Chemical biology approaches to identify novel anti-pseudomonas aeruginosa agents

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**CHEMICAL BIOLOGY APPROACHES TO
IDENTIFY NOVEL ANTI-PSEUDOMONAS
AERUGINOSA AGENTS**

JULY FONG

SCHOOL OF BIOLOGICAL SCIENCES

2019

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NOVEL ANTI-PSEUDOMONAS AERUGINOSA
AGENTS**

JULY FONG

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement
for the degree of Doctor of Philosophy

2019

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The contributions of the co-authors are as follows:

- Prof. Wu Bin, Prof. Haibin Su, and Prof. Yang Liang provided the initial project initiation and overall project direction.
- Dr. Chaodong Zhang provided mathematical modeling simulation and calculation.
- Dr. Renliang Yang and Zhao Zhi Boo purified the enzyme AiiA and provided its hydrolysis activity.
- I carried out all the biological assays, data analysis, and interpretation.
- The manuscript draft was prepared by Chaodong Zhang (simulation part) and me (microbiology part).
- Prof. Tan Soon Keat, Dr. Thomas E. Nielsen, Prof. Michael Givskov, Prof. Xue-Wei Liu, Prof. Wu Bin, Prof. Haibin Su, and Prof. Yang Liang assisted in the data analysis, discussion, and manuscript revision.

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LIST OF PAPERS

Fong, J.; Chen, L.; Nielsen, T. E.; Yang, L.; Givskov, M., Zinc pyrithione and its derivatives as novel quorum sensing inhibitor and virulence attenuator of *P. aeruginosa*. **2019**, (Pending patent application)

Fong, J.; Mortensen, K. T.; Nørskov, A.; Qvortrup, K.; Yang, L.; Tan, C. H.; Nielsen, T. E.; Givskov, M., Itaconimides as novel quorum sensing inhibitors of *Pseudomonas aeruginosa*. *Front. Cell. Infect. Microbiol.* **2019**, 8, 443

Fong, J.; Zhang, C.; Yang, R.; Boo, Z. Z.; Tan, S. K.; Nielsen, T. E.; Givskov, M.; Liu, X.-W.; Bin, W.; Su, H.; Yang, L., Combination therapy strategy of quorum quenching enzyme and quorum sensing inhibitor in suppressing multiple quorum sensing pathways of *P. aeruginosa*. *Sci. Rep.* **2018**, 8, 1155.

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ABSTRACT

The emergence of multidrug-resistance pathogens has called for alternative therapies to treat bacterial infections. *Pseudomonas aeruginosa* is an opportunistic pathogen that often causes persistent infections associated with highly antibiotic-resistance and biofilms formation. In this study, various chemical biology approaches were utilized to interfere with bacterial cell-to-cell communication, termed quorum sensing (QS), to address drug resistance problems currently observed worldwide. Many bacteria employ QS to govern the production of virulence factors as well as the formation of biofilms. Instead of targeting components essential for growth and stimulate the development of resistant traits, chemical attenuation of QS could preferentially block QS-regulated cascades of bacterial gene expression, thus leading to attenuation of virulence.

In many cases, bacteria employ multiple QS systems, which pose a challenge to develop novel inhibitors. In the first study, two different classes of QS interfering agents, such as quorum sensing inhibitor (QSI) and quorum quenching enzyme (QQE), were studied to suppress multiple pathways of QS of *P. aeruginosa*. The two QS interfering agents have been studied independently and work in different ways. Using mathematical modeling and biochemical assays, the results from this study suggested a novel approach of utilizing combination therapy of QSI and QQE, and also the importance to develop inhibitors that could suppress all QS systems simultaneously and block pathogenesis.

In the second study, a drug repurposing approach was utilized to identify novel functions of FDA-approved drugs as QSI against *P. aeruginosa*. Given the slow pace of the new antibiotics discovery, repurposing of old drugs has been seen as an attractive strategy to recognize the new function of drugs outside their scope of original medical application, with the advantages of safer toxicity profiles, well-published clinical data, and lower cost and time. Five drugs with excellent QSI activities, namely benzbromarone (uricosuric agent), 8-quinolinol sulfate monohydrate (antineurodegenerative, anticancer, and antidiabetic agent), cetylpyridium chloride (antiseptic agent), zinc pyrithione (antidandruff and antifungal agent), and carbimazole (hyperthyroidism drug) were successfully identified. The application of the compounds *in vivo* using peritoneal silicone implant models on mice

study was also demonstrated. Overall, this study represents how drug repurposing approach could be used to identify repurposable compounds as novel QS-interfering compounds to attenuate the virulence of *P. aeruginosa*.

In the last chapter, a small chemical library based on zinc pyrithione was constructed as part of Structure-Activity Relationship (SAR) study. Novel metal-containing compounds were successfully synthesized and characterized as QSI of *P. aeruginosa*. SAR study yielded analogs that could reduce the production of QS-regulated virulence factors, as well as successfully eradicate colistin-resistant cells in *P. aeruginosa* biofilms when used together with the antibiotic. Transcriptomic profiling using RNA sequencing as well as experimental adaptive evolution study reveal the working mechanism of the compounds. As ionophore, the compounds could deliver metal ions through lipid membrane, and once inside the cells, the released metal ions interact with several targets simultaneously and also affect metal homeostasis. Both *fur* and *zur* pathways are affected upon treatment with compounds. The findings presented in this study show potentials of chemical attenuation for treating *P. aeruginosa* infections.

ABBREVIATION

QS	Quorum sensing
QSI	Quorum sensing inhibitor
QQE	Quorum quenching enzyme
SAR	Structure-Activity Relationship
DNA	Deoxyribonucleic acid
eDNA	Extracellular DNA
RNA	Ribonucleic acid
mRNA	Messenger RNA
sRNA	Small RNA
EPS	Extracellular polymeric matrix
LPS	Lipopolysaccharide
c-di-GMP	Cyclic diguanylate monophosphate
AHL	Acyl homoserine lactone
BHL	N-butanoyl-L-homoserine lactone
PQS	<i>Pseudomonas</i> quinolone signal
HHQ	4-hydroxy-2-heptylquinoline
PVD	Pyoverdine
T3SS	Type III Secretion System
T6SS	Type VI Secretion System
TCS	Two-component system
MIC	Minimum inhibition concentration
SDS	Sodium dodecyl sulfate
SNP	Single-nucleotide polymorphism
SNV	Single-nucleotide variant
FDA	Food and Drug Administration
ZnP	Zinc pyrithione
CoP	Cobalt pyrithione
QSM	8-quinolinol sulfate monohydrate
CPC	Cetylpyridium chloride
IR	Infrared radiation
ICP-MS	Inductively coupled plasma mass spectrometry
LCMS	Liquid chromatography mass spectrometry
SPR	Surface Plasmon Resonance
HRMS	High resolution mass spectrometry
TLC	Thin layer chromatography
DMSO	Dimethyl sulfoxide
DCM	Dichloromethane
MeOH	Methanol
NaOH	Sodium hydroxide
m-CPBA	meta-Chloroperoxybenzoic acid
HCl	Hydrogen chloride
HMTT	3-hydroxy-4-methyl-2(3H)-thiazolethione
HQC	8-hydroxy-2-quinolinecarboxaldehyde
HMQ	2-(hydroxymethyl)-3-quinolinol

1 INTRODUCTION

1.1 Pathogenicity and antimicrobial resistance of *P. aeruginosa*

The major obstacle faced in the current clinical microbiology is the slow development of new drugs to compete with the rapid evolution of multidrug resistance bugs. The ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* species) are commonly associated with life-threatening nosocomial infections amongst critically ill and immunocompromised patients.¹ They present a great challenge in 21st-century modern medicine, with limited treatment options once resistance diagnosed and developed. It is therefore critical to take pre-emptive measures in studying and developing effective therapies for this pressing issue.

P. aeruginosa is a rod-shaped Gram-negative bacterium which can thrive in diverse environmental conditions. *P. aeruginosa* (or *Bacillus pyocyaneus* during its early discovery) was only successfully isolated in pure culture until 1882 by Carle Gessard from green pus.² Scientists in the 18th century have observed blue-green coloration in the wounds of patients, but yet to identify the causative agents.³ It was later discovered that the blue-green characteristic color of *P. aeruginosa* was derived from its virulence factors pyocyanin. On top of that, it also produces various pigments like pyorubin (red), pyomelanin (black), and pyoverdin (yellow-green or brown and fluorescent) that makes *P. aeruginosa* distinctive to characterize. In certain cases, the strain also produces extracellular polysaccharide called alginate, which causes the colony to appear as mucoid.^{1b}

P. aeruginosa is seldom present as normal microbial flora in humans, although it can cause high morbidity and mortality once infections occur in patients. *P. aeruginosa* is the third leading cause of gram-negative infections,⁴ which includes nosocomial pneumonia, urinary tract infection, chronic wounds, and patients with compromised host defenses.⁵ Infection sites can be soft tissue (from burns, open wounds, postsurgery), bacteremia (immunocompromised patients), diabetic foot (from diabetes, impaired microvascular circulation), respiratory/pneumonia (from cystic fibrosis, mechanical ventilation, chronic obstructive pulmonary disease, old age), corneal infection (from extended contact lens wear, contaminated contact lens solution), hot tub rash (from improperly cleaned hot tubs), etc.⁶ On top of that, it could also form biofilms on chronic

wounds as well as on medical devices like implants, catheters, tubes, artificial hip, and many more.⁷

What makes *P. aeruginosa* an important pathogen to be studied? As an opportunistic pathogen, *P. aeruginosa* possesses a large arsenal of weapons, which technically covers every type of bacterial virulence factors. These include exotoxins and proteases, type III secretion system (T3SS), pili, flagella, alginate, and iron uptake siderophores (pyochelin and pyoverdin). It is also recalcitrant towards many antimicrobial treatments due to its ability to express multiple resistance mechanisms, including enzymes and efflux pumps (Table 1-1).^{3, 8} *P. aeruginosa* has been listed by World Health Organization (WHO) among the critical priority pathogens that require attention for antibiotics resistance development.⁹ U.S.A. Centres for Disease Control (CDC) estimated that more than 20,000 deaths per year are attributed to antibiotic resistance cases.¹⁰

Table 1-1 Resistance mechanisms in *P. aeruginosa*. The data was collected from different literature sources.^{6, 11}

Resistance class	Mechanism	Examples
Intrinsic	Efflux pumps	MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexGHI-OpmD, MexJK, MexMN, MexPQ-OpmE, MexVW, MexXY-OprM.
	Outer membrane impermeability	OprF, OprD, OprB.
	β -lactamases	AmpC.
Acquired	Targeted mutation	DNA gyrase, DNA topoisomerase, MexZ.
	Horizontal transfer	Metallo- β -lactamases, extended spectrum β -lactamase.
Adaptive	Membrane changes	Lipid A modification, AmpC regulation.

The pathogenesis of *P. aeruginosa* is also contributed by its ability to form biofilms, which affects up to 65% infectious diseases caused by *P. aeruginosa*.¹² In the biofilms mode of life, bacteria are inherently more resistant towards antibiotic treatment and host immune system. At different phases of *P. aeruginosa* infection – from colonization to chronic and acute infection – the process depends mostly on cell-to-cell signaling called QS, which operates on a density-dependent manner via synthesis of small diffusible signal molecules.¹³ Altogether, the QS signals control 5-10% of the *P. aeruginosa* genome, which in turn regulates virulence factor expression, biofilms formation, type III secretion system (T3SS), colonization of host, and many more (Figure 1-1).¹⁴

Figure 1-1 Virulence mechanisms of *P. aeruginosa* through various exotoxins, proteases, adhesins, effector proteins during infections. Image was taken from Ref. 15 with modification.

In the following section of the literature review, the emphasis will revolve around biofilms formation and QS of *P. aeruginosa*. As QS governs various patterns of genes expression to control virulence and biofilm formation, it has been proposed that interfering with the communication system could be a promising strategy for the control and prevention of bacterial infections.¹⁶ The different factors affecting biofilms formation in *P. aeruginosa* will be discussed, followed by the QS pathways and regulation.

1.2 Biofilms formation in *P. aeruginosa*

The initial phase of biofilm formation begins with the attachment of bacteria cells to a substratum. The bacteria-surface adhesion involves a complexity of factors including surface sensing, bacteria physiology (cell appendages and motility), surface chemistry and the physical forces of interaction.¹⁷ Attachment takes place in two stages; the reversible and irreversible stages. During attachment, cell to cell interactions (QS) are also critical for biofilm formation through the development of high cell density populations.¹⁸ Once attached, bacteria will grow into microcolonies and slowly develop into mature biofilms. Eventually, some cells would detach, disperse and colonize other surfaces to repeat the biofilms life cycle (Figure 1-2).

Figure 1-2 Schematic diagram of biofilms life cycle in *P. aeruginosa*. Image was drawn using ChemBioDraw Ultra.

EPS matrix

In the biofilm mode of life, bacterial cells are enclosed within a matrix of extracellular polymeric substances (EPS). EPS is the hallmark of biofilms and still considered as “dark matter” as we are still lacking the tools to isolate and study the exact compositions without disrupting the matrix.¹⁹ Nevertheless, the role in EPS in the biofilms lifestyle is undeniably important. Major components of comprises of EPS are exopolysaccharides, proteins, extracellular DNA (eDNA), lipids or surfactants, and macromolecules that are self-produced by the cells.²⁰ The composition determines the microenvironment of the biofilm cells, such as porosity, density, water content, charge, sorption, hydrophobicity, architecture and mechanical stability. For example, mutants that cannot synthesize exopolysaccharides are severely compromised or unable to form mature biofilms.²⁰

In *P. aeruginosa*, the exopolysaccharide Psl acts as ‘molecular glue’, which forms a fibre-like matrix and promotes bacterial cell-cell and cell-surface interactions. In the study done by Wang *et al.*, eDNA and Psl physically interact to form the web of Psl-eDNA fibres which acts as a skeleton to allow bacterial adhesion and grow.²¹ Psl has been shown to interact not only with eDNA from *P. aeruginosa*, but also *S. aureus* and genomic DNA from human neutrophils.²¹ Pel is another polysaccharide produced by *P. aeruginosa* that is composed of cationic amino sugars. A study done by Allesen-Holm *et al.* showed eDNA is localized in the stalk of the biofilm (*vide supra*),²² and Jennings *et al.* further demonstrated that the localization is contributed to the binding with Pel polysaccharide.²³ In the *P. aeruginosa* biofilms, the pH ranges from 5.5 to 7 from the center of the microcolony to the bulk fluid. It was proposed that Pel is positively charged in the biofilm stalk, hence facilitating the ionic interactions with eDNA.

The interactions of eDNA with polysaccharides would benefit biofilms in many ways. Formation of DNA-Psl/Pel fibers can provide enhanced backbone support, allow bacteria to attach and hence facilitating the growth of the biofilm. The association of eDNA with Psl/Pel may also offer protection towards agents that specifically target one component of the biofilms matrix. It has been shown that DNase treatment cannot disrupt mature, aged biofilms.²⁴ The eDNA-Psl interaction may also increase the survival of *P. aeruginosa* in multispecies or host infections as Psl could interact with DNA from other bacteria cells as well as neutrophils.

Roles of filamentous Pf bacteriophage (Pf phage)

Another key component affecting *P. aeruginosa* biofilms development is the Pf phage. Recent paper by Sweere et al. reported Pf phages were present in almost 70% of *P. aeruginosa*-infected chronic wounds in human and mice.²⁵ Further mechanistic study on the roles of Pf phage in the pathogenesis of *P. aeruginosa* infections revealed the endocytosis of Pf phage by mammalian immune cells and triggered the Toll-like receptor 3 (TLR3)–and TIR domain–containing adapter-inducing interferon- β (TRIF)–dependent type I interferon production.²⁵

Biofilm experiments of *P. aeruginosa in vitro* have shown that Pf1-like phage (designated Pf4) have been linked with the appearance of small colony variants (SCVs) and affect the biofilms structure. Bacteriophage-mediated SCVs exhibit enhanced attachment and microcolony development as compared to the wild type.²⁶ On another hand, biofilms of Pf4 phage-deficient mutant were less stable and did not form the hollow voids as compared to the wild type.²⁷ It was later shown that Pf phages contribute to the biofilms formation through interaction with matrix polymers and form liquid crystalline organization, which further increase viscosity and tolerance towards desiccation and antibiotics.²⁸

Biofilms and resistance to antimicrobial agents

One striking way in which bacteria in biofilms are distinguished from their planktonic counterparts is their protection from environmental stress and pressure (chemicals, antibiotics, immune response, etc). They are also highly resistant to antimicrobials treatment, which could reach up to 1000-folds than the planktonic bacteria.²⁹ Many factors contribute to the tenacious and robust resistance of biofilm properties, notably

the EPS protective barrier and the components inside the matrix, horizontal gene transfer, and the presence of persister cells.

The complexity of EPS and the components inside the matrix has provided an excellent protection layer to the cells that lead to resistant to antimicrobial agents through multiple mechanisms. The matrix consisted of a niche microenvironment, with different diffusion rate, oxygen and nutrient availability, and other mechanical properties. It has been reported that oxygen limitation and low metabolic activity in the biofilms matrix contribute to tolerance of *P. aeruginosa* towards antibiotics ciprofloxacin and tobramycin.³⁰ Oxygen was found to penetrate 50 – 90 µm from the air interface, and this oxic zone correlates to metabolically active cells. In certain cases, antibiotic targeting growth, protein synthesis and metabolism would lose functions and render ineffective towards biofilm cells. This often includes biofilms harboring drug-tolerant persister cells, which are dormant, neither growing nor dying when exposed to bactericidal concentration of antibiotics.³¹

Next, biofilms matrix also renders successful penetration of antimicrobial agents. Certain antibiotics could penetrate *P. aeruginosa* biofilms matrix (e.g. ofloxacin and ciprofloxacin), while aminoglycosides (e.g. tobramycin and gentamicin) have a retarded penetration. Nevertheless, those antibiotics fail to kill biofilms cell,^{30, 32} revealing complex mechanisms on how to successfully delivery drugs and treat biofilms-associated infections. Alternatively, accumulation of waste and byproducts could also alter pH in the biofilms interior, which could antagonize the action of antibiotic. Aminoglycoside has been reported to be less effective in anaerobic than in aerobic conditions.³³ Stress response in the osmotic environment could also lead to changes in gene expression of porins proportion, which resulted in reduced cell envelope permeability and increased antibiotic resistance.³⁴

In addition, eDNA has been shown to play important roles in biofilms defense. Due to its highly anionic character, eDNA can bind to divalent metal cations, notably Mg^{2+} , Mn^{2+} , Ca^{2+} and Zn^{2+} .³⁵ eDNA has been reported to induce antibiotic resistance because of its cation chelating capability under cation limiting condition (ie. biofilms and other infection sites like CF lung).³⁵ The author postulated that the presence of DNA in the extracellular matrix of biofilms creates a localized cation-limited environment, which leads to the induction of lipopolysaccharide (LPS) modification genes and resistance to

antimicrobials. Wilton *et al.* also showed that eDNA could acidify planktonic and biofilm cultures. Acidic pH has long been correlated to increased resistance of aminoglycosides (2- to 8-fold), and the sensitivity can be restored by neutralizing the pH. The author proposed that the accumulation of eDNA in biofilms lead to acidic pH, an environmental cue that activates PhoPQ and PmrAB signalling, which eventually resulted in increased aminoglycoside resistance.³⁶ On the other hand, Chiang *et al.* attributed the tolerance towards aminoglycosides to the protective shield effect of eDNA instead of genetic factors due to the activation of *pmr* genes in *P. aeruginosa*.³² eDNA binds to aminoglycosides and cationic antimicrobial peptides via electrostatic interactions, and this may act as a sink that sequesters antibiotics in the biofilms. Exogenously added DNA increased the survival of biofilms toward antibiotics treatment. Using *pmrF* mutant, the effect of eDNA on the aminoglycoside tolerance was shown to be independent on the Pmr system. They also showed DNA release-deficient QS mutant were susceptible to aminoglycoside treatment but became resistant when exogenous DNA was added into the biofilms. The unique physicochemical properties of eDNA contribute to the defence of the biofilm; a protective role exhibited by the eDNA either via direct binding, chelation, or the trigger of genetic response related to pathogenicity and resistance to antimicrobials.

In the biofilms lifestyle, where cells are often kept in close proximity, it is often considered as a hot spot for horizontal gene transfer (HGT). HGT has been considered as the most important factor in the spreading virulence and antibiotic resistance genes.³⁷ As cells are compacted and enclosed together, it is easier to acquire transmissible genetic elements at accelerated rates. The high-density and well-organized diverse microbial community, along with EPS matrix that concentrates various chemical compounds, contributes to the likelihood of HGT. It has been shown that conjugation and transformation occur at higher frequencies in biofilm communities than planktonic state.³⁸ The chance for transformation was greater near the biofilm substratum, where cell density was greatest.^{38b} This phenomenon has also been observed in the human oral microbiome, which consisted of a rich collection of microorganisms existing as multispecies biofilms.³⁹ Geng *et al.* conducted the comparative genomics and phylogenetic analysis of *Streptococcus parasanguinis* FW213, and showed that five genomic islands (GIs) acquired from HGT event are involved in virulence, bacteriocins production, adherence, biofilm formation and resistance to host immune systems.⁴⁰ In

Porphyromonas gingivalis, natural transformation is also a major mechanism for HGT that contributes to its survival and persistence in the human oral environment.⁴¹ Overall, this highlights the roles of HGT in bacteria adaptive evolution towards external pressures imposed by the host and environment.

Figure 1-3 Summary of different mechanisms of antibiotic resistance employed by *P. aeruginosa* growing as biofilms. Image was taken from Ref. 42.

Regulation of biofilm development in *P. aeruginosa*

Recent genetic and molecular approaches have shown that bacteria biofilms display unique gene-expression patterns and undergo profound changes at different stages of biofilms development. Global gene expression of *P. aeruginosa* biofilms grown on different media, mode and surfaces have been accomplished using microarray and RNA sequencing. The first microarray study on *P. aeruginosa* biofilms identified 73 genes that are differentially expressed, with *rpoS* being identified to be involved in morphology and antibiotic sensitivity of biofilms.⁴³ Study done by Waite *et al.* also stated the contribution of QS and RpoS regulated genes.⁴⁴ Hentzer *et al.* concluded that biofilm cells have similar transcriptomic profiles to stationary phase cells, suggesting that biofilm development involves series of adaptive responses (ie. anaerobic, stress response, iron-limitation stresses).⁴⁵ Using microscopy and proteomics, Sauer *et al.* reported distinct protein pattern and phenotypes at different stages of biofilms development. Motility, alginate production, and QS were amongst the genes that were

differentially regulated during biofilms development.⁴⁶ The revelation highlights that biofilms formation is complex, multifaceted, requires intercellular signaling, and highly regulated (Figure 1-4).

Figure 1-4 Model of effectors involved in *P. aeruginosa* biofilms development. Image was taken from Ref. 7. Reprinted with permission from AAAS.

Recent study by Lee *et al.* demonstrated that c-di-AMP (cAMP) signaling and type IV pili (TFP) play important roles in the early stage of biofilms development.⁴⁷ Using multigenerational, single-cell tracking time-lapse microscopy, the authors were able to provide key determinants of transition from reversible to irreversible attachment, as well as multigenerational analysis of the cells involved in the initial attachment step. For irreversible attachment to occur, multiple reversible attachment/detachment events (such as sensing, division, and detachment cycles) are required to create a planktonic population with robust cAMP-TFP-based memory of the surface. The cells were then able to return and better adapted to colonize the surface. The overall “adaptive” time-delayed adhesion requires cAMP and TFP, which are critical for surface sensing mechanism. The study done by Barken *et al.* also suggested the role of TFP, in addition to flagellum-mediated motility and quorum sensing-controlled DNA release, in the formation of mature multicellular structures in *P. aeruginosa* biofilms. Using the genetic approach, they demonstrated that *P. aeruginosa pilA* mutant, *fliM* and *cheY* mutants (flagellum-associated motilities), and *pilAlasRrhIR* mutant (quorum sensing mutant which releases less DNA as compared to wild type) were cap formation deficient.⁴⁸ It was proposed that the interaction between eDNA and type IV pili serves as a guide for motile subpopulation to migrate towards the cap, and subsequently forms the mushroom-shaped structures in *P. aeruginosa* biofilms.⁴⁸

Biofilms and c-di-GMP signaling

One important signal molecules that play a critical role in switching between planktonic and biofilms lifestyle is the secondary messenger cyclic-di-GMP (c-di-GMP). In *P. aeruginosa* biofilms, approximately 75 – 110 pmol of c-di-GMP / mg of total cell extract are estimated, while planktonic cells contain less than 30 pmol/mg.⁴⁹ The importance of c-di-GMP in the bacterial world has opened doors to many exciting research studies in the 21st century, where many groups are still trying to understand and unravel the systems biology of c-di-GMP. Other physiological functions of c-di-GMP include reduced motility (through expression and activity of flagella, swimming and swarming)⁵⁰, reduced virulence factors (in *Vibrio cholerae*)⁵¹, synthesis of various adhesins and exopolysaccharides⁵², and cell cycle progression (in *Caulobacter crescentus*).⁵³ The correlation between c-di-GMP and biofilms formation is not just conserved in *P. aeruginosa*, but also across different species like *Escherichia coli* and *Salmonella enterica* serovar Typhimurium.⁵⁴

In *P. aeruginosa* biofilms, the concentration of c-di-GMP is regulated by two enzymes, firstly is diguanylate cyclases (DGCs) containing GGDEF domain, which synthesizes two molecules of GTP to c-di-GMP. Next, it is degraded into 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) and 2 GMP by phosphodiesterases (PDEs) containing EAL or HD-GYP domains. These amino acid motifs are essential for their enzymatic activities and determine the c-di-GMP turnover and widespread in several other bacteria. The *P. aeruginosa* genome encodes one of the highest numbers of DGCs and PDEs: 18 GGDEF, 5 EAL, 16 GGDEF/EAL, and 3 HD-GYP predicted proteins.⁵⁵ The conundrum lies among the proteins that contain two domains with opposite enzymatic activities, and it was postulated that they are differentially regulated by environmental cues and/or intracellular signals.⁵⁶ For instance, activation of PDE DipA by interaction with the c-di-GMP effector BdlA in response to starvation induces dispersion.⁵⁷

Coordination action of c-di-GMP signaling pathway, which requires discrete roles of DGCs and PDEs, orchestrates the different stages of biofilms formation and during infection (Figure 1-5). Overexpression of DGC (WspR, YfiN, or TbpB) induces small colony variant (SCV) morphology, which often found in clinical isolates from cystic fibrosis patients.⁵⁸ In addition, there are also various c-di-GMP receptors (binding target) and effectors (proteins whose activity changes allosterically upon c-di-GMP binding and regulate a defined interacting target protein) that have been discovered to play crucial cellular functions (Table 1-2).

Table 1-2 The c-di-GMP binding effector components. The data was collected from different literature sources.^{55, 59}

Domain	Name	Functions
RNDEG	<i>pelD</i>	Pellicle formation
PelD	<i>pelE</i>	EPS synthesis
PilZ	<i>alg44</i>	Alginate synthesis
PilZ	<i>pilZ</i>	Twitching motility
FleQ	<i>fleQ</i>	Activator of flagellar gene expression and Pel synthesis
Unknown	<i>brlR</i>	Biofilm resistance
GDSIF, EVL	<i>fimX</i>	Twitching motility and biofilm formation

Figure 1-5 DGCs and PDEs involved in various stages of biofilms development in *P. aeruginosa*. The lower panel illustrates different two-component regulatory systems that are known to control biofilms formation. Image was taken from Ref. 59.

There is another layer of complexity for the biofilms regulation and signaling pathways in *P. aeruginosa*. c-di-GMP has been reported to cross-talk with other signaling pathways, such as 3', 5'-cyclic adenosine monophosphate (cAMP) and QS. cAMP has been associated with the ability of *P. aeruginosa* to establish acute infection.⁶⁰ cAMP-dependent transcription factor (Vfr) positively regulates expression of numerous acute virulence factors (e.g. type II and T3SS, TFP, as well as QS).⁶¹ The two second messengers were found to be spatially organized in the biofilms, where high c-di-GMP levels and low cAMP levels were found in the deeper layers of biofilms. It was later

reported that c-di-GMP could inhibit cAMP-Vfr signaling pathway and resulted in decreased virulence factors production.⁶² Recent paper by Chua *et al.* shows dispersal cells have low c-di-GMP content and induced QS system which leads to high production of virulence factors like rhamnolipid and pyocyanin.⁶³ Ueda and Wood identified the novel link that connects c-di-GMP, QS, Pel polysaccharide and biofilms formation via tyrosine phosphatase TpbA.⁶⁴ The cross-talk concept highlights different mechanisms employed by *P. aeruginosa* to control biofilms formation and virulence.

The next literature review will look at how QS works in *P. aeruginosa*. This collective social behavior is a widespread phenomenon in bacteria to coordinate gene expression to obtain group-specific benefits. Although QS in *P. aeruginosa* is the well-studied up to date, its role in biofilms development is still debatable. We will discuss the roles of QS in both planktonic and biofilms stage, as well as regulation of this complex network system.

1.3 QS in *P. aeruginosa*

The early evidence that showed bacteria respond to environmental cues was described in the early 1970s, where *Vibrio harveyi* activates the luminescence genes (*lux*) in the late exponential and early stationary growth phase.⁶⁵ Further studies on *Vibrio fischeri*, *E. coli*, and many more organisms revealed that bacteria produced signal molecules termed as autoinducer, which accumulates in the environment and upon reaching certain threshold, they bind to the receptor and the complex would then bind to DNA-binding regions of several other transcription factors.⁶⁶ Ever since its discovery, the cell-density dependent phenomena, which is now termed as QS, has gained a lot of attention considering the wide extent of how QS governs sociomicrobiology behaviors of bacteria in the context of pathogenicity, biofilms formation, and host immune response.

QS regulation

P. aeruginosa utilizes acyl homoserine lactones (AHLs) as signal molecules, which comprises the LuxR-type homologs LasRI and RhlRI. LasI synthase is responsible for the synthesis of *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C12-HSL), which will bind to its receptor LasR. The 3-oxo-C12-HSL-LasR complex would then bind to the conserved DNA sequence in the promoter region (*lux* box) and subsequently activate genes expression under QS control, including its synthase LasI which will complete the

positive regulation feedback for its induction. The *las* system also positively regulates *rhl* system, where RhII directs the synthesis of *N*-butanoylhomoserine lactone (C4-HSL) that would bind to its receptor RhIR.⁶⁷ The *rhl* system is also positively autoregulated upon activation of C4-HSL-RhIR complex. The third signaling molecule is non-AHL and 2-alkyl-4-quinolones (AQs) based system called “pseudomonas quinolone signal” (PQS), which is intertwined between the *las* and *rhl* systems.⁶⁸ PQS is synthesized from its precursor 2-heptyl-4-hydroxyquinoline (HHQ), which is under *pqsABCDE* operon. Under aerobic conditions, it requires *pqsH* via oxidation of HHQ. The PQS system is under positive regulation of PqsR, which is known to regulate *pqsABCDE*-phnAB expression.⁶⁹

The *las* system is often regarded on top of the hierarchy, which controls a large regulon of downstream genes, including the *rhl* and PQS system. Nonetheless, the *rhl* system could also inhibit the expression of *pqsR* and *pqsABCD*, which encodes PQS precursor biosynthesis pathways. The PQS-PqsR complex could sequentially activate *rhlRI* and connect the whole signaling loop. Lastly, the fourth signal molecule called Integrative Quorum Sensing Signal (IQS) has been reported, which could overtake the central *las* system under phosphate depletion condition⁷⁰. The high degree of interconnectivity and complex signaling pathways shows how QS is tightly modulated in *P. aeruginosa* to ensure a correct balance of signal molecules and precise regulation of QS-dependent genes (Figure 1-6).

Figure 1-6 QS network in *P. aeruginosa*. There are currently reported four QS pathways, such as *las*, *rhl*, *pqs* and IQS. The structure of signal molecules are shown respectively. The autoinduction is shown in red arrows, whereby black arrows show positive or negative regulation. Image was taken from Ref. 71.

