, diffuse into cells and drive luxR dimerization and the expression of the bioluminescence cassette through promoter P_{luxI}. In contrast, active enzymes can hydrolyze AHLs, avoid quorum sensing and quench

bioluminescence signals. Therefore, the huPON2 variants exhibiting weak bioluminescence signals indicate enhanced quenching ability, and are selected for further characterizations (**Figure 3-1c**).

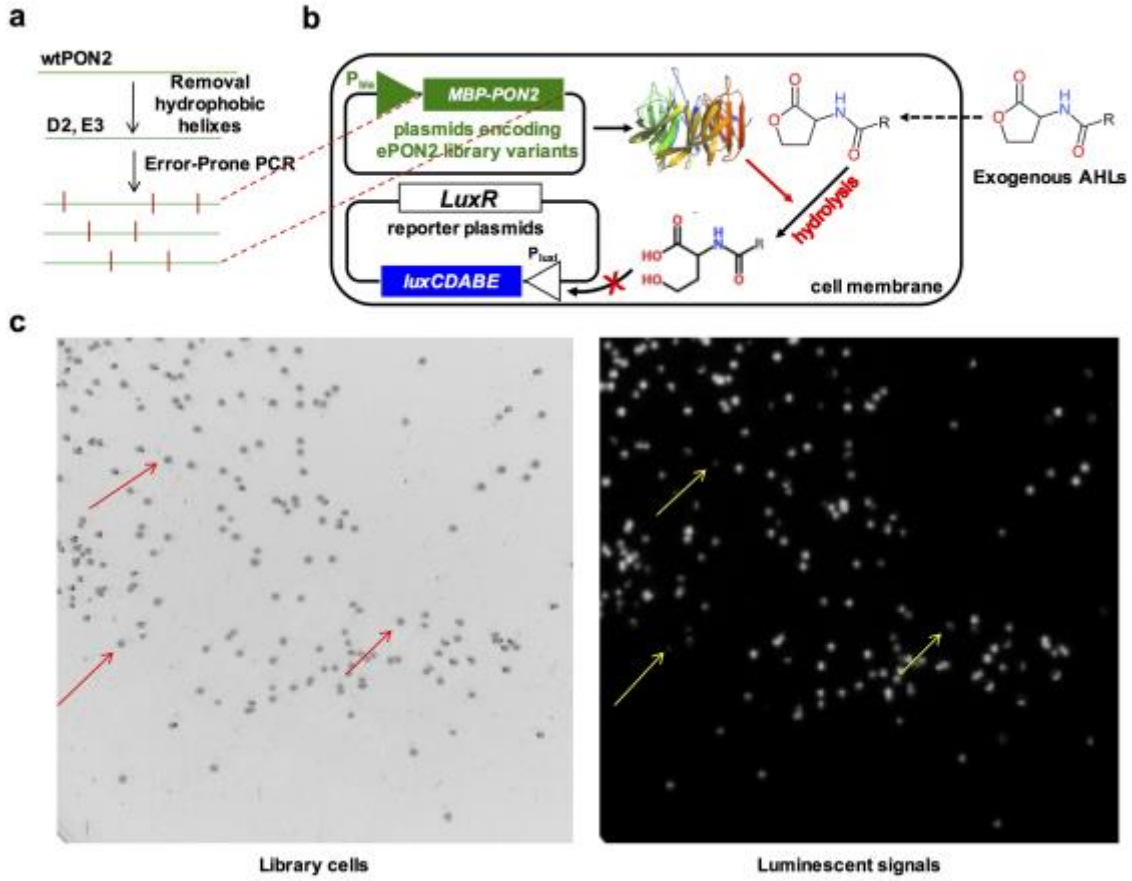

Figure 3-1. Directed evolution of huPON2 variants with increased quorum quenching activity. (a) Random mutagenesis of soluble huPON2 by error-prone PCR (b) The screening system composes of bioluminescence reporter and huPON2 library plasmids. Exogenous AHLs drive expression of bioluminescence gene cassette luxCDABE, while active PON2 mutants hydrolyze AHLs resulting in weak bioluminescence signals. (c) Representative images of screening showing in bright-field (left) and bioluminescence channel (right). Arrows indicate quorum quenching colonies.

In the first round, ~2 × 10^5 library colonies were screened on agar plates supplement with 100 nM 3-oxo-C_{12}HSL, and 550 colonies with quenched luminescent signals were identified. These clones were re-screening on agar plates with 100 nM or 500 nM 3-oxo-C_{12}HSL, and six clones exhibiting consistent QQ activities, i.e. weaker signals than D2 and E3 and similar signal intensities as aiiA, were isolated. Images of a representative re-screening are shown in **Figure 3-2**. Sequencing results revealed these six clones were unique. Genes of these isolated clones were mixed for error-prone PCR to generate the second-round library with ~10^6 variants. 480 clones were selected from 8 × 10^5 colonies on 100 nM 3-oxo-C_{12}HSL plates. Rescreening with 100 nM and 500 nM 3-oxo-C_{12}HSL identified one more clone showing improved quenching ability.

Mutations of isolated clones were summarized in **Table 3-1**. Sequencing results revealed that among the six mutants from the first round, E3E8 and E3A9 had their origin of E3, and D2H5, D2F9, D2G7 and D3H7 had their origin of D2. Clone 1A3 from the second round was derived from D3H7 with six additional mutations.

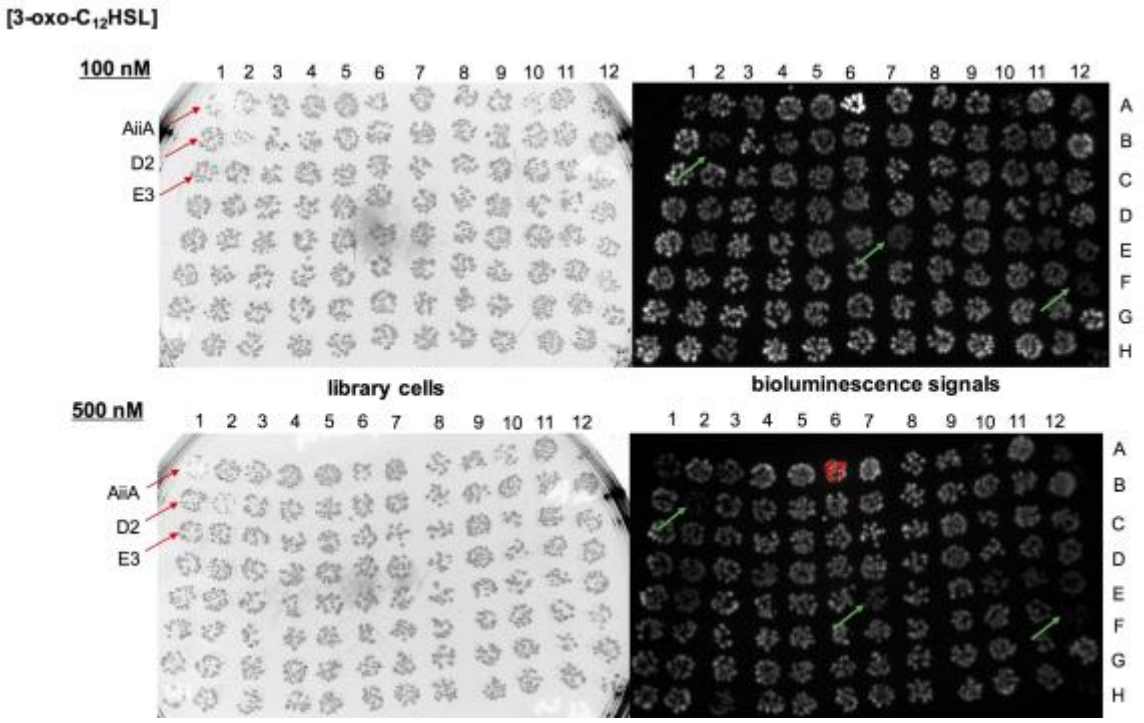

Figure 3-2. Results of re-screening with 100 or 500 nM 3-oxo-C_{12}HSL. Library mutants with weaker bioluminescent signals than D2 and E3 groups were pointed by green arrows. aiiA, D2 and E3 served as controls (red arrows).