QS controls 5-10% of the *P. aeruginosa* genome, which in turn regulates virulence factor expression, biofilms formation, type III secretion system (T3SS), colonization of host, and many more.¹⁴ The *las* system activates transcription of genes responsible for virulence such as *lasB* encoding elastase, protease *apr*, and exotoxin A *toxA*.⁷² Elastase, also called LasB protease and pseudolysin, is a major elastolytic zinc-metalloprotease and the most abundant secreted proteases of *P. aeruginosa*.⁷³ The *rhl* system controls the production of several exoproducts like elastase, rhamnolipids, hydrogen cyanide, phenazine and pyocyanin. Rhamnolipid was also demonstrated to cause rapid necrotic killing of polymorphonuclear (PMNs) leukocytes, which serves as a mechanism for immune evasion and a prerequisite for the development of chronic *P. aeruginosa* infections.⁷⁴ Rhamnolipid has also been reported to facilitate swarming motility⁷⁵ and affect biofilm architecture.⁷⁶ The PQS system regulates genes associated with virulence factors like elastase, pyocyanin, hydrogen cyanide, PA-IL lectin and rhamnolipids. Pyocyanin (PCN) is a phenazine redox-active toxic metabolite and readily recovered from the sputum of CF patients.⁷⁷ Virulence of *P. aeruginosa* during airway infections requires PCN biosynthesis and plays a critical role in *P. aeruginosa* infection.

To date, several regulatory systems have been reported to control QS gene expression in *P. aeruginosa* (Table 1-3). The two-component system (TCS) GacA/GacS regulates a large set of acute and chronic virulence-associated genes (including QS, T3SS, T6SS, biofilms formation, motility) through its control over small RNAs (sRNAs) RsmY/RsmZ.⁷⁸ The RsmY/RsmZ represses global posttranscriptional regulator RsmA, which in turn represses the production of AHL signal molecules. The other sRNAs like PrrF1, PrrF2, and PhrS control PQS biosynthesis pathways through base-pairing mechanism to activate the open reading frame where *pqsR* is translationally coupled. The PhrS activates PqsR in oxygen-limiting condition and requires oxygen responsive regulator ANR,⁷⁹ while PrrF1 and PrrF2 respond through iron-limiting condition and under the regulation of *fur* (ferric uptake regulator).⁸⁰

Table 1-3 Super-regulators of QS in *P. aeruginosa*¹⁵

Regulator	Mechanism of action
AlgR2	Negative transcriptional regulator of <i>lasR</i> and <i>rhlR</i>

DksA	Negative transcriptional regulator of <i>rhlI</i>
GacA/GacS	Positive transcriptional regulator of <i>lasR</i> and <i>rhlR</i>
MvaT	Negative transcriptional regulator (global regulation)
QscR	Negative regulator of LasR
QslA	Negative regulator of LasR and PqsR
QteE	Negative post-translational regulator of LasR and RhlR
RpoN	Negative transcriptional regulator of <i>lasIR</i> and <i>rhlIR</i>
RpoS	Negative transcriptional regulator of <i>rhlI</i>
RsaL	Negative transcriptional regulator of <i>lasI</i>
RsmA	Negative transcriptional regulator of <i>lasI</i>
Vfr	Positive transcriptional regulator of <i>lasR</i> and <i>rhlR</i>
VqsR	Positive transcriptional regulator of <i>lasI</i>

QS and environmental responses

P. aeruginosa can sense and respond to various environmental cues when subjected to different environmental stress conditions (i.e. low phosphate, starvation, hypoxia, and iron depletion). There have been numerous pieces of evidence that environmental triggers can influence QS system. For instance, IQS is activated under phosphate depletion conditions and can overtake the *las* system. The PQS system is also activated, which further enhances the expression of virulence factors production like rhamnolipids, phenazines, pyochelin, pyoverdine, cyanide, exotoxin A and LasA protease. A high killing rate was observed in low phosphate medium using *Caenorhabditis elegans* and mouse models, which was attributed to phosphate signaling (PhoB), the MvfR–PQS pathway of quorum sensing, and the pyoverdine iron acquisition system.⁸¹ The PQS-Fe(III) complex was lethal to the worms and resulted in the “red death” phenomenon, which demonstrates the cross-talk between phosphate, iron acquisition and QS in *P. aeruginosa*. The PQS molecule also serves as an iron trap, which would subsequently be internalized into the cells by the siderophores.⁸² In low iron condition, the Fur-dependent sRNAs genes *prfF1* and *prfF2* expression was de-repressed, and they inhibit expression of *antABC* genes encoding anthranilate degradation enzymes AntABC, precursor of PQS.⁸³ Overall, this leads to increased concentration of HHQ and PQS and illustrates how iron depletion could modulate QS and bacterial virulence through the PQS system in *P. aeruginosa*.

Under anaerobic condition, hydrogen cyanide biosynthesis genes *hcnABC* were activated by anaerobic regulator ANR as well as LasR and RhlR.⁸⁴ Hydrogen cyanide (HCN) is a potent virulence factor that could cause cellular respiration arrest and potent

inhibitor of cytochrome *c* oxidase and metalloenzymes. ANR has been reported to be important co-regulator of QS-regulated genes under anaerobic condition.⁸⁵

Under starvation stress, the stringent response is activated via ppGpp (penta- and tetra-phosphorylated guanosine), which coordinates physiological adaptation under nutritional starvation.⁸⁶ The stringent response has been associated with virulence, resistance to UV radiation, regulation of oxidative stresses, and antibiotic tolerance in biofilms in *P. aeruginosa*. ppGPP is synthesized by stringent response protein RelA and SpoT, which further activate early transcription of *las* and *rhl* system that is premature and independent of cell-density.⁸⁷ ppGpp has also been reported to modulate QS, and negatively regulates the biosynthesis of HHQ and PQS signals. It is also required for the full expression of both *las* and *rhl* system.⁸⁶

QS and biofilms

Several studies have reported the importance of QS genes in the biofilm mode of growth.^{18, 88} In *lasI* and *lasIrhII* double mutants, where neither of the QS signals is present, thin biofilms are formed and they appeared to be undifferentiated. However, upon addition of synthetic 3-oxo-C12-HSL, the architecture of the normal biofilms could be restored. Davies *et al.* showed that QS did not play many role in the initial stage of attachment and growth, where cells are still freely swimming.¹⁸ It is during microcolonies development, that cells reach higher population density and QS activates the biofilms growth and maturation in a coordinated manner.

The architecture of *P. aeruginosa* biofilms depends on the interconnectivity of the components inside the EPS matrix and the biochemical processes occur within it. QS has been reported to regulate the release of eDNA, which plays diverse roles to support the formation and development of the biofilm. Using mutant strains that are deficient in the QS pathways (*lasIrhII*, *pqsA*, *pqsL*), Holm *et al.* observed that the biofilms formed by the mutants contained less eDNA than normal biofilms, and also were more susceptible to treatment with detergent like sodium dodecyl sulphate (SDS).²² Whitchurch *et al.* have shown that treatment with DNase can abolish young biofilms of *P. aeruginosa*.²⁴ Pyocyanin, virulence factors under PQS regulation in *P. aeruginosa*, has been shown to intercalate with DNA nitrogenous base pair regions and increase the DNA solution viscosity.⁸⁹ Viscoelasticity is one of the key properties associated with the biofilm under infection settings, like the sputum of cystic fibrosis (CF) patients.

DNA has also been known as a conductive biopolymer. It has been reported that pyocyanin enhances the electron transfer along the DNA molecule.⁸⁹ This could help cells to maintain a basal rate of respiration for energy harvesting and maintain cellular redox homeostasis in the biofilms.

On top of that, the *rhl* system presented multiple roles in biofilms formation in *P. aeruginosa* through the control of swarming and twitching motilities, as well as rhamnolipids and lectins production.⁹⁰ The swarming motility is responsible for surface motility during the early stage of biofilms development.⁹¹ Strains with active swarming form flat and uniform biofilms, whereby strains with limited swarming form structured biofilms. Type IV pili, which is important for the adherence to and colonization of eukaryotic cell surfaces, is required for twitching motility. Using time-lapse microscopy, O'Toole and Kolter showed that flagella-mediated motility is important for irreversible attachment, and type IV pili is required for microcolonies formation.⁹² The twitching motility, which is mediated by type IV pili, is regulated by the *rhl* system under iron-limited medium.⁹³ The studies overall further establish the important regulatory link between QS and iron in biofilms formation of *P. aeruginosa*.

Rhamnolipids, major virulence factors under control of the *rhl* system, contribute to biofilms development through several roles. They are required to form microcolonies in the initial phase and facilitate migration-dependent structural development of three-dimensional mushroom-shaped in *P. aeruginosa* biofilms.^{88b} Rhamnolipids are also required to maintain water channels surrounding microcolonies through intercellular interaction, which are important for biofilms architecture.⁷⁶ In *rhlA* and *rhlAB* mutants, where rhamnosyltransferase whose function in rhamnolipid synthesis is absent, flat and unstructured biofilms are formed and it was proposed that the biosurfactant could affect the cell-to-cell and cell-to-surface interactions.^{76, 88a} Lastly, rhamnolipid-mediated dispersals have also been demonstrated, where strains producing excess rhamnolipids exhibit hyper-detaching properties.^{88c}

QS has also been linked to the production of glucose-rich exopolysaccharide (PEL) that builds the biofilm matrix through the *las* system. It was proposed that LasIR positively regulates the expression of the tyrosine phosphatase TpbA, which dephosphorylates DGC TpbB and leads to decreased c-di-GMP production. This resulted in decreased expression of *pel* genes, which is essential polysaccharide matrix formation, and

reduced biofilms formation.⁶⁴ This is the first study that connects QS, c-di-GMP, and biofilms formation in *P. aeruginosa*.

1.4 Interfering with QS as antivirulence therapy

With the ever-increasing threat of antimicrobial resistant (AMR) strains and slow development of new antibiotics, the future of healthcare seems bleak. Major pharmaceutical companies have withdrawn their antibiotics research projects, leaving only Merck, Roche, GlaxoSmithKline, and Pfizer with ongoing antibiotic programs.⁹⁴ With only 12 antibiotics have been approved since 2000,⁹⁵ it is quite a paradox reality. Scientists are now trying to look for alternatives to tackle the superbug threat, which could kill as much as 10 million people by 2050 if left unresolved.

It has been proposed that QS interference could serve as an alternative therapy to treat bacterial infections.^{16,96} Since the bacteria growth is not inhibited and the virulence traits are diminished, there would be milder selective pressure for antimicrobial resistance development. Hentzer *et al.* demonstrated how halogenated furanone compound from marine alga *Delisea pulchra* could reduce virulence and increase susceptibility towards tobramycin clearance of *P. aeruginosa* biofilms using mouse pulmonary infection model. By blocking QS and attenuate bacterial virulence, the compounds could reduce bacterial counts in the lung and promoted clearance by the immune system.¹⁶

There are two possible pathways for chemical attenuation of bacterial virulence, either by interfering with the QS signaling pathways (signal generator or receptor), or intercepting with the signal molecules (AHL)⁹⁷. Enzymes that inactivate QS signals are called quorum quenching enzymes (QQE), while chemicals that disrupt QS pathways and reduce the expression of QS-controlled genes are called quorum sensing inhibitors (QSI)⁹⁸. Dong *et al.* first demonstrated how QQE could be used to control bacterial infections in plants.⁹⁹ The enzyme encoded by *aiiA* gene isolated from Gram-positive *Bacillus* species is capable of inactivating AHL signals through hydrolysis of the ester bond of the homoserine lactone ring and quench QS signaling. Given that many bacteria employ QS systems, compounds targeting QS could be used in many fields, such as medicine, agriculture, industrial membrane bioreactors, aquaculture and crop production^{98, 100}.

Natural food sources have also shown promising QSI properties. Taking inspiration from foods with health-promoting effects, Jakobsen *et al.* discovered iberin, an active ingredient from horseradish, could effectively target *las* and *rhl* QS systems in *P. aeruginosa*.¹⁰¹ They also identified ajoene derived from garlic to inhibit genes controlled by QS. Further DNA microarray studies revealed that ajoene targets sRNAs RsmY/Z and demonstrated promising *in vitro* and *in vivo* effects in promoting the clearance of bacteria. Mice studies using pulmonary and peritoneal silicone implant models showed ajoene promoted faster clearance of bacteria as compared to control groups.¹⁰² It is currently in development for clinical trial by the company Neem Biotech. Wu *et al.* showed that ginseng extract (0.5 – 2.0%) could prevent biofilms formation and destroy 7-day old mature biofilms of *P. aeruginosa*. The ginseng extract did not affect the growth of bacteria, but could enhance swimming, twitching and reduced swarming motility. The mode of action is still unclear, but ginseng treatment has seen promising therapeutic results on chronic *P. aeruginosa* lung infections, and potential therapy for *P. aeruginosa* biofilms infections.¹⁰³

Tan *et al.* utilized structure-based virtual screening and molecular docking approach to identify five structurally unrelated QSI of *P. aeruginosa* from natural compounds. Among the promising compounds is G1, which reduced the expression of several quorum-sensing-regulated genes, and also eDNA release, which is important for *P. aeruginosa* biofilms architecture.¹⁰⁴ Recently, terein, which was identified from *Aspergillus terreus*, was found to reduce QS-regulated virulence factors as well as biofilms formation via antagonizing QS receptors.¹⁰⁵ Interestingly, this compound also reduced intracellular c-di-GMP levels by decreasing the activity of DGC. The compound also showed antivirulence effects in *C. elegans* and mouse model. It is the first dual inhibitor that decreases QS signaling and c-di-GMP metabolism in *P. aeruginosa*.

Some antibiotics have been reported to have QSI activity in addition to their antimicrobial activities, such as macrolide azithromycin, the β -lactam ceftazidime and the fluoroquinolone ciprofloxacin.¹⁰⁶ Azithromycin (AZM), one of the macrolide classes of antibiotics, has been reported to inhibit quorum sensing circuit at sub-MIC concentration and inhibit virulence and biofilm formation.¹⁰⁷ Beneficial effects were

observed in pulmonary infections and CF patients in many clinical trials data upon treatment of low-dose of AZM (2 ug/mL).¹⁰⁸

Synthetic QSIs

With the battles against bacterial resistance in modern healthcare settings, development of novel compounds to treat persistent bacterial infections remain an active, growing research area with tremendous potentials await to be discovered. Many scientists take inspiration from nature in the hope to find the new antimicrobial compounds. Hentzer *et al.* constructed halogenated furanone compounds, and identified furanone C-30 as promising QSI of *P. aeruginosa*. The synthetic furanone could block QS, reduce virulence factors, and promote bacteria detachment in *P. aeruginosa* biofilms.¹⁶ Recently, our group also reported synthetic disulfide compounds, which bear similar structure as ajoene with much potent IC₅₀ values against *P. aeruginosa* QS bioreporter strains.¹⁰⁹ The compounds were able to reduce major QS-regulated virulence factors production and successfully inhibit the establishment of *P. aeruginosa* biofilms in implant-associated infection in the mice model.

Solid-phase synthesis has also yielded several successful derivatives of QSIs. Geske *et al.* reported a microwave-assisted solid-phase synthetic route to generate natural and unnatural AHLs. Due to their structural similarity to AHL, the compounds were able to inhibit LasR and also biofilms formation of *P. aeruginosa*.¹¹⁰ Hansen *et al.* also reported several triazole-containing AHL derivatives synthesized from the combination of solution and solid-phase synthesis methods. In the study, they identified 13 compounds with antagonistic LasR activity, and 9 compounds with agonistic activity.¹¹¹ The findings highlight the potential utility of combinatorial methods to discover novel small molecules as modulators of QS.

To date, there have been numerous findings of synthetic QSIs that could block virulence and biofilms formation.¹¹² O'Loughlin *et al.* reported a new class of compounds meta-bromo-thiolactone (mBTL) that inhibits both LasR and RhIR.¹¹³ The compounds efficiently reduced pyocyanin production as well as biofilms formation, and improved survival in *C. elegans* model. Lu *et al.* reported the antagonists of PqsR, which could efficiently repress the production of pyocyanin but not so much on elastase and rhamnolipid.¹¹⁴ Our group has also recently identified itaconimide as an important

scaffold for QSI. The itaconimide analogs could target multiple QS pathways and reduce virulence factors production, and also successfully eradicated biofilms formation when used in combination with tobramycin. Interestingly, its stereoisomer citraconimide did not show any QSI activity.¹¹⁵ The representative summary of synthetic QSIs can be found in Figure 1-7.

Figure 1-7 Representative of natural and synthetic QSIs discovered in the recent years.

On top of that, compounds targeting antivirulence signaling could also be used in combination with other well-known or novel antibiotics to enhance the antimicrobial property and promote clearance of biofilms. For instance, biofilms grown in the presence of furanone C-30 were more susceptible to treatment with 0.1% SDS as well as tobramycin, as compared to non-furanone-treated biofilms.¹⁶ Chua *et al.* also demonstrated a combination treatment between macrolide erythromycin and colistin

could eradicate and repress the development of antibiotic-resistant subpopulations.¹¹⁶ The overall findings suggested potential therapies targeting QS mechanism to combat biofilm-associated infections.

1.5 Current research gaps and objectives of study

Till date, non-native AHLs remain the most extensively studied class of synthetic QSIs,¹¹⁷ with several SAR studies been carried out around AHLs backbone and halogenated furanones.^{112, 117} However, the AHL is susceptible to hydrolysis at physiological pH and degradable by mammalian lactonases,¹¹⁸ and the halogenated furanones suffer from high toxicity.¹¹⁹ The resulting side products are either inactive against QS or toxic, thus limiting the use of such molecules as pharmaceutical agents.¹²⁰ Nevertheless, the hunt for novel scaffold important for the treatment of infectious diseases is still ongoing. The exploration of new compound classes that are structurally distinct from native AHLs is needed to ultimately deliver clinical candidates. Issues such as toxicity, stability under aqueous conditions, carcinogenic property need to be addressed.

Despite significant progress in the field of drug discovery in QSI and anti-biofilm compounds, we are yet to witness the application of such compounds in practical settings. There are still some outstanding questions in the quest to search for a new class of compounds as modulators for QS. What type of QSI should be developed? High-affinity noncompetitive inhibitors of LuxR-type proteins, competitive inhibitors, or irreversible inhibitors?¹²¹ And which type would be more useful *in vivo*? How specific should the QSIs be? Are there any limitations in developing one specific type of inhibitor (i.e. LasR inhibitor)? What type of compounds should be screened for QSI activity? And will there any rooms to allow *de novo* designs that are structurally distinct from existing scaffolds? This thesis aims to address such questions through chemical biology approaches.

Objectives and thesis organization

In chapter 2, the concept of combining QQE and QSI to target multiple QS pathways in *P. aeruginosa* will be introduced. Bacteria often employ multiple QS pathways, as have been seen in *P. aeruginosa* as the model organism. This poses a challenge as most compounds developed are highly specific targeting one pathway.¹²² Both QQE and QSI

have been studied independently, each with their own advantages and drawbacks. This study will be our proof-of-concept to demonstrate the importance of developing compounds with broad targets by combining two QS interfering agents with different mechanism of actions in suppressing multiple QS pathways. This work has been published in Scientific Reports, titled “Combination Therapy Strategy of Quorum Quenching Enzyme and Quorum Sensing Inhibitor in Suppressing Multiple Quorum Sensing Pathways of *P. aeruginosa*”.¹²³

In chapter 3, a drug-repurposing approach was utilized to identify potential antivirulence drugs that could target multiple QS pathways. Screening of chemical library consisted of marketed US Food and Drug Administration (FDA)-approved compounds was performed against QS bioreporter strains. Several promising compounds that could suppress QS-activated virulence phenotypes in *P. aeruginosa*, including elastase, pyocyanin and rhamnolipid production, were identified. In addition, the efficacy of the compounds *in vivo* was evaluated using mouse implant-biofilm infection.

In chapter 4 and 5, I will focus on one promising class of compounds identified from the screening. A small focused library was constructed as part of SAR study to develop a new series of metal compounds analogs, which exhibit excellent QSI activities and reduced toxicity to murine macrophages. Lastly, the mechanism study of the compounds was evaluated using system biology and adaptive evolution approach. The overall findings highlight the utility of various chemical biology approaches to identify novel antivirulence lead compounds for *P. aeruginosa* infections.

2 COMBINATION THERAPY STRATEGY OF QUORUM QUENCHING ENZYME (QQE) AND QUORUM SENSING INHIBITOR (QSI) IN SUPPRESSING MULTIPLE QUORUM SENSING PATHWAYS OF *P. AERUGINOSA*

In this study, the concept of combining QQE and QSI was explored to disrupt both *las* and *rhl* AHL signaling and signal reception capacities, and reduce the pathogenicity of *P. aeruginosa*. The presence of multiple QS systems in individual bacterial species poses a challenge in QS interference strategy since multiple QS systems might have different induction dynamics during the bacterial growth. The two AHL-QS systems in *P. aeruginosa* are also highly adaptable and capable of responding to changing environmental stress conditions.^{70, 124} Combinational therapy could provide multiple points of attack to broaden the coverage and completely block the QS systems, which could significantly attenuate the survival of *P. aeruginosa* under stress conditions.¹²⁵ The two classes of QS disrupting agents have been studied independently, each with their own advantages and drawbacks. Small molecules as QSIs have well-known chemical structures, which in turn would allow structural activity and relationship (SAR) study and biological activity modification (i.e. pharmacodynamics and pharmacokinetics properties). The molecules can also diffuse into the cells and target the receptors, in contrast to QQE that act extracellularly to degrade AHLs.¹²⁶ For this study, we used G1 as QSI, which is a specific inhibitor to the LuxR-type receptor of *P. aeruginosa*.¹⁰⁴ Because of their distinct molecular structures and functional mechanisms, it would be interesting to explore the possible combination effect of QQE and QSI molecule in suppressing multiple *P. aeruginosa* QS pathways.

2.1 Materials and Methods

2.1.1 Bacterial strains and media

Bacteria were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). Media used for biological assay was ABTGC (AB minimal medium supplemented with 0.2% glucose and 0.2% casamino acids) to minimize fluorescence interference¹⁰⁹. Bacterial strains used in this study are shown in Table 2-1.

Table 2-1 Bacterial strains used in this study

Strains	Relevant genotype and/or characteristics
PAO1	<i>P. aeruginosa</i> wild type ¹²⁷

PAO1- <i>gfp</i>	GFP-tagged PAO1 ¹²⁸
PAO1- <i>lasB-gfp</i>	PAO1 containing <i>lasB-gfp</i> (ASV) reporter fusion ¹²⁷
PAO1- <i>pqsA-gfp</i>	PAO1 containing <i>pqsA-gfp</i> (ASV) reporter fusion ¹²⁸
PAO1- <i>rhlA-gfp</i>	PAO1 containing <i>rhlA-gfp</i> (ASV) reporter fusion ¹²⁸
PAO1 Δ <i>lasI</i> Δ <i>rhlI</i>	PAO1 QS deficient <i>lasI</i> and <i>rhlI</i> double mutant ¹⁶
PAO1 Δ <i>lasI</i> Δ <i>rhlI</i> - <i>lasB-gfp</i>	PAO1 <i>lasI</i> and <i>rhlI</i> mutant containing <i>lasB-gfp</i> (ASV) reporter fusion ¹²⁹
PAO1 Δ <i>lasI</i> Δ <i>rhlI</i> - <i>rhlA-gfp</i>	PAO1 <i>lasI</i> and <i>rhlI</i> mutant containing <i>rhlA-gfp</i> (ASV) reporter fusion ¹²⁹

2.1.2 Expression and purification of QQ enzyme AiiA

The gene coding for AiiA was cloned into pET-47b(+) vector (BamHI-HindIII sites). The expression vector was transformed into *E. coli* BL21(DE3) competent cells (New England Biolabs, USA). The cells were grown in 2L of LB media supplied with 35 mg/L Kanamycin at 37°C. The expression of the protein was induced with 0.5 mM of Isopropyl β -D-1-thiogalactopyranoside (IPTG) when OD₆₀₀ reached 0.6. The cells were grown overnight at 18°C. The harvested cells were resuspended in 50 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% (v/v) CHAPS, 10 % (v/v) glycerol) and lysed by passing the homogenized cells through an Emulsiflex-C3 (Avestin, USA) high-pressure apparatus at 15,000 psi three times. The cell lysate was centrifuged at 25,000 g for 25 min. The supernatant was then applied to Ni-NTA gravity column (Bio-rad) equilibrated with lysis buffer. After extensive washing with lysis buffer, the bound proteins were eluted with lysis buffer containing increasing concentration of imidazole (0 - 300 mM). The eluted fractions were analyzed with 15% of SDS-PAGE and fractions containing the desired protein were pooled and dialyzed against lysis buffer. The final concentration of the protein was measured using Bradford Assay.

2.1.3 Enzymatic assay of AiiA

The 3-oxo-C12-HSL hydrolysis activity of AiiA was tested with 500 μ M 3-oxo-C12-HSL, 30 μ M AiiA in reaction buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.0). After 30 min of reaction at 30°C, the reaction mixture was monitored at 215 nm by analytic C18 reverse phase HPLC column (Jupitar, 5 μ , 300Å, 250x4.6 mm) with a flow rate of 0.5 mL/min (Gradient: 0-100% buffer B (90% acetonitrile, 10% H₂O, 0.05% TFA) in buffer A (100% H₂O, 0.05% TFA) for 50 min).

2.1.4 Model of LasR/I circuit

The models in this study simulated batch cultures according to the experimental setup. The LasR/I QS circuit of *P. aeruginosa* is shown in Supplementary Figure 1. QSI binds to LasR similarly as AHL, but in this case only AHL can stabilize the LasR¹³⁰. Since our focus of the present study was whether QQ enzyme and QSI have synergistic effect in inhibiting QS, some complex features in the QS network were simplified to make the model easier to implement and reduce computational cost. For instance, the interactions of LasR/I QS circuits with other cellular components, such as the binding of 3-oxo-C12-HSL to RhlR¹³¹ were not included in the network studied in this work. Both heterogeneity and asynchronization of cells were beyond the scope of the modeling in this work. The final component concentrations of cells in batch culture were adapted in the computation of AHL concentration. As the response time of QS switching is much faster than the time required for culture growth¹³², the final component concentrations can be approximated by the stationary concentrations with the final cell volume fraction ρ . The AHL concentration was considered to be homogeneous inside and outside cells due to its large diffusion coefficient¹³³. Vfr was assumed to be some large enough constant since it is normally expressed in experimental strains of this study. QSI concentration was taken as a constant considering its relative big value. A maximum concentration of AHL $A_{\max}$ was set in the model to avoid very large concentrations of components caused by the accumulation of stable AHL in batch cultures¹³⁴. The reactions of QS network are shown in Table 2-2. QQ enzyme and QSI are written as Q_Q and Q_I to avoid confusion when necessary.

Table 2-2 Biochemical reactions in *P.aeruginosa* LasR/I circuit.

Reaction	Description	Rate
$I \rightarrow I + A$	Production of A	$\frac{V_A I}{K_A + I}$
$A \rightarrow \text{null}$	Natural decay of A	$d_A A$
$R + A \rightarrow P$	Combination of R and A	$k_{RA} R A$
$P \rightarrow R + A$	Dissociation of P	$d_P P$
$R \rightarrow \text{null}$	Decay of LasR protein	$d_R R$
$r \rightarrow r + R$	Translation of lasR mRNA	$k_r r$
$\text{null} \rightarrow r$	Transcription of lasR	$r_0 + V_r Z \left(\frac{1 - e^{-\beta V}}{K_{r1} + Z} + \frac{e^{-\beta V}}{K_{r2} + Z} \right)$
$r \rightarrow \text{null}$	Decay of lasR mRNA	$d_r r$
$i \rightarrow i + I$	Translation of lasR mRNA	$k_i i$

$I \rightarrow \text{null}$	Decay of LasI protein	$d_I I$
$\text{null} \rightarrow i$	Transcription of lasI	$i_0 + \frac{V_i Z}{K_i + Z}$
$i \rightarrow \text{null}$	Decay of lasI mRNA	$d_i i$
$2P \rightarrow Z$	Dimerization of P	$k_Z P^2$
$Z \rightarrow 2P$	Dissociation of Z	$d_Z Z$
$R + Q_I \rightarrow F$	Combination of R and QSI	$k_{RQ} R Q_I$
$F \rightarrow R + Q_I$	Dissociation of F	$d_F F$
$F \rightarrow Q$	Degradation of R in F	$d_R F$
$A \rightarrow \text{null}$	Degradation of A by QQ	$\eta(Q_Q) A$

The ordinary differential equations of the QS network are listed in equations 1 to 8. Stationary QS components were solved using the steady state condition. When there are multiple stable stationary solutions, the state with smallest concentrations was chosen as the outcome presented in this work. The parameters of *P. aeruginosa* LasR/I QS circuit were firstly estimated from reported values in the literature, then optimized to enable the switching behaviour of the QS network observed in experiments (Supplementary Table S1)^{71, 132}.

$$\frac{dA}{dt} = \rho \frac{V_A I}{K_A + I} + \rho d_P P - \rho k_{RA} R A - d_A A - (1 - \rho) \eta(Q_Q) A \quad 1$$

$$\frac{dR}{dt} = k_r r + d_P P - k_{RA} R A - d_R R + d_F F - k_{RQ} R Q_I \quad 2$$

$$\frac{dF}{dt} = k_{RQ} R Q_I - d_F F - d_R F \quad 3$$

$$\frac{dr}{dt} = \frac{r_0}{V_b} + \frac{V_r Z}{V_b} \left(\frac{1 - e^{-\beta V}}{K_{r1} + Z} + \frac{e^{-\beta V}}{K_{r2} + Z} \right) - d_r r \quad 4$$

$$\frac{dP}{dt} = k_{RA} R A - d_P P + 2d_Z Z - 2k_Z P^2 \quad 5$$

$$\frac{dZ}{dt} = k_Z P^2 - d_Z Z \quad 6$$

$$\frac{di}{dt} = \frac{i_0}{V_b} + \frac{V_i}{V_b} \frac{Z}{K_i + Z} - d_i i \quad 7$$

$$\frac{dI}{dt} = k_i i - d_I I \quad 8$$

2.1.5 Reporter gene assay

Stock solution of G1 was prepared by dissolving appropriate amount of chemicals in DMSO to make final concentration of 10 mM, aliquoted into small Appendorf tube and stored at -20°C until further usage. The compound was then dissolved in ABTGC

medium to the working concentration and 100 μ L of this solution was pipetted into first rows of 96-well microtiter dish (Nunc, Denmark). Two-fold serial dilution was made to the rest of the rows, leaving the last two rows empty for blank and solvent control. Next, 50 μ L of AiiA diluted in ABTGC media was added into each well. Overnight culture of *P. aeruginosa* reporter strain PAO1-*lasB-gfp* was diluted to optical density at 600 nm (OD_{600}) of 0.02 (approximately 2.5×10^8 CFU/mL). 100 μ L of the bacterial suspension was added to each well and the plate was incubated for at least 16 hours at 37°C. GFP fluorescence (excitation at 485 nm, emission at 535 nm) and OD_{600} readings were recorded every 15 mins using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). For the IC_{50} value calculation, it was determined at the time point where inhibition started to occur (between 4-6 hours) using GraphPad Prism 6 software. All assays were done in triplicate manner.

2.1.6 Rhamnolipid quantification assay

Rhamnolipid production was quantified using Orcinol according to original protocol by Koch *et al.* with modifications.¹³⁵ Subculture of *P. aeruginosa* was diluted 100 times from overnight culture into fresh ABTGC medium ($OD_{600} = 0.1$). Treated cultures were added with test compounds to give final concentration of 10 μ M. The cultures were grown for 24 hours at 37 °C, shaking condition (200 rpm). The final cell density was measured at 600 nm (OD_{600}) using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). Supernatants were collected by centrifuging at 10,000 rpm for 10 mins and extracted with diethyl ether (twice). Organic fractions were concentrated to yield white solids. It was then re-suspended in deionized water and added with 0.19% (w/v) orcinol in 50% H_2SO_4 . The resulting mixture was incubated at 80 °C for 30 mins to give yellow-orange solution. After cooling to room temperature, the absorbance was measured at 421 nm and results were normalized with final OD_{600} values.

2.2 Results

2.2.1 Mathematical modeling shows combination effects between QQE and QSI on LasR/I circuit.

When only QQE was present, simulation results showed a switching behavior between two steady states of low and high levels of AHL concentration (Figure 2-1A). The AHL degradation rate by QQE is represented by $\eta(QQ)$. When QQE concentration is low and $\eta(QQ)$ is small, the stationary AHL concentration is high. However, when $\eta(QQ)$

exceeds a threshold ($2.8 \times 10^{-4} s^{-1}$), the stationary AHL concentration suddenly decreases to an insignificant value. Similar switching behaviors have been observed in the simulation response curves of QS components to population size,¹³⁶ cell volume fraction,¹³¹ or to external AHL concentration.¹³⁷ Switching behaviors of QS networks have also been observed experimentally at individual cell level.^{71, 132}

When only QSI was present, similar switching behavior was observed and shown in Figure 2-1B. As QSI and AHL bind to LasR competitively, the inhibiting effect of QSI is less efficient. Irreversible QSIs like halogenated furanones¹³⁸ that induce degradation of the LasR receptor protein can inhibit QS more effectively (simulation data not shown). When combined, QQE and QSI can enhance the inhibiting effects of each other (Figure 2-1C and D). 0.5 μM QSI alone has very little effect, but it can reduce the minimum QQE rate required to turn off QS up to four-fold. Similarly, adding a small amount of QQE ($\eta(QQ) = 0.5 \times 10^{-4} s^{-1}$) can reduce the minimum QSI concentration required to turn off QS up to 20-fold. The stationary AHL concentration decreased as compared to single treatment using QSI.

Figure 2-1 Simulation results of stationary AHL concentration to QQ and QSI. (A) $\eta(QQ)$, (B) QSI, (C) $\eta(QQ)$ at different QSI concentrations, and (D) QSI at different $\eta(QQ)$ values.

3D plot of stationary AHL to $\eta(QQ)$ and QSI is shown in Figure 2-2A. A clear boundary between QS on and off states was observed, which is shown in Figure 2-2B. This boundary curve is “U”-shaped which means QQE and QSI have a synergistic effect in inhibiting QS¹³⁹. $\eta(QQ)$ is assumed to be proportional to QQE in this simulation. However, if other enzymatic dynamics such as the Michaelis–Menten equation¹⁴⁰ was used, $\eta'(QQ) > 0$ and $\eta''(QQ) \leq 0$ are satisfied. If we change the $\eta(QQ)$ axis to QQE in Fig. 2-2B, the curve will still be “U”-shaped and the conclusions of the simulation will remain the same.

Figure 2-2 Simulation QS states to QQ and QSI. (A) 3D stationary AHL concentration to $\eta(QQ)$ and QSI, (B) 2D map of QS on and off states to $\eta(QQ)$ and QSI.

2.2.2 Combination treatment of QSI and QQE inhibits *las* and *pqs* expression of *P. aeruginosa* bioreporter strains.

To validate the mathematical modeling results, the combination effects of QSI and QQE were tested using *P. aeruginosa* QS bioreporter strain PAO1-*lasB*-*gfp*.¹⁴¹ In this study, AiiA was chosen as QQE in combination with G1, a small molecule of QSI which binds to LasR and RhIR¹⁰⁴ as the models (Figure 2-3A). The elastase encoding *lasB* gene is controlled by LasR¹⁴², hence any reduction in the fluorescence signals would indicate the presence of 3-oxo-C12-HSL antagonist. Both G1 and AiiA were tested at different concentration gradients to generate the dose-dependent curves, which are used to calculate the IC₅₀ values. Most importantly, in support of the non-growth inhibitory antimicrobial principle¹⁶, neither G1 nor AiiA affected the growth rate of the bacteria

(Supplementary Figure 2). The growth measured as OD₆₀₀ was used as control of the non-growth inhibitory concept.

Combination effects of G1 and AiiA on *lasB-gfp* expression are summarized in Figure 2-3. The effects were also observed in dose-dependent manner (Figure 2-3C, Supplementary Fig. 3). Both G1 and AiiA inhibited *lasB-gfp* expression in dose-dependent manner with IC₅₀ values of $1.36 \pm 0.08 \mu\text{M}$ and $6.88 \pm 0.46 \mu\text{g/ml}$ respectively. Promising results were obtained in combinational therapy of AiiA and G1, where the IC₅₀ values were significantly reduced to nanomolar range. The IC₅₀ value calculated for G1 when combined with 32 $\mu\text{g/mL}$ of AiiA was $4.45 \pm 0.15 \text{ nM}$ (Figure 2-3D).

Figure 2-3 (A) Structure of G1. (B) Combination effects of G1 and AiiA on PAO1-*lasB-gfp* reporter strain. (C) Dose-response effects of G1 (50 μM) added with AiiA (32 $\mu\text{g/ml}$ and 16 $\mu\text{g/ml}$) on *lasB-gfp*. (D) IC₅₀ values of the single and combination treatments on *lasB-gfp*, G1 ($1.36 \pm 0.08 \mu\text{M}$), AiiA ($6.88 \pm 0.46 \mu\text{g/ml}$), G+A 32 $\mu\text{g/ml}$ ($4.45 \pm 0.15 \text{ nM}$). Calculation was performed using GraphPad Prism 6 software, taken at

the time point between 4-6 hours where the inhibition started to occur. (E) Dose-pair effects of G1 and AiiA on *lasB-gfp*. The concentration of DMSO and buffer correspond to the highest test concentration used in the assays. All experiments were done in triplicate manner, only representative data are shown.

Next, I investigated if the combination treatment would also affect the PQS system: the third intercellular signaling mechanism of *P. aeruginosa* that regulates numerous virulence factors, including those involved in iron scavenging and apoptosis of host cells.^{82, 143} PQS is under positive regulation of LasR and negative regulation of RhIR.¹⁴³⁻¹⁴⁴ PQS has also been detected in the lungs of cystic fibrosis patients¹⁴⁵ and reported to suppress host innate immune responses through nuclear factor- κ B pathway.¹⁴⁶

For this experiment, the compounds were tested against *pqsA-gfp* reporter strain. The biosynthesis of PQS and other classes of alkyl quinolones requires genes encoded by the *pqsABCDE* and *phnAB* operons.¹⁴⁷ Combination treatment reduced *pqsA-gfp* expression, and the effects were also observed in a dose-dependent manner (Figure 2-4A and B, Supplementary Fig. 4). The IC₅₀ value of combined treatment was reduced up to five-fold as compared to single treatments (Figure 2-4C). To confirm the significance of our data, 2-way ANOVA analysis was performed and multiple comparisons test indicated significant p values across various combination doses for different reporter strains (Supplementary Table 2).

Figure 2-4 (A) Combination effects of G1 and AiiA on PAO1-*pqsA-gfp* reporter strain. (B) Dose-response effects of G1 (50 μ M) added with AiiA (32 μ g/ml and 16 μ g/ml) on *pqsA-gfp*. (D) IC₅₀ values of the single and combination treatments on *pqsA-gfp*, G1

($3.08 \pm 0.72 \mu\text{M}$), AiiA ($15.6 \pm 0.2 \mu\text{g/ml}$), G+A $32 \mu\text{g/ml}$ ($0.63 \pm 0.06 \mu\text{M}$). Calculation was performed using GraphPad Prism 6 software, taken at the time point between 4-6 hours where the inhibition started to occur. Experiments were done in triplicate manner, only representative data are shown

2.2.3 Competitive binding of QSI G1 to LasR and RhlR.

Next, I examined the effects of QSI on the *rhl* QS system, which regulates many QS-dependent virulence factors once activated upon formation of RhlR-C4-HSL,^{67c, 148} The AiiA has been experimentally shown to degrade C4-HSL.¹⁴⁹ Previous experiments done in our group also showed that G1 was able to inhibit *rhl* system more effectively in *P. aeruginosa lasR* mutant but not the *rhl* system in the PAO1 wild type.¹⁰⁴ Thus it was hypothesized that G1 has a different binding affinity to LasR than RhlR in the PAO1 wild type and its intracellular concentration is not high enough to repress both LasR and RhlR simultaneously.

To test this hypothesis, I examined the competitive binding efficacy of G1 with 3-oxo-C12-HSL and C4-HSL using a QS deficient *P. aeruginosa ΔlasIΔrhlI* double mutant which can respond to the addition of exogenous AHLs (3-oxo-C12-HSL and C4-HSL respectively). In this setting, only one QS system is activated at one time. The reporter strains showed dose-dependent curves when supplemented with a different concentration of 3-oxo-C12-HSL and C4-HSL (Figure 2-5A and B). Next, using the same setting, $50 \mu\text{M}$ of G1 was added together with the exogenous AHLs and the expression profiles were monitored for up to 16 hours (Supplementary Fig. 5).

When $50 \mu\text{M}$ of G1 was added together with 3-oxo-C12-HSL, reduction in *lasB-gfp* was only observed when the concentration of 3-oxo-C12-HSL is less than $2.5 \mu\text{M}$ (Figure 2-5C). However, G1 was able to reduce *rhlA-gfp* expression with all tested C4-HSL concentrations up to the basal level (Figure 2-5D). Comparing the data with previously reported findings¹⁰⁴, it was proposed that G1 has a higher affinity to RhlR than LasR. However because most of the intracellular G1 was ‘consumed’ due to LasR abundance, hence its effect to inhibit *rhl* QS in the PAO1 wild type was abolished due to earlier induction of *las* QS than the *rhl* QS during growth.

Figure 2-5 (A) Dose-dependent curves of QS deficient $\Delta lasI\Delta rhII$ double mutant harboring *lasB-gfp* supplemented with 3-oxo-C12-HSL (AHL). (B) Dose-dependent curves of QS deficient $\Delta lasI\Delta rhII$ double mutant harboring *rhIA-gfp* supplemented with C4-HSL (BHL). (C) Expression of the $\Delta lasI\Delta rhII$ double mutant harboring *lasB-gfp* when G1 was added together with AHL. (D) Expression of the $\Delta lasI\Delta rhII$ double mutant harboring *rhIA-gfp* when G1 was added together with BHL. Experiments were done in triplicate manner, only representative data are shown. Error bars are means $\pm$ SDs. ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, two-way ANOVA test.

2.2.4 AiiA enhances inhibition of G1 on *rhl* QS system in *P. aeruginosa*.

Since strong inhibition effects on *las* system was observed, it was hypothesized that the combination therapy strategy could also be applied to the *rhl* system. AiiA could act extracellularly and degrade 3-oxo-C12-HSL, therefore less amount of 3-oxo-C12-HSL would enter the cell and activate the LasR production. Low abundance of LasR protein would ‘consume’ less amounts of G1 hence more of these molecules could bind with RhIR.

To test the hypothesis, PAO1-*rhIA-gfp* bioreporter strain was used to evaluate the combination effects. The *rhIA* is the first gene of the *rhIAB* operon that codes for the rhamnolipid biosynthesis¹⁴⁸. Similar findings were observed where the *rhIA-gfp* activity was highly suppressed in the combination treatment (Figure 2-6A). The effects were also observed in dose-dependent manner (Figure 2-6B, Supplementary Fig. 6). IC₅₀

value calculated for the combination treatment between G1 and AiiA for the *rhIA-gfp* expression is $1.38 \pm 0.16 \mu\text{M}$, much lower than the single treatments (IC_{50} for G1 = $3.65 \pm 0.95 \mu\text{M}$ and AiiA = $17.8 \pm 1.8 \mu\text{g/ml}$, Supplementary Fig. 7).

Next, I investigated if the combination effects could reduce the virulence of *P. aeruginosa*. For this, the combination treatment was tested on the rhamnolipid production, as it is one of the key QS-regulated virulent factors in the early stages of infection. Rhamnolipid promotes infiltration of respiratory epithelial cells¹⁵⁰ and promote rapid necrotic killing of polymorphonuclear (PMNs) leukocytes.⁷⁴ Rhamnolipid is also critical in each stage of biofilm formation and contribute to the structure of biofilms.^{88a, 88b}

In the rhamnolipid assay, overnight culture of PAO1 was adjusted to OD_{600} 0.01 and grown in the presence of AiiA, G1 and combination of AiiA and G1 for 18 hours. The rhamnolipid was then extracted and quantified using the orcinol assay. Treatment with AiiA alone did not fully decrease rhamnolipid production. However, when combined with G1, the rhamnolipid production was almost diminished to a similar level to the QS-defective $\Delta\text{lasI}\Delta\text{rhlI}$ mutant (Figure 2-6C). The findings correlate well with the results obtained from inhibition of *rhIA-gfp* bioreporter strain. The experimental results showed promising application of QQE and QSI in reducing virulence factors associated with host infection.

Figure 2-6 Effects of QQ and QSI on the *rhl* system. (A) Combination effects of G1 and AiiA on PAO1-*rhIA-gfp* reporter strain. (B) Dose-response effects of G1 (50 μM) added with AiiA (32 $\mu\text{g/ml}$) on *rhIA-gfp*. (C) Effects on rhamnolipid production when tested at final concentration of 50 μM (for G1), and 32 $\mu\text{g/mL}$ (for AiiA). Same amount of DMSO and buffer were used as positive control. PAO1 $\Delta\text{lasI}\Delta\text{rhlI}$ was used as

negative control. Experiments were done in triplicate manner. Error bars are means $\pm$ SDs. *** = $p < 0.001$. **** = $p < 0.0001$, Student's t test.

2.3 Discussion

Over the years, the emergence of multidrug-resistant bacteria and shortage of new antibiotics have been seen as critical issues and the greatest threat to human health. The antivirulence approach has been long considered as an alternative in controlling the pathogenicity and reducing resistance development¹⁶. Bacterial QS systems have been proposed as ideal targets for the development of antivirulence drugs. However, no QS interference compounds have been applied clinically till date. Many of the QSIs are developed via targeting the AHL-mediated QS systems. The co-existence of multiple AHL QS systems in individual bacterial species might thus be a challenge for the application of QSIs in clinical settings.

In this study, mathematical modeling of *P. aeruginosa* LasR/I QS network in batch culture indicated that the combination therapy of QQE and QSI has combinatorial effects in suppressing the *las* QS. Simulation results show that very large $\eta(QQ)$ or QSI concentration was needed to inhibit QS. Strong combination effect was observed between QQE and QSI. Interestingly, switching of QS circuit in the simulations was not observed in experiments. This might be due to simplification of the mathematical model, which assumes every cell is homogeneous and synchronized. In the single-cell study of QS signaling in *V. fischeri*, switching behaviour was observed while tracking single cells but the population level fluorescence was a graded response.¹³² This could also be similar to the case of the lactose utilization network, where switching was observed at the single cell but not at the population level.¹⁵¹

The experimental results showed promising application of QQE and QSI based on different bioreporter assays. The AHL-dependent QS system has been an attractive target to control bacterial pathogenicity as it controls the expression of a wide range of virulence genes. The AiiA enzyme has been reported to show high specificity and preference towards different signal molecules (acyl chain length and substitution)¹⁴⁹ and demonstrated to reduce the concentration of 3-oxo-C12-HSL based on our HPLC analysis (Supplementary Figure 8). In some cases, the degradation of QS signal alone is not sufficient to completely diminish and block the QS activities¹²⁶. AiiA could abolish and effectively quench the AHL signal molecules, however it was surprising to see its

much lesser effect on the PQS system, which was also under *las* regulation. Combination treatment with G1 resulted in significant reduction of *lasB*, *pqsA*, and *rhlA-gfp* expression as compared to single treatment of both AiiA and G1. This work demonstrated that combining two classes of QSI and QQE could provide multiple points of attacks and efficient blockade of QS-mediated signaling pathways.

In the previous study done in our group, G1 has been shown to interact and compete with AHL to inhibit LasR in *P. aeruginosa*¹⁰⁴. In *P. aeruginosa* PAO1 strain, where both *las* and *rhl* circuits exist, G1 could only inhibit *las* system but not *rhl* system. However, when *lasR* was mutated, G1 could effectively inhibit *rhl* system. In the competitive binding assay using bioreporter strains, it was demonstrated that QSIs might have different affinities to QS receptor proteins. G1 could inhibit *lasB-gfp* expression only when concentration of 3-oxo-C12-HSL was smaller than 1.25 μ M. However, the inhibition effect of G1 to *rhlA-gfp* was still significant even when the concentration of C4-HSL was 10 μ M. We also observed the transcription rates of *las* system was activated first before *rhl*. The results explain why despite of its higher binding affinity to RhlR, G1 would still bind to LasR as the *las* system is activated first in PAO1. But in the case of *lasR* mutant, G1 has higher competitiveness to C4-BHL and could inhibit *rhl* system effectively.

The results also suggested why AiiA could enhance the inhibition effects of G1 on *rhl* system, as shown by the experimental data. Assuming the intracellular concentration of G1 is constant, G1 would have to competitively bind to both LasR and RhlR. In this case, addition of AiiA to quench both 3-oxo-C12-HSL and C4-HSL resulted in lower abundance of LasR and therefore G1 could specifically bind to inhibit RhlR. Thus combination of QQE and QSIs might not only enhance the efficacy of QSIs, but also expand the targeting systems of QSIs as many bacteria have more than one *lux* QS systems.

Although LasR regulator has long been considered essential for full virulence of *P. aeruginosa*,¹⁵² loss-of-function *lasR* mutants occur frequently in the natural environment¹⁵³, individuals suffering from pneumonia, wound infections¹⁵⁴ and in cystic fibrosis patients.¹⁵⁵ In the *lasR* mutants, the QS-regulated virulence factors continue to be expressed. There has also been reports that the *rhl* system could override the hierarchy of QS network in a non-functional *las* system.¹⁵⁶ Recent studies also

showed that RhlR plays critical roles as QS regulator using *Drosophila melanogaster* oral infection model¹⁵⁷ and controls pathogenesis and biofilm development¹⁵⁸. This highlights the importance to develop new strategies that target multiple QS pathways, instead of a specific QS system to control pathogenicity. This could be achieved in two ways; firstly through exploitation of natural or synthetic compounds that have broad targets on QS pathways. The synthetic disulfide-containing ajoene analogs are examples of such class of compounds demonstrated to inhibit QS in *P. aeruginosa*.¹⁰⁹ Secondly, we propose new concept of combining two classes of QS interfering agents with different mechanism of actions in suppressing multiple QS pathways.

In conclusion, the combination effects of QQE and QSI compound have been demonstrated *in vitro* in this study. Mathematical modeling showed enhanced QS inhibiting effects on AHL concentration when QQE (AiiA) and QSI (G1) were applied together. I have also provided better understanding and elucidated QS network interaction with G1 in this work. The implication of this study represents a novel approach of utilizing QS-interfering compounds to impede virulence and block pathogenesis. Future work will aim to evaluate the effectiveness of the combination therapy *in vivo*, either using inhibitors targeting QS receptors or QQ interfering agents.

3 TEACHING OLD DRUG NEW TRICKS: DRUG REPURPOSING APPROACH TO IDENTIFY NOVEL QSIS OF *P. AERUGINOSA*

Although many quorum sensing inhibitors (QSIs) have been identified in the last decades, the antivirulence strategies have not been subjected to large-scale clinical trials yet.¹⁵⁹ In this study, a drug repurposing approach was applied to identify potential antivirulence drugs against *P. aeruginosa*. Drug repurposing (also called drug repositioning, reprofiling or re-tasking) has become an attractive proposition because it could reduce time, cost and risk associated with new compounds.¹⁶⁰ The main advantage is the well-studied mechanism of action, pharmacology, formulation, toxicity profiles, safety and adverse drug reaction issues. On top of that, repurposed drugs may find other applications and reveal new targets to be further exploited. There have been several success stories: Viagra, which was previously developed as angina medication, is used to treat erectile dysfunction; azidothymidine, which failed as chemotherapy drug but now used as HIV therapy; Thalidomide, a sedative which eventually found its new purposes in multiple myeloma and subsequently led to discovery of its successful derivatives, such as lenalidomide.¹⁶⁰⁻¹⁶¹

In this study, 1600-FDA approved compounds under Pharmakon 1600 library would be used for screening against *P. aeruginosa* QS bioreporter strains. The effects on virulence factors production and biofilms formation would also be evaluated. Lastly, as the compounds come from marketed US Food and Drug Administration (FDA)-approved drugs, which safety issues have been extensively studied and considered in humans, the potential application as antivirulence drugs would also be explored using mice infection model.

3.1 Materials and Methods

3.1.1 Bacterial strains and media

Bacteria were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). Media used for biological assay was ABTGC (AB minimal medium supplemented with 0.2% glucose and 0.2% casamino acids) to minimize fluorescence interference¹⁰⁹. Bacterial strains used in this study are shown in Table 3-1.

Table 3-1 Bacterial strains used in this study

Strains	Relevant genotype and/or characteristics
PAO1	<i>P. aeruginosa</i> wild type ¹²⁷
PAO1- <i>gfp</i>	GFP-tagged PAO1 ¹²⁸
PAO1- <i>lasB-gfp</i>	PAO1 containing <i>lasB-gfp</i> (ASV) reporter fusion ¹²⁷
PAO1- <i>pqsA-gfp</i>	PAO1 containing <i>pqsA-gfp</i> (ASV) reporter fusion ¹²⁸
PAO1- <i>rhlA-gfp</i>	PAO1 containing <i>rhlA-gfp</i> (ASV) reporter fusion ¹²⁸
PAO1 Δ <i>lasI</i> Δ <i>rhlI</i>	PAO1 QS deficient <i>lasI</i> and <i>rhlI</i> double mutant ¹⁶
Δ <i>wspF</i> Δ <i>pelA</i> Δ <i>pslBCD</i> - <i>cdrA-gfp</i>	PAO1 <i>pelA</i> , <i>pslBCD</i> , and <i>wspF</i> deletion mutants carrying <i>cdrA-gfp</i> (ASV) reporter plasmid ¹⁶²

3.1.2 Screening of QSIs

Screening library used in this study is Pharmakon1600, which consisted of 1600 known drugs from US and International Pharma. The library was contributed by Experimental Therapeutics Centre (ETC, A*STAR, Singapore). Each well consisted of 2 μ L of 10 mM stock concentration in DMSO. The compounds were then transferred to 96-well plates and diluted to 10 μ M as working concentration. Screening was performed using PAO1-*lasB-gfp* as reporter strain. GFP fluorescence (excitation at 485 nm, emission at 535 nm) and OD₆₀₀ readings were recorded every 15 mins using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). The first hit compounds were then validated again at different dose-dependent concentration to calculate the IC₅₀ values. All assays were done in triplicate manner.

3.1.3 Virulence factors production

For the elastase assay, overnight culture of *P. aeruginosa* wild type and Δ *lasI* Δ *rhlI* mutant strain were diluted in 5 mL of ABTGC medium to a final optical density at 600 nm (OD₆₀₀) of 0.01. Compounds were added to give final concentration of 10 μ M. The cultures were grown for 24 hours at 37 °C, shaking condition (200 rpm). 0.8 mL culture supernatants were sampled and centrifuged at 10,000 rpm for 10 mins. Elastase activity was measured by using EnzChekElastase assay kit (Invitrogen, USA), which consisted of BODIPY fluorophore (FL)-labeled DQ elastin conjugate and yields fluorescent fragments upon cleavage by elastase enzyme.¹⁰⁴ Fluorescence signal was recorded every 10 mins with Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland; excitation 490 nm, emission 520 nm) for three hours.

For pyocyanin quantification assay, overnight culture of *P. aeruginosa* wild type and $\Delta lasI\Delta rhII$ mutant strain was standardized to OD₆₀₀ of 0.01 and diluted 100 times into 25 mL of fresh Kings Medium in 250 mL flask. Compounds were added to give final concentration of 10 μ M. The cultures were grown for 48 hours at 37 °C, shaking condition (200 rpm). The cultures were monitored every 24 hours to observe any colour changes from light yellow to greenish yellow solution in untreated flask. Mutant strain $\Delta lasI\Delta rhII$ was used as negative control. The final cell density was measured at 600 nm (OD₆₀₀) using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). The cultures were then centrifuged for 10 mins at 10,000 rpm and 7.5 mL of the supernatants were transferred into new Falcon tubes. Pyocyanin extraction was conducted by extracting with chloroform (3 mL) and 0.2 M HCl (1.5 mL). The top aqueous layer of HCl containing pyocyanin was pipetted into microtiter plate and absorbance was measured at 520 nm. The data was normalized by dividing OD₅₂₀ reading with the final OD₆₀₀ values.

Rhamnolipid assay was done as previously described. Briefly, subculture of *P. aeruginosa* was diluted 100 times from overnight culture into fresh ABTGC medium (OD₆₀₀ = 0.01). Treated cultures were added with test compounds to give final concentration of 10 μ M. The cultures were grown for 24 hours at 37 °C, shaking condition (200 rpm). The final cell density was measured at 600 nm (OD₆₀₀) using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). Supernatants were collected by centrifuging at 10,000 rpm for 10 mins and extracted with diethyl ether (twice). Organic fractions were concentrated to yield white solids. It was then re-suspended in deionized water and added with 0.19% (w/v) orcinol in 50% H₂SO₄. The resulting mixture was incubated at 80 °C for 30 mins to give yellow-orange solution. After cooling to room temperature, the absorbance was measured at 421 nm and results were normalized with final OD₆₀₀ values.

3.1.4 Ethics

The animal model protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanyang Technological University under the permit number A-0228 AZ. Animal experiments were performed in accordance to the NACLAR Guidelines of Animal and Birds (Care and Use of Animals for Scientific Purposes) Rules by Agri-Food & Authority (AVA) of Singapore.

3.1.5 Implant infection model

Female BALB/c mice were purchased from InVivos Singapore at 8 weeks of age. Mice are maintained on water and standard mouse chow *ad libitum* for 7 days before the experiment. All animal experiments were conducted in compliance with the guide for the Care and Use of laboratory animals (National Research Council) under Nanyang Technological University Institutional Animal Care and Use Committee (IACUC) protocol number ARF SBS/NIE-A18047, which was approved by the Nanyang Technological University-IACUC. The experiment was conducted as previously reported.¹⁶³ Silicone tubes (inner diameter 3.0 mm, outer diameter 6.0 mm, wall thickness 1.0 mm) were cut to a length of 3 mm. The implants were placed in 10 mL 0.9% NaCl in the Erlenmeyer flasks with overnight culture of *P. aeruginosa* (OD₆₀₀ of 0.1) and incubated for 18 h at 37 °C, shaking condition (110 rpm).

Mice were anesthetized by subcutaneous injections in the groin area with 200 µL cocktail of Ketamine HCl and Xylazine HCl (2 mL Ketamine HCl (100 mg/mL), 0.5 mL Xylazine HCl (20 mg/mL) and 7.5 mL saline). The implant was inserted into the right side of the peritoneal cavity near the groin area. The drugs were prepared by dissolving compounds in DMSO to give final concentration of 10 mM before further diluted 1000 times in a 20% vehicle solution [(2-hydroxypropyl)-β-cyclodextrin dissolved in 0.9% NaCl] to a final concentration of 10 µM. 200 µL of this solution was then injected at the site of implantation after certain hours of infection. In the control group, only the vehicle solution was given. On the next day, the mice were sacrificed to remove the implants and also the spleens to investigate the spread of systemic infection. CFU/mL determination of implants and spleens was conducted as follows: implants were placed in Eppendorf tube containing 1 mL 0.9% NaCl and sonicated in ice water bath for 10 mins (5 mins of degassing followed by 5 mins of sonication) to disrupt the biofilm. The spleens were removed from the mouse peritoneum and homogenized with homogenizer (Bio-Gen Pro200, Pro Scientific) to disrupt the tissues and release any bacterial cells. Next, the samples were serially diluted, plated on LB plates and incubated at 37 °C overnight. CFU/mL was calculated by multiplying the average number of colonies by the dilution factor and divided by the volume in mL.

3.2 Results

3.2.1 Screening of 1600 FDA-approved drugs

Screening system based on *P. aeruginosa* PAO1-*lasB-gfp* bioreporter strain was used to identify potential novel QSI from 1,600 FDA-approved compounds with known biological activities (Pharmakon1600). Compounds were screened at 10 μ M concentration, which was diluted 1000x from its stock concentration 10 mM, and contained the final concentration of 0.1% DMSO. When a compound inhibits the expression of QS-controlled *lasB-gfp* fusion, a decrease in GFP would be observed. The growth of the reporter strain (optical density of 600 nm (OD₆₀₀)) was monitored to ensure that the compounds would not affect the growth rate. The output fluorescence reading was normalized with the cell density (GFP/OD₆₀₀) and measured over time. To investigate the effects on other QS pathways, PAO1-*rhlA-gfp* and *pqsA-gfp* were also used as bioreporter strains. Both *rhlA* and *pqsA* are the first genes of the *rhlAB* operon and *pqsABCDE* operon that code for the production of rhamnolipid and PQS precursor molecules.¹⁶⁴ To eliminate false positive results, a *gfp*-tagged *P. aeruginosa* was used as a control.

Based on the screening results, 16 compounds were found to exert antibiotics effect (growth-inhibiting) on *P. aeruginosa*. 10 out of 16 are known antibiotics, mostly from fluoroquinolone class of compounds. The six novel antimicrobial compounds are fluorouracil (anticancer drug), linezolid and rivaroxaban (anticoagulant), thimerosal (preservative in drugs and vaccine products), phenylmercuric acetate (disinfectant and antifungal), and benzonatate (non-narcotic cough medicine).

Interestingly, several macrolides (telithromycin and azithromycin) were also identified as potential c-di-GMP modulator through screening with *P. aeruginosa* Δ *wspF* Δ *pelA* Δ *pslBCD*-*cdrA-gfp*, which have high c-di-GMP levels. The biofilms matrix adhesin CdrA is under the regulation of c-di-GMP, hence it will be a good indicator as c-di-GMP reporter strain. This finding was published by another group, which reported azithromycin regulates c-di-GMP through PA2567 that has PDE activity.¹⁶⁵ The results here reveal another function of versatile macrolides, which could also attenuate QS in *P. aeruginosa* at sub-MIC concentration.^{107a}

For the QSIs, 24 hits were identified based on the screening with PAO1-*lasB-gfp*. However, not all compounds can be purchased from local chemical distributors. For the next stage of studies, we decided to focus on five compounds that have the lowest IC₅₀

values among the hit compounds and readily available in Singapore. The five potential QSIs are benzbromarone (uricosuric agent), 8-Quinolinol sulfate monohydrate (antineurodegenerative, anticancer, and antidiabetic agent), cetylpyridium chloride (antiseptic agent), zinc pyrithione (antidandruff and antifungal agent), and carbimazole (hyperthyroidism drug). The summary of all hit compounds can be found in Table 3-2.

Table 3-2 Summary of hit compounds from screening with PAO1 bioreporter strains.

No.	Name	Structure	Functions
Antibiotics			
1	Fluorouracil		Anticancer drug (colon cancer, esophageal cancer, stomach cancer, pancreatic cancer, breast cancer, and cervical cancer)
2	Linezolid		Anticoagulant
3	Rivaroxaban		Anticoagulant
4	Thimerosal		Preservative in biological, vaccines and drug products
5	Phenylmercuric acetate		Disinfectant, antifungal
6	Benzonatate		Non-narcotic cough medicine
QSIs (PAO1-<i>lasB</i>-gfp)			
7	Phenelzine sulfate		Antidepressant drug

8	Phenindione		Synthetic anticoagulant; vitamin K antagonist
9	Roxithromycin		Macrolide antibiotic; also has antimalarial activity.
10	Rifaximin		Antibiotic
11	Rifampin		Antibiotic, mostly used to treat tuberculosis
12	Azithromycin		Macrolide antibiotic
13	Nisoldipine		High blood pressure drug (calcium channel blockers)

14	Benzbromarone		Uricosuric agent
15	Mianserin		Antidepressant drug
16	Aurothioglucose		Rheumatoid arthritis drug
17	Erdosteine		Mucolytic for acute bronchitis
18	Broxaldine		Antifungal, anti-infective
19	Carbimazole		Hyperthyroidism drug
20	Nifuroxazide		Antibiotic
21	Triclosan		Antibacterial and antifungal agent
22	Dichlorophen		Anticestodal, fungicide, germicide and antimicrobial agent
23	Cetylpyridinium chloride		Antiseptic
24	Zinc pyrithione		Antifungal agent

25	Hexachlorophene		Disinfectant, antiseptic
26	Oxyguinoline hemisulfate		Disinfectant, antiseptic
27	Benzalkonium chloride		Disinfectant, antiseptic
28	Thonzonium bromide		Surfactant for ear drops, antifungal
29	Pyrvinium pamoate		Antiparasitic drug
30	Oxyclozanide		Antiparasitic drug
c-di-GMP (<i>ΔwspFΔpelAΔpslBCD-cdrA-gfp</i>)			
31	Telithromycin		Macrolide antibiotic

32	Azithromycin		Macrolide antibiotic
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3.2.2 Inhibition assay with *P. aeruginosa* QS bioreporter strains

From the previous screening results, I decided to focus on the five compounds as they are structurally distinct from currently reported QSIs. Literature searches from the database also did not show any precedent reports regarding their QSI activities in *P. aeruginosa*. The five compounds were tested on their inhibition of *lasB-gfp*, and they were found to inhibit *lasB-gfp expression* in a dose-dependent manner without affecting growth at 10 μ M. The compounds were also further tested on other QS bioreporter strains *rhlA-gfp* and *pqsA-gfp*. To ensure that the compounds did not affect GFP production and give false positive results, the compounds were also tested with PAO1-*gfp* as control and did not observe any reduction in the fluorescence output signals (Supplementary Figure 9). Promising results were obtained for all five compounds with three different QS reporter strains (Figure 3-1).