Table 3-1 Library variants mutations and their locations

clone	mutations	mutation locations
E3E8	L53P, I192T	L53P on the β-sheet, I192T on the loop
E3A9	E109K, P137S	Both on the loops
D2H5	G10D, S168P, K197E	All on the loops
D2F9	N202D	On the β-sheet
D2G7	D131G, N171S, A272T	All in the tunnel
D3H7	E196G, N262D	E196G on the loop, N262G on the β-sheet
1A3	(E196G, N262D) + (H38Y, Y90N, S114T, Y154N, F167Y, L284Q)	All of them either on the outside of β-sheet or loops.

Figure 3-3 shows the locations of these mutations on huPON2 D2/E3 structures. Most mutants are located on protein surface either on β-sheet or the loops linking β-sheets. These mutations are away from the catalytic pocket, yet could aid protein expression, such as hydrophobic to hydrophilic mutations I192T in E3E8, or promote subtle changes on the overall folding. However, all three mutations D131G, N171S and A272T on D2G7 are located in the tunnel of the propeller folding, which might benefit substrate binding or catalytic reactions.

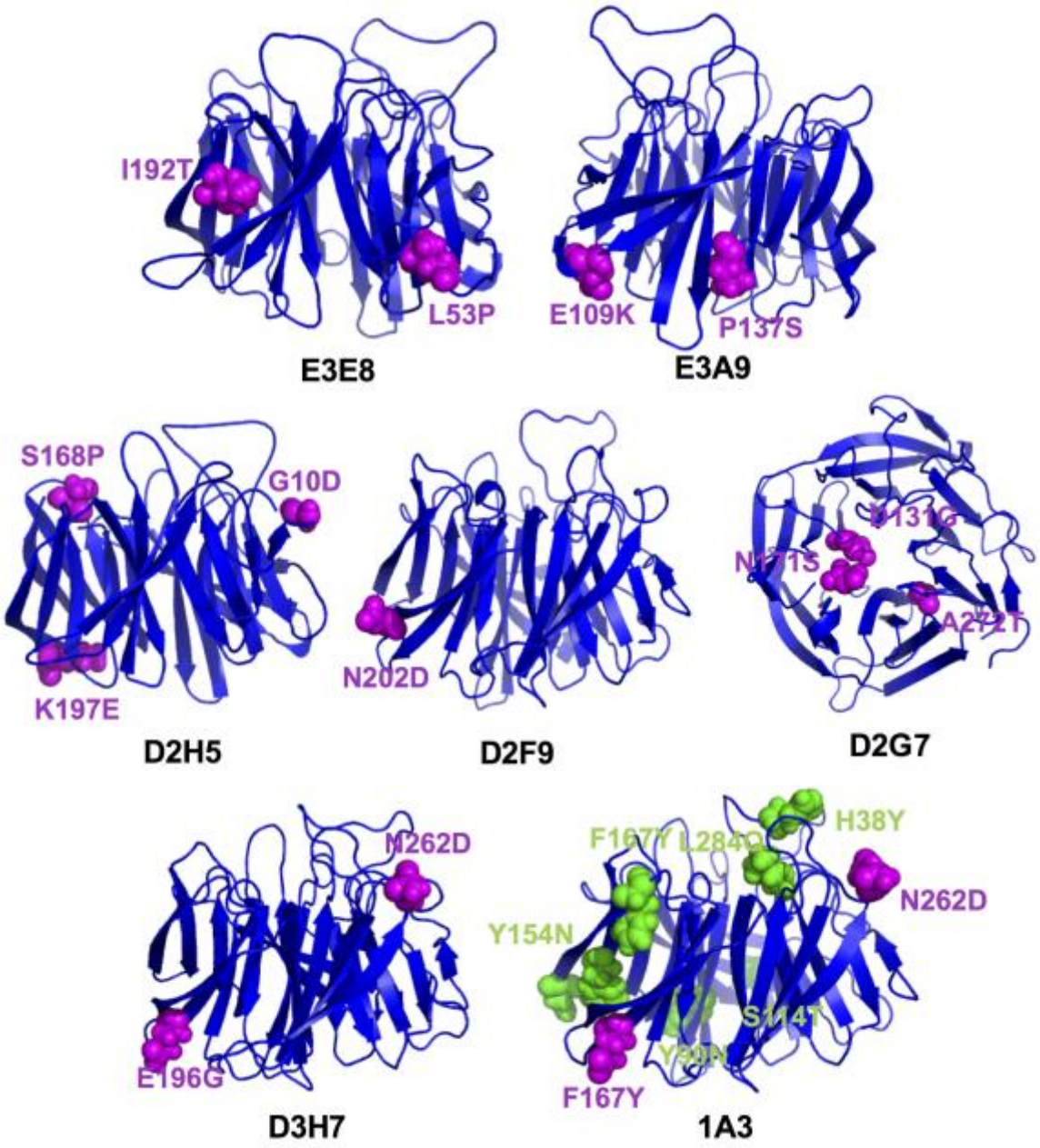

Figure 3-3. Mutation locations of isolated huPON2 mutations. Mutated residues are labeled in either purple (the first round) or green (the second round). Most mutations are on the protein surface, while for except D2G7 mutations are inside the tunnel.

3.4.2 Isolated PON2 mutants exhibited activity toward a broad range of lactones

Even library variants were screened with 3-oxo-C_{12}HSL, it is interesting to evaluate isolated variants on different AHLs. Five AHLs at two concentrations were used in bioluminescence assays on agar plates: 100 nM / 1 μM C_4HSL, C_{12}HSL and 3-oxo-C_{12}HSL, and 10 / 100 nM C_6HSL and 3-oxo-C_6HSL (natural signaling molecule of LuxR, thus lower concentrations to even exposure time). As results shown in **Figure 3-4**, in the presence of 100 nM 3-oxo-C_{12}HSL, five mutates D2H5, D3H7, E3E8, E3A9 and 1A3 displayed enhanced quenching abilities, while D2F9 and D2G7 exhibited relatively weaker quenching than D2 and E3. With 1 μM 3-oxo-C_{12}HSL, signal intensities with all variants increased but following the same pattern as 100 nM 3-oxo-C_{12}HSL. When C_{12}HSL was used, aiiA showed increased signal intensity, indicating it had lower catalytic activity on C_{12}HSL than 3-oxo-C_{12}HSL. HuPON2 variants exhibited similar quenching capacity toward 3-oxo-C_{12}HSL. As the natural substrate for luxR receptor, 3-oxo-C_6HSL and C_6HSL stimulated strong signals. Isolated variants also illustrated significant quenching abilities toward 3-oxo-C_6HSL and C_6HSL. Among them, C_6HSL was more sensitive for enzymatic hydrolysis by huPON2 mutants. In the presence of 100 nM and 1μm C_4HSL, signals of D2H5, D3H7, E3E8, E3A9, and 1A3 were completely quenched. Overall, E3E8 and E3A9

exhibited the highest quorum quenching abilities compatible to aiiA, against all five tested AHLs.