A.

Benzbromarone
Molecular Weight: 424.09

B.

8-Quinololin sulfate
monohydrate (QSM)
Molecular Weight: 243.23

Figure 3-1 Structure and dose-dependent curves of compounds against QS bioreporter strains PAO1-*lasB-gfp*, *rhlA-gfp* and *pqsA-gfp*. (A) benzbromarone (B) 8-quinolinol sulfate monohydrate (QSM), (C) cetylpyridium chloride (CPC), (D) zinc pyrithione (ZnP), (E) carbimazole. DMSO 0.1% was used as vehicle control. The experiments were done in triplicate manner, only representative data are shown.

IC₅₀ values were then calculated from the dose-response curves when inhibition started to occur (4-8 hours). The IC₅₀ values represent half of the concentration required to inhibit the biosynthesis rate of GFP due to AHL induction.¹⁰⁴ The values could be used as a comparison for the effectiveness of compounds in reducing the gene expression of QS bioreporter strains. The five compounds have promising results, with IC₅₀ values in the low micromolar range. The summary can be found in Table 3-3.

Table 3-3 Summary of IC₅₀ values (μM) for different QS bioreporter strains. IC₅₀ calculation was based on three biological replicates and performed using GraphPad Prism 6 software.

Compounds	<i>lasB-gfp</i>	<i>rhlA-gfp</i>	<i>pqsA-gfp</i>
Benzbromarone	2.10 ± 0.11	2.72 ± 0.11	0.78 ± 0.01
8-Quinolinol sulfate monohydrate (QSM)	2.30 ± 0.14	7.86 ± 0.02	4.71 ± 0.14
Cetylpyridium chloride (CPC)	6.64 ± 2.39	12.0 ± 1.1	3.92 ± 0.27

Zinc pyrithione (ZnP)	1.20 ± 0.03	1.06 ± 0.10	1.82 ± 0.07
Carbimazole	6.27 ± 2.64	5.91 ± 0.08	21.4 ± 1.3

Based on the IC₅₀ values, it could be postulated that the compounds were able to affect all three QS pathways. As mentioned in the previous chapter, the development of compounds that have broad multiple QS targets are more advantageous, since many pathogens employ different QS pathways. Indeed, *lasR* mutants are commonly found in patients suffering from cystic fibrosis.¹⁵³⁻¹⁵⁵ Interestingly, carbimazole has not much effect on the PQS system. As PQS is under control of the *las* system, the QS inhibition could be an off-target of the compound. Similarly, CPC also has not much effect on *rhlA-gfp*. The overall results suggest that the compounds could hit one or more targets on top of the QS signaling cascade, which resulted in the downregulation of all three QS pathways. The best compound is ZnP, which has the lowest IC₅₀ values for all three reporter strains: 1.20 ± 0.03 μ M for *lasB-gfp*, 1.06 ± 0.10 μ M for *rhlA-gfp*, and 1.82 ± 0.07 μ M for *pqsA-gfp* (Figure 3-2).

Figure 3-2 IC₅₀ values of ZnP (A) 1.20 ± 0.03 μ M for *lasB-gfp* (B) 1.06 ± 0.10 μ M for *rhlA-gfp* (C) 1.82 ± 0.07 μ M for *pqsA-gfp*. Calculation was performed using GraphPad Prism 6 software. The experiments were done in triplicate manner; only representative data are shown.

3.2.3 Effects on virulence factors production

In this study, the effects of five compounds were tested on QS-regulated virulence factors, namely elastase, rhamnolipid and pyocyanin. The coordinated expression of virulence genes allows *P. aeruginosa* to overcome host defenses during acute infection when they are produced at a high enough level.¹³ The importance of *las* and *rhl* systems to induce virulence genes has been described, where they were abolished in the QS double mutant $\Delta lasI \Delta rhlI$ strain.¹⁴²

Figure 3-3 Effects of ZnP on virulence. (A) Elastase activity (B) Rhamnolipid production (C) Pyocyanin production. Compound was tested at final concentration of 10 μ M. PAO1 $\Delta lasI\Delta rhII$ was used as negative control. Error bars represent standard deviation of three measurements. * = $p < 0.1$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, Student's t test.

Upon treatment with compounds at 10 μ M, we could see the different effects of the five compounds on elastase production. CPC, QSM and ZnP could suppress elastase levels to almost the same level as mutant strains $\Delta lasI\Delta rhII$. Benzbromarone could reduce around 50% elastase level, whereby no effect was observed upon the addition of carbimazole (Figure 3-3A). For rhamnolipid production, most of the compounds could reduce up to 60% as compared to the wild type PAO1. Carbimazole and CPC are less efficient in reducing rhamnolipid levels as compared to the other compounds (Figure 3-3B). Based on the bioreporter strains assay, CPC and carbimazole indeed have higher IC_{50} values on *rhlA-gfp* as compared to the other compounds. On the other hand, most of the compounds were able to reduce pyocyanin levels up to 25% comparing to the PAO1 control (Figure 3-3C).

The overall results demonstrated the efficacy of the compounds in reducing virulence factors production, which plays critical role in *P. aeruginosa* infection. Some

compounds demonstrate better profiles in reducing specific virulence factors, and some may even lose activities in the others. Further studies are required to investigate the mechanism of the compounds, which are clearly distinct from one another.

3.2.4 *In vivo* application using mice infection model

The implication of QS-virulence genes in *in vivo* studies has been demonstrated in many studies, including pneumonia,¹⁶⁶ burn wound infection model,¹⁶⁷ corneal infections,^{152, 168} and chronic lung infection.¹⁶⁹ QS-deficient mutants like *lasI*, *rhlI*, double mutant *lasI rhlI*, and *lasR* have been reported to have significantly reduced virulent as compared to the wild type.^{16, 170} In a neonatal mouse model of pulmonary infection, *lasR* mutant could colonize the lung but didn't cause any mortality as compared to the wild type *P. aeruginosa* strain.¹⁷¹ In an *in vivo* foreign-body infection model, treatment with QS inhibitor furanone C-30 resulted in the faster clearing of the implants colonized with the wild type *P. aeruginosa*.¹⁷²

The ability of the compounds to suppress QS *in vivo* was investigated using mice model for implant-associated infection. Silicone implants coated with *P. aeruginosa* biofilms were surgically inserted into the peritoneum of mice and treated with compounds (10 μ M) or DMSO control. Day 2 post-infection, the mice were sacrificed and the implants were extracted to quantify the content of bacteria. Since the compounds used in this study are FDA-approved drugs that have passed clinical trials, they should be safe to be used in the mice study. Moreover, studies on their toxicity profiles can be found in the literature search.

Bacterial counts of *P. aeruginosa* from the mice study showed that treatment with the compounds (200 μ L of 10 μ M) could reduce bacterial counts in both implants and spleen. Treatment with DMSO alone didn't have any significant effects. Benzbromarone, ZnP, and CPC were able to reduce bacterial loads from the implants up to 3-log reduction. QSM was able to reduce bacterial counts up to 4-log reduction. No significant difference was observed upon treatment with carbimazole. However, there was an apparent reduction of bacterial counts in the spleen (2-log reduction), which shows that carbimazole could retard bacterial dissemination to the spleen. Altogether, treatment with the compounds could weaken the biofilms formation on the implants as well as facilitate host immune system in clearing the biofilm infections. The results further confirm the potential application of the compounds as antivirulence therapy.

Figure 3-4 Bacterial counts on (A) implants and (B) spleen after challenged with silicone implants coated with *P. aeruginosa* that would develop into biofilms. One day post infection (dpi), the mice were treated with compounds (10 μ M). DMSO 0.1% was used as vehicle control. Mice were sacrificed on two dpi, and bacterial loads on implants and spleens were quantified. Statistical difference was tested by Mann-Whitney U test using GraphPad Prism 6 software. ** = $p < 0.01$, * = $p < 0.1$.

3.3 Discussion

This study demonstrates how a drug repurposing approach leads into the discovery of benzbromarone, ZnP, CPC, QSM and carbimazole as QSI against *P. aeruginosa*. Benzbromarone is a uricosuric drug used to treat hyperuricemia in patients with gout. It is to date the most potent uricosuric drug tested in clinical practice.¹⁷³ However, after several reports of serious hepatotoxicity, the drug was withdrawn in 2003 by Sanofi-Synthelabo. Nevertheless, studies done by Lee *et al.* suggested that the adverse effects of the drug are relatively infrequent but potentially severe, and the usage of benzbromarone to treat gout would still be recommended.¹⁷⁴ Currently the drug is still marketed in some countries outside the USA. ZnP is widely known as an active ingredient in many anti-dandruff shampoos. Other than antifungal properties, it also has antibacterial properties against *Streptococcus* and *Staphylococcus*, and also used to treat some mild forms of dermatitis (e.g. seborrhoea, dandruff).¹⁷⁵ CPC is an oral antiseptic drug and mostly used in mouthwashes, toothpaste, lozenges, etc. It also has a broad antimicrobial spectrum on gram positive bacteria and yeast. CPC has been considered as an alternative ingredient other than chlorhexidine to prevent gingivitis and as oropharyngeal antiseptic.¹⁷⁶ QSM and its derivatives have been long studied as

important moieties in drug development because of its wide array of biological properties, such as antibiotic, anticancer, antituberculosis and antimalarial drugs. The heterocyclic could form complexes with metal ions, hence it has good lipid solubility and active against certain bacteria and fungi.¹⁷⁷ Carbimazole is used to treat hyperthyroidism. It is a pro-drug that would be converted to its active form methimazole, which would then prevent iodine from binding with the thyroid peroxidase enzyme.¹⁷⁸

Results from QS bioreporter strains show that the compounds could efficiently inhibit *las*, *rhl* and *pqs* QS system in a dose-dependent manner. The antivirulence activities have also been evaluated, and the compound could inhibit the production of important QS-virulence factors such as elastase, rhamnolipid and pyocyanin. Lastly, the applications of the compounds *in vivo* has also been demonstrated. One of the clear advantages of the drug repurposing approach is the availability of mechanism studies, toxicology, and clinical data from literature reviews. When treated with 200 μ L of 10 μ M, the compounds were able to reduce bacterial loads on the implants and spleens. The most promising results were found in groups treated with benzbromarone, ZnP, CPC and QSM. Nevertheless, carbimazole was able to reduce bacterial counts on the spleen. One possible explanation could be the pharmacokinetic (PK) and pharmacodynamic (PD) profiles of the drug, where carbimazole needs to be metabolized into its active form. In human, carbimazole is mostly taken orally and rapidly metabolized in the intestine. The drug is also hydrolyzed rapidly in the blood and undergoes oxidative decomposition in the liver and thyroid.¹⁷⁸

In conclusion, this study reported novel functions of FDA-approved drugs benzbromarone, ZnP, CPC, QSM and carbimazole as QSI against *P. aeruginosa*. The compounds were able to inhibit multiple QS pathways in *P. aeruginosa* and also reduced virulence factors production. The application of the drugs *in vivo* using mice implant model was also demonstrated. The implication of this study represents how drug repurposing approach could be used to identify novel QS-interfering compounds in attenuating virulence of the superbug *P. aeruginosa*. Future work will aim to investigate the mechanism of the compounds as QSI, as well as SAR study to further improve their bioactivities.

4 ZINC PYRITHIONE AND ITS METAL DERIVATIVES AS QSI AND VIRULENCE ATTENUATOR OF *P. AERUGINOSA*

In the previous study, I have shown the QSI activities of benzobromarone, ZnP, CPC, QSM and carbimazole, which were discovered from the screening of 1600 FDA-approved drugs. In this study, the focus will be given on one particular compound, ZnP, which exhibits excellent QSI activities *in vitro* and *in vivo*. The antifungal and antimicrobial actions of ZnP have been well-described¹⁷⁹, nevertheless its potential usage as QSI has not been reported. For this chapter, a small focused chemical library would be constructed and structural-activity relationship (SAR) study would be conducted to explore novel candidate and improve bioactivities. A new series of metal compounds analogs were developed, which exhibit excellent QSI activities and show reduced toxicity to murine macrophages. Effects on the biofilms have also been investigated using flow cells. Lastly, I have investigated the mode of actions of compounds using system biology and adaptive evolution approaches. The overall findings highlight the utility of chemical biology approach to identify novel antivirulence lead compounds for *P. aeruginosa* infections.

4.1 Materials and Methods

4.1.1 Bacterial strains and media

Bacteria were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). Media used for biological assay was ABTGC (AB minimal medium supplemented with 0.2% glucose and 0.2% casamino acids) to minimize fluorescence interference.¹⁰⁹ Bacterial strains used in this study are shown in Table 4-1.

Table 4-1 Bacterial strains used in this study

Strains	Relevant genotype and/or characteristics
PAO1	<i>P. aeruginosa</i> wild type ¹²⁷
PAO1- <i>gfp</i>	GFP-tagged PAO1 ¹²⁸
PAO1- <i>lasB-gfp</i>	PAO1 containing <i>lasB-gfp</i> (ASV) reporter fusion ¹²⁷
PAO1- <i>pqsA-gfp</i>	PAO1 containing <i>pqsA-gfp</i> (ASV) reporter fusion ¹²⁸
PAO1- <i>rhlA-gfp</i>	PAO1 containing <i>rhlA-gfp</i> (ASV) reporter fusion ¹²⁸
PAO1- <i>rsmY-gfp</i>	PAO1 containing <i>rsmY-gfp</i> (ASV) reporter fusion ¹⁸⁰
PAO1- <i>rsmZ-gfp</i>	PAO1 containing <i>rsmZ-gfp</i> (ASV) reporter fusion ¹⁸⁰
PAO1 Δ <i>lasI</i> Δ <i>rhII</i>	PAO1 QS deficient <i>lasI</i> and <i>rhII</i> double mutant ¹⁶

4.1.2 Chemical synthesis

Reagents were purchased from Alfa Aesar and Sigma Aldrich and used directly without further purification. Mass spectrometric data were recorded on a ThermoFinnigan PolarisQ MS / ThermoFinnigan LCQ Fleet MS. High resolution MS was done using Waters Q-ToF Premier Mass Spectrometer (Waters, USA). The values were reported in the unit of mass to charge ratio (m/z). ^1H and ^{13}C NMR spectra were recorded using a BrukerAvance III 400 (400 MHz) spectrometer. Chemical shifts (ppm) were recorded with residual solvent peak as internal reference. NMR data can be found in Supplementary Fig. 14. IR was done using Shimadzu IR Prestige-21 (Shimadzu, Japan). IPC-MS was performed using ELAN DRC-e with Meinhard[®] Nebulizer (PerkinElmer, USA). Purification of products via column chromatography was done on Merck 60 (0.040 – 0.063 mm mesh silica gel). Thin layer chromatography (TLC) was performed using Merck 60 F254 silica gel plates. Visualization was carried out with UV lamp and KMnO_4 stain. Melting points (mp) were recorded on Buchi B-54 melting point apparatus and uncorrected.

General procedure for the synthesis of metal pyrithione

Step 1 A solution of m-CPBA (75%) (15 mmol) was firstly dissolved in dichloromethane (DCM). The solution was then added dropwise to a stirred solution of pyridine (10 mmol) in DCM at 0°C . After addition, the reaction mixture was heated to reflux for 4h. Excess K_2CO_3 was added to the reaction mixture and stirred for further 2h. After filtration and concentration, the crude product was purified by flash chromatography on silica gel (DCM:MeOH, 20:1) to give colorless oil (70% yield).

Step 2 A solution of phosphorus oxychloride (6 mmol) in DCM (5 mL) was added dropwise to a stirred solution of pyridine-N-oxide (5 mmol) and triethylamine (6 mmol)

in DCM (10mL) at 0 °C. The reaction mixture was stirred for 30 minutes at room temperature and then refluxed for further 1h. The mixture was poured into water (10 mL) and neutralized with 2N NaOH. The organic layer was separated and aqueous layer was extracted with DCM. The combined DCM was washed brine, dried and concentrated at reduced pressure to give colorless liquid (85% yield).

Step 3 A solution of 2-chloropyridine oxide (2 mmol) was added into 1.5 mL of water in a two-neck flask. The mixture was allowed to heat to 95 °C before the addition of aqueous solution containing 1.5 mmol of sodium sulfide and 2.2 mmol of sodium hydrosulfide. After addition was completed, the reaction was stirred at 95 °C for 30 minutes. Next, reaction mixture was poured into water and neutralized with 1M HCl and extracted with DCM. The combined organic layer was washed with brine and dried over MgSO₄. The solvents were removed via rotary evaporation, and the crude product was purified via flash chromatography (DCM) to give pale white solid (38% yield).

Step 4 An aqueous solution of metal salts (1.5 mM) was added to an aqueous solution of 2-mercaptopyridine-N-oxide (3.0 mM) and NaOH. Reaction was stirred at room temperature until precipitation was formed. The solids were collected by filtration and washed with MeOH.

Zinc pyrithione (1). White solid; 96% yield; mp 261 °C. ¹H NMR (400 MHz, DMSO): δ 8.42 (dd, *J* = 6.6, 1.1 Hz, 1H), 7.61 (dd, *J* = 8.2, 1.8 Hz, 1H), 7.33 (m, 1H), 7.01 (td, *J* = 7.1, 1.9 Hz, 1H); ¹³C NMR (101 MHz, DMSO): δ 159.5, 137.4, 129.4, 128.6, 118.0; IR (neat) ν_{max} 1145, 1200, 1458, 1543 cm⁻¹; HRMS (ESI, *m/z*) calc for [M+H]⁺ = 316.9397, found [M+H]⁺ = 316.9400.

Cobalt pyrithione (2). Green solid; 89% yield; mp 263 °C. ¹H NMR (400 MHz, DMSO): δ 8.47 (d, *J* = 6.1 Hz, 1H), 7.44 (d, *J* = 7.9 Hz, 1H), 7.20 (t, *J* = 7.3 Hz, 1H), 6.98 (d, *J* = 5.9 Hz, 1H); ¹³C NMR (101 MHz, DMSO): δ 161.3, 137.6, 128.4, 124.1, 118.1; IR (neat) ν_{max} 1153, 1192, 1458, 1543 cm⁻¹; HRMS (ESI, *m/z*) calc for [M+H]⁺ = 311.9437, found [M+H]⁺ = 311.9434.

Nickel pyrithione (3). Brown solid; 60% yield; mp 259 °C. ¹H NMR (400 MHz, DMSO): δ 8.75 (d, *J* = 6.6 Hz, 1H), 7.61 (dd, *J* = 8.3, 1.4 Hz, 1H), 7.50 (m, 1H), 7.17 (td, *J* = 7.0, 1.6 Hz, 1H); ¹³C NMR (101 MHz, DMSO): δ 153.9, 139.4, 131.5, 130.9, 120.4; IR (neat) ν_{max} 1145, 1184, 1462 1546 cm⁻¹; HRMS (ESI, *m/z*) calc for [M+H]⁺ = 310.9459, found [M+H]⁺ = 310.9459.

Copper pyrithione (4). Pale Green solid; 82% yield; mp 262 °C. IR (neat) ν_{max} 1149, 1196, 1458, 1543 cm⁻¹; HRMS (ESI, *m/z*) calc for [M+H]⁺ = 315.9401, found [M+H]⁺ = 315.9406

Vanadium oxide pyrithione (5). Purple solid; 78% yield; mp 151 °C. IR (neat) ν_{max} 1142, 1184, 1458, 1550 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 319.9494$, found $[\text{M}+\text{H}]^+ = 319.9492$.

Bis((5-fluoropyridin-2-yl)thio)di(λ 1-oxidaneyl)cobalt (6). Green solid; 40% yield; mp 59 °C. HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 347.2200$, found $[\text{M}+\text{H}]^+ = 348.2200$.

General procedure for the synthesis of metal - pyridine N-oxide complexes

An aqueous solution of metal salts (1.5 mM) was added to an aqueous solution of pyridine N-oxide (3.0 mM) and NaOH. Reaction was stirred at room temperature until precipitation was formed. The solids were collected by filtration and washed with MeOH.

di(λ 1-oxidaneyl)bis(pyridin-2-yloxy)zinc (7). White solid; 97% yield; mp 330 °C. IR (neat) ν_{max} 1184, 1524, 1630, cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 284.9854$, found $[\text{M}+\text{H}]^+ = 284.9859$.

di(λ 1-oxidaneyl)bis(pyridin-2-yloxy)cobalt (8). Purple solid; 98% yield; mp 291 °C. IR (neat) ν_{max} 1180, 1371, 1518, 1628 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 279.9894$, found $[\text{M}+\text{H}]^+ = 279.9900$.

di(λ 1-oxidaneyl)bis(pyridin-2-yloxy)copper (9). Blue solid; 92% yield; mp 297 °C. IR (neat) ν_{max} 1184, 1232, 1502, 1626 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 283.9858$, found $[\text{M}+\text{H}]^+ = 283.9851$.

di(λ 1-oxidaneyl)bis(pyridin-2-yloxy)nickel (10). Green solid; 92% yield; mp 336 °C. IR (neat) ν_{max} 1180, 1367, 1516, 1628 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 278.9916$, found $[\text{M}+\text{H}]^+ = 278.9923$.

General procedure for the synthesis of metal - isoquinoline N-oxide complexes

An aqueous solution of metal salts (1.5 mM) was added to an aqueous solution of isoquinoline N-oxide (3.0 mM) and NaOH. Reaction was stirred at room temperature until precipitation was formed. The solids were collected by filtration and washed with MeOH.

Bis(isoquinolin-3-ylthio)di(λ 1-oxidaneyl)zinc (11). White solid; 40% yield; mp 59 °C. IR (neat) $\nu_{\max}$ 1180, 1367, 1516, 1628 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 417.8060$, found $[\text{M}+\text{H}]^+ = 310.06$.

Bis(isoquinolin-3-ylthio)di(λ 1-oxidaneyl)cobalt (12). Green solid; 74% yield; mp 260 °C. IR (neat) $\nu_{\max}$ 1180, 1560, 1615 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 411.9750$, found $[\text{M}+\text{H}]^+ = 411.9763$.

General procedure for the synthesis of metal - quinoline N-oxide complexes

An aqueous solution of metal salts (1.5 mM) was added to an aqueous solution of quinoline N-oxide (3.0 mM) and NaOH. Reaction was stirred at room temperature until precipitation was formed. The solids were collected by filtration and washed with MeOH.

Di(λ 1-oxidaneyl)bis(quinolin-2-ylthio)cobalt (13). Green solid; 65% yield; mp 235 °C. IR (neat) $\nu_{\max}$ 1022, 1190, 1495, 1547 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 411.9750$, found $[\text{M}+\text{H}]^+ = 411.9768$.

General procedure for the synthesis of 3-hydroxy-4-methyl-2(3H)-thiazolethione (HMTT) metal complexes

An aqueous solution of metal salts (1.5 mM) was added to an aqueous solution of HMTT (3.0 mM) and NaOH. Reaction was stirred at room temperature until precipitation was formed. The solids were collected by filtration and washed with MeOH.

Zn(MTT)₂ (14). White solid; 90% yield; mp 275 °C. IR (neat) $\nu_{\max}$ 1038, 1317, 1373, 3100 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 356.8838$, found $[\text{M}+\text{H}]^+ = 356.8839$.

Co(MTT)₂ (15). Purple solid; 92% yield; mp 228 °C. IR (neat) $\nu_{\max}$ 1030, 1323, 1377, 3080 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 351.8879$, found $[\text{M}+\text{H}]^+ = 351.8865$.

Cu(MTT)₂ (16). Pale green solid; 80% yield; mp 247 °C. IR (neat) ν_{max} 1037, 1323, 1394, 1589, 3095 cm⁻¹; HRMS (ESI, m/z) calc for [M+H]⁺ = 355.8843, found [M+H]⁺ = 355.8813.

Ni(MTT)₂ (17). Yellow solid; 83% yield; mp 263 °C. IR (neat) ν_{max} 1040, 1374, 1684, 3100 cm⁻¹; HRMS (ESI, m/z) calc for [M+H]⁺ = 350.8900, found [M+H]⁺ = 350.8905.

General procedure for the synthesis of ethyl xanthate metal complexes

An aqueous solution of metal salts (1.5 mM) was added to an aqueous solution of ethyl xanthate (3.0 mM) and NaOH. Reaction was stirred at room temperature until precipitation was formed. The solids were collected by filtration and washed with MeOH

Bis((ethoxycarbonothioyl)thio)zinc (18). White solid; 96% yield; mp 281 °C. IR (neat) ν_{max} 1002, 1148, 1637, 3423 cm⁻¹; HRMS (ESI, m/z) calc for [M+H]⁺ = 306.8933, found [M+H]⁺ = 306.8923.

Bis((ethoxycarbonothioyl)thio)cobalt (19). Green solid; 98% yield; mp 314 °C. IR (neat) ν_{max} 1005, 1254, 1367, 3476 cm⁻¹; HRMS (ESI, m/z) calc for [M+H]⁺ = 301.8974, found [M+H]⁺ = 301.8985.

Bis((ethoxycarbonothioyl)thio)nickel (20). Brown solid; 93% yield; mp 233 °C. IR (neat) ν_{max} 1030, 1223, 1367, 1464, 2957 cm⁻¹; HRMS (ESI, m/z) calc for [M+H]⁺ = 300.8995, found [M+H]⁺ = 300.8996

General procedure for the synthesis of 8-hydroxy-2-quinolinecarboxaldehyde (HQC) metal complexes

An aqueous solution of metal salts (1.5 mM) was added to an aqueous solution of HQC (3.0 mM) and NaOH. Reaction was stirred at room temperature until precipitation was formed. The solids were collected by filtration and washed with MeOH.

Zn(HQC)₂ (21). Brown solid; 78% yield; mp 118 °C(decompose.). IR (neat) ν_{max} 1056, 1109, 1468, 1701cm⁻¹; HRMS (ESI, m/z) calc for [M+H]⁺ = 409.0167, found [M+H]⁺ = 409.0190.

Co(HQC)₂ (22). Brown solid; 63% yield; mp 122 °C(decompose.). IR (neat) $\nu_{\max}$ 1069, 1103, 1464, 1701 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 404.0207$, found $[\text{M}+\text{H}]^+ = 404.0211$

Cu(HQC)₂ (23). Red solid; 82% yield; mp - °C(decompose.). IR (neat) $\nu_{\max}$ 1111, 1456, 1701 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 408.0171$, found $[\text{M}+\text{H}]^+ = 408.0174$.

Ni(HQC)₂ (24). Yellow solid; 80% yield; mp 128 °C(decompose.). IR (neat) $\nu_{\max}$ 1070, 1105, 1464, 1701 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 403.0229$, found $[\text{M}+\text{H}]^+ = 403.0224$.

General procedure for the synthesis of 2-(hydroxymethyl)-3-quinolinol (HMQ) metal complexes

Step 1. Aldehyde HQC (1 mmol) was dissolved in 10 mL methanol, followed by addition of NaBH_4 (1.5 mmol). The reaction mixture was allowed to stir under room temperature and monitored by TLC. Upon completion, aqueous NH_4Cl solution was added to quench the reaction. The mixture was extracted with ethyl acetate, the combined organic layer was washed with brine and dried over MgSO_4 . After concentration, the crude product was purified by flash chromatography on silica gel (ethyl acetate:hexane, 1:3), to give product as light brown solids (94% yield).

Step 2. An aqueous solution of metal salts (1.5 mM) was added to an aqueous solution of HMQ (3.0 mM) and NaOH . Reaction was stirred at room temperature until precipitation was formed. The solids were collected by filtration and washed with MeOH .

Co(HMQ)₂ (25). Deep brown solid; 72% yield; mp 241 °C. IR (neat) $\nu_{\max}$ 1047, 1107, 1473, 3045 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 408.0520$, found $[\text{M}+\text{H}]^+ = 408.0529$.

Cu(HQC)₂ (26). Light brown solid; 86% yield; mp 219 °C. IR (neat) $\nu_{\max}$ 1056, 1111, 1473, 2930, 3423 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 412.0484$, found $[\text{M}+\text{H}]^+ = 412.0494$.

Ni(HQC)₂ (27). Green solid; 87% yield; mp 238 °C. IR (neat) $\nu_{\max}$ 1045, 1110, 1475, 3215 - 3406 cm^{-1} ; HRMS (ESI, m/z) calc for $[\text{M}+\text{H}]^+ = 407.0542$, found $[\text{M}+\text{H}]^+ = 407.0540$.

Zn(HQC)₂ (28). Yellow solid; 80% yield; mp 236 °C. IR (neat) ν_{max} 1064, 1109, 1475, 3043 cm⁻¹; HRMS (ESI, m/z) calc for [M+H]⁺ = 413.0480, found [M+H]⁺ = 413.0500.

4.1.3 Bioreporter and QS virulence assays

Reporter gene assay was done as described in the previous chapters. Stock solution of the synthesized compounds were prepared by dissolving appropriate amount of chemicals in DMSO to make final concentration of 10 mM, aliquoted into small Appendorf tube and stored at -20°C until further usage. The compound was then further diluted in ABTGC medium to the working concentration and tested with respective QS bioreporter strains. GFP fluorescence (excitation at 485 nm, emission at 535 nm) and pyoverdine (excitation at 405 nm, emission at 465 nm) was measured using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). IC₅₀ value calculation was done using GraphPad Prism 6 software. The elastase, rhamnolipid and pyocyanin assays were also done as described in the previous chapters. All assays were done in triplicate manner.

4.1.4 Biofilms flow experiment

GFP-tagged *P. aeruginosa* were grown in ABTG medium and flowed through flow chambers as previously described.¹⁸¹ Each flow chamber is consisted of three-channel flow cells that were supplied with a flow of medium and oxygen, while waste medium would be directed into a waste flask. Briefly, overnight cultures were diluted 1,000 times in ABTG medium and injected into each channel for 1 h incubation time without flow. Next, the medium was allowed to flow into the flow cells and the velocity was maintained at 0.2 mm/s using Cole-Palmer peristaltic pump. Biofilms were grown for 72 h before treatment with compounds for further 48 h. To visualize dead cells, 300 µL of propidium iodide (PI) stain was injected into each flow cells. Biofilm images were taken with LSM confocal laser scanning microscope (Carl Zeiss, Germany) at 20x objective lens. Microscopy images were processed with IMARIS software (Bitplane AG, Zurich, Switzerland). Experiments were done in triplicate manner and repeated at least twice to confirm the results.

4.1.5 Cytotoxicity assay

Toxicity assay was done as previously reported.¹⁰⁹ Murine macrophage RAW264.7 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies), supplemented with 10% fetal bovine serum (Gibco) at 37 °C and 5 % CO₂. The cells

were passaged into 96-well microplates, with each well containing 1×10^4 macrophages. After 16 h, the cells were washed with phosphate-buffered saline (PBS) and treated with compounds at varying concentration in 100 μ L DMEM. The plate was incubated at 37 °C and 5 % CO₂ for further 16 h. Resazurin was then added into each well to reach final concentration of 10 μ M for cell viability measurement. The live cells would be able to convert the dye to red colour, whereby dead cells would remain as blue colour. Absorbance was taken at 595 nm using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). Experiments were done in triplicate manner and repeated at least twice to confirm the results.

4.1.6 Implant infection model

The implant infection model was done as described in the previous chapter. The animal model protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanyang Technological University under the permit number A-0228 AZ. Female BALB/c mice were purchased from InVivos Singapore at 8 weeks of age. Mice are maintained on water and standard mouse chow *ad libitum* for 7 days before the experiment. All animal experiments were conducted in compliance with the guide for the Care and Use of laboratory animals (National Research Council) under Nanyang Technological University Institutional Animal Care and Use Committee (IACUC) protocol number ARF SBS/NIE-A18047, which was approved by the Nanyang Technological University-IACUC.

4.1.7 RNA preparation for RNA-Seq

PAO1 were grown in 1 mL ABTGC medium in each well (triplicates) within a 24-well microplate (Nunc). Compounds and vehicle control (DMSO) were added in each well, and the cells were grown in 37 °C, 200 rpm shaking. Bacterial cells were harvested at the late-log phase (early stationary phase) where QS genes are highly activated. Bacterial cells were treated with RNA Protect Reagent (Qiagen, Netherlands) to maintain the integrity of RNA. Next, total RNA was extracted using RNeasy Mini Kit (Qiagen, Netherlands), followed by Turbo DNA-free Kit (Thermo Fisher Scientific, Lithuania) to remove genomic DNA contaminant from total RNA. DNA contamination was assessed with Qubit 2.0 Fluorometer (Invitrogen, Austria) using dsDNA High Sensitivity (HS) assay (PicoGreen dye). Afterwards, ribosomal RNA was depleted with

Ribo-Zero rRNA removal kit (Illumina, USA). The integrity of total RNA was assessed with Agilent TapeStation System (Agilent Technologies, UK).