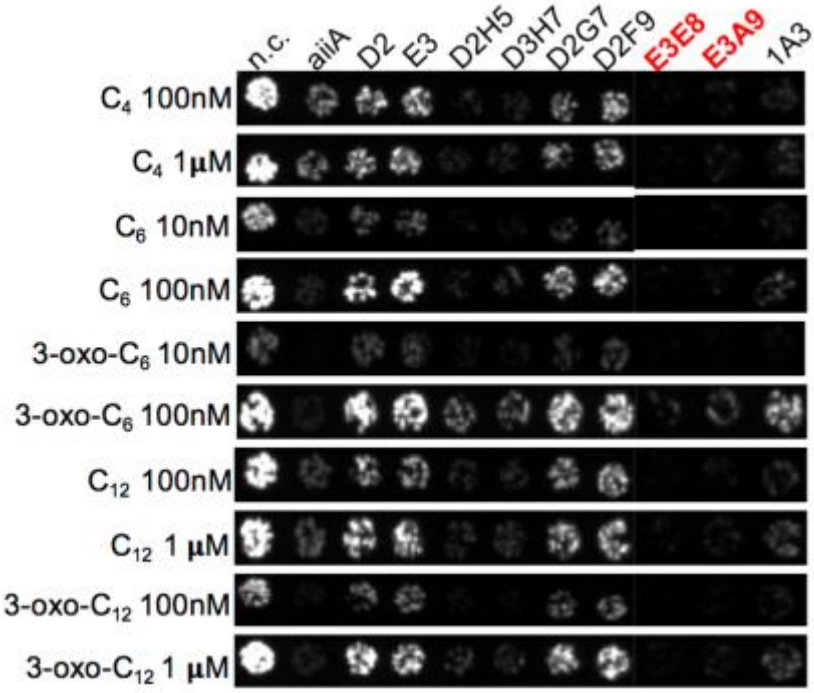

Figure 3-4: Library variants quorum quenching against different AHLs. Negative control (n.c.) was empty vector without enzyme gene. aiiA was an efficient lactonase having broad and effective lactonase activity. D2 and E3 were the soluble huPON2 as the templates for these library variants.

3.4.3 HuPON2 Variants AHLs Hydrolytic Activity

Due to the strong luminescence quenching abilities, D2H5, E3E8, D3H7, and 1A3 were purified, and their kinetic constants toward four AHLs were obtained (**Table 3-2**). Compared to E3, E3E8 exhibited 1.8-, 3.1-, 3.2-fold increase of turnover rates (k_{cat}) toward C_4HSL, C_6HSL, and 3-oxo-C_{12}HSL, and the binding affinities remained the same. The k_{cat}/K_m values of D3H7 and 1A3

toward all tested AHLs had a 19- to 86.5-fold increase compared to D2. The turnover rates of D3H7 improved for 11.3-, 12.3-, 11.4-, and 40.8-fold for C_4HSL, C_6HSL, 3-oxo-C_6HSL and 3-oxo-C_{12}HSL, also its binding affinities increased 3.6-, 6.9-, 9.1-fold toward C_4HSL, C_6HSL, 3-oxo-C_6HSL. 1A3 had the most improved k_{cat} among all tested AHLs. The k_{cat} of D2H5 also showed about 10-fold turnover rates enhancement to all tested AHLs.

Table 3-2 Specific Lactonase Activates of huPON2 Variants

	C_4HSL			C_6HSL			3-oxo-C_6HSL			3-oxo-C_{12}HSL		
	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1}-min^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1}-min^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1}-min^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1}-min^{-1})
E3	1.28	2.45	1.91	0.49	2.24	4.57	1.29	1.63	1.26	0.15	1.22	8.13
E3E8	1.45	4.43	3.06	0.65	6.86	10.55	1.21	1.6	1.32	0.16	3.9	24.38
D2	2.53	0.3	0.12	2.7	0.22	0.08	4.57	0.2	0.04	0.12	0.23	1.92
D3H7	0.7	3.39	4.84	0.39	2.7	6.92	0.5	2.27	4.54	0.24	9.39	39.13
1A3	1.3	5.63	4.33	1.22	5.11	4.19	6.69	8.18	1.22	0.38	13.93	36.66
D2H5	2.4	2.67	1.11	1.02	3.51	3.44	2.99	2.24	0.75	0.22	1.2	5.45

3.4.4 HuPON2 Variants Expression Levels

To confirm the enhanced QQ was due to the improvement of enzyme catalysis activity but not the enzyme concentrations, we measured the expression levels for isolated variants in the reporter strain. Compared to D2 and E3, the majority of isolated clones exhibited similar expression level (**Figure 3-4**), while 1A3 had higher and D2G7 had lower expression levels. However, the differences were 2-fold or less, thus protein expression did not contribute significantly.

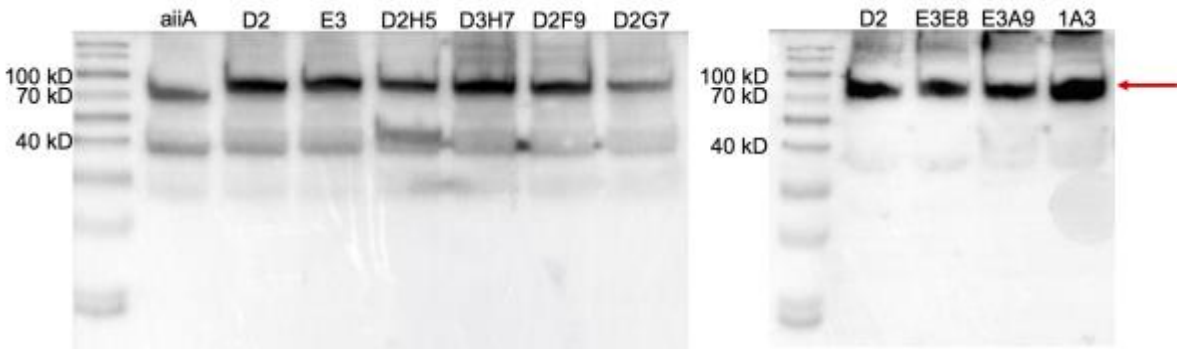

Figure 3-5. Western blot analysis for library variants expression levels. aiiA fusion protein (~73.4 kD) was used as a reference to compare the difference. HuPON2 (~78 kD) library variants (red arrow) have similar expression level compared to D2.

3.4.4 Inhibition of *P. aeruginosa* Swimming Motilities.

The library mutants were subcloned into a shuttle plasmid, and transformed into *P. aeruginosa* PAO1 strain. After 24h culture, the images were taken for the *P. aeruginosa* swimming motility (**Figure 3-6)**. *P. aeruginosa* carrying the empty vector covered large area, 20.4 ± 4.4 cm^2 on the plate, while *P. aeruginosa* expressing aiiA as the positive control markedly showed a 83% decrease of the spreading area, suggesting a strong QQ bioactivity. D2 and E3 exhibited 44% and 47% decreases of the motilities. *P. aeruginosa* expressing huPON2 mutants implied similar or increased motilities compared to D2 and E3, which was not consistent with the bioluminescence quenching assays or kinetics measurements. These data might result from different stabilities and expression levels of huPON2s in *P. aeruginosa*.

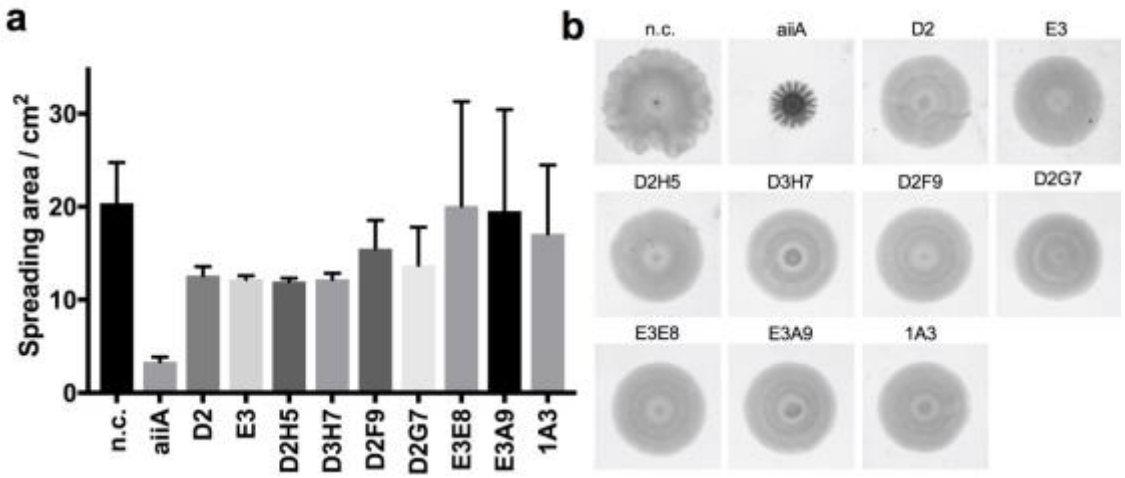

Figure 3-4. Reduction of *P. aeruginosa* swimming motility with huPON2 library variants. (a) Quantitative measurements of spreading zone areas after 24h culture. (b) Representative 0.3% agar plates showing PAO1 spreading zones. *P. aeruginosa* was transformed with vector plasmid (n.c.), aiiA, D2, E3, library variants: D2H5, D3H7, D2F9, D2G7, E3E8, E3A9, 1A3.