4.1.8 RNA Seq and data analysis

RNA-Seq experiment was done as previously reported.¹⁸² Gene expression analysis (three biological replicates) was conducted by Illumina RNA sequencing (RNA-Seq technology). The double-stranded cDNAs were reverse-transcribed using NEBNext RNA first and second strand synthesis module (NEB, USA). The cDNAs were then processed with Illumina's TrueSeq Stranded mRNA protocol. The libraries were then pooled and sequenced using the Illumina HiSeq 2500 platform with a paired-end protocol and read lengths of 100 nucleotides.

For the data analysis, the sequence reads were assembled and analyzed with CLC Genomics Workbench 8.5 (CLC Bio Qiagen, USA) using the "RNA-Seq and expression analysis" function. The PAO1 genome (<http://www.ncbi.nlm.nih.gov/nuccore/110645304>) was utilized as the reference genome for the assembly. Next, the total gene reads were saved and subjected to the DESeq2 package (R/Bioconductor).¹⁸³ For statistical analysis, a hierarchical clustering analysis was done with negative binomial test using the DESeq2 package of R/Bioconductor. Heat map and PCA plots were generated using R/Bioconductor (fold change > 2, p-values < 0.05). Transcripts were stringently determined as differentially expressed when having a fold change larger than 5 and an adjusted p-value smaller than 0.05.

4.1.9 Inductively Coupled Plasma Mass Spectroscopy (ICP-MS)

Overnight culture of *P. aeruginosa* wild type and evolved strains were diluted in 15 mL of ABTGC medium to a final optical density at 600 nm (OD₆₀₀) of 0.01. For treated group, compounds were added to give final concentration of 10 µM. The cultures were grown for 16 hours at 37 °C, shaking condition (200 rpm). The cells were centrifuged at 10,000 g for 3 mins and washed twice with 1 mL of PBS. Cell pellets were then resuspended in 500 µL of HNO₃ (69 %) and sonicated for 2 x 15 mins in ice-filled water bath (37 Hz, 100% power). The samples were then adjusted and diluted to 15 mL of 1% HNO₃. ICPMS analysis was conducted in Central Environmental Science and Engineering Laboratory (CEE, NTU, Singapore). The results were normalized with protein concentration and reported as average results from three biological replicates.

4.2 Results

4.2.1 Structural-Activity Relationship (SAR) study

To improve bioactivities, a small library of ZnP derivatives was constructed as part of the SAR study. Many bioactive small molecules have incorporated metal complexes to improve their therapeutic activity, such as cisplatin, salvarsan, auranofin, etc.¹⁸⁴ It was proposed that complexation to metal scaffolds could improve the physicochemical properties of the ligand. The SAR study consisted of two major parts, where firstly different aromatic groups were modified to analyze the effects of ligands, and secondly several metals other than zinc were explored to study the effects of different metals on QSI activities. The lead compound ZnP is numbered as **1**. The summary of the compounds can be found in Table 4-2.

Table 4-2 SAR study and QSI activities of ZnP and its metal analogs*

Compound	Inhibitor Structure	IC ₅₀ (μM)	Compound	Inhibitor Structure	IC ₅₀ (μM)
1 X = Zn		1.20 ± 0.03	14 X = Zn		-
2 X = Co		2.44 ± 0.37	15 X = Co		4.83 ± 0.14
3 X = Ni		2.21 ± 0.18	16 X = Cu		7.50 ± 0.59
4 X = Cu		3.80 ± 0.92	17 X = Ni		-
5 X = V		1.73 ± 0.18	18 X = Zn		-
6 X = Co		8.80 ± 0.11	19 X = Co		-
7 X = Zn		-	20 X = Ni		-
8 X = Co		22.0 ± 2.6	21 X = Zn		5.31 ± 0.54
9 X = Cu		23.3 ± 2.4	22 X = Co		6.07 ± 0.83
10 X = Ni		-	23 X = Cu		8.79 ± 0.42
11 X = Zn		-	24 X = Ni		6.42 ± 0.48
12 X = Co		4.75 ± 2.39	25 X = Co		2.52 ± 0.04
13 X = Co		3.14 ± 0.94	26 X = Cu		4.58 ± 0.59
			27 X = Ni		6.90 ± 0.24
			28 X = Zn		11.7 ± 1.0

* IC₅₀ values based on PAO1-*lasB-gfp*. Calculations were based on three biological replicates and performed using GraphPad Prism 6 software.

Several novel compounds with different metal and ligand substituents were successfully synthesized. SAR study revealed the compounds that could deliver the lowest IC₅₀ values were derived from the original pyridithione motifs (compound **1** - **5**). Interestingly, substituting the sulfur ligand with oxygen reduced the QSI activities, whereas no inhibition was observed with the Zn and Ni analogs (**7** - **10**). We also tried to increase the lipophilicity of the compounds through quinoline and isoquinoline moieties, but no improvement was observed (**11** - **13**). Addition of extra aldehyde (**21** - **24**) or alcohol

functional groups (**25** – **28**) on the naphthalene side-chain seems to reduce hydrophobicity and provided overall good IC₅₀ values across different metal analogs. We also explored five-membered ring ligands (5-methyl-3-thiazole analogs, **14** - **17**), however only the cobalt and copper-containing analogs showed some inhibition of QSI activities. Replacing the aromatic ligand to the linear side chain completely obstructed the bioactivities (**18** – **20**). As a control for SAR study, metal salts only (ZnSO₄, CoBr₂, NiCl₂, CuSO₄, VOCl₃) were also tested but no QSI activity was observed at test concentration (10 μM, Supplementary Fig. 10).

4.2.2 Effects of metal analogs on *rhl* and PQS system

Across all the metal series, the cobalt analogs provided the most promising results with different ligand substituents. A few potential compounds were tested on QS bioreporter strains PAO1 *rhlA-gfp* and *pqsA-gfp* to see if they also affect other QS pathways. The results showed that the compounds could reduce the expression of *rhlA-gfp* in a dose-dependent manner (Figure 4-1A). The most promising compound was found to be cobalt-containing pyrithione (**2**, CoP), which IC₅₀ was calculated to be 4.10±0.02 μM (Figure 4-1B and C). As PQS system is also controlled by the *las* system, we also investigated the effect of synthesized metal compounds on *pqsA-gfp*. Dose-dependent effects were observed upon treatment with metal compounds (Figure 4-1D). Similarly, CoP was found to efficiently reduce the expression of *pqsA-gfp* with IC₅₀ values of 3.06±0.08 μM (Figure 4-1E and F). The summary of IC₅₀ values of metal analogs for *rhlA-gfp* and *pqsA-gfp* can be found in Table 4-3.

Figure 4-1 (A) Dose-response curve on *P. aeruginosa rhlA-gfp*, (B) dose-dependent curve and (C) IC₅₀ values of compound 2 (CoP) on *rhlA-gfp*. (D) Dose-response curve

on *P. aeruginosa pqsA-gfp*, (E) dose-dependent curve and (F) IC₅₀ values of compound **2** (CoP) on *pqsA-gfp*. Calculation was performed using GraphPad Prism 6 software. All experiments were done in triplicate manner, only representative data are shown.

Table 4-3 IC₅₀ values (μM) of metal analogs for *rhlA-gfp* and *pqsA-gfp*

Compound	<i>rhlA-gfp</i>	<i>pqsA-gfp</i>
ZnP	1.06±0.10	1.82±0.07
CoP	4.10±0.02	1.10±0.09
12	15.2±0.5	1.00±0.03
13	12.0±0.1	1.73±0.02
15	6.29±0.15	1.09±0.10
22	3.43±0.26	2.47±0.14
25	3.74±0.02	1.90±0.24

4.2.3 Effects of metal analogs on virulence factors production

Next, the effects of compounds on virulence factors production were also measured, where it showed the reduction of elastase, rhamnolipid and pyocyanin productions. Treatment with CoP was able to reduce elastase activity almost similar to *ΔlasIΔrhlI* level (Figure 4-2A). Compounds **13**, **22**, and **25** were able to reduce elastase level almost 70% as compared to the untreated PAO1 wild type as control. No apparent reduction was observed for compounds **12** and **15** on elastase level.

For other virulence factors production, compounds **2**, **12**, **15**, and **25** were seen to be most promising in reducing rhamnolipid and pyocyanin levels (Figure 4-2B and C). Compounds **13** and **22** did not show any significant reduction on both rhamnolipid and pyocyanin levels. The overall results demonstrated the application of novel metal analogs synthesized in this study as broad-target QSI and virulence attenuator in *P. aeruginosa*.

Figure 4-2 Effects of compounds on virulence factors production (A) elastase, (B) rhamnolipid and (C) pyocyanin production. Compounds were tested at final concentration of 10 μ M. PAO1 QS mutant strain *ΔlasIΔrhlI* was used as negative control. All experiments were done in triplicate manner, only representative data are shown. Error bars are means $\pm$ SDs. ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, Student's t test.

4.2.4 Effects of metal analogs on *P. aeruginosa* biofilms formation.

Given that QS contributes to the regulation of gene expression in biofilms, I examined the combination of metal compounds and colistin in clearing the colistin-resistant subpopulations in *P. aeruginosa* biofilms. Colistin has been used as one of the last-resort polymyxin antibiotics for drug-resistant Gram-negative bacteria, including *P. aeruginosa*.¹⁸⁵ Treatment of colistin alone wasn't able to kill the resistant cap-forming subpopulation in the outer layer of biofilm grown in flow cells.¹⁸⁶ Combination therapy between colistin and other antibiotics have been reported and show promising results in the lungs of CF patients.¹⁸⁷

Biofilms were grown on flow cells for three days before adding compounds, colistin, and combination of compounds and colistin on day 4. As seen from the confocal image, treatment with compounds alone at 10 μ M didn't eradicate the biofilm formation. In the colistin-treatment biofilms, only the stalk-forming subpopulations were killed due to the membrane-targeting action and the surviving antibiotic-resistant cells were able to migrate to the top layer. Lastly, combination treatment with compounds and colistin lead to the total eradication of biofilms, including the colistin-resistant subpopulations (Figure 4-3). The overall results indicate the potential of our new compounds as antivirulence therapy, as well as *P. aeruginosa* biofilms agents.

Figure 4-3 *P. aeruginosa* PAO1 tagged with GFP was grown in flow cell biofilms for three days. On the subsequent day, the biofilms were treated with DMSO control (0.1%), compounds (10 μM), colistin (10 $\mu\text{g/mL}$), and combination of compounds and colistin. Panel A shows flow cell experiments for ZnP, and panel B showed flow cell experiments for CoP. Experiments were done in triplicate manner, only representative images are shown. Live cells appear green and dead cells appear red. Scale bar, 50 μm .

4.2.5 Cytotoxic effects of metal analogs on macrophages

As the compounds synthesized in this study are mostly novel with unknown properties, I decided to evaluate their toxicity profile using murine macrophages. I first tested the pyrithione ligand with different metal substituents (**1 – 5**) and discovered that most compounds in this series except cobalt are quite toxic at their working concentration and above ($> 10 \mu\text{M}$, Figure 4-4A). Through optimization from SAR study, we managed to obtain less toxic series of compounds (**25 – 28**) that have low toxicity (up to 100 μM , Figure 4-4B). Lastly, I also did a comparison with selected metal analogs that have better QSI profiles and the results show most of the analogs are not cytotoxic up to 40 μM (Figure 4-4C). The results indicated that the metal analogs developed in this study could be further used for subsequent *in vivo* experiments at their relevant concentration. Through the optimization of SAR study, we also obtained several promising analogs with improved toxicity profiles.

Figure 4-4 Cytotoxic effect of various metal compounds on murine macrophages. DMSO was used as vehicle control. Compounds were tested at various concentrations (100 μM and subsequent dilution). Experiments were done in triplicate manner. Error bars are means $\pm$ SDs.

4.2.6 *In vivo* application of metal analogs

The ability of metal analogs to suppress QS *in vivo* was investigated using implant-associated infection in mice model. Due to the limited number of mice can be used for this study, we decided to focus on CoP and compound **25**, which have lower toxicity profiles and IC_{50} values as compared to other analogs. Silicone implants coated with *P. aeruginosa* biofilms were surgically inserted into the peritoneum of mice and treated with compounds and DMSO control on day 2 post-infection. For the treatment, 200 μL of compounds dissolved in the vehicle solution (20% 2-hydroxypropyl- β -cyclodextrin dissolved in 0.9% NaCl) were injected per mice in the morning and after 6 hours. Day 3 post-infection, the mice were sacrificed and the implants and spleens were extracted to quantify the content of bacteria.

Figure 4-5 Bacterial counts on (A) implants and (B) spleen after challenged with silicone implants coated with *P. aeruginosa* that would develop into biofilms. One day post infection (dpi), the mice were treated with compounds. The dosage for high concentration is 1 mg/kg and low concentration is 0.1 mg/kg. DMSO 0.1% was used as control. Mice were sacrificed on two dpi, and bacterial loads on implants and spleens were quantified. Statistical difference was tested by Mann-Whitney U test using GraphPad Prism 6 software. * = $p < 0.1$, ** = $p < 0.01$, *** = $p < 0.001$.

Based on the implants infection on mice, treatment with compounds CoP and **25** could reduce CFU counts on the implants (Figure 4-5A). By optimizing the dose of the compounds at high concentration (1 mg/kg), the efficacy of the treatment could be improved where we see clearance of bacteria in the implants. Treatment with the compounds could also prevent dissemination of bacteria into the spleen, as shown in the CFU counts (Figure 4-5B). This data further demonstrates how treatment with antivirulence compounds could help the host immune system to clear bacterial infection.

4.2.7 Transcriptional analysis of *P. aeruginosa* treated with ZnP and CoP

To evaluate the underlying mechanisms of compounds on *P. aeruginosa*, we decided to employ RNA sequencing (RNA-Seq) to study the gene expression and changes in overall mRNA expression. For this study, *P. aeruginosa* cultures were grown in the presence of 10 μ M ZnP, CoP and DMSO control, and harvested at the late-log phase when QS genes were highly activated. For the RNA-Seq results, a cutoff of ± 2 -fold change was set for the gene expression. The complete list of the genes being affected can be found in Supplementary Table 3 and 4.

Principal component analysis (PCA) plot and heat map show complete different gene expression profiles for DMSO control and treated groups (Figure 4-6). The overviews of the genes being upregulated and downregulated were also categorized based on the

gene functional classification For ZnP, many genes related to pathogenesis and phenazine biosynthetic process were downregulated. A huge set of genes for transcription factor activity was also downregulated. Zinc transmembrane transporter activity and membrane-associated genes were upregulated (Supplementary Figure 11). For CoP, genes associated with pathogenesis and nitrogen compound metabolic process were mostly downregulated. Metabolic process and isoprenoid catabolic process were mostly upregulated (Supplementary Figure 12).

Figure 4-6 (A) Heat map showing genes being up- and down-regulated for more than 2-folds between control and treatment groups. The Z-score shows the standardization of each gene expression level between the two different groups. (B) Principal component analysis (PCA) plot showing the expression profiles of *P. aeruginosa* between control and treatment with ZnP (C) and CoP (D). Each dot indicates one biological replicate with different colors representing the sample source.

For ZnP, a total of 257 genes were upregulated and 228 genes were downregulated. Among the upregulated genes, the prominent ones are in particular membrane proteins (Tyrosine porin *opdT*, 253-fold; multidrug efflux outer membrane protein *oprN* 5-fold), Type III repressor A (*ptrA*, 244-fold), heavy metals efflux system (*czcABCRS*, 54 – 87-fold), efflux pumps (*mexEF*, 5 - 17-fold), Hcp-secretion island-I encoded T6SS (*hcpI*, *vgrGI*; 15 – 16-fold), heat-shock proteins (*hslUV*, 6-8-fold; *hptG*, 8.4-fold; *ibpA*, 9.4-

fold). As expected, QS-regulated virulence genes were mostly downregulated, e.g. chitin-binding protein *cbpD*, hydrogen cyanide synthase *hcnABC*, protease *lasA* and elastase *lasB*, lectin *lecAB*, anthranilate synthase *phnAB*, phenazine *phzABCDEFGM*, PQS *pqsABCDE*, C4-HSL synthase *rhlI* and rhamnolipid biosynthase *rhlABC*. We also observed Zn uptake regulation gene *zur* and Zn transporter *znuABC* are downregulated (5- and 4-fold respectively), which play important roles in maintaining homeostasis of intracellular Zn level. Other genes found to be downregulated include T3SS which is also regulated by QS (translocator protein *popBD* and membrane protein *pcrV*), superoxide dismutase *sodM*, and redox-sensitive transcriptional activator *soxR* (Table 4-4).

For CoP, 47 genes had increased expression and 76 genes had decreased expression. Most of the up-regulated genes belong to the metabolic process (59.3%) and cellular process (33.3%). As expected, most of the downregulated genes are virulence-related, e.g. chitinase *chiC*, protease *lasA*, elastase *lasB*, pyochelin and phenazine biosynthesis pathways, and rhamnolipid biosynthase *rhlAB*. Interestingly, different efflux pumps were involved as compared to ZnP, where *mexGHI-opmD* were downregulated 2 – 3-fold. Another important finding is that *phrS* was found to be downregulated 2-fold in CoP, but not in ZnP. The small RNA PhrS has been reported to activate PqsR synthesis, which further regulates synthesis of PQS and pyocyanin.⁷⁹ The phenazine biosynthesis pathway was found to be mostly downregulated (4-fold, Table 4-4).

Table 4-4 Selected up- and downregulated genes of *P. aeruginosa* upon treatment with ZnP and CoP*

Gene name	ZnP		CoP		Gene product description
	Fold change	p value	Fold change	p value	
<i>cbpD</i>	-5.92	9.96E-116	-2.87	1.22E-122	Chitin-binding protein CbpD precursor
<i>chiC</i>	-6.36	9.01E-54	-4.52	6.20E-204	Chitinase
<i>hcnA</i>	-11.19	1.17E-08	NA	NA	Hydrogen cyanide synthase HcnA
<i>hcnB</i>	-6.14	9.85E-37	NA	NA	Hydrogen cyanide synthase HcnB
<i>hcnC</i>	-6.05	8.21E-44	NA	NA	Hydrogen cyanide synthase HcnC
<i>lasA</i>	-5.05	7.53E-72	-2.99	4.46E-126	LasA protease precursor
<i>lasB</i>	-36.09	8.18E-264	-2.29	2.91E-72	Elastase LasB
<i>lecA</i>	-52.08	1.42E-72	NA	NA	PA-1 galactophilic lectin
<i>lecB</i>	-10.00	2.31E-54	NA	NA	Fucose-binding lectin PA-IIL
<i>lipA</i>	-4.46	9.25E-40	NA	NA	Lactonizing lipase precursor

<i>phnA</i>	-13.00	1.91E-228	NA	NA	Anthranilate synthase I
<i>phnB</i>	-3.24	1.38E-68	NA	NA	Anthranilate synthase II
<i>phhR</i>	NA	NA	-2.97	3.56E-30	Transcriptional regulator PhhR
<i>phrS</i>	NA	NA	-2.12	1.82E-08	Small regulatory RNA, activator of PqsR synthesis
<i>phzA1</i>	-9.07	7.85E-05	NA	NA	Probable phenazine biosynthesis protein
<i>phzA2</i>	-21.00	2.95E-06	-4.82	3.89E-48	Phenazine biosynthesis protein
<i>phzB1</i>	-10.46	3.82E-27	NA	NA	Probable phenazine biosynthesis protein
<i>phzB2</i>	-6.26	1.20E-17	-4.19	5.38E-121	Phenazine biosynthesis protein
<i>phzC1</i>	-10.18	1.77E-04	NA	NA	Phenazine biosynthesis protein
<i>phzC2</i>	-3.55	4.22E-02	-4.25	3.09E-38	Phenazine biosynthesis protein PhzC
<i>phzD1</i>	-9.42	1.07E-03	NA	NA	Phenazine biosynthesis protein PhzD
<i>phzD2</i>	-4.63	1.87E-02	NA	NA	Phenazine biosynthesis protein PhzD
<i>phzE2</i>	-15.10	2.19E-02	NA	NA	Phenazine biosynthesis protein PhzE
<i>phzF2</i>	-88.49	3.30E-02	NA	NA	Phenazine biosynthesis protein
<i>phzG2</i>	-4.36	1.79E-03	-3.53	1.62E-35	Probable pyridoxamine 5-phosphate oxidase
<i>phzM</i>	-5.60	1.23E-50	NA	NA	Phenazine biosynthesis protein
<i>piv</i>	-7.75	5.48E-50	NA	NA	Protease IV; PvdS-regulated endoprotease
<i>pqsA</i>	-20.37	2.10E-197	NA	NA	Coenzyme A ligase
<i>pqsB</i>	-23.70	0	NA	NA	3-oxoacyl-(Acyl carrier protein) synthase III (PqsB)
<i>pqsC</i>	-16.11	1.19E-168	NA	NA	PqsC
<i>pqsD</i>	-13.74	4.34E-175	NA	NA	2-heptyl-4(1H)-quinolone synthase PqsD
<i>pqsE</i>	-11.81	1.37E-194	NA	NA	Quinolone signal response protein
<i>psrA</i>	4.09	2.97E-48	NA	NA	Transcriptional regulator PsrA
<i>rhlA</i>	-27.39	1.21E-159	-2.20	4.45E-64	Rhamnosyltransferase chain A
<i>rhlB</i>	-10.35	1.54E-82	-2.36	2.29E-71	Rhamnosyltransferase chain B
<i>rhlC</i>	-3.52	9.67E-31	NA	NA	Rhamnosyl transferase 2
<i>rhlI</i>	-4.44	1.60E-90	NA	NA	Autoinducer synthesis protein RhlI
<i>cobM</i>	NA	NA	-2.06	8.28E-04	Precorrin-3 methylase
<i>cobN</i>	3.69	8.20E-22	-2.45	8.66E-18	Cobalamin biosynthetic protein CobN
<i>czcA</i>	54.43	2.07E-281	NA	NA	RND divalent metal cation efflux transporter CzcZ
<i>czcB</i>	48.58	9.23E-98	NA	NA	RND divalent metal cation efflux membrane fusion protein CzcB precursor
<i>czcC</i>	87.03	1.05E-40	NA	NA	Outer membrane protein precursor
<i>czcR</i>	96.96	3.30E-147	NA	NA	Metal-inducible TCS transduction system
<i>czcS</i>	27.87	4.88E-165	NA	NA	CzcS sensor protein
<i>dkcA2</i>	-77.05	7.80E-77	NA	NA	RNA polymerase-binding transcription factor
<i>fpr</i>	NA	NA	-2.48	2.95E-56	Ferredoxin--NADP+ reductase

<i>fptA</i>	NA	NA	-3.68	8.82E-185	Fe(III)-pyochelin outer membrane receptor precursor
<i>fpvB</i>	-3.11	4.02E-34	NA	NA	Ferripyoverdin receptor, iron uptake
<i>np20</i>	-5.07	1.62E-69	NA	NA	Zur, zinc uptake regulation protein
PA3601	166.93	0	2.73	4.24E-42	50S ribosomal protein L31 type B, <i>rpmE2</i>
PA4837	-128.12	0	2.50	4.41E-18	TonB-dependent outer membrane protein, <i>zrmA</i>
<i>pchA</i>	NA	NA	-4.46	2.20E-181	Salicylate biosynthesis isochorismate synthase
<i>pchB</i>	NA	NA	-4.29	6.09E-141	Salicylate biosynthesis protein PchB
<i>pchC</i>	NA	NA	-3.78	1.27E-21	Pyochelin biosynthetic protein PchC
<i>pchD</i>	NA	NA	-4.26	9.09E-170	Pyochelin biosynthesis protein PchD
<i>pchE</i>	NA	NA	-5.37	1.39E-193	Dihydroaeruginic acid synthetase
<i>pchF</i>	NA	NA	-5.11	2.85E-176	Pyochelin synthetase
<i>pchG</i>	NA	NA	-4.22	9.83E-172	Pyochelin biosynthetic protein PchG
<i>pcrH</i>	-3.07	1.28E-09	NA	NA	Regulatory protein PcrH
<i>ppyR</i>	-6.80	4.67E-10	NA	NA	Psl and pyoverdin operon regulator
<i>pvcB</i>	-3.71	3.66E-03	NA	NA	Pyoverdin biosynthesis protein
<i>pvdJ</i>	4.54	2.87E-52	NA	NA	Probable non-ribosomal peptide synthetase
<i>pvdS</i>	-3.70	7.32E-35	NA	NA	Sigma factor; activate production of pyoverdine
<i>znuC</i>	-4.52	2.83E-59	NA	NA	Zinc transport protein ZnuC
<i>hcpI</i>	16.55	9.81E-234	NA	NA	Hcp secretion island I (HSI-I) T6SS
<i>icmF1</i>	6.92	2.50E-80	NA	NA	Hcp secretion island I (HSI-I) T6SS
<i>pcrV</i>	-3.31	1.97E-14	NA	NA	T3SS secretion protein
<i>popB</i>	-3.48	5.12E-10	NA	NA	Translocator protein, T3SS
<i>popD</i>	-3.13	2.06E-08	NA	NA	Translocator protein, T3SS
<i>tssB1</i>	11.1	2.85E-100	NA	NA	Hcp secretion island I (HSI-I) T6SS
<i>vgrG1</i>	15.96	3.79E-113	NA	NA	Hcp secretion island I (HSI-I) T6SS
<i>mexE</i>	17.40	9.03E-56	NA	NA	RND multidrug efflux membrane fusion protein MexE
<i>mexF</i>	5.22	1.57E-66	NA	NA	RND multidrug efflux membrane fusion protein MexF
<i>mexG</i>	NA	NA	-2.59	1.76E-14	RND multidrug efflux membrane fusion protein MexG
<i>mexH</i>	NA	NA	-2.79	7.87E-16	RND multidrug efflux membrane fusion protein MexH
<i>mexI</i>	NA	NA	-2.24	3.76E-19	RND multidrug efflux membrane fusion protein MexI
<i>opmD</i>	NA	NA	-2.13	5.90E-05	Probable outer membrane protein precursor
<i>oprD</i>	-11.38	8.62E-141	NA	NA	Outer membrane porin protein OprD precursor
<i>oprN</i>	5.47	3.15E-14	NA	NA	Multidrug efflux outer membrane protein OprN precursor
<i>pilA</i>	4.08	1.17E-33	NA	NA	Type 4 fimbrial precursor PilA
<i>pilC</i>	3.07	4.92E-32	NA	NA	Type 4 fimbrial assembly protein PilC

<i>pilD</i>	3.33	9.45E-35	NA	NA	Type 4 prepilin-like proteins leader peptide-processing enzyme
<i>sodM</i>	-4.90	1.95E-109	NA	NA	Superoxide dismutase
<i>soxR</i>	-3.21	4.18E-21	NA	NA	Redox-sensitive transcriptional activator SoxR

* Blue is QS-regulated genes, green is metal ions transport and siderophore genes, pink is T3SS and T6SS-related genes, purple is efflux pump and porin associated genes.

Regardless of the structure similarity, RNA-Seq analysis suggested that ZnP and CoP seem to produce different effects on the gene expression of *P. aeruginosa*. Among upregulated genes, only eight genes showed increased expression in both treatments (*aroP2*, *gcvP2*, *gcvH2*, *glyA2*, PA0863, PA1297, PA1298, *phhB*). They correspond to glycine and tetrahydrobiopterin biosynthesis pathway, as well as metal transporter. For the downregulated genes, there are 21 genes show decreased expression in both treatments (*cbpD*, *chiC*, *lasA*, *lasB*, *opmD*, PA0236, PA1869, PA2067, PA2068, PA2069, PA2134, PA3601, PA4141, PA4142, *phzA2*, *phzB2*, *phzC2*, *phzG2*, *piv*, *rhlA*, *rhlB*). QS-associated virulence factors were found to be downregulated in both compounds, such as chitinase, elastase, protease IV (PIV), rhamnolipid and phenazine. PA0236 is a transcriptional factor with DNA-binding site and unknown function. PA3601 (*rpmE2*) encodes zinc-independent paralogs of ribosomal protein L31 and usually expressed in low Zn condition. Interestingly, we also observe downregulation of PA4141 and PA4142, encoding a putative T1SS in *P. aeruginosa*.¹⁸⁸

4.2.8 Effects on small RNAs

To further evaluate the molecular mechanism of the compounds, the ZnP and CoP were tested on bioreporter strains carrying *rsmY-gfp* and *rsmZ-gfp*. The role of small RNAs (sRNAs) RsmY and RsmZ in QS and biofilms formation, whose expression is also controlled by GacA-GacS, has been widely studied in *P. aeruginosa*. As the compounds affect the expression of many QS-related genes, it was hypothesized that Gac/Rsm signaling cascade could be affected.

Figure 4-7 Dose-dependent curves of ZnP incubated with *P. aeruginosa* PAO1-*rsmY-gfp* (A) and PAO1-*rsmZ-gfp* (B), and CoP incubated with PAO1-*rsmY-gfp* (C) and PAO1-*rsmZ-gfp* (D). DMSO 0.1% was used as control. The experiments were done in triplicate manner; only representative data are shown.

Based on the bioreporter strains assay, ZnP was observed to induce both *rsmY* and *rsmZ-gfp* expression. The results are quite intriguing as increased in small RNAs would affect QS production as well, but we observed downregulation in QS genes upon treatment with ZnP. CoP did not affect *rsmY* but decreased *rsmZ-gfp* expression in a dose-dependent manner. This experiment further shows that the two compounds could operate through different mechanisms, and the striking differences could be caused by the particular metal ions (zinc or cobalt).

4.2.9 Influence on metal homeostasis - effects on intracellular metal concentrations

Since the compounds contain metals (either zinc or cobalt), it was hypothesized that they could interfere with the intracellular metal homeostasis and affect virulence production of *P. aeruginosa*. Transcriptional analysis of ZnP reveals that it affects zinc uptake pathway. RNA-Seq data shows that metal cation transporter (*czcABC*) was upregulated (up to 97-fold), while the zinc ABC transporter (*znuC*) and the zinc uptake

regulation (*zur*) were downregulated (up to 5-fold). RNA-Seq data of ZnP also shows massive downregulation in the gene clusters controlled by *cnt* operon (PA4834 - PA4837), in contrast to CoP where they were upregulated. Transcriptomic data of other Zur-regulated genes were shown in Table 4-5.

Table 4-5 Gene expression of Zur regulated genes in *P. aeruginosa*¹⁸⁹

Locus tag	Gene	ZnP		CoP		Gene product description
		Fold change	p value	Fold change	p value	
PA0781	<i>omr</i>	-53.71	7.03E-172	2.69	4.42E-11	Zn-regulated outer membrane receptor
PA1921		-7.58	1.78E-52	NA	NA	S-adenosyl-L-methionine-dependent methyltransferase-like
PA1922		-103.21	7.68E-95	NA	NA	Probable outer membrane protein; TonB-dependent outer membrane protein
PA1923		-33.91	1.74E-76	NA	NA	CobN cobaltochelataase subunit
PA1924		-9.16	2.33E-24	NA	NA	Biopolymer transporter ExbD
PA1925		-7.53	9.22E-39	NA	NA	Hypothetical protein
PA3600	<i>rpmJ</i>	-118.65	9.91E-222	2.86	3.16E-17	50S ribosomal protein L36 2
PA3601	<i>rpmE2</i>	-166.93	0.00E+00	2.73	4.24E-42	50S ribosomal protein L31 type B
PA4063		-77.46	6.23E-180	2.83	1.23E-15	Zn ²⁺ periplasmic binding protein
PA4064		-7.35	1.32E-43	NA	NA	ABC transporter nucleotide binding protein
PA4065		-9.61	2.13E-107	NA	NA	Lipoprotein release ABC transporter permease
PA4066		-5.79	1.28E-49	NA	NA	Lipoprotein; Zn ²⁺ periplasmic binding protein
PA4834	<i>cntI / zrmD</i>	-7.86	5.96E-109	2.24	5.17E-04	Putative membrane transporter
PA4835	<i>cntM / zrmC</i>	-23.41	1.56E-139	2.10	1.28E-06	Hypothetical protein
PA4836	<i>cntL / zrmB</i>	-63.99	2.29E-178	2.50	4.41E-18	Probable nicotianamine synthase protein (important divalent metal chelator)
PA4837	<i>cntO / zrmA</i>	-128.12	0.00E+00	2.24	5.17E-04	Probable outer membrane protein; TonB-dependent outer membrane protein
PA2946		3.17	1.62E-47	3.17	1.62E-47	Hypothetical protein; Putative cobalt transporter subunit (CbtA)
PA5498	<i>znuA</i>	-8.61	5.38E-89	NA	NA	Zn ²⁺ ABC transporter substrate-binding protein
PA5499	<i>zur</i>	-5.07	1.62E-69	NA	NA	zur; Zinc uptake regulation protein
PA5500	<i>znuC</i>	-4.52	2.83E-59	NA	NA	Zinc ABC transporter, ATP-binding protein ZnuC
PA5534		-21.79	1.23E-128	NA	NA	Hypothetical protein
PA5535		-20.37	8.28E-227	NA	NA	Cobalamin synthesis protein

PA5536	<i>dksA2</i>	-77.05	7.80E-77	NA	NA	RNA polymerase-binding transcription factor dksA2, DnaK suppressor protein
PA5537		-5.6	5.33E-33	NA	NA	Glutamine synthetase
PA5538	<i>amiA</i>	-54.6	7.33E-305	NA	NA	Peptidoglycan amidase AmiA
PA5539	<i>folE2</i>	-86.57	6.34E-139	NA	NA	GTP cyclohydrolase I type 2
PA5540	<i>can</i>	-36.68	2.36E-168	2.16	1.29E-03	Carbonate dehydratase
PA5541	<i>pyrQ</i>	-8.57	1.09E-197	2.35	2.74E-04	Probable dihydroorotase

To further show that the addition of the compounds could affect metal homeostasis, I next investigate the effects of the compounds on the intracellular metal concentration. The metal compounds were added into *P. aeruginosa* culture and incubated overnight. DMSO 0.1% was used as control. Next, the cultures were separated using centrifugation and the cell pellets were lysed. The intracellular and supernatant portions were subjected to inductively coupled plasma mass spectrometry (ICP-MS) to measure the metal concentration. The results were then normalized with protein levels.