3.5 Discussion

HuPON2 hydrolyzes a broad range of Gram negative bacteria quorum sensing molecule AHLs, which are necessary for pathogenic biofilm formation, pro-inflammatory gene expression, and leukocyte proliferation[21-22]. After solubility improvement in chapter 2, this study aims to engineer huPON2 for enhanced activity by random mutations followed by bioluminescence screening. From > 10^6 variants, initial screening identified hundreds of clones, but re-screening confirmed 7 clones consistently exhibited enhanced QQ activity. The false positive circumstance has been reported previously in a similar screening system[23]. One alternative approach is to replace the bioluminescent reporter gene cassette with a suicide gene, such as CcdB[24]. In the absence of hydrolase,

AHLs bind to regulator luxR and induce the expression of CcdB, resulting in cell death; in the presence of the active huPON2 mutants, hydrolysis of AHLs avoids CcdB expression and leads to cell growth. This modification will generate a positive selection and likely reduce false positives.

To illustrate the quorum quenching bioactivity of huPON2 variants, huPON2 variants were constantly expressed in *P. aeruginosa*. Even though the variants showed enhanced activity in vitro, they did not improve inhibition toward *P. aeruginosa* swimming motility. The possible reasons for this could be different expression levels and stabilities in *P. aeruginosa*. Further studies, i.e. application of purified proteins mixed with *P. aeruginosa* culture for mobility tests need to be performed for fully addressing the question.

Despite the abundance of membrane-associated enzymes, the mechanism on how membrane binding stabilizes these enzymes and stimulates their catalysis remains largely unknown. Even the surface hydrophobic patches are away from catalytic pocket, removing them could impact on the catalytic activity. Recent studies suggested that the stability and enzymatic activity of serum PON1 were dramatically stimulated when associated with high-density lipoprotein (HDL) particles[25]. Such interaction with HDL promotes the formation of a network of hydrogen bonds, ensuring the precise alignment of Asn168 (a key catalytic residue in the catalytic pocket) with the catalytic calcium and the lactone substrate. Mutations toward key residues in the long-range interactions reduce

stability and activity of PON1 lactonase[25]. The essentiality of this network needs to be further confirmed on PON2. With extended interactions, site-directed mutagenesis can be performed to enhance the evolution process.

3.6 Conclusion

To improve the lactonase activity of huPON2, random mutagenesis followed by bioluminescence screening was performed to and several clones exhibiting significant quenching ability were isolated. Particularly, the k_{cat}/K_m values of D3H7 and 1A3 toward tested AHLs were significantly improved comparing to D2. However, *P. aeruginosa* swimming mobility tests did not display the expected enhancement. Applications of purified huPON2 variants on *P. aeruginosa* should be further examined. Using CcdB suicide gene can be an alternative approach to screen the library clones. Overall, this study, along with others, suggests that enhancement of quorum quenching activities can be introduced by subtle, remote mutations. Given the relative ease of activity improvement, applications of huPONs are promising due to their human origin and their hydrolysis activities toward a broad range of QS AHLs.

3.7 Reference

Uncategorized References

1. Furlong, C. E., Genetic variability in the cytochrome P450-paraoxonase 1 (PON1) pathway for detoxication of organophosphorus compounds. *J Biochem Mol Toxic* **2007,** *21* (4), 197-205.

2. Bhattacharyya, T.; Nicholls, S. J.; Topol, E. J.; Zhang, R. L.; Yang, X.; Schmitt, D.; Fu, X. M.; Shao, M. Y.; Brennan, D. M.; Ellis, S. G.; Brennan, M. L.; Allayee, H.; Lusis, A. J.; Hazen, S. L., Relationship of paraoxonase 1 (PON1) gene polymorphisms and functional activity with systemic oxidative stress and cardiovascular risk. *Jama-J Am Med Assoc* **2008,** *299* (11), 1265-1276.

3. Zintzaras, E.; Hadjigeorgiou, G. M., Association of paraoxonase 1 gene polymorphisms with risk of Parkinson's disease: a meta-analysis. *J Hum Genet* **2004,** *49* (9), 474-481.

4. Furlong, C. E.; Cole, T. B.; Jarvik, G. P.; Pettan-Brewer, C.; Geiss, G. K.; Richter, R. J.; Shih, D. M.; Tward, A. D.; Lusis, A. J.; Costa, L. G., Role of paraoxonase (PON1) status in pesticide sensitivity: Genetic and temporal determinants. *Neurotoxicology* **2005,** *26* (4), 651-659.

5. Draganov, D. I.; La Du, B. N., Pharmacogenetics of paraoxonases: a brief review. *N-S Arch Pharmacol* **2004,** *369* (1), 78-88.

6. Draganov, D. I.; Teiber, J. F.; Speelman, A.; Osawa, Y.; Sunahara, R.; La Du, B. N., Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *Journal of lipid research* **2005,** *46* (6), 1239-47.

7. Juhas, M.; Eberl, L.; Tummler, B., Quorum sensing: the power of cooperation in the world of Pseudomonas. *Environ Microbiol* **2005,** *7* (4), 459-71.

8. Shamir, R.; Hartman, C.; Karry, R.; Pavlotzky, E.; Eliakim, R.; Lachter, J.; Suissa, A.; Aviram, M., Paraoxonases (PONs) 1, 2, and 3 are expressed in human and mouse gastrointestinal tract and in Caco-2 cell line: Selective secretion of PON1 and PON2. *Free Radical Bio Med* **2005,** *39* (3), 336-344.

9. Levy, E.; Trudel, K.; Bendayan, M.; Seidman, E.; Delvin, E.; Elchebly, M.; Lavoie, J. C.; Precourt, L. P.; Amre, D.; Sinnett, D., Biological role, protein expression, subcellular localization, and oxidative stress response of paraoxonase 2 in the intestine of humans and rats. *American journal of physiology. Gastrointestinal and liver physiology* **2007,** *293* (6), G1252-61.

10. Willcox, M. D.; Zhu, H.; Conibear, T. C.; Hume, E. B.; Givskov, M.; Kjelleberg, S.; Rice, S. A., Role of quorum sensing by Pseudomonas aeruginosa in microbial keratitis and cystic fibrosis. *Microbiology* **2008,** *154* (Pt 8), 2184-94.

11. Stoltz, D. A.; Ozer, E. A.; Ng, C. J.; Yu, J. M.; Reddy, S. T.; Lusis, A. J.; Bourquard, N.; Parsek, M. R.; Zabner, J.; Shih, D. M., Paraoxonase-2 deficiency enhances Pseudomonas aeruginosa quorum sensing in murine tracheal epithelia. *Am J Physiol Lung Cell Mol Physiol* **2007,** *292* (4), L852-60.

12. Josse, D.; Ebel, C.; Stroebel, D.; Fontaine, A.; Borges, F.; Echalier, A.; Baud, D.; Renault, F.; le Maire, M.; Chabrieres, E.; Masson, P., Oligomeric states of the detergent-solubilized human serum paraoxonase (PON1). *Journal of Biological Chemistry* **2002,** *277* (36), 33386-33397.

13. Aharoni, A.; Gaidukov, L.; Yagur, S.; Toker, L.; Silman, I.; Tawfik, D. S., Directed evolution of mammalian paraoxonases PON1 and PON3 for bacterial expression and catalytic specialization. *Proceedings of the National Academy of Sciences of the United States of America* **2004,** *101* (2), 482-487.

14. Harel, M.; Aharoni, A.; Gaidukov, L.; Brumshtein, B.; Khersonsky, O.; Meged, R.; Dvir, H.; Ravelli, R. B.; McCarthy, A.; Toker, L.; Silman, I.; Sussman, J. L.; Tawfik, D. S., Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. *Nature structural & molecular biology* **2004,** *11* (5), 412-9.

15. Sarkar, M.; Harsch, C. K.; Matic, G. T.; Hoffman, K.; Norris, J. R., 3rd; Otto, T. C.; Lenz, D. E.; Cerasoli, D. M.; Magliery, T. J., Solubilization and humanization of paraoxonase-1. *Journal of lipids* **2012,** *2012*, 610937.

16. Stevens, R. C.; Suzuki, S. M.; Cole, T. B.; Park, S. S.; Richter, R. J.; Furlong, C. E., Engineered recombinant human paraoxonase 1 (rHuPON1) purified from Escherichia coli protects against organophosphate poisoning. *Proceedings of the National Academy of Sciences of the United States of America* **2008,** *105* (35), 12780-4.

17. Li, X. C.; Wang, C.; Mulchandani, A.; Ge, X., Engineering Soluble Human Paraoxonase 2 for Quorum Quenching. *ACS Chem Biol* **2016,** *11* (11), 3122-3131.

18. Kovach, M. E.; Elzer, P. H.; Hill, D. S.; Robertson, G. T.; Farris, M. A.; Roop, R. M., 2nd; Peterson, K. M., Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **1995,** *166* (1), 175-6.

19. Choi, K. H.; Kumar, A.; Schweizer, H. P., A 10-min method for preparation of highly electrocompetent Pseudomonas aeruginosa cells: Application for DNA fragment transfer between chromosomes and plasmid transformation. *J Microbiol Meth* **2006,** *64* (3), 391-397.

20. Lindsay, A.; Ahmer, B. M., Effect of sdiA on biosensors of N-acylhomoserine lactones. *Journal of bacteriology* **2005,** *187* (14), 5054-8.

21. Zimmermann, S.; Wagner, C.; Muller, W.; Brenner-Weiss, G.; Hug, F.; Prior, B.; Obst, U.; Hansch, G. M., Induction of neutrophil chemotaxis by the quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone. *Infection and immunity* **2006,** *74* (10), 5687-5692.

22. Smith, R. S.; Kelly, R.; Iglewski, B. H.; Phipps, R. P., The Pseudomonas autoinducer N-(3-oxododecanoyl) homoserine lactone induces cyclooxygenase-2 and prostaglandin E2 production in human lung fibroblasts: implications for inflammation. *J Immunol* **2002,** *169* (5), 2636-42.

23. Chow, J. Y.; Xue, B.; Lee, K. H.; Tung, A.; Wu, L.; Robinson, R. C.; Yew, W. S., Directed evolution of a thermostable quorum-quenching lactonase from the amidohydrolase superfamily. *The Journal of biological chemistry* **2010,** *285* (52), 40911-20.

24. Olieric, N.; Kuchen, M.; Wagen, S.; Sauter, M.; Crone, S.; Edmondson, S.; Frey, D.; Ostermeier, C.; Steinmetz, M. O.; Jaussi, R., Automated seamless DNA co-transformation cloning with direct expression vectors applying positive or negative insert selection. *Bmc Biotechnol* **2010,** *10*.

25. Ben-David, M.; Sussman, J. L.; Maxwel, C. L.; Szeler, K.; Kamerlin, S. C. L.; Tawfik, D. S., Catalytic Stimulation by Restrained Active-Site Floppiness-The Case of High Density Lipoprotein-Bound Serum Paraoxonase-1. *Journal of molecular biology* **2015,** *427* (6), 1359-1374.

4 Chapter 4 The Effect of Quorum Quenching Molecules on *Pseudomonas Aeruginosa* PAO1 Biofilm Formation

4.1 Abstract

Quorum sensing (QS) is a chemical communication process that bacteria use to regulate collective behaviors. Disabling quorum sensing circuits with small molecules and quorum quenching (QQ) enzymes has been proposed as a potential strategy to prevent bacterial pathogenicity. QQ enzymes have been of interest as they may act as an effective catalytic process for degrading QS signals without the need to enter the cell. This study focused on two QS inhibitors - (Z-)-4-bromo-5-(bromomethylene)-2(5H)-furanone (C-30) and 4-nitropyridine-*N*-oxide (NPO), and two QQ enzymes - the lactonase aiiA and engineered human paraoxonase 2 (huPON2) E3 - for their ability to degrade the signaling molecule acyl-homoserine lactones (AHLs). 0.05 mM of brominated furanone C-30 and NPO can sufficiently inhibit *P. aeruginosa* biofilm formation. Enzymatic QQ of AiiA reduced the amount of biofilm for both *P. aeruginosa* and chronic wound swabs. In particular, the potential of human-based PONs on inhibiting biofilm formation in the human pathogen *Pseudomonas aeruginosa* was highlighted.

4.2 Introduction

Pathogenic bacteria minimize host immune responses by delaying the production of tissue-damaging virulence factors until sufficient bacteria have amassed and are prepared to overwhelm the host immune system and establish infection[1-2]. *Pseudomonas aeruginosa* is a human pathogen involved in many infections, including both community-acquired and hospital-acquired infections, such as otitis, keratitis, wound and burn infections, pneumonia, and urinary tract infections[3-4]. *P. aeruginosa* uses a molecular communication system, referred to as quorum sensing (QS), to regulate the expression of up to 10% of its genome. Most importantly, the QS system synchronizes the production of virulence factors, including pyocyanin and proteases[5], antibiotics, motility[6] and biofilm formation[7].

Regarding the importance of bacterial communication in the development of virulence, strategies for QS disruption, known as quorum quenching (QQ), have emerged to disrupt bacteria's commensal network. QQ chemical molecules and QQ enzymes have been particularly considered because unlike traditional antibiotics they do not generate a harsh selection pressure, which may stimulate resistance [8-11]. By structurally mimicking QS molecules, QQ compounds prevent bacteria from perceiving endogenous QS molecules as competitive inhibitors [12]. For instance, halogenated furanones can target the *P. aeruginosa las* and *rhl* systems, and significantly reduce biofilm reduction[5, 13]. On the other hand, QQ enzymes can directly degrade communication signals[14-15]. Plants engineered to

express AHL-lactonase demonstrated a capability for substantially enhanced resistance to *E. carotovora* infection[21]. In addition, a recombinant *E. coli* which producing aiiA was encapsulated inside the lumen of a microporous hollow fiber membrane and alleviated biofouling on the surface of the filtration membrane[22]. Furthermore, aiiA has a broad-range of specificity for hydrolyzing the lactone rings of AHLs[16], resulting in inhibition of *P. aeruginosa* biofilm formation[17]. In this chapter, effectiveness of four QQ agents, furanone C-30, 4-nitropyridine-*N*-oxide (NPO), aiiA, and huPON2 E3 were investigated on *P. aeruginosa* PAO1 biofilm formation, which was regulated AHL-dependent QS (3-oxo-C_{12}HSL and C_4HSL).

4.3 Materials and Methods

4.3.1 Bacterial Strains and Growth Conditions

P. aeruginosa PAO1 and chronic wound swabs were kindly provided by Dr. Colin Manoil of the University of Washington, Seattle, and Dr. Manuela Martins-Green of the University of California, Riverside, respectively. *P. aeruginosa* PAO1 was inoculated from a single colony and cultivated in LB broth overnight at 37 °C with shaking at 225 rpm. Subsequently, the inoculum was diluted in M9 minimal media to OD_{600} = 0.05. 200 μL of the dilutions were cultured in 96-microplate at 37 °C without agitation. M9 minimal media was prepared with 2 ml 1 M $MgSO_4$, 0.1 mL 1 M $CaCl_2$, 20 mL 20% (w/v) glucose and 478 mL M9 salts solution (12.8 g Na_2HPO_4, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, 478 mL H_2O).