Figure 4-8 Concentration of metal ions in the presence / absence of compound (ZnP or CoP). Intracellular and supernatant of the cells were measured using ICPMS. Experiments were done using three biological replicates, only representative data were shown. Error bars are means $\pm$ SDs.

From the ICP-MS results, we could observe a very high level of metal concentration both intracellularly and in the supernatant as compared to the non-treated condition. Metal ions were higher in the supernatant as compared to the intracellular conditions. The results show that the metal compounds could indeed enter the cells and potentially act as metal ions importer (Figure 4-8).

Next, the efficiency of the compounds to import metal ions was compared with commonly used metal salts, such as zinc bromide (ZnBr_2) and cobalt bromide (CoBr_2). The metal salts are commonly used reagents to study the effects and implications of specific metal ions to the growth of bacteria. As part of the SAR study, I also included

some of the metal analogs synthesized in this study that did not have QSI activities. Compounds **7** and **8** are also pyrrhione-based ligand, with the sulfur atom replaced with oxygen. This minor tweak resulted in the loss of QSI activities. Compounds **18** and **19** have a linear ligand, and this also resulted in no QSI activities. It was hypothesized that the loss of QSI activity could be due to the inability of compounds to import the metal ions to the intracellular compartment.

Figure 4-9 Intracellular concentration of metal ions upon addition with different metal salts and compounds. (A) Comparison between ZnP, ZnBr₂, and different metal analogs, (B) CoP, CoBr₂, and different metal analogs. (C) Metal concentrations in the supernatant of ZnP, ZnBr₂, and different metal analogs. (D) Metal concentrations in the supernatant of CoP, CoBr₂, and different metal analogs. Experiments were done using three biological replicates, only representative data were shown. *** = $p < 0.001$, **** = $p < 0.0001$, Student's t test. Error bars are means $\pm$ SDs.

Treatment with metal salts only (ZnBr₂ and CoBr₂) at 10 μ M did not affect much of the intracellular metal concentration as compared to the DMSO control and untreated PAO1 wild type. As observed from the previous experiment, there is a significant increase in the intracellular metal concentration when treated with ZnP and CoP at 10 μ M (Figure 4-9A and B). Treatment with compound **7** and **8** did not change intracellular metal concentrations. Interestingly, addition of compound **18** resulted in even higher intracellular Zn concentration as compared to ZnP. This phenomenon was not observed

in compound **19**. Not much difference in the supernatant of different metal analogs, except for compounds **18** and **19** (Figure 4-9C and D).

To further confirm the role of metal ions, the QSI activities of metal compounds were tested with and without the presence of metal chelator like EDTA using QS bioreporter strains (PAO1-*lasB-gfp*). EDTA was found to diminish the QSI activities of ZnP and CoP, and the effects could be observed in the dose-dependent manner (Figure 4-10A and B). Increasing the concentration of EDTA restored the *lasB-gfp* expression almost to the wild type levels. As a control, EDTA itself did not affect *lasB-gfp* expression (Figure 4-10C). Lastly, I also look at the levels of pyoverdinin, an important iron siderophore and virulence factor produced by *P. aeruginosa*. When PAO1 was treated with metal chelator like EDTA, it was expected to observe higher level of pyoverdinin (PVD) production to compensate the loss of metal ions. Here, we observed that addition of EDTA increased PVD levels as compared to PAO1 wild type (Figure 4-10F). Treatment with ZnP and CoP reduced PVD production, and addition of EDTA reversed the effects (Figure 4-10D and E).

Figure 4-10 Effects of ZnP and CoP with / without EDTA on QS bioreporter strains PAO1-*lasB-gfp*. Dose-dependent curve of EDTA with ZnP 5 μ M (A), CoP 5 μ M (B), and EDTA only (C). PVD measurement of EDTA with ZnP 5 μ M (D), CoP 5 μ M (E), and EDTA only (F). Single treatments were marked with (-) as controls (ZnP only, DMSO only, DMSO+EDTA only). GFP fluorescence (excitation at 485 nm, emission at 535 nm) and pyoverdinin (excitation at 405 nm, emission at 465 nm) were measured

using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). Experiments were done in triplicate manner, only representative data was shown.

4.3 Discussion

The discovery of cisplatin as an anticancer drug in 1965 has opened a new class of pharmacophore which incorporates transition metal and metal-binding groups in the drug design. Such class of compounds is called metallodrugs, where transition-metal-based complexes undergo ligand exchange with the target protein at the active site to form drug-target covalent bond. In all cases, the mechanism of actions can be traced from the metal-ligand interactions with the metalloenzymes to inhibit the functionality. Several compounds belong to this class have found their way into clinical trials and approved by FDA for treatment of various diseases, such as auranofin (gold compound), RAPTA-T (ruthenium-based agent), Raltegravir (magnesium-chelated drugs), Vorinostat (zinc-containing complex), etc.¹⁹⁰

In this study, a series of novel derivatives based on one metallodrug named zinc pyrithione (ZnP) were reported. A small library has been constructed, where the metal center is incorporated into the ligand. By exploring various transition metals and organometallic ligands, novel metal analogs were successfully synthesized and characterized. The compounds were then tested for their QSI activities in *P. aeruginosa*. SAR study reveals that the thiol group in the pyrithione ligand is essential for its inhibition activity. Substituting the 6-membered ring with the 5-membered ring (compounds **14** – **17**) or linear ligands (compounds **18** – **20**) diminished the effects. Through optimization from SAR study, we also managed to obtain less toxic series of compounds (**25** – **28**) that have better toxicity profiles as compared to original pyrithione ligands. Zinc and cobalt-containing compounds were found to be less toxic in murine macrophages, as compared to copper, nickel and vanadium complexes.

Among the best candidates, a few compounds were selected to compare their QSI activities on virulence factors production. Both compounds **12** and **15** were able to reduce rhamnolipid levels, but no effect was observed on the elastase level. In contrast, compound **22** was able to reduce the elastase level, but not so much on rhamnolipid and pyocyanin. It was interesting to see how the substitution of the aldehyde to alcohol group could significantly improve the QSI activities. Compound **25** was found to successfully reduce all three virulence factors production.

The overall results propose that the compounds could have different target of master regulators that control QS. On top of that, the physical properties of the compounds must also be considered (ie. solubility, stability, dissociation constant, etc). The different ligands could contribute to different chelation effects on the metal ions, and might affect the complex dissociation and concentration in the cells. Further investigation on the coordination chemistry and the dynamics of metal-ligand interactions would be beneficial in elucidating the mechanism of the compounds.

Next, the efficacy of the compounds on biofilms formation in *P. aeruginosa* was assessed. Based on the biofilms study using flow cells condition, we observed treatment with the compounds alone could not eliminate mature *P. aeruginosa* biofilms but was capable of eradicating colistin-resistant cells when used together with the antibiotic. The combined effect of ZnP and colistin in clearing the flow cell biofilms could be attributed to the attenuation of several QS genes that have roles in biofilm development by ZnP. Previous studies have demonstrated that biofilms grown with defective QS systems are more vulnerable to antibiotic treatments and PMNs phagocytosis.^{102, 170, 191} It was proposed that once stalk subpopulations were killed by colistin, surviving antibiotic-resistant microcolonies formed through QS-regulated factors on the top of the dead biofilm.^{187c} Utilizing compounds that inhibit the cell-to-cell signaling such as ZnP and CoP could increase the biofilm susceptibility towards treatment with colistin and help to improve the effectiveness of the antibiotic.

In chapter 3, promising results for the potential treatment of ZnP in clearing biofilms using mice implant models have been demonstrated. The preliminary testing with CoP and compound **25** shows both compounds could eradicate the biofilms infection. The results further show how targeting QS and reducing virulence could help host immune system to fight against bacterial infection. This strategy could be an alternative approach for the treatment of *P. aeruginosa* infections and complement the conventional antibiotics remedy.

The next puzzle remained to be solved is the mechanism of action of the metal compounds. Ever since its discovery, many groups have investigated how ZnP could exert its antifungal and antimicrobial mechanism. Firstly, ZnP could inhibit yeast growth through copper influx and inactivation of Fe-S protein,^{179b} and induce iron starvation and DNA damage in *Saccharomyces cerevisiae*.¹⁹² ZnP could also stimulate

cellular import of Zn^{2+} and efficiently inhibit RNA Polymerase of coronavirus and arterivirus.¹⁹³ Recently, ZnP has been discovered to inhibit aminoglycoside acetyltransferase (AAC) resistance enzymes in various bacteria strains (*A. baumannii*, *E. coli*, and *K. pneumoniae*). Combination treatment with amikacin and tobramycin were able to reduce MIC values in several clinical isolates harboring the resistance genes.¹⁹⁴ The inhibitory activity was derived from the inhibition of the AAC from the metal salts. The versatility of ZnP in various biological applications has prompted further investigation on the mechanism and how the compounds could affect QS in *P. aeruginosa*.

Next, I utilized Illumina HiSeq to analyze transcriptomic profiles of *P. aeruginosa* upon treatment with ZnP and CoP. RNA-Seq results show QS-regulated genes were downregulated, including the *las*, *rhl*, and PQS genes. Stringent response and stress-response genes were also found to be downregulated. Unfortunately, no master regulators were identified to be affected. Small RNA PhrS, which is an activator of PqsR synthesis, was found to be downregulated in the CoP-treated group.

Bacteria employ a set of genes to regulate metal homeostasis in response to its availability in the environment. It could be achieved through control of metal-sensing regulatory transcription factor, such as Fur for iron and Zur for zinc. As expected, heavy metal efflux system and *zur* regulation pathways were found to be affected upon treatment with metal compounds. The presence of excess zinc increased the expression of the metal efflux pump CzcABC, and decreased expression of the zinc transport protein ZnuC, implying bacteria respond to maintain homeostasis. The first report on how transcriptional regulator CzcRS, which is inducible by metal, modulates QS in *P. aeruginosa* was reported by Dieppois *et al.*¹⁹⁵ Chromosome immunoprecipitations (ChIP) experiments showed localization of CzcR on *lasI* promoter, but not on the other *rhl* and PQS regulations. Elastase, rhamnolipid and biofilms production was impaired in ΔczcRS mutant; however, the exact mechanism on how CzcR controls QS is still unclear.

RNA-Seq data of ZnP also shows huge downregulation in the gene clusters controlled by *cnt* operon (PA4834 - PA4837), whereby CoP shows only slight upregulation (Table 4-5). Interestingly, this particular cluster was also studied and published at the same time by two groups independently, where they were also called as *zrmDCBA*.^{189, 196} The four

genes were part of the operon that is under control of *zur* repressor and highly induced in zinc deficiency condition. They are involved in the biosynthesis of metallophores called pseudopaline, a stereoisomer of staphylopine, found in *Staphylococcus aureus*.¹⁹⁷ The importance of *cnt* (*zrm*) cluster has been reported in several studies, where it facilitates zinc acquisition both in culture and during infection, maintains zinc homeostasis, virulence factors production, and establishes acute and systemic infections in mice.^{189, 196-197} It has been proposed that such metallophores and the *cnt* importer roles resemble a parallel function of siderophore-mediated iron uptake in *P. aeruginosa*.

Another interesting finding is upregulation of the T6SS genes in ZnP-treated group. The T6SS is multi-component machinery employed by bacteria for inter-bacterial competition, host attack and virulence.¹⁸⁸ On top of the defense and weaponry machinery, the role of T6SS in metal ions uptake has also been elucidated. As eukaryotic hosts often activate the ‘nutritional immunity’ to sequester metal ions, bacterial species engage the T6SS to acquire metal ions.¹⁹⁸ T6SS-4 of *Yersinia pseudotuberculosis* was reported to participate in Zn^{2+} acquisition during high oxidative stress condition. The author postulated that the activation of T6SS-4 in metal acquisition is only activated when classical transporters (like *ZnuCB*) are not sufficient. Mutation of T6SS-4 and *znuCB* resulted in mutant strains that were avirulent in mice infection model.¹⁹⁸ A recent paper by Lin *et al.* has also reported the role of T6SS effector (TseF) for iron acquisition in *P. aeruginosa*. TseF could be incorporated into outer membrane vesicles (OMVs) containing PQS- Fe^{3+} , recognized by cell surface receptors FptA or OprF to deliver iron into the cells.¹⁹⁹ The exact mechanism is still unknown; nonetheless regulation of T6SS by Fur or iron has also been reported in *E. coli*, *Edwardsiella tarda*, *Burkholderia mallei* and *Burkholderia pseudomalleis*.²⁰⁰

Based on RNA-Seq analysis, siderophore encoding PVD and pyochelin genes were found to be downregulated in both groups. This implies that the treatment of ZnP and CoP might affect iron uptake system in *P. aeruginosa*. The possibility of excess zinc and cobalt to affect other metal ions pathway has been reported. In *S. pneumoniae*, exposure to excess Zn(II) impaired the ability for Mn(II)-binding protein PsaA to bind with manganese, causing the bacteria to become hypersensitive to oxidative stress.²⁰¹ Metal intoxication has also been reported to cause enzyme mismetallation leading to inactivation.²⁰² For instance, Fe-S clusters regulates many aspects of cellular processes

in bacteria and particularly vulnerable to metal intoxication.²⁰³ In *Bacillus subtilis*, excessive copper resulted in an imbalance of iron metabolism by interfering with Fe-S cluster formation.^{203a}

Several studies have shown how metal ions affect virulence across different bacteria species.^{202, 204} Mutants lacking metal efflux genes have been found to be less virulent in different host model.²⁰⁵ In *P. aeruginosa*, zinc homeostasis affects the expression of QS-related virulence traits, swarming and swimming motility, as well as biofilms formation.^{195, 206} The zinc uptake was also found to interfere with iron uptake via synthesis of pyoverdine, and important for colonizing human lungs.²⁰⁶ The correlation between *zur* and virulence has also been described *in vivo*. Δzur did not affect the growth of *P. aeruginosa* but significantly reduce virulence on neutropenic mice.²⁰⁷ Gallium, which is transition metal similar to iron, has also been reported to interfere with iron-related metabolic processes and is able to kill planktonic and biofilms of *P. aeruginosa*.²⁰⁸

Previous study by Reeder *et al.* have shown ZnP could inhibit yeast growth through copper influx and inactivation of Fe-S proteins.^{179b} Similarly, Yasokawa *et al.* also reported ZnP causes iron starvation in *S. cerevisiae*.¹⁹² Transcriptomic analysis from this study corroborates with previously reported mechanisms of ZnP in other organisms. Therefore, I conclude that the metal compounds ZnP and CoP act as ionophores, which could transport metal ions across the lipid membrane into the cells and cause an imbalance in metal homeostasis in *P. aeruginosa*, which mediates their effects to the QS system and leads to the attenuation of virulence. Further studies are required to investigate the exact molecular mechanism of how metal ions control QS in *P. aeruginosa*. This study further highlights how interfering with metal homeostasis could be used as an alternative strategy to control QS and attenuate bacterial virulence.

5 ADAPTIVE EVOLUTION STUDY OF METAL PYRITHIONE

As discussed in Chapter 1 literature review section, *P. aeruginosa* is an excellent adapter towards many environmental stresses, including antimicrobial resistance through various mechanisms. Its common strategies include mutations in drug targets, modulation of efflux pump activities, enzymatic modification of drugs, and decreasing membrane permeability. In most cases, bacteria could develop spontaneous mutations that lead to modification of the target, so that the drugs no longer bind to it.²⁰⁹ To further study the molecular mechanism of our compounds, I decided to use adaptive evolution assay to trace the mutation occurred across the genome, which could be potentially the drug target. Next, molecular genetic approaches were used to reverse the mutation and observe the phenotypes. To confirm the results, the putative target protein was purified and subjected to the binding assay with the compounds. Overall, various chemical biology tools were utilized to shed some lights on the molecular mechanism of novel metal compounds in *P. aeruginosa*.

5.1 Materials and Methods

5.1.1 Bacterial strains and media

Bacteria were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 0.5% NaCl). Media used for biological assay was ABTGC (AB minimal medium supplemented with 0.2% glucose and 0.2% casamino acids) to minimize fluorescence interference.¹⁰⁹ Bacterial strains used in this study are *P. aeruginosa* wild type PAO1 and the evolved strains as described in the following section.

5.1.2 Evolution assay

Overnight of *P. aeruginosa* wild type culture was diluted to OD 0.01 in ABTGC medium. Compounds at sub-MIC concentration (treated group) and DMSO (control group, contains the same concentration as the treated group) were added in the bacterial cultures and grown for 24 hours at 37 °C, shaking condition (200 rpm). The cultures were passaged every day to fresh media (OD 0.01) with the same concentration of compounds and DMSO control, while their growth was monitored every day. Experiments were halted when resistance has developed over time. Evolved strains were stored in 25% glycerol under -80 °C as frozen stocks for further experiments. Experiments were done with three biological replicates for each group.

5.1.3 Whole genome sequencing

Three single colonies from each biological replicates were inoculated in ABTGC medium for 24 hours at 37 °C, shaking condition (200 rpm). Genomic DNA from each isolate was purified using Blood and Cell Culture DNA Midi Kit (Qiagen). Whole genome sequencing was done using Illumina HiSeq 2500 platform to assemble the full-length genomes and identify SNPs from the evolution assay. The assembled genomes were then processed using CLC Genomic Workbench 8.5 (CLC Bio, Qiagen, USA).

5.1.4 Detection of nucleotide differences

Nucleotide differences were done using CLC Genomic Workbench 8.5 (CLC Bio, Qiagen, USA). Paired-end reads from each group in FASTQ formats were mapped to the PAO1 genome (<http://www.ncbi.nlm.nih.gov/nuccore/110645304>) as reference. The reads from the treated group were then compared with control group to generate lists of SNPs and short indels. A frequency cut-off of more than 80% was set to minimize false SNPs due to sequencing error.

5.1.5 Transformation of evolved strains

Plasmid extraction from QS reporter strains (*lasB*, *rhlA*, and *pqsA-gfp*) was done using Nucleospin® Plasmid EasyPure (Macherey-Nagel, Germany). The plasmids were then transformed into evolved strains by electroporation. The transformed strains were then lysed with cracking buffer and verified with gel electrophoresis. Gel images can be found in Supplementary Figure 13.

5.1.6 Complementation of *mexS*

The wild type *mexS* gene was amplified from PAO1 genomic DNA using primers *mexSCOM_F_BamHI* (5'- CTGAGGATCCGCACTGCAGAGGTTTGCG-3') and *mexSCOM_R_HindIII* (5'- ACTGAAGCTTTCTGTGGATCTGAAACCGCG-3'), and inserted into BamHI and HindIII sites in pUCP18 vector to construct *mexS* complementation plasmid pUCP18::*mexS*. The pUCP18::*mexS* plasmid was transformed into evolved strains by electroporation.

5.1.7 Surface Plasmon Resonance (SPR)

For SPR experiment, proteins of interest were prepared in binding buffer (ie. PBS pH 8.0). Small molecules were prepared in DMSO as 100 mM stock solution. SPR experiments were done using Biacore T-200 biosensor with research grade

carboxymethylated (CM5S) sensor chips (Biacore, Sweden). Carboxymethylated CM5S chips were activated using 70 μL of 0.2 M N-ethyl-N'-(dimethylaminopropyl)carbodiimide and 0.05 M N-hydroxysuccinimide in a 1:1 ratio. 100 μL of MexS in sodium acetate solution (0.1 mg/mL, pH 4) were injected over the activated surface for 10 minutes at a flow rate of 10 $\mu\text{L}/\text{min}$ and reached approximately immobilization level of 17000 response units. Next, 100 μM stock solutions of compounds were prepared in PBS pH 8.0 with 5% DMSO. 2-fold serial dilutions were performed in the same buffer to eight concentration ranging from 0.78 to 100 μM . The solutions were then injected over immobilized proteins for 50 s at flow rate of 10 $\mu\text{L}/\text{min}$, and dissociation of the bound analytes was monitored for 3 mins after the end of the injection. The chip surface was regenerated with PBS (pH 8.0) for 100 s. All assays were done in triplicate manner, and average values were reported.

5.1.8 Quantification of AHLs from bacterial cells

Overnight culture of *P. aeruginosa* wild type and evolved strains were diluted in 15 mL of ABTGC medium to a final optical density at 600 nm (OD_{600}) of 0.01. The cultures were grown for 16 hours at 37 °C, shaking condition (200 rpm). The cells were centrifuged at 10,000 g for 3 mins and washed twice with 1 mL of 1 mM ice-cold ammonium acetate. The cells were then resuspended in 1 mL of ice-cold AMW solution (40 % Acetonitrile / 40 % Methanol / 20 % H_2O) and sonicated for 2 x 15 mins in ice-filled water bath (37 Hz, 100% power). Afterwards, the cultures were centrifuged at 20,000 g for 10 mins at 4 °C. The step can be repeated if necessary to remove all cell debris. Lastly, the supernatant was collected and concentrated to remove organic solvent. 100 – 200 μL of AMW solution was added into the dried samples and subjected to LCMS for AHL quantification. The LCMS analysis was performed in Singapore Phenome Centre (Lee Kong Chian School of Medicine, NTU, Singapore). The concentrations of 3-oxo-C12-HSL and C4-HSL were normalized with proteins level and reported as average results from three biological replicates.

5.1.9 Pyoverdinin quantification

Subculture of *P. aeruginosa* was diluted 100 times from overnight culture into fresh ABTGC medium ($\text{OD}_{600} = 0.1$). Treated cultures were added with test compounds to give final concentration of 10 μM . The cultures were grown for 24 hours at 37 °C, shaking condition (200 rpm). Supernatants were collected by centrifuging at 10,000 rpm

for 3 mins. Pyoverdin was measured in supernatants by recording the fluorescence (excitation at 405 nm, emission at 465 nm) using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). Experiments were done in triplicate manner.

5.2 Results

5.2.1 Adaptive evolution assay

For the adaptive evolution study, *P. aeruginosa* PAO1 were grown in the presence of the compounds ZnP, CoP and DMSO as solvent control in ABTGC minimal medium. Conventionally, the adaptive evolution exposes bacteria towards different drug / antibiotic concentration with slow gradient increment. The evolved strains were then analysed and compared with the ancestor for differences in the mutations, growth profiles, phenotypes and genotypes. In this study, the bacteria were exposed with fixed concentration of the compounds at sub-MIC level (20 μ M) as mild selection pressure. The growth was monitored every three days to check if resistance has developed. After 21 days of exposure to ZnP, the strains have evolved resistance at 40 μ M. No increment in MIC values was detected in both DMSO and PAO1 control strains (Figure 5-1). The evolved strains of ZnP and DMSO were stored in frozen stock for the subsequent genomic experiment. A similar experiment was also done for CoP, where the strains were evolved in the presence of sub-MIC concentration (20 μ M). The experiments were halted at day 26, where CoP-evolved strains show an increased growth curve up to 100 μ M (Figure 5-2).

Figure 5-1 Growth profiles of strains evolved with ZnP and DMSO. *P. aeruginosa* PAO1 was used as reference strain. ZnP was added at various concentrations to the strains and the growth was monitored for 16 hours using Tecan Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). Experiments were done in triplicate manner, only representative data were shown.

Figure 5-2 Growth profiles of strains evolved with CoP and DMSO. *P. aeruginosa* PAO1 was used as reference strain. CoP was added at various concentrations to the strains and the growth was monitored for 16 hours using Tecan Tecan Infinite 200 Pro

plate reader (Tecan Group Ltd, Männedorf, Switzerland). Experiments were done in triplicate manner, only representative data were shown.

5.2.2 Genomic comparison of evolved strains

From the adaptive evolution assay, total genomic DNA was harvested from the evolved strains. Next, I utilized Illumina HiSeq to compare and determine the single-nucleotide polymorphisms (SNPs) and short indels between our evolved strains, DMSO control strains and PAO1 reference strain. For ZnP-evolved strains, mutations were found on *phoQ* and *mexS* in two different populations and *rne* mutation in one population. For CoP-evolved strains, *mexS* mutation was also identified across all three different populations. Intriguingly, iron pathway and pili were also affected in CoP-evolved strains. The results were summarized in Table 5-1.

Table 5-1 Summary of mutations identified from the evolved strains

Popula tion	Chromosome	Region	Type	Refer ence	Allele	Refere nce allele	Amino acid change	Non- synony mous	NC_002516 (Gene)
ZnP									
1	NC_002516	3333256	SNV	G	A	No	Val126Met	Yes	<i>rne</i>
	NC_002516	1279140	SNV	T	G	No	Val260Gly	Yes	<i>phoQ</i>
2	NC_002516	2806658	SNV	C	T	No	Gly238Ser	Yes	<i>mexS</i>
	NC_002516	2806865	SNV	G	T	No	Leu169Ile	Yes	<i>mexS</i>
3	NC_002516	1279140	SNV	T	G	No	Val260Gly	Yes	<i>phoQ</i>
	NC_002516	2806658	SNV	C	T	No	Gly238Ser	Yes	<i>mexS</i>
CoP									
	NC_002516	2806555	SNV	T	G	No	Asp272Gly	Yes	<i>mexS</i>
1	NC_002516	5351958	SNV	G	A	No	Ala41Val	Yes	<i>fur</i>
	NC_002516	5676046	SNV	T	C	No	Thr605Pro	Yes	<i>pilQ</i>
	NC_002516	2806555	SNV	T	G	No	Asp272Gly	Yes	<i>mexS</i>
2	NC_002516	2807227	SNV	C	G	No	Arg48Pro	Yes	<i>mexS</i>
	NC_002516	5351958	SNV	G	A	No	Ala41Val	Yes	<i>fur</i>

	NC_002516	5351838	SNV	A	G	No	Leu81Pro	Yes	<i>fur</i>
	NC_002516	5676046	SNV	T	C	No	Thr605Pro	Yes	<i>pilQ</i>
	NC_002516	5351838	SNV	G	-	No	Gln1022fs	Yes	<i>pilYI</i>
	NC_002516	2806555	SNV	T	G	No	Asp272Gly	Yes	<i>mexS</i>
3	NC_002516	5351958	SNV	G	A	No	Ala41Val	Yes	<i>fur</i>
	NC_002516	5676046	SNV	T	C	No	Thr605Pro	Yes	<i>pilQ</i>

Most of the proteins identified in the adaptive evolution contain metal-binding sites and are involved in the metal pathway. Ribonuclease E RNase E is an endoribonuclease that plays the role in maintaining the composition of mRNA. RNase E cleaves RNA internally, and although the catalytic site does not require metal ions, the zinc-link is essential in maintaining the quaternary structure and effective catalytic activation.²¹⁰ The role of RNase E in global gene regulation and cellular homeostasis through their degradation and processing of RNA species make them an attractive therapeutic target. The mutation observed in the ZnP-evolved strains was found in the N-terminal catalytic domain, and the implication of this SNV is still unknown. The first and only study that links RNase E with QS was reported by Baumgardt *et al.*, where it could control AHL production in *Sinorhizobium meliloti*.²¹¹ The RNase E specifically targets 5' untranslated region of *sinI* mRNA, the AHL synthase in *S. meliloti*. Unfortunately, the RNase E is an essential gene in *E. coli* and *P. aeruginosa*, and we were unable to generate a knockout *rne* mutant for this study.

Another interesting finding from the genomic sequencing of the evolved strains is the mutations found in MexS across two different compounds. The MexS protein is involved in the regulation of the transcriptional regulator MexT as well as MexEF-OprN efflux pumps. Clinical strains carrying *mexS* mutation have also been reported to exhibit downregulation of MexAB-OprM, QS, virulence factors production, and biofilms formation.²¹² MexS also has a Zn-binding site domain, which further support the hypothesis that the metal compounds could import metal ions and once released, the metal ions interact with different possible target proteins including MexS.

5.2.3 Evolved strains express low QS phenotypes

Several clinical strains with *mexS* point mutations have been reported to exhibit low virulence production and loss of QS. Nevertheless, it is still unknown which loci are important to impair the MexS function and upregulate *mexEF-oprN* expression. Next, I investigated the phenotypes of ZnP and CoP-evolved strains, and look at QS-regulated virulence factors production. Indeed, the evolved strains exhibit different morphologies, where they are less green and smaller as compared to the PAO1 wild type strain.

Using LCMS, the intracellular concentration of 3-oxo-C12-HSL and C4-HSL from the evolved strains were determined. The extracellular concentration was determined from the supernatant portion using QS bioreporter strains that correspond to specific C12 and C4-AHL. The results show that evolved strains have a much lower level of 3-oxo-C12-HSL and C4-HSL in the supernatant portion as compared to DMSO-evolved strains and PAO1 wild type. Interestingly, the intracellular concentration of 3-oxo-C12-HSL is higher in the evolved strains. No difference was observed in the C4-HSL levels as the molecules can freely diffuse in and out of the cells (Figure 5-3).

As we observed low levels of 3-oxo-C12-HSL and C4-HSL, the QS activities of the evolved strains were further investigated using the plasmids from QS monitor strains. The QS reporter plasmids (*lasB*, *rhlA*, and *pqsA-gfp*) were transformed into the evolved strains and monitored over 16 hours. Interestingly, the evolved strains did not express any QS genes and the GFP levels remained constant at the basal level (Figure 5-3C). The results further show that the mutations found in the evolved strains lead to the loss of QS activities, as demonstrated from the concentration of signal molecules as well as the transcription of QS-controlled *lasB*, *rhlA*, and *pqsA* genes.

Figure 5-3 Concentration of 3-oxo-C12-HSL and C4-HSL from ZnP-evolved strains (ZnP-1, ZnP-2, ZnP-3), CoP-evolved strains (CoP-1, CoP-2, CoP-3), DMSO-evolved strains (DMSO-1, DMSO-2, DMSO-3) and PAO1 wild type strains. (A) Extracellular concentration from the supernatant was determined using QS bioreporter strains $\Delta lasI \Delta rhlI$ double mutant harboring *lasB-gfp* for 3-oxo-C12-HSL and *rhlA-gfp* for C4-HSL. (B) Intracellular concentration from the cell pellets was determined using LCMS and normalized with protein levels. (C) Transformation of evolved strains with QS reporter plasmid (*lasB*, *rhlA*, *pqsA-gfp*). The GFP expression was measured using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland) with excitation wavelength at 485 nm and emission wavelength at 535 nm. Experiments were done in triplicate manner, only representative data was shown. Error bars are means $\pm$ SDs.

The evolved strains were further transformed with the *mexS* gene from PAO1 wild type, and the AHL levels and elastase production were then measured. Complementation with the *mexS* gene restored the AHL levels to the same level as the wild type. Overexpressing *mexS* in the PAO1 wild type strain did not have any significant effect

(Figure 5-4A and B). Similarly, elastase levels were found to be lower in the evolved strains as compared to the DMSO-evolved strains and PAO1 wild type. Complementation with the *mexS* gene restored the elastase level back to the same level as the wild type (Figure 5-4C). On top of that, complementation with *phoQ* gene in the ZnP-evolved strains did not restore elastase levels, suggesting that only mutation in *mexS* gene is responsible for the loss of QS phenotypes (Figure 5-4D).