All the solutions were sterilized and stored at 4 °C prior to mixing. For chronic wound swabs, 5 μL stock was inoculated to 5 mL LB broth overnight culture at 37 °C with shaking at 225 rpm. The inoculum was then diluted in 200 μL LB media for a starting OD_{600} of 0.05, for cultured in 96-microplate at 37 °C without agitation for 12h.

4.3.2 Expression and Purification of Lactonases

Enzyme production was performed using *E. coli* BL21 (DE3) carrying plasmid pMAL-tac-c5x-aiiA or pMAL-tac-c5x-E3. In brief, cells were grown in 2.5 mL LB medium supplemented with 100 μg/ml ampicillin and 0.5 mM $CoCl_2$ at 37 °C overnight. The inoculum was added to 250 mL LB media, cultured for 2 - 3h until OD_{600} reached 0.5−0.6 for induction with 0.2 mM IPTG at room temperature overnight. Cells were harvested by centrifugation, resuspended in column buffer (20 mM Tris-HCl at pH 7.4, 100 mM NaCl, 0.5 mM $CoCl_2$), and lysed by sonication. Cell lysates were clarified by centrifugation at 10000×g for 30 min. MBP-aiiA was captured by an amylose resin column (NEB). After wash with column buffer, MBP-aiiA or MBP-E3 was eluted with 10 mM maltose in column buffer. The eluted fractions containing fusion proteins were dialyzed overnight at 4 °C against 20 mM Tris-HCl at pH 7.4, 5 mM NaCl, and 0.5 mM $CoCl_2$. Purified protein samples were aliquoted in 20% glycerol and stored at

−80°C before use. For E3 purification, the metal ion supplement was 1 mM $CaCl_2$ instead of 0.5 mM $CoCl_2$ for aiiA.

4.3.3 Quantitative biofilm-disruption assay

The effects of chemicals and lactonases on the biofilm formation of *P. aeruginosa* PAO1 or chronic wound swabs were measured using microtiter plate assay. Various concentrations of chemical inhibitors (Z-)-4-bromo-5-(bromomethylene)-2(5H)-furanone (C-30) and 4-nitropyridine-*N*-oxide (NPO), or lactonases MBP-aiiA and MBP-E3, were added to fresh *P. aeruginosa* culture and chronic wound swabs culture in 96-well microtiter plates (Costar, USA). After incubation for 12h unless specifically mentioned, the medium was discarded, and the plates were gently washed three times by submerging in a water reservoir to remove the weakly adherent planktonic cells. Plates were gently tapped on paper towels and were air-dried prior to staining. The adherent biofilms were stained with 225 μL 0.1% crystal violet for 10 min, followed by solution discard and washing three times with water. The plates were then dried in an oven at 60 °C for 15 min, and the bacteria-bound crystal violet was dissolved in 250 μL 95% ethanol. OD_{590} was measured using a microtiter absorbance reader (BioTek).

4.4 Results and Discussion

4.4.1 Effects of Chemical Molecules on the Inhibition of *P. aeruginosa* PAO1 Biofilm Formation.

The sensitivities of *P. aeruginosa* PAO1 towards chemical inhibitors (Z-)-4-bromo-5-(bromomethylene)-2(5H)-furanone (C-30)[5] and 4-nitropyridine-*N*-oxide (NPO)[18] were tested by monitoring the biofilm formation every two hours between 4-12 hours. Contrast to the continual development of biofilm without QS inhibitors, when 0.05 mM C-30 was applied to 2.5×10^7 cfu, biofilm formation was dramatically decreased during 4-10 hours, and at 10h only 5% biofilm was measured compared to the cell culture without QQ compounds (**Figure 4-1**). 0.05 mM NPO reduced the biofilm formation significantly as well, and at 10h, 55% less biofilm was formed than control culture without QQ compounds. When the concentration of C-30 or NPO was increased to 0.5 mM, *P. aeruginosa* PAO1 cell growth was completely impeded, and thus this concentration is not appropriate for biofilm assays. And at 0.005 mM, NPO and C-30 reduced the amount of *P. aeruginosa* PAO1 biofilm by 41% and 34% respectively. Therefore, 0.05 mM C-30 was used as positive control in this study.

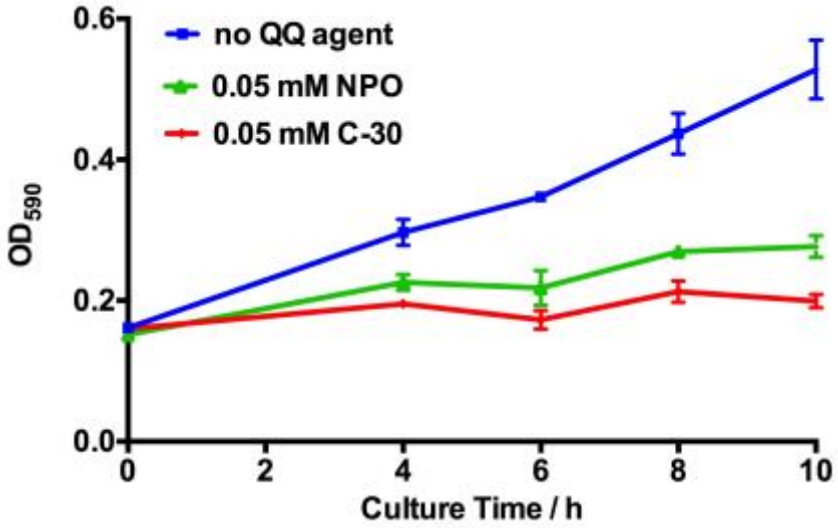

Figure 4-1. Effects of chemical inhibitors on *P aeruginosa* PAO1 biofilm formation. 0.05 mM C-30 or NPO were used.

4.4.2 Evaluating Effects of QQ Proteins on *P. aeruginosa* Biofilm Formation

QQ agents and control agent (BSA) were added after either 6h, 8h, or 10h culture of 2.5×10^7 cfu *P. aeruginosa*. When no QQ agent was added, biofilm formation is dominant with a OD_{590} ranging from 0.89 to 0.97. When 5.4 μM BSA was applied, *P. aeruginosa* biofilm formations were increased by 10-35% compared to no agent group. 50 μM C-30 adding at 6h decreased the biofilm formation by 64%, while adding it at 8h or 10h reduced biofilm by 40% or 22%, indicating early treatment is more beneficial. Applying 5.4 μM purified aiiA at 6h reduced biofilm formation by 56%. And applying aiiA at later stage was less effective (8 % and 7% reduction at 8h and 10h respectively), which was as

consistent with C-30 results. As expected, a low dosage of aiiA (1.1 μM) was less effective to impede the development of biofilm.

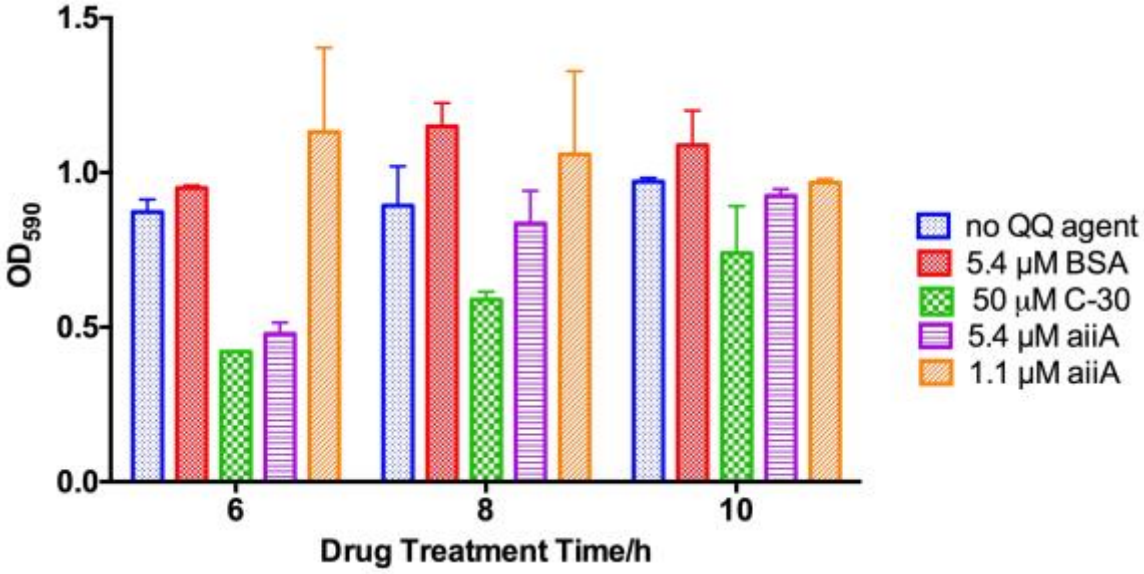

Figure 4-2. The inhibition effect of aiiA on *P. aeruginosa* PAO1 biofilm formation. BSA, C-30, or aiiA were added at either 6h, 8h, or 10h to the *P aeruginosa* culture. Biofilm formations were examined all together at 12h.

The effect of huPON2 E3 on *P aeruginosa* biofilm formation was tested likewise. 50 μM C-30, 5.4 μM aiiA, 17 μM or 34 μM of E3 were added at 6h to the *P aeruginosa* culture and biofilm formations were tested at 12h (**Figure 4-3**). C-30 and aiiA inhibited the biofilm formation by 64% and 45%. When 34 μM of E3 was utilized, the biofilm could be reduced by 32%. However, 17 μM of E3 did not impede the biofilm formation likely due to low dosage.

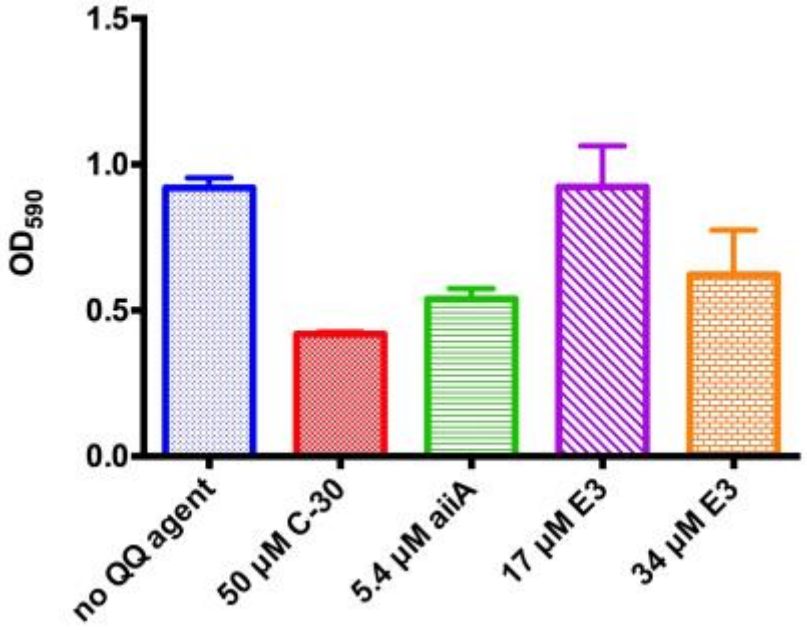

Figure 4-3. The effect of engineered huPON2 E3 on *P. aeruginosa* biofilm formation. 50 μM C-30, 5.4 μM aiiA, 17 μM and 34 μM of E3 were added at 6h to the *P aeruginosa* culture and biofilm formations were tested quantitatively at 12h.

4.4.3 Evaluating Effects of QQ Enzymes on Chronic Wound Swab Biofilm Formation

Unlike *P aeruginosa* cultured in M9 minimal media, chronic wound swabs were cultured in LB media to maintain the strain diversity. BSA, C-30, aiiA and huPON2-E3, were applied at 6h, and the biofilms were quantitatively examined at 12h. 5.4 μM aiiA reduced the biofilm by 33%, however, 50 μM C-30 and 5.4 μM huPON-E3 did not effectively inhibit the development of biofilm, likely due to the complexity of the bacterial community present in the wound swabs. As a broad spectrum lactonase able to hydrolyze multiple AHLs, aiiA was capable to impede

the biofilm formation of wound swabs. This result suggests two factors for a successful QQ enzyme therapy: high activity and broad substrate specificity.

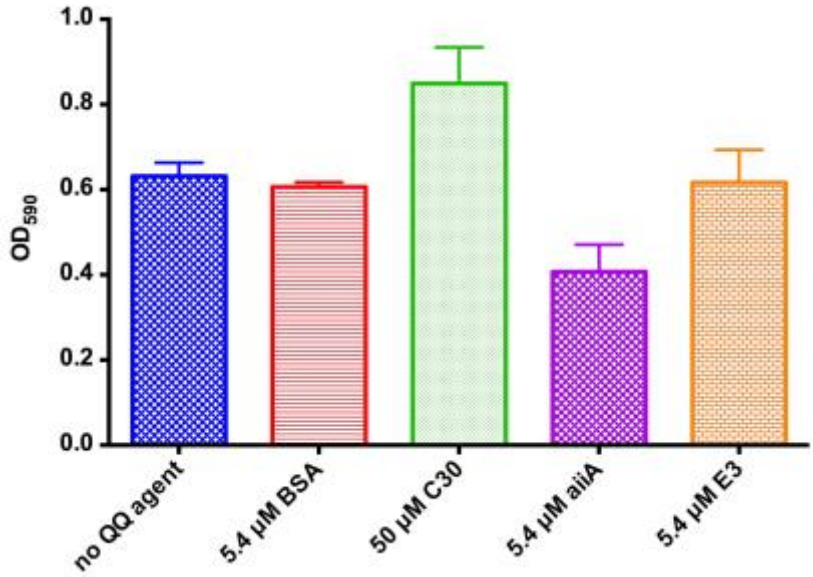

Figure 4-4. Enzymatic inhibition of biofilm on chronic wound swabs.

This study shed light on the potential of applying heterologous enzymes to human infections. From a biocontrol point of view, combining the QQ approach with other treatments, such as antibiotics, to obtain a synergistic effect is another potential strategy that could possibly increase the susceptibility of bacteria to antibiotic treatment.

4.5 Conclusion

In this chapter, the efficacy of QS inhibitors and QQ enzymes on pathogens' biofilm development was tested. 0.05 mM of brominated furanone C-30 and NPO can sufficiently inhibit *P. aeruginosa* biofilm formation. Enzymatic QQ of AiiA reduced the amount of biofilm for both *P. aeruginosa* and chronic wound swabs. Engineered huPON2 E3 exhibited promising QQ results when applied to pure *P. aeruginosa* culture. Further characterization of QQ enzymes on chronic wounds are still needed test their efficacy.

4.6 References

Uncategorized References

1. Costerton, J. W.; Stewart, P. S.; Greenberg, E. P., Bacterial biofilms: A common cause of persistent infections. *Science* **1999,** *284* (5418), 1318-1322.

2. Davey, M. E.; O'toole, G. A., Microbial biofilms: from ecology to molecular genetics. *Microbiol Mol Biol R* **2000,** *64* (4), 847-+.

3. Stover, C. K.; Pham, X. Q.; Erwin, A. L.; Mizoguchi, S. D.; Warrener, P.; Hickey, M. J.; Brinkman, F. S. L.; Hufnagle, W. O.; Kowalik, D. J.; Lagrou, M.; Garber, R. L.; Goltry, L.; Tolentino, E.; Westbrock-Wadman, S.; Yuan, Y.; Brody, L. L.; Coulter, S. N.; Folger, K. R.; Kas, A.; Larbig, K.; Lim, R.; Smith, K.; Spencer, D.; Wong, G. K. S.; Wu, Z.; Paulsen, I. T.; Reizer, J.; Saier, M. H.; Hancock, R. E. W.; Lory, S.; Olson, M. V., Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature* **2000,** *406* (6799), 959-964.

4. Driscoll, J. A.; Brody, S. L.; Kollef, M. H., The epidemiology, pathogenesis and treatment of Pseudomonas aeruginosa infections. *Drugs* **2007,** *67* (3), 351-368.