Figure 5-4 Complementation of the *mexS* gene in the evolved strains restored 3-oxo-C12-HSL (A) and C4-HSL levels (B). The evolved strains of ZnP and CoP were also found to have lower elastase production. Complemented strains restored elastase back to the PAO1 wild type levels. (C). Complementation with *phoQ* did not restore elastase production (D). Experiments were done in triplicate manner, only representative data was shown. Error bars are means $\pm$ SDs.

5.2.4 Effects on intracellular metal concentrations and iron uptake siderophore

Genomic analysis of the adaptive evolution assays also revealed that the iron pathway encoded by the *fur* gene is affected in CoP-evolved strains. The finding corroborates with the RNA-Seq data presented in the previous chapter, where the compounds interfere with the metal uptake pathway and cellular homeostasis without affecting growth. Reeder et al. reported ZnP could increase cellular copper levels and inactivate

Fe-S cluster-containing proteins in *S. cerevisiae*^{179b}, hence it would be also interesting to examine how our compounds could affect Fe and Cu level in *P. aeruginosa*.

Figure 5-5 (A) Intracellular concentration of metal ions and (B) PVD levels upon treatment with ZnP. (C) Intracellular concentration of metal ions and (D) PVD levels upon treatment with CoP. Zinc (Zn), Fe (iron), and copper (Cu) concentrations were measured using ICPMS, where (-) represents control groups and (+) represents treatment groups. The metal concentration was shown as ppb and normalized with protein concentration (ug/ml). Error bars are means ± SDs. * = p < 0.1, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001, Student's t test.

Based on the results, both zinc and cobalt concentrations were still found to be higher in the treated group of both PAO1 and evolved strains. This indicated that the metal pyrrithiones were still able to enter and deliver metal ions in the evolved strains. Nevertheless, evolved strains had lower intracellular metal concentration as compared to the PAO1 wild type, which could be due to MexS mutation. Iron levels were higher in ZnP-treated group, but lower in the CoP-treated group. This could be due to the different effects of the metal ions on PAO1. Interestingly, copper levels were also found

to be higher in the treated group, and highest in the evolved strains (Figure 5-5A and C). Lastly, the PVD measurement of the evolved strains showed lower level as compared to the PAO1 wild type. Treatment with ZnP and CoP reduced PVD levels in both PAO1 and evolved strains. The reduction was lesser in the CoP-evolved strains, which could be due to the additional *fur* mutation (Figure 5-5B and D).

5.2.5 Binding of metal ions to MexS

The correlation of MexS/MexT functions in regulating MexEF-OprN efflux pumps, QS and T3SS prompted the basis to further investigate the interaction between MexS and the metal compounds. For this, the biophysical method Surface Plasmon Resonance (SPR) was used to examine the binding affinities of MexS to the compounds. As controls for the experiments, the pyrithione ligand (without any metal ions) and free metal salts (ZnBr₂ and CoBr₂) were used.

Preliminary results show the binding interaction between the metal complexes and MexS. Structural information on MexS has shown the presence of Zn-binding site (NCBI Conserved Domain Search). Results from SPR experiments revealed that cobalt could also bind with MexS in a dose-dependent manner. This is the first report that shows MexS could interact with different metal ions other than zinc. In the absence of metal ions (ie. Pyrithione), no signal was detected (Figure 5-6).

Figure 5-6 Sensorgrams showing the interaction of MexS with CoP, ZnP, pyrithione, CoBr₂ and ZnBr₂ from concentration of 0.78 to 100 μ M. Experiments were done in triplicate manner.

Based on the dose-response curves, the data were fitted to the steady-state model of the evaluation software of Biacore T-200 (Figure 5-7). The binding constant (K_D values) calculated between MexS and ZnP, CoP, ZnBr₂, and CoBr₂ were 5.43, 76.65, 9.33, and 6.48 μ M respectively. Based on the results, it can be deduced that the interaction of the compounds with MexS was attributed to the presence of metal ions. The K_D values for CoP was higher, which could be due to less efficient of dissociation between complex and free metal ions form. The overall findings further support the hypothesis that the compounds act as metal importer to the cells. Once released, the metal ions interact with target proteins to regulate downstream processes. Further studies are required to understand the exact mechanism of how the compounds interact with MexS and other putative target proteins.

Figure 5-7 Steady state equilibrium fitting based on the dose-response curve for the interaction between CoP, ZnP, pyrithione, CoBr₂, ZnBr₂ and MexS. The values were calculated using Biacore T-200 software. Experiments were done in triplicate manner, only representative data was shown.

5.3 Discussion

Adaptive evolution study in the laboratory setting has been commonly used to study the impacts of bacterial response to the environment. By exposing the bacteria towards different growth condition, nutrient availability, temperature, as well as drug and antibiotic concentration for successive days, one could study fundamental evolutionary

principles of the bacteria, isolate the evolved strains and investigate their phenotypes and genotypes. On top of that, it would also be useful to explore the evolutionary potential of *P. aeruginosa* to develop resistance and its mechanism towards the metal compounds.

Genomic analysis of the evolved strains revealed several mutations across exposure to the zinc and cobalt pyrithione, such as ribonuclease E (*rne*), two-component sensor PhoQ (*phoQ*), probable oxidoreductase MexS (*mexS*), ferric uptake regulator (*fur*), and fimbrial assembly protein PilQ (*pilQ*). It is intriguing to see the mutations found across all populations involved metal ions as well as antibiotic resistance genes. RNase E requires metal ions (zinc and magnesium) to form stable quaternary complexes for its function, whereby MexS also contains a Zn-binding site. PhoQ is part of the two-component system (TCS) PhoPQ that has been linked to cationic antimicrobial peptide polymyxin B resistance as well as virulence and biofilms formation. The type IV pili is also employed to sense surfaces environmental conditions during biofilms formation. In *Neisseria gonorrhoeae*, mutation in *pilQ1* resulted in the suppression of hemoglobin receptor mutant and allowed the bacteria to grow on hemoglobin as a sole source of iron. On top of that, PilQ has also been shown to increase resistance towards antimicrobial compounds by forming a channel that allows entry of heme and antibiotics.²¹³ In cyanobacteria, the major pili protein PilA1 could act as bacterial nanowires and facilitate electron donation to extracellular electron acceptors, showing the novel function of PilA1 in iron acquisition.²¹⁴

The adaptive evolution studies have provided the knowledge of several possible mechanisms on how the compounds could affect QS and attenuate virulence. The discovery of MexS mutation in this study further leads us to hypothesize that the compounds could affect QS through the crossroad of efflux pumps and QS regulation network. Other than its role in antimicrobial resistance, efflux pumps have also been proposed to transport QS signal molecules.²¹⁵ MexAB-OprM has been reported to be involved in the active transport of 3-oxo-C12-HSL.²¹⁶ The *mexAB-oprM* is actively transcribed in the stationary phase, where QS is also switched on. The transcription could be enhanced by the addition of exogenous C4-HSL and repressed by transcriptional regulator MexT, which also positively regulates *mexEF-oprN* in *P. aeruginosa*.²¹⁷ Intriguingly, strains expressing *mexAB-oprM* produce lesser virulence factors and avirulent in mice models.²¹⁸

Similar phenomena have been observed in strains expressing *mexEF-oprN*, where they also have reduced pyocyanin production.²¹⁹ The MexEF-OprN is able to export kynurenine and HHQ, the precursor of PQS signal molecules. Strains expressing *mexEF-oprN* were found to have reduced QS genes from the transcriptional analysis, and impaired virulence in *C. elegans* model.²²⁰ No detectable fitness cost was observed in strains with overexpression of *mexEF-oprN*. The MexEF-OprN also negatively regulates T3SS, which is also an important virulence mechanism during host infection.²²¹ The observed defect in T3SS was due to the lack of expression of the *exsA* gene in response to environmental signals.

In this study, the evolved strains were found to express low QS and elastase production. The intracellular concentration of 3-oxo-C12-HSL was also found to be lower in the evolved strains. *In vitro*-selected *mexS* mutants have been shown to exhibit low QS-dependent virulence factors production and T3SS. The phenotypes were attributed to the activation of MexEF-OprN that exports HHQ, a precursor of PQS molecules, as well as downregulation of T3SS through MexT.²¹² Under normal condition (wild type cells), transcriptional regulator MexT was normally quiescent due to suppression by MexS. Mutation in MexS produces active MexT, a LysR-type transcriptional regulator that modulates expression of *mexEF-oprN*, virulence factors and motility. MexT could also activate expression of *mexS* and downregulate several T3SS, pyocyanin production and early attachment in MexEF-OprN independent manner.²²² Transcriptional analysis of overexpressing *mexT* strains revealed novel cellular function of MexT regulon, which most likely involved in broad-spectrum detoxification or nutrient-scavenging in *P. aeruginosa*. One of the regulons includes PA4354 – PA4355 – *xenB* encoding an ArsR-type transcriptional regulator, which are involved in the detoxification of heavy metal ions.²²³ Overall studies demonstrate that MexT plays a more global regulatory role in modulating virulence than just efflux pumps.

There are still some questions remain unanswered. Uwate *et al.* have reported that *P. aeruginosa* can utilize either of the two routes of *mexEF-oprN* expression (MexS-mediated or MexS-bypassed pathways).²²⁴ There is still no direct evidence in this study to show if the regulation of *mexEF-oprN* is through which mechanism. Further studies are required to investigate fully the effects of the compounds on the crossroad between

efflux pumps and QS pathways. The mechanism of *mexS* mutation would be further explored, in particular its role in QS, metal intoxication, and resistance mechanism.

Based on the previous RNA-Seq results, we have observed that treatment with the compounds could affect genes transcription involved in metal uptake pathways. The intracellular metal concentration was also increased upon addition of the compounds. Adaptive evolution study also shows that iron pathway (*fur*) is affected. In *P. aeruginosa*, *fur* is considered an essential gene which controls iron uptake, storage and maintenance of iron homeostasis. The importance of iron in *P. aeruginosa* is further exemplified by the fact that 6% of the genome are iron-responsive, which includes regulatory genes, genes involved in metabolic processes, oxidative stress protection (superoxide dismutase), siderophores and their cognate receptors, and virulence.²²⁵

To evaluate the effects of the compounds on iron uptake system, the siderophore PVD levels were measured from the overnight culture of *P. aeruginosa* treated with the compounds. The results showed that the PVD level decreased in the treatment cultures. Consistent with this finding, RNA-Seq analysis also showed downregulation in iron-dependent sigma factor *pvdS* (ZnP group) and pyochelin biosynthesis pathways (CoP group). Both RNA-Seq and adaptive evolution assays further confirm the hypothesis that the compounds could disturb metal homeostasis without affecting their fitness level and attenuate virulence of *P. aeruginosa*.

To answer the penultimate question, what happened when the compounds enter the cells? It is very possible that once inside the cells, the released metal ions could interact with several targets simultaneously. SPR experiments have shown binding of metal ions with MexS, which could activate expression of *mexEF-oprN* through MexT-mediated or MexS-bypassed pathways, and resulted in reduced QS genes, T3SS, and virulence factors production. On top of that, released zinc and cobalt ions in the cell could also disrupt metal homeostasis. Both *fur* and *zur* pathways are affected upon treatment with compounds. Siderophore production (PVD) is also reduced in the treatment groups.

With the critical roles of iron for the pathogens to establish infection, it has been proposed to target bacterial iron acquisition as a therapeutic strategy. Novel treatment strategies include “Trojan horse” approaches including iron-mimetic compounds (e.g. galium), interfering with siderophore import machinery (e.g. siderophore-antibiotic

conjugates), and using iron chelators (e.g. lactoferrin, EDTA).²²⁶ Galium (Ga^{3+}) has a similar ionic radius to Fe^{3+} , and it could be used to confuse pathogens and compete with iron for cellular processes. Galium has been shown to inhibit iron uptake system through repression of *pvdS* gene, and is able to prevent biofilms and disperse established ones. The gallium-based therapeutics have also seen promising results in mice models.²⁰⁸

As FDA-approved drugs, the metal compounds hold promising action as novel antivirulence compounds. Experiments done in this study have shed light on the mechanism of ZnP and CoP. The findings presented in the study provided encouraging results to discover the potential candidates for treating *P. aeruginosa* infections. Future work will aim to provide the molecular mechanisms of the metal compounds as antivirulence compounds, as well as looking into the pharmacological properties of the compounds.

6 CONCLUSION

Overall, this study has demonstrated the strategy of antivirulence approach to complement the conventional antimicrobial development. The first study demonstrated the application of combination treatment of QSI and QQE in suppressing multiple pathways of QS in *P. aeruginosa*. The second study demonstrated how drug repurposing approach leads to the discovery of five repurposed drugs as QSI in *P. aeruginosa*. Promising results were obtained from both *in vitro* and *in vivo* studies, demonstrated how drug repurposing approach could be used to identify novel QS-interfering compounds in attenuating virulence of the superbug *P. aeruginosa*. I focused on one particular class called metal pyrithione, and generated potent novel metal analogs as QSI against *P. aeruginosa*. The compounds could inhibit the production of important QS-virulence factors such as elastase, rhamnolipid and pyocyanin. By interfering with QS, the compounds could also repress the development of antibiotic-resistant subpopulation and facilitates the antibiotic clearance of biofilms. The data presented in this study shows encouraging results for the development of QSIs as antivirulence drugs to treat *P. aeruginosa* infections in future.

Lastly, I have also demonstrated various chemical biology tools used in this study to investigate QS in *P. aeruginosa*. Utilizing Next-Generation Sequencing (NGS) and adaptive evolution assay, both studies revealed that the compounds target multiple metal-containing proteins and affect metal homeostasis. We have provided further evidence on the roles of metal ions in QS and virulence attenuation in *P. aeruginosa*. Another interesting highlight is the mutation of MexS found across the evolved strains. The mechanism of MexS mutation and its role in QS is worthy of future exploration. On top of that, there is still much more to learn about how bacteria maintain the metal ion homeostasis in the case of metal intoxication.

As the progress of antibiotic discovery slowly diminished, it is critical to continue searching for alternative and novel strategies to fight the drug resistance challenges. Using our metal-containing compounds, the overall results suggested that it is possible to attenuate virulence by disrupting the metal homeostasis. Key questions remain to be answered, such as the mechanisms of metal homeostasis during infection as well as host-pathogen interactions.

7 CONTRIBUTIONS

For chapter 2, Prof. Yang Liang, Asst. Prof. Wu Bin, Dr Chaodong Zhang and I initiated the project to study the combinational therapy of QQE and QSI for *P. aeruginosa*. Dr Chaodong Zhang was responsible for the mathematical modeling and equations, while I performed all the biological experiments to validate the results. The enzyme AiiA was purified by Dr Renliang Yang and Zhao Zhi Boo from Asst. Prof. Wu Bin's lab. I analyzed the data and overall results from this study, with guidance from Prof. Yang Liang.

For chapter 3, Prof. Yang Liang, Prof. Michael Givskov and I initiated the project to use drug repurposing approach to identify potential QSI and anti-biofilm agents for *P. aeruginosa*. The screening and all phenotypic assays were done by me. May Margarette Salido Delos Santos and I performed the mice experiment. I analyzed the data and overall results from this study.

For chapter 4, Prof. Yang Liang, Dr Thomas E. Nielsen, Dr Chen Li and I initiated the SAR study of the metal compounds. The design of SAR study was done by Dr Chen Li and me. Dr Chen Li assisted with chemical synthesis and characterization. I performed the QS bioreporter and phenotypic assays. Dr Joey Tan and I performed the flow biofilms assay and confocal microscopy. I performed the cell culture and toxicity assay. May Margarette Salido Delos Santos and I performed the mice experiment. Seng Zi Jing performed the RNA Seq experiment, and I performed the analysis with initial guidance of Dr Ding Yichen and Dr Cai Zhao. I prepared the samples for IPMS and analyzed the data. The ICPMS was done by Maria Chong Ai Shing from Central Environmental Science and Engineering Laboratory, NTU. I analyzed the data and overall results from this study.

For chapter 5, Prof. Yang Liang and I initiated the adaptive evolution study to study the mechanism of metal compounds. I performed the evolution assay and extracted the genomic DNA of the evolved strains. I performed the whole genome sequencing and analysed the data. I designed and constructed the plasmid used in this study. I prepared the samples and designed the experiments for SPR and LCMS. The SPR experiment was done by SPR Biosensing platform at NTU, assisted by Dr Chia Wei Sheng. The LCMS analysis was assisted by Metabolomics team from Lee Kong Chian School of

Medicine, NTU. I conducted the pyoverdin quantification. I analyzed the data and overall results from this study.

8 PERMISSIONS

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4536890009741. Sherrard, L. J.; Tunney, M. M.; Elborn, J. S., Antimicrobial resistance in the respiratory microbiota of people with cystic fibrosis. *Lancet* **2014**, 384, 703-713.

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10. APPENDIX FIGURES AND TABLES

Supplementary Table 1 Parameters used for modeling LasR/I circuit

Parameter	Description	Value	Unit	Reference
V_b	Average cell volume	1.37	μm^3	1
V_A	AHL max production rate	181.476	nM/s	2
K_A	Half rate constant of AHL production	1585.6	nM	optimized
k_{RA}	Combination rate of LuxR and AHL	3.2×10^{-5}	$\text{nM}^{-1}\text{s}^{-1}$	3
d_P	Dissociation rate of P	6.265×10^{-3}	s^{-1}	3
r_0/V_b	Basal transcription rate of lasR	2.24×10^{-4}	nM/s	3
i_0/V_b	Basal transcription rate of lasI	7.52×10^{-5}	nM/s	3
d_r	Degradation rate of lasR mRNA	4.286×10^{-4}	s^{-1}	2b, 4
d_i	Degradation rate of lasI mRNA	5.682×10^{-4}	s^{-1}	2b, 4
V_r/V_b	Activated transcription rate of lasR	0.021	nM/s	3b
V_i/V_b	Activated transcription rate of lasI	0.083	nM/s	3b
K_{r1}	Half rate constant of lasR transcription with Vfr	4.125	nM	3b, 5
K_{r2}	Half rate constant of lasR transcription without Vfr	50	nM	assumed
K_i	Half rate constant of lasI transcription	122.43	nM	3b
k_r	Translational rate of lasR	7.794×10^{-3}	s^{-1}	3
k_i	Translational rate of lasI	7.15×10^{-3}	s^{-1}	3
d_R	Degradation rate of LasR	6.139×10^{-4}	s^{-1}	3, 6
d_I	Degradation rate of LasI	8.294×10^{-4}	s^{-1}	3, 6
d_A	Natural degradation rate of AHL	3.0×10^{-6}	s^{-1}	7
k_Z	Dimerization rate of P	2.803×10^{-6}	$\text{nM}^{-1}\text{s}^{-1}$	3

d_Z	Dissociation rate of Z	0.0191	s^{-1}	³
k_{RQ}	Combination rate of LasR and QSI	3.23×10^{-5}	$nM^{-1}s^{-1}$	use K_{RA}
d_F	Dissociation rate of F	6.265×10^{-3}	s^{-1}	use d_P
A_{max}	Max AHL concentration allowed in model	20	μM	assumed
ρ	Final cell volume fraction	0.3	1	estimated

Supplementary Table 2. Two-way ANOVA analysis of the dose-response effects between single and combination treatment of G1 (50 μ M) and AiiA (32 μ g/mL and 16 μ g/mL) at QS reporter strains. Calculations were done using GraphPad Prism 6 software based on Bonferroni's multiple comparisons test. ns = $p > 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p \leq 0.0001$.

Concentration (μ M)	<i>lasB-gfp</i>			<i>pqsA-gfp</i>			<i>rhlA-gfp</i>	
	DMSO vs G1	G1 vs G+A 32	G1 vs G+A 16	DMSO vs G1	G1 vs G+A 32	G1 vs G+A 16	DMSO vs G1	G1 vs G+A 32
0	***	ns	**	ns	ns	ns	ns	ns
1.5625	****	****	****	****	****	****	****	****
3.125	****	****	****	****	****	****	****	****
6.25	****	****	****	****	****	****	****	****
12.5	****	****	****	****	****	****	****	****
25	****	****	****	****	****	****	****	****
50	****	****	***	****	****	****	****	****

Supplementary Table 3 Summary of overall genes expression upon treatment with ZnP

Gene name	Fold change	p value	Gene product description
<i>amiA</i>	-54.60	7.33E-305	Peptidoglycan amidase AmiA
<i>armR</i>	-3.49	3.69E-03	antirepressor for MexR
<i>arnF</i>	-3.41	2.50E-09	Probable 4-amino-4-deoxy-L-arabinose-phosphoundecaprenol flippase subunit ArnF
<i>aroP2</i>	48.81	1.55E-238	Aromatic amino acid transport protein
<i>bdhA</i>	-3.52	1.06E-22	3-hydroxybutyrate dehydrogenase
<i>capB</i>	7.23	1.75E-51	Cold-shock protein
<i>catA</i>	26.25	9.20E-38	Catechol 1,2-dioxygenase
<i>catC</i>	3.07	1.15E-03	Muconolactone delta-isomerase
<i>cbpD</i>	-5.92	9.96E-116	Chitin-binding protein
<i>ccoO2</i>	4.11	4.67E-12	Cbb3-type cytochrome C oxidase subunit II
<i>chiC</i>	-6.36	9.01E-54	Chitinase
<i>chpB</i>	3.65	1.50E-38	Protein-glutamate methylesterase
<i>clpB</i>	10.40	8.39E-242	Chaperone protein ClpB.
<i>clpV1</i>	15.62	6.47E-186	Probable ClpA/B-type chaperone
<i>cobN</i>	3.69	8.20E-22	Aerobic cobaltochelate subunit CobN
<i>cysT</i>	3.05	1.47E-08	Putative sulfate transport system permease protein CysT
<i>czcA</i>	54.43	2.07E-281	RND divalent metal cation efflux transpoter CzcZ
<i>czcB</i>	48.58	9.23E-98	RND divalent metal cation efflux membrane fusion protein CzcB precursor
<i>czcC</i>	87.03	1.05E-40	CzcC; outer membrane protein precursor
<i>dapB</i>	5.50	3.52E-54	Dihydrodipicolinate reductase
<i>desB</i>	12.92	2.87E-85	Fatty acid desaturase; associated with various virulence factors; control HAQ synthesis
<i>dhcA</i>	-4.30	2.79E-26	Probable CoA transferase, subunit A
<i>dhcB</i>	-3.83	6.15E-23	Probable CoA transferase, subunit B
<i>dnaJ</i>	8.98	1.59E-130	Chaperone protein DnaJ.
<i>dnaK</i>	12.80	4.17E-247	Chaperone protein DnaK (heat shock protein).
<i>dsbC</i>	3.03	5.48E-37	DsbC, Thiol:disulfide interchange protein
<i>exbD1</i>	5.09	3.31E-09	Transport protein
<i>exoY</i>	-3.31	3.40E-10	Adenylate cyclase; toxin, activates GAP and ADPRT; TTSS
<i>fahA</i>	11.89	5.04E-100	Fumarylacetoacetase
<i>fdhE</i>	3.50	2.57E-12	Protein FdhE homolog
<i>fdnG</i>	3.41	5.11E-41	Formate dehydrogenase-O, major subunit
<i>fdnH</i>	10.52	2.74E-107	Formate dehydrogenase-O iron-sulfur subunit.

<i>fdnI</i>	12.42	1.14E-61	Nitrate-inducible formate dehydrogenase.
<i>femI</i>	-11.94	2.42E-118	Probable sigma-70 factor
<i>femR</i>	-9.83	1.56E-38	Sigma factor regulator
<i>fepB</i>	-6.74	3.62E-29	Ferrienterobactin-binding periplasmic protein precursor FepB
<i>fepC</i>	-6.17	6.57E-26	Ferric enterobactin transport protein
<i>fepD</i>	-3.01	5.08E-05	Ferric enterobactin transport protein FepD.
<i>fhp</i>	-11.58	1.59E-80	Energy metabolism
<i>fleR</i>	-4.08	4.48E-72	Sigma-54 dependent transcriptional regulator/response regulator; regulate motility and adhesion to mucin.
<i>flhF</i>	-3.01	3.08E-81	Flagellar biosynthesis protein FlhF.
<i>fliE</i>	-3.25	3.42E-23	Flagellar hook-basal body complex protein FliE.
<i>fliF</i>	-3.34	5.38E-84	Flagellar M-ring protein.
<i>flp</i>	-3.07	4.11E-09	Hypothetical protein
<i>folP</i>	3.11	1.07E-48	Dihydropteroate synthase (DHPS).
<i>foxI</i>	-6.74	5.38E-89	Probable ECF sigma-70 factor
<i>foxR</i>	-4.10	7.96E-13	Anti-sigma factor; Fe ²⁺ dicitrate sensor
<i>fpvB</i>	-3.11	4.02E-34	Probable TonB-dependent receptor; second ferric pyoverdine receptor
<i>ftsJ</i>	3.25	1.43E-49	Ribosomal RNA large subunit methyltransferase E.
<i>fumC1</i>	-5.89	9.44E-122	Fumarate hydratase
<i>gbuA</i>	6.49	3.64E-07	Guanidinobutyrase.
<i>gcvH2</i>	18.90	3.84E-143	Glycine cleavage system protein H2
<i>gcvP2</i>	23.89	3.54E-139	Glycine cleavage system protein P2
<i>gcvT2</i>	4.35	1.59E-130	Glycine cleavage system protein T2
<i>glpD</i>	10.77	2.82E-110	Glycerol metabolism
<i>glpF</i>	12.43	3.39E-116	Glycerol uptake facilitator protein.
<i>glpK</i>	3.26	1.73E-53	Glycerol kinase 2.
<i>glyA2</i>	39.53	1.49E-276	Serine hydroxymethyltransferase
<i>groEL</i>	5.27	4.92E-79	60 kDa chaperonin (GroEL protein) heat shock protein.
<i>groES</i>	4.90	1.21E-82	10 kDa chaperonin (GroES protein)
<i>grpE</i>	10.01	1.75E-95	Heat shock protein
<i>hasAp</i>	-4.04	1.25E-53	Heme acquisition protein
<i>hcnA</i>	-11.19	1.17E-08	Hydrogen cyanide synthase HcnA
<i>hcnB</i>	-6.14	9.85E-37	Hydrogen cyanide synthase HcnB
<i>hcnC</i>	-6.05	8.21E-44	Hydrogen cyanide synthase HcnC
<i>hcpI</i>	16.55	9.81E-234	Hcp secretion island I (HSI-I) T6SS
<i>hmgA</i>	25.10	1.94E-134	homogentisate 1,2-dioxygenase
<i>hpd</i>	109.20	0.00E+00	4-hydroxyphenylpyruvate dioxygenase
<i>hslU</i>	8.71	3.18E-98	Heat shock protein

<i>hslV</i>	6.36	5.62E-39	ATP-dependent protease subunit HslV.
<i>htpG</i>	8.40	9.64E-100	Heat shock protein
<i>hutH</i>	-3.01	1.56E-26	histidine amino acid permease
<i>hutU</i>	-3.83	8.97E-50	urocanase
<i>ibpA</i>	9.44	2.40E-93	heat-schock protein
<i>icmF1</i>	6.92	2.50E-80	icmF1; Hcp secretion island I (HSI-I) T6SS
<i>idh</i>	4.15	1.23E-93	Isocitrate dehydrogenase.
<i>iscR</i>	3.17	4.71E-59	Iron-sulfur cluster assembly transcription factor IscR
<i>lasA</i>	-5.05	7.53E-72	LasA protease precursor
<i>lasB</i>	-36.09	8.18E-264	Elastase LasB
<i>lecA</i>	-52.08	1.42E-72	PA-1 galactophilic lectin
<i>lecB</i>	-10.00	2.31E-54	Fucose-binding lectin PA-III
<i>lipA</i>	-4.46	9.25E-40	Influence regulation of pyoverdinin production and expression of sigma factor PvdS
<i>lon</i>	3.22	2.87E-61	Lon protease
<i>maiA</i>	10.25	5.67E-59	Maleyacetate isomerase
<i>metE</i>	61.46	1.91E-286	5-methyltetrahydropteroyltrimethyl- homocysteine S-methyltransferase
<i>metH</i>	3.20	6.00E-101	Methionine synthase
<i>metK</i>	3.70	7.32E-49	S-adenosylmethionine synthase
<i>metR</i>	4.01	2.43E-45	Transcriptional regulator MetR
<i>mexE</i>	17.40	9.03E-56	RND multidrug efflux membrane fusion protein MexE
<i>mexF</i>	5.22	1.57E-66	RND multidrug efflux membrane fusion protein MexF
<i>mifS</i>	3.97	2.47E-17	MifS-MifR Two-Component System
<i>mmsA</i>	-10.86	4.39E-144	Methylmalonate-semialdehyde dehydrogenase (MSDH)
<i>mmsB</i>	-9.64	1.84E-90	3-hydroxyisobutyrate dehydrogenase
<i>morA</i>	3.56	1.83E-55	Motility regulator
<i>msbA</i>	3.86	3.04E-59	Lipid A export ATP-binding/permease protein MsbA
<i>nalD</i>	3.86	1.77E-51	HTH-type transcriptional repressor BepR (NalD)
<i>napB</i>	3.35	6.76E-09	Cytochrome c-type protein NapB precursor
<i>nosY</i>	5.03	4.03E-02	Membrane protein NosY.
<i>np20</i>	-5.07	1.62E-69	zur; Zinc uptake regulation protein
<i>nrdB</i>	3.05	8.56E-22	Ribonucleoside-diphosphate reductase subunit beta.
<i>nuoA</i>	6.47	5.67E-70	NADH-quinone oxidoreductase subunit A 2.
<i>nuoI</i>	3.90	9.24E-33	NADH Dehydrogenase I chain I
<i>nuoJ</i>	3.16	1.46E-21	NADH Dehydrogenase I chain J
<i>nuoM</i>	3.30	1.20E-50	NADH Dehydrogenase I chain M

<i>nuoN</i>	3.66	1.13E-43	NADH Dehydrogenase I chain N
<i>opdT</i>	253.12	0.00E+00	Tyrosine porin OpdT
<i>opmD</i>	-3.48	2.92E-08	Probable RND/outer membrane efflux protein precursor
<i>oprD</i>	-11.38	8.62E-141	Outer membrane porin protein OprD precursor; import of basic amino acids and carbapenem antibiotics
<i>oprN</i>	5.47	3.15E-14	Multidrug efflux outer membrane protein OprN precursor
PA0041	3.11	9.81E-42	Probable hemagglutinin
PA0052	-7.33	2.92E-71	Hypothetical protein
PA0070	8.80	2.00E-121	Hypothetical protein
PA0072	10.19	4.54E-14	Hypothetical protein
PA0073	13.55	2.23E-08	Probable ATP-binding component of ABC transporter.
PA0076	12.15	4.30E-19	Hypothetical protein
PA0078	6.30	5.08E-23	Hypothetical protein
PA0079	3.52	5.96E-27	Hypothetical protein
PA0080	5.10	6.53E-21	Hypothetical protein
PA0082	5.16	1.27E-27	Hypothetical protein
PA0083	11.11	2.85E-100	<i>tssB1</i> ; Hcp secretion island I (HSI-I) T6SS
PA0084	12.25	5.76E-129	EvpB family type VI secretion protein.
PA0086	6.67	1.82E-28	Hypothetical protein
PA0087	5.10	1.90E-11	Hypothetical protein
PA0088	9.65	8.31E-56	Hypothetical protein
PA0089	10.20	1.33E-29	Hypothetical protein
PA0092	4.39	5.47E-24	Hypothetical protein
PA0097	4.25	9.95E-18	Hypothetical protein
PA0098	4.02	3.19E-09	Hypothetical protein
PA0099	6.65	1.00E-64	Hypothetical protein
PA0101	3.61	2.00E-15	Hypothetical protein
PA0122	-17.42	1.00E-193	Hypothetical protein
PA0123	-4.57	8.53E-53	Probable transcriptional regulator; cytoplasmic membrane.
PA0126	4.04	1.54E-15	Hypothetical protein
PA0144	-3.55	4.11E-13	Hypothetical protein
PA0149	-3.36	8.25E-05	Probable sigma-70 factor
PA0161	3.60	1.74E-12	Hypothetical protein
PA0201	7.71	9.64E-29	Hypothetical protein
PA0202	5.77	2.11E-20	Probable amidase
PA0236	-3.98	1.14E-04	Probable transcriptional regulator
PA0264	4.42	6.20E-52	Hypothetical protein
PA0279	-3.45	1.48E-05	Probable transcriptional regulators, ydfF