5. Hentzer, M.; Wu, H.; Andersen, J. B.; Riedel, K.; Rasmussen, T. B.; Bagge, N.; Kumar, N.; Schembri, M. A.; Song, Z. J.; Kristoffersen, P.; Manefield, M.; Costerton, J. W.; Molin, S.; Eberl, L.; Steinberg, P.; Kjelleberg, S.; Hoiby, N.; Givskov, M., Attenuation of Pseudomonas aeruginosa virulence by quorum sensing inhibitors. *Embo Journal* **2003,** *22* (15), 3803-3815.

6. Daniels, R.; Vanderleyden, J.; Michiels, J., Quorum sensing and swarming migration in bacteria. *Fems Microbiology Reviews* **2004,** *28* (3), 261-289.

7. Whiteley, M.; Bangera, M. G.; Bumgarner, R. E.; Parsek, M. R.; Teitzel, G. M.; Lory, S.; Greenberg, E. P., Gene expression in Pseudomonas aeruginosa biofilms. *Nature* **2001,** *413* (6858), 860-864.

8. Dong, Y. H.; Wang, L. H.; Zhang, L. H., Quorum-quenching microbial infections: mechanisms and implications. *Philos T R Soc B* **2007,** *362* (1483), 1201-1211.

9. Kalia, V. C., Quorum sensing inhibitors: An overview. *Biotechnology Advances* **2013,** *31* (2), 224-245.

10. Tang, K. H.; Zhang, X. H., Quorum Quenching Agents: Resources for Antivirulence Therapy. *Mar Drugs* **2014,** *12* (6), 3245-3282.

11. Fetzner, S., Quorum quenching enzymes. *J Biotechnol* **2015,** *201*, 2-14.

12. Ueda, A.; Attila, C.; Whiteley, M.; Wood, T. K., Uracil influences quorum sensing and biofilm formation in Pseudomonas aeruginosa and fluorouracil is an antagonist. *Microb Biotechnol* **2009,** *2* (1), 62-74.

13. Hentzer, M.; Riedel, K.; Rasmussen, T. B.; Heydorn, A.; Andersen, J. B.; Parsek, M. R.; Rice, S. A.; Eberl, L.; Molin, S.; Hoiby, N.; Kjelleberg, S.; Givskov, M., Inhibition of quorum sensing in Pseudomonas aeruginosa biofilm bacteria by a halogenated furanone compound. *Microbiol-Sgm* **2002,** *148*, 87-102.

14. Bzdrenga, J.; Daude, D.; Remy, B.; Jacquet, P.; Plener, L.; Elias, M.; Chabriere, E., Biotechnological applications of quorum quenching enzymes. *Chemico-biological interactions* **2017,** *267*, 104-115.

15. Remy, B.; Plener, L.; Poirier, L.; Elias, M.; Daude, D.; Chabriere, E., Harnessing hyperthermostable lactonase from Sulfolobus solfataricus for biotechnological applications. *Sci Rep-Uk* **2016,** *6*.

16. Wang, L. H.; Weng, L. X.; Dong, Y. H.; Zhang, L. H., Specificity and enzyme kinetics of the quorum-quenching N-acyl homoserine lactone lactonase (AHL-lactonase). *Journal of Biological Chemistry* **2004,** *279* (14), 13645-13651.

17. Wang, Y.; Dai, Y.; Zhang, Y.; Hu, Y. B.; Yang, B. Y.; Chen, S. Y., Effects of quorum sensing autoinducer degradation gene on virulence and biofilm formation of Pseudomonas aeruginosa. *Sci China Ser C* **2007,** *50* (3), 385-391.

18. Cady, N. C.; McKean, K. A.; Behnke, J.; Kubec, R.; Mosier, A. P.; Kasper, S. H.; Burz, D. S.; Musah, R. A., Inhibition of Biofilm Formation, Quorum Sensing and Infection in Pseudomonas aeruginosa by Natural Products-Inspired Organosulfur Compounds. *Plos One* **2012,** *7* (6).

19. Dong, Y. H.; Xu, J. L.; Li, X. Z.; Zhang, L. H., AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and

attenuates the virulence of Erwinia carotovora. *Proceedings of the National Academy of Sciences of the United States of America* **2000,** *97* (7), 3526-3531.

20. Shrout, J. D.; Chopp, D. L.; Just, C. L.; Hentzer, M.; Givskov, M.; Parsek, M. R., The impact of quorum sensing and swarming motility on Pseudomonas aeruginosa biofilm formation is nutritionally conditional. *Molecular Microbiology* **2006,** *62* (5), 1264-1277.

21. Dong, Y. H.; Wang, L. H.; Xu, J. L.; Zhang, H. B.; Zhang, X. F.; Zhang, L. H., Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature* **2001,** *411* (6839), 813-817.

22. Oh, H. S.; Yeon, K. M.; Yang, C. S.; Kim, S. R.; Lee, C. H.; Park, S. Y.; Han, J. Y.; Lee, J. K., Control of membrane biofouling in MBR for wastewater treatment by quorum quenching bacteria encapsulated in microporous membrane. *Environ Sci Technol* **2012,** *46* (9), 4877-84.

23. Migiyama, Y.; Kaneko, Y.; Yanagihara, K.; Morohoshi, T.; Morinaga, Y.; Nakamura, S.; Miyazaki, T.; Hasegawa, H.; Izumikawa, K.; Kakeya, H.; Kohrogi, H.; Kohno, S., Efficacy of AiiM, an N-Acylhomoserine Lactonase, against Pseudomonas aeruginosa in a Mouse Model of Acute Pneumonia. *Antimicrob Agents Ch* **2013,** *57* (8), 3653-3658.

24. Gupta, P.; Chhibber, S.; Harjai, K., Efficacy of purified lactonase and ciprofloxacin in preventing systemic spread of Pseudomonas aeruginosa in murine burn wound model. *Burns* **2015,** *41* (1), 153-162.

5 Chapter 5 Concluding Remarks

This dissertation adopted both rational design and combinatory approach to engineering human paraoxonase 2 for its soluble expression while retaining its functions. Two clones, D2 and E3, were characterized for their quorum quenching bioactivities by bioluminescent quenching reporter assay, direct hydrolysis of Gram-negative bacteria quorum sensing signals, and inhibition of *P. aeruginosa* swimming and swarming motilities.

D2 and E3 were further engineered for improving their enzymatic activity by random mutagenesis and bioluminescence screening. Five clones with improved bioluminescent quenching ability were isolated. Particularly mutant D3H7 (from the first round) and 1A3 (from the second round) elicited the highest lactonase activity toward 3-oxo-C_{12}HSL. Yet compared to D2 and E3, endogenously expressed huPON2 variants did not exhibit enhanced activity in *P. aeruginosa* biofilm formation assays.

Finally, the efficacy of quorum sensing inhibitors C-30 and NPO, quorum quenching enzymes including lactonase aiiA and huPON2 E3 was evaluated on *P. aeruginosa* pure culture and chronic wound swabs. Inhibitors and aiiA significantly inhibited biofilm formation, and the effect of E3 on chronic wound swab biofilm formation still needs to be analyzed. This experiment provided us a closer look at how to utilize quorum quenching enzymes in repressing a pathogen's biofilm formation. Combination therapy with both antibiotics and QQ

agent could be a promising approach for limiting the proliferation, virulence and resistance emergence of pathogens.

Uncategorized References

1. Defoirdt, T.; Boon, N.; Bossier, P., Can bacteria evolve resistance to quorum sensing disruption? *PLoS Pathog* **2010,** *6* (7), e1000989.

2. Sandoz, K. M.; Mitzimberg, S. M.; Schuster, M., Social cheating in Pseudomonas aeruginosa quorum sensing. *Proceedings of the National Academy of Sciences of the United States of America* **2007,** *104* (40), 15876-15881.

3. Defoirdt, T.; Sorgeloos, P.; Bossier, P., Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology* **2011,** *14* (3), 251-258.