PA0311	3.16	3.67E-03	Hypothetical protein
PA0397	3.45	3.54E-82	Cation efflux system protein CzcD.
PA0445	3.18	3.24E-02	Probable family 20 transposase.
PA0457	3.17	2.40E-13	Hypothetical membrane protein
PA0471	-4.96	6.64E-37	Probable transmembrane sensor, <i>fiuR</i> .
PA0472	-4.69	2.10E-71	<i>fiuI</i> ; Probable sigma-70 factor
PA0529	3.47	8.47E-17	conserved Hypothetical protein
PA0539	4.44	2.56E-15	Hypothetical protein
PA0547	4.91	5.37E-76	Probable transcriptional regulator.
PA0561	3.60	1.82E-22	Hypothetical protein
PA0730	3.02	3.15E-21	<i>phaG</i> ; (R)-3-hydroxydecanoyl-ACP:CoA transacylase (synthesis of polyhydroxyalkanoic acid).
PA0746	-3.05	2.18E-40	Probable acyl-CoA dehydrogenase.
PA0747	-4.33	1.69E-113	Probable aldehyde dehydrogenase
PA0779	6.60	1.01E-148	Lon protease.
PA0781	-53.71	7.03E-172	<i>omr</i> ; Zn-regulated outer membrane receptor
PA0797	-9.70	1.72E-75	<i>prpR</i> ; propionate catabolism transcriptional regulator
PA0800	-3.36	4.09E-03	Hypothetical protein
PA0839	7.79	6.75E-30	Probable transcriptional regulator
PA0840	3.83	4.94E-38	Probable oxidoreductase
PA0863	11.56	3.86E-98	Probable oxidoreductase
PA0952	13.47	2.44E-78	Hypothetical protein
PA0961	4.29	7.62E-04	Probable cold-shock protein
PA0975	4.24	3.30E-54	Probable radical activating enzyme
PA0976	4.39	1.82E-75	7-cyano-7-deazaguanine synthase (<i>queC</i>)
PA1026	70.33	1.30E-160	Hypothetical protein
PA1027	7.25	2.01E-116	Probable aldehyde dehydrogenase
PA1028	4.06	1.32E-48	Probable oxidoreductase.
PA1166	-3.01	2.68E-26	Hypothetical protein
PA1190	-3.49	1.19E-17	Hypothetical protein
PA1199	-3.70	9.37E-38	Probable lipoprotein
PA1213	-5.11	1.00E-10	Taurine catabolism dioxygenase TauD; clavaminic acid synthetase
PA1214	-5.87	4.22E-19	Asparagine synthase
PA1215	-5.73	2.40E-22	Hypothetical protein
PA1216	-8.15	3.05E-57	Hypothetical protein; methyltransferase
PA1217	-4.56	4.45E-44	Probable 2-isopropylmalate synthase
PA1218	-7.35	1.26E-54	PhyH; phytanoyl-CoA dioxygenase family protein
PA1219	-5.11	4.97E-07	Hypothetical protein
PA1220	-14.72	2.31E-20	Hypothetical protein

PA1221	-13.65	5.96E-62	Hypothetical protein; non-ribosomal peptide synthetase
PA1274	3.25	4.07E-03	Hypothetical protein
PA1285	-3.49	4.55E-15	Probable transcriptional regulator
PA1289	-5.54	4.82E-33	Hypothetical protein
PA1297	11.65	7.87E-84	Probable metal transporter.
PA1298	3.57	3.88E-11	Probable MFS transporter
PA1299	-4.19	3.61E-35	Hypothetical protein
PA1300	-7.09	9.36E-93	Probable sigma-70 factor, ECF subfamily/transcriptional regulators
PA1301	-5.14	2.36E-112	Probable transmembrane sensor
PA1416	3.69	1.54E-23	Hypothetical protein
PA1417	4.09	2.36E-08	Thiamine pyrophosphate enzyme binding domain
PA1418	4.22	1.40E-07	Sodium/solute symporters
PA1419	4.57	5.61E-06	Nitrate reductase; nucleobase transmembrane transporter
PA1420	3.51	1.50E-04	Hypothetical protein
PA1506	6.60	7.34E-09	Hypothetical protein
PA1518	-3.46	2.54E-28	5-hydroxyisourate hydrolase
PA1519	-3.15	3.20E-11	Xanthine/uracil/thiamine/ascorbate permease family protein
PA1540	4.57	1.11E-09	Hypothetical protein
PA1541	20.68	2.23E-45	Probable drug efflux transporter
PA1549	3.36	2.08E-28	Probable cation-transporting P-type ATPase.
PA1550	3.30	5.69E-24	Hypothetical protein
PA1551	4.10	1.69E-35	Probable ferredoxin.
PA1559	-6.92	1.73E-104	Hypothetical protein
PA1560	-5.15	1.94E-41	Hypothetical protein
PA1653	3.87	3.74E-07	Probable transcriptional regulator
PA1654	3.98	8.52E-32	Probable aminotransferase
PA1655	3.30	5.33E-28	Probable glutathione S-transferase
PA1743	5.18	1.21E-03	Putative membrane protein
PA1744	3.81	3.31E-06	Hypothetical protein
PA1784	-6.34	2.03E-41	Hypothetical protein
PA1842	3.12	1.37E-23	Hypothetical protein
PA1869	-6.44	1.18E-22	Acyl carrier protein 2, acpP2
PA1914	-5.98	5.71E-18	Conserved Hypothetical protein
PA1921	-7.58	1.78E-52	Methyltransferase
PA1922	-103.21	7.68E-95	Probable outer membrane protein; TonB-dependent outer membrane protein
PA1923	-33.91	1.74E-76	CobN cobaltochelataase subunit
PA1924	-9.16	2.33E-24	Biopolymer transporter ExbD

PA1925	-7.53	9.22E-39	Hypothetical protein
PA1942	33.88	3.42E-124	Hypothetical protein
PA1955	-3.04	1.60E-02	Hypothetical protein
PA1964	5.85	2.67E-58	Putative ABC transporter ATP-binding protein YbiT
PA1967	-3.48	9.02E-41	Hypothetical protein
PA1970	23.86	9.03E-56	Hypothetical protein
PA2004	-3.45	2.28E-21	Putative transporter YxjC
PA2006	7.64	4.22E-33	Probable major facilitator superfamily (MFS) transporter
PA2010	5.37	2.45E-41	Probable transcriptional regulator
PA2017	-3.06	3.70E-13	Hypothetical protein
PA2021	-3.64	3.03E-15	Hypothetical protein
PA2030	-3.16	5.70E-49	Hypothetical protein
PA2031	-3.63	1.97E-51	Hypothetical protein
PA2033	-9.98	4.14E-186	Probable encode protein homologous to siderophore-interacting protein
PA2034	-8.16	9.99E-72	Hypothetical protein; magnesium protoporphyrin O-methyltransferase
PA2049	4.80	5.65E-29	Hypothetical protein
PA2063	5.31	1.15E-12	Hypothetical protein
PA2067	-4.06	1.03E-13	Probable hydrolase
PA2068	-5.51	1.85E-22	Probable MFS transporter
PA2069	-30.05	5.84E-87	Probable carbamoyl transferase
PA2134	-5.19	7.55E-13	Probable outer membrane protein
PA2135	-3.93	6.71E-09	Probable transporter
PA2146	-3.53	2.04E-16	Conserved Hypothetical protein
PA2148	-3.61	2.86E-18	Hypothetical protein
PA2167	-3.16	4.13E-27	Hypothetical protein
PA2203	4.79	4.26E-03	Probable amino acid permease.
PA2204	7.14	1.49E-80	Probable binding protein component of ABC transporter
PA2260	-6.24	6.77E-45	Probable sugar phosphate isomerase
PA2261	-7.04	6.44E-40	Probable 2-ketogluconate kinase
PA2262	-5.05	3.21E-34	Probable 2-ketogluconate transporter
PA2263	-7.92	8.63E-40	Probable 2-hydroxyacid dehydrogenase
PA2321	-3.47	6.88E-56	Gluconokinase
PA2322	-4.81	1.22E-61	Gluconate permease
PA2367	3.49	2.29E-15	Hypothetical protein
PA2368	3.12	8.83E-04	Hypothetical protein
PA2369	3.12	7.77E-13	Hypothetical protein
PA2370	4.69	2.14E-12	Hypothetical protein
PA2371	3.90	2.42E-38	Probable ClpA/B-type protease

PA2384	-3.35	1.45E-66	Hypothetical protein
PA2415	-3.27	1.06E-15	Hypothetical protein; membrane protein
PA2433	-4.34	8.96E-29	Hypothetical protein
PA2437	-5.06	1.97E-06	Membrane protease subunit of HflC family
PA2438	-4.44	6.78E-17	Membrane protease subunit of HflC family
PA2439	-5.20	3.17E-13	Membrane protease subunit of HflK family
PA2440	3.65	5.03E-08	Hypothetical protein
PA2441	4.17	4.09E-39	Hypothetical protein
PA2487	3.07	1.04E-08	Hypothetical protein
PA2506	720.90	7.54E-110	Hypothetical protein
PA2523	96.96	3.30E-147	CzcR; metal inducible two-component signal transduction systems
PA2524	27.87	4.88E-165	CzcS sensor protein
PA2525	3.39	5.19E-24	Outer membrane efflux protein OpmB
PA2536	4.09	2.40E-39	Probable phosphatidate cytidylyltransferase
PA2537	4.55	7.25E-28	Probable acyltransferase.
PA2538	3.01	4.08E-04	Hypothetical protein
PA2539	4.10	1.82E-17	Conserved Hypothetical protein
PA2550	4.05	7.80E-50	Probable acyl-CoA dehydrogenase
PA2564	-4.73	2.02E-44	Hypothetical protein
PA2565	-3.83	3.11E-14	Hypothetical protein
PA2566	-11.76	9.33E-115	Conserved hypothetical protein; NAD(FAD)-dependent dehydrogenases
PA2567	3.08	3.25E-27	Hypothetical protein
PA2588	-5.53	2.83E-85	Probable transcriptional regulator
PA2636	4.96	6.10E-06	Hypothetical protein
PA2684	3.14	3.21E-26	Conserved Hypothetical protein; Rhs family protein
PA2696	-6.33	2.32E-30	Probable transcriptional regulator
PA2729	5.41	6.49E-20	Hypothetical protein
PA2748	5.67	1.32E-63	Methionine aminopeptidase (MAP)
PA2753	3.03	2.04E-07	Hypothetical protein
PA2757	5.12	2.39E-04	Hypothetical protein
PA2758	5.17	1.23E-39	Probable transcriptional regulator
PA2759	18.47	3.65E-124	Hypothetical protein
PA2773	3.31	7.06E-10	Hypothetical protein
PA2790	10.70	3.36E-42	Hypothetical protein
PA2791	13.18	1.44E-26	Hypothetical protein
PA2792	3.89	3.69E-10	Hypothetical protein
PA2806	3.36	2.43E-20	NADPH-dependent 7-cyano-7-deazaguanine reductase
PA2807	38.91	1.75E-148	Hypothetical protein
PA2927	-3.23	1.37E-20	Hypothetical protein; phospholipase

PA2937	-4.18	8.16E-42	Hypothetical protein
PA2938	-3.15	4.62E-35	Probable aminopeptidase
PA2939	-3.64	1.85E-24	Probable transporter
PA2942.1	3.28	1.12E-10	Probable magnesium chelatase.
PA2946	3.17	1.62E-47	Hypothetical protein; Putative cobalt transporter subunit (CbtA)
PA2947	3.14	2.46E-03	Hypothetical protein
PA3009	5.58	1.08E-40	Hypothetical protein
PA3229	11.88	8.05E-144	Hypothetical protein
PA3230	3.05	2.13E-41	Conserved hypothetical protein
PA3273	-3.03	4.83E-13	Hypothetical protein
PA3326	-3.78	8.09E-59	ClpP2; Probable Clp-family ATP-dependent protease
PA3328	-3.31	7.74E-06	Probable FAD-dependent monooxygenase
PA3410	-3.83	1.66E-23	Probable sigma-70 factor
PA3412	12.40	9.40E-38	Hypothetical protein
PA3414	-3.31	1.34E-10	Hypothetical protein
PA3444	5.33	3.37E-08	Conserved Hypothetical protein
PA3445	6.38	1.67E-21	Putative aliphatic sulfonates-binding protein (<i>ssuA</i>)
PA3446	6.95	3.64E-18	Conserved Hypothetical protein
PA3450	5.72	2.69E-44	Probable antioxidant protein/adaptation, protection
PA3518	3.82	3.97E-07	Hypothetical protein; coenzyme PQQ biosynthesis protein
PA3519	8.07	1.19E-24	Hypothetical protein
PA3522	3.46	1.84E-21	Probable RND efflux transporter
PA3523	4.39	8.04E-18	Probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein.
PA3530	-3.74	9.07E-74	Hypothetical protein
PA3567	-3.43	6.37E-40	NADPH:quinone reductase (Oxidoreductase) (Zinc-type alcohol dehydrogenase-like protein).
PA3568	-8.95	7.68E-129	Probable acetyl-CoA synthetase
PA3588	3.51	1.14E-19	Probable porin.
PA3599	-15.16	8.67E-199	Probable transcriptional regulator
PA3600	-118.65	9.91E-222	50S ribosomal protein L36 2
PA3601	-166.93	0.00E+00	50S ribosomal protein L31 type B
PA3690	137.04	0.00E+00	Probable metal-transporting P-type ATPase
PA3709	-5.84	2.15E-77	Probable major facilitator superfamily (MFS) transporter
PA3710	-5.32	4.10E-129	Probable GMC-type oxidoreductase
PA3727	10.61	2.92E-41	Hypothetical protein
PA3728	4.89	4.35E-103	Hypothetical protein; chromosome segregation ATPases

PA3729	3.84	2.33E-50	Conserved hypothetical protein; putative membrane protein
PA3734	-6.10	3.27E-65	Hypothetical protein
PA3785	-3.13	5.76E-43	Hypothetical protein
PA3899	-5.84	1.20E-39	Probable sigma-70 factor
PA3900	-4.69	1.33E-13	Probable transmembrane sensor; ; Fe ²⁺ dicitrate sensor
PA3920	20.81	5.41E-164	Probable metal transporting P-type ATPase
PA3931	4.22	2.22E-11	Conserved hypothetical protein
PA3938	3.11	2.02E-06	Probable periplasmic taurine-binding protein precursor
PA3939	6.79	2.60E-09	Hypothetical protein; acyl-CoA dehydrogenase
PA3971	3.07	1.96E-16	Hypothetical protein
PA4022	18.65	1.09E-54	<i>aldA1</i> ; Aldehyde dehydrogenase
PA4023	10.34	5.08E-23	Probable transport protein.
PA4063	-77.46	6.23E-180	Zn ²⁺ periplasmic binding protein
PA4064	-7.35	1.32E-43	ABC transporter nucleotide binding protein
PA4065	-9.61	2.13E-107	Lipoprotein release ABC transporter permease
PA4066	-5.79	1.28E-49	Lipoprotein; Zn ²⁺ periplasmic binding protein
PA4078	-3.30	3.99E-30	Probable nonribosomal peptide synthetase
PA4129	-3.04	4.43E-08	Hypothetical protein
PA4133	-3.13	1.33E-28	Cytochrome c oxidase subunit (cbb3-type)
PA4141	-58.58	2.70E-114	Hypothetical protein
PA4142	-20.19	2.96E-56	Probable secretion protein
PA4143	-8.02	6.72E-55	Probable toxin transporter
PA4144	-6.57	3.88E-13	Probable outer membrane protein precursor
PA4155	-5.35	2.86E-33	Hypothetical protein
PA4156	-5.31	6.16E-39	Probable TonB-dependent receptor
PA4170	-4.73	3.40E-21	Hypothetical protein; thioredoxin reductase
PA4287	3.29	7.42E-09	Hypothetical protein
PA4321	3.95	1.13E-21	Hypothetical protein
PA4322	3.36	2.87E-21	Hypothetical protein
PA4354	3.81	4.58E-19	Conserved Hypothetical protein
PA4357	-4.13	6.65E-13	Hypothetical protein
PA4377	-5.38	1.14E-19	Putative secreted protein.
PA4387	6.79	1.60E-54	Conserved hypothetical protein
PA4467	-4.49	1.55E-135	Hypothetical protein; Probable divalent heavy-metal cations transporter
PA4469	-4.58	2.92E-71	Hypothetical protein
PA4471	-11.43	9.05E-163	Hypothetical protein
PA4489	3.49	7.00E-79	Hypothetical protein
PA4515	-4.39	1.74E-62	Conserved hypothetical protein; Fe(II)-dependent oxygenase superfamily protein

PA4524.1	5.62	1.73E-02	Nicotinate-nucleotide pyrophosphorylase (<i>nadC</i>)
PA4570	-18.75	3.22E-193	Hypothetical protein; regulate RsmW production; iron limitation
PA4573	-3.27	5.98E-28	Hypothetical protein
PA4622	3.68	3.93E-09	Probable major facilitator superfamily (MFS) transporter
PA4623	82.43	9.47E-125	Hypothetical protein
PA4648	-4.00	1.54E-23	Hypothetical protein
PA4649	-3.32	6.03E-15	Hypothetical protein
PA4677	-3.59	3.82E-54	Hypothetical protein
PA4683	8.86	4.44E-77	Hypothetical protein
PA4709	-3.03	2.03E-33	Probable hemin degrading factor
PA4746	3.50	3.85E-18	Ribosome maturation factor RimP.
PA4753	4.40	1.10E-26	Probable RNA-binding protein
PA4773	-5.26	1.18E-35	<i>speH</i> , S-adenosylmethionine decarboxylase proenzyme
PA4774	-5.24	5.59E-28	<i>speE2</i> , Polyamine aminopropyltransferase 2
PA4782	-4.97	5.31E-88	Putative secreted protein
PA4783	4.10	4.52E-13	Putative inner membrane transporter <i>yedA</i>
PA4817	6.64	5.07E-10	Hypothetical protein
PA4818	16.78	2.24E-19	Hypothetical protein
PA4819	13.84	1.74E-14	Probable glycosyl hydrolase
PA4820	3.22	2.66E-05	Hypothetical protein
PA4834	-7.86	5.96E-109	Putative membrane transporter, zinc/cobalt uptake, <i>zrmD</i>
PA4835	-23.41	1.56E-139	Hypothetical protein, zinc/cobalt uptake, <i>zrmC</i>
PA4836	-63.99	2.29E-178	Probable nicotianamine synthase protein (important divalent metal chelator), <i>zrmB</i>
PA4837	-128.12	0.00E+00	Probable outer membrane protein; TonB-dependent outer membrane protein, <i>zrmA</i>
PA4838	-10.12	1.71E-86	Hypothetical membrane protein
PA4844	4.66	4.73E-18	Probable chemotaxis transducer
PA4881	75.13	4.25E-109	Hypothetical protein
PA4889	13.23	3.14E-35	Probable oxidoreductase
PA4896	-3.68	2.51E-27	Probable sigma-70 factor, ECF subfamily
PA4902	-3.31	7.91E-13	Probable transcriptional regulator
PA4925	-3.50	3.35E-33	Hypothetical protein
PA4962	4.38	1.29E-05	Aminomethyltransferase
PA4985	4.21	1.82E-08	Hypothetical protein
PA4986	3.39	2.76E-18	Probable oxidoreductase
PA5081	14.10	5.89E-08	Hypothetical protein

PA5082	61.59	2.98E-17	<i>dguC</i> ; Probable binding protein component of ABC transporter
PA5083	50.02	2.12E-12	<i>dguB</i> ; conserved Hypothetical protein
PA5084	20.99	5.53E-07	Probable oxidoreductase
PA5096	-3.06	1.32E-24	Probable binding protein component of ABC transporter
PA5115	4.43	4.57E-16	Hypothetical protein
PA5220	-5.74	1.42E-81	Hypothetical protein
PA5228	3.11	5.08E-23	Probable periplasmic taurine-binding protein.
PA5362	3.27	7.41E-50	Hypothetical protein
PA5403	3.68	4.44E-05	Probable transcriptional regulator.
PA5467	3.61	3.66E-10	Hypothetical protein
PA5475	3.69	7.02E-21	Probable N-Acetyltransferase activity
PA5498	-8.61	5.38E-89	ZnuA, Zn ²⁺ ABC transporter substrate-binding protein
PA5532	-3.11	3.02E-11	CobW, cobalamin biosynthesis protein
PA5534	-21.79	1.23E-128	Hypothetical protein
PA5535	-20.37	8.28E-227	Cobalamin synthesis protein
PA5536	-77.05	7.80E-77	RNA polymerase-binding transcription factor dksA2.
PA5537	-5.60	5.33E-33	Glutamine synthetase
PA5539	-86.57	6.34E-139	folE2, GTP cyclohydrolase I type 2
PA5540	-36.68	2.36E-168	Carbonate dehydratase
<i>pcoA</i>	13.08	6.55E-84	Hypothetical protein
<i>pcoB</i>	20.66	1.56E-38	Copper resistance protein B.
<i>pcrH</i>	-3.07	1.28E-09	Regulatory protein PcrH; chaperone, T3SS, virulence
<i>pcrV</i>	-3.31	1.97E-14	T3SS membrane protein
<i>phhA</i>	57.00	6.69E-155	Phenylalanine-4-hydroxylase
<i>phhB</i>	19.75	1.04E-103	Pterin-4-alpha-carbinolamine dehydratase
<i>phhC</i>	19.06	4.52E-169	Aromatic amino acid aminotransferase
<i>phnA</i>	-13.00	1.91E-228	Anthranilate synthase I
<i>phnB</i>	-3.24	1.38E-68	Anthranilate synthase II
<i>phzA1</i>	-9.07	7.85E-05	Probable phenazine biosynthesis protein
<i>phzA2</i>	-21.00	2.95E-06	Phenazine biosynthesis protein
<i>phzB1</i>	-10.46	3.82E-27	Probable phenazine biosynthesis protein
<i>phzB2</i>	-6.26	1.20E-17	Phenazine biosynthesis protein
<i>phzC1</i>	-10.18	1.77E-04	Phenazine biosynthesis protein
<i>phzC2</i>	-3.55	4.22E-02	Phenazine biosynthesis protein PhzC
<i>phzD1</i>	-9.42	1.07E-03	Phenazine biosynthesis protein PhzD
<i>phzD2</i>	-4.63	1.87E-02	Phenazine biosynthesis protein PhzD
<i>phzE2</i>	-15.10	2.19E-02	Phenazine biosynthesis protein PhzE
<i>phzF2</i>	-88.49	3.30E-02	Phenazine biosynthesis protein

<i>phzG2</i>	-4.36	1.79E-03	Probable pyridoxamine 5-phosphate oxidase
<i>phzM</i>	-5.60	1.23E-50	Phenazine biosynthesis protein
<i>pilA</i>	4.08	1.17E-33	Type 4 fimbrial precursor PilA
<i>pilC</i>	3.07	4.92E-32	Type 4 fimbrial assembly protein PilC
<i>pilD</i>	3.33	9.45E-35	Type 4 prepilin-like proteins leader peptide-processing enzyme.
<i>piv</i>	-7.75	5.48E-50	Protease IV; PvdS-regulated endoprotease
<i>pmtA</i>	-5.90	4.94E-28	Phospholipid methyltransferase
<i>popB</i>	-3.48	5.12E-10	Translocator protein; T3SS
<i>popD</i>	-3.13	2.06E-08	Translocator protein; T3SS
<i>ppkA</i>	8.74	2.42E-106	Serine/threonine protein kinase
<i>pppA</i>	8.11	1.79E-27	Probable phosphoprotein phosphatase
<i>ppyR</i>	-6.80	4.67E-10	Psl and pyoverdine operon regulator, PpyR
<i>pqsA</i>	-20.37	2.10E-197	coenzyme A ligase
<i>pqsB</i>	-23.70	0.00E+00	3-oxoacyl-(Acyl carrier protein) synthase III (PqsB).
<i>pqsC</i>	-16.11	1.19E-168	PqsC
<i>pqsD</i>	-13.74	4.34E-175	2-heptyl-4(1H)-quinolone synthase PqsD
<i>pqsE</i>	-11.81	1.37E-194	Quinolone signal response protein
<i>pra</i>	-3.70	3.34E-49	Protein activator
<i>prpB</i>	-5.74	2.35E-55	methylisocitrate lyase
<i>prpC</i>	-5.16	4.87E-68	2-methylcitrate synthase
<i>psrA</i>	4.09	2.97E-48	Transcriptional regulator PsrA (transcriptional activator of RpoS)
<i>ptrA</i>	244.20	4.14E-120	Pseudomonas type III repressor A; copper tolerance.
<i>ptxR</i>	-3.25	8.41E-07	Transcriptional regulator
<i>ptxS</i>	-4.10	4.44E-75	Transcriptional regulator; exotoxin A regulatory gene
<i>pvcB</i>	-3.71	3.66E-03	Pyoverdine biosynthesis protein
<i>pvdJ</i>	4.54	2.87E-52	Probable non-ribosomal peptide synthetase
<i>pvdS</i>	-3.70	7.32E-35	Sigma factor; activate production of pyoverdine
<i>pyrQ</i>	-8.57	1.09E-197	Probable dihydroorotase
<i>rcpC</i>	-3.18	4.21E-23	Probable type II secretion system protein
<i>rfaD</i>	3.36	1.59E-11	ADP-L-glycero-D-manno-heptose-6-epimerase
<i>rhlA</i>	-27.39	1.21E-159	Rhamnosyltransferase chain A
<i>rhlB</i>	-10.35	1.54E-82	Rhamnosyltransferase chain B
<i>rhlC</i>	-3.52	9.67E-31	Rhamnosyl transferase 2
<i>rhlI</i>	-4.44	1.60E-90	Acyl-homoserine-lactone synthase
<i>rho</i>	7.13	6.32E-119	Transcription termination factor Rho
<i>rimJ</i>	3.06	5.70E-12	Ribosomal-protein-alanine acetyltransferase.
<i>rmf</i>	-5.88	2.05E-102	Ribosome modulation factor
<i>sahH</i>	3.04	1.60E-26	Adenosylhomocysteinase

<i>sbp</i>	5.65	6.59E-26	sulfate transport system
<i>sdaA</i>	3.59	8.25E-98	L-serine dehydratase
<i>sodM</i>	-4.90	1.95E-109	Superoxide dismutase
<i>soxR</i>	-3.21	4.18E-21	Transcriptional activator SoxR; activated by phenazine.
<i>vgrG1</i>	15.96	3.79E-113	Hcp secretion island I (HSI-I) T6SS
<i>wzx</i>	3.04	1.14E-22	Potential multiple membrane spanning domains (Wzx).
<i>xylS</i>	4.10	3.67E-13	Transcriptional regulator XylS; positive controls expression of genes on the TOL plasmid.
<i>znuC</i>	-4.52	2.83E-59	Zinc ABC transporter, ATP-binding protein ZnuC

Supplementary Table 4 Summary of overall gene expression upon treatment with CoP

Gene name	Fold change	p value	Gene product description
<i>acoB</i>	2.32	6.08E-05	acetoin catabolism protein AcoB
<i>aroP2</i>	3.00	4.45E-64	aromatic amino acid transport protein AroP2
<i>cbpD</i>	-2.87	1.22E-122	chitin-binding protein CbpD precursor
<i>chiC</i>	-4.52	6.20E-204	chitinase
<i>cobM</i>	-2.06	8.28E-04	precorrin-3 methylase
<i>cobN</i>	-2.45	8.66E-18	cobalamin biosynthetic protein CobN
<i>csaA</i>	2.16	1.46E-08	CsaA protein
<i>cysD</i>	-2.20	2.43E-12	ATP sulfurylase small subunit
<i>cysT</i>	-2.55	8.20E-05	sulfate transport protein CysT
<i>fahA</i>	-3.06	5.28E-88	fumarylacetoacetase
<i>fpr</i>	-2.48	2.95E-56	ferredoxin--NADP+ reductase
<i>fptA</i>	-3.68	8.82E-185	Fe(III)-pyochelin outer membrane receptor precursor
<i>fptB</i>	-3.19	9.18E-52	transport of Fe ³⁺ -pyochelin
<i>gcvH2</i>	5.02	1.14E-43	glycine cleavage system protein H2
<i>gcvP2</i>	8.84	1.42E-241	glycine cleavage system protein P2
<i>glcD</i>	3.27	1.48E-36	glycolate oxidase subunit GlcD
<i>glcE</i>	3.98	3.03E-13	glycolate oxidase subunit GlcE
<i>glcF</i>	4.72	7.80E-31	glycolate oxidase subunit GlcF
<i>glyA2</i>	13.42	3.72E-79	serine hydroxymethyltransferase
<i>hmgA</i>	-2.90	1.41E-93	homogentisate 1,2-dioxygenase
<i>lasA</i>	-2.99	4.46E-126	LasA protease precursor
<i>lasB</i>	-2.29	2.91E-72	elastase LasB
<i>lipC</i>	2.82	1.17E-09	lipase LipC
<i>liuA</i>	2.91	6.21E-103	probable acyl-CoA dehydrogenase
<i>liuB</i>	3.67	2.21E-177	probable acyl-CoA carboxytransferase beta chain
<i>liuC</i>	3.39	8.85E-90	probable enoyl-CoA hydratase/isomerase
<i>liuD</i>	3.39	1.02E-148	3-methylcrotonoyl-CoA carboxylase, alphasubunit
<i>liuE</i>	3.38	1.97E-132	3-hydroxy-3-isohexenylglutaryl-CoA/hydroxy-methylglutaryl-CoA lyase
<i>liuR</i>	2.51	1.28E-57	regulator of <i>liu</i> genes
<i>maiA</i>	-3.11	5.17E-67	maleylacetoacetate isomerase
<i>mexG</i>	-2.59	1.76E-14	RND multidrug efflux membrane fusion protein MexG
<i>mexH</i>	-2.79	7.87E-16	probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein precursor
<i>mexI</i>	-2.24	3.76E-19	probable Resistance-Nodulation-Cell

			Division (RND) efflux transporter
<i>opdP</i>	2.41	9.76E-17	glycine-glutamate dipeptide porin OpdP
<i>opmD</i>	-2.13	5.90E-05	probable outer membrane protein precursor
PA0113	2.20	2.82E-09	cyoE; protoheme IX farnesyltransferase
PA0236	-2.17	6.13E-05	probable transcriptional regulator
PA0284	-4.02	1.27E-10	uncharacterized protein
PA0781	2.69	4.42E-11	omr; Zn-regulated outer membrane receptor transcriptional regulator
PA0863	14.66	4.12E-196	probable oxidoreductase.
PA1214	2.29	2.31E-09	asparagine synthase
PA1215	2.00	1.59E-07	hypothetical protein
PA1218	2.03	1.07E-10	PhyH; phytanoyl-CoA dioxygenase family protein
PA1271	-2.03	1.23E-07	probable TonB-dependent receptor
PA1297	26.83	8.56E-230	probable metal transporter.
PA1298	22.84	3.09E-124	probable MFS transporter
PA1299	10.04	9.43E-58	hypothetical protein
PA1869	-2.14	1.71E-13	Acyl carrier protein 2, acpP2.
PA2006	-4.48	2.59E-52	Probable major facilitator superfamily (MFS) transporter.
PA2010	-2.62	8.33E-19	Probable transcriptional regulator.
PA2062	-3.04	1.38E-18	Probable pyridoxal-phosphate dependent enzyme
PA2066	-2.93	2.52E-29	hypothetical protein
PA2067	-2.91	1.57E-25	probable hydrolase
PA2068	-3.32	4.02E-61	probable MFS transporter
PA2069	-3.97	6.55E-124	probable carbamoyl transferase
PA2134	-2.08	1.15E-13	probable outer membrane protein
PA2154	-2.11	5.03E-04	hypothetical protein
PA2169	-2.27	3.91E-13	hypothetical protein
PA2204	-3.33	4.23E-23	probable binding protein component of ABC transporter
PA2556	2.56	2.65E-38	conserved hypothetical protein; NAD(FAD)- dependent dehydrogenases
PA2557	3.60	4.79E-127	hypothetical protein
PA2691	2.51	9.48E-28	uncharacterized protein
PA2945	-2.42	1.24E-17	CobW, involved in cobalt reduction leading to cobalt(I) corrinoids
PA2946	-2.12	6.86E-10	hypothetical protein; Putative cobalt transporter subunit (CbtA)
PA3446	-2.46	1.19E-04	conserved hypothetical protein
PA3450	-5.86	6.45E-40	probable antioxidant protein/adaptation, protection

PA3568	2.64	7.35E-101	probable acetyl-CoA synthetase
PA3600	2.86	3.16E-17	50S ribosomal protein L36 2.
PA3601	2.73	4.24E-42	50S ribosomal protein L31 type B, <i>rpmE</i>
PA3709	2.71	3.92E-54	probable major facilitator superfamily (MFS) transporter.
PA4063	2.83	1.23E-15	Zn ²⁺ periplasmic binding protein
PA4141	-2.27	3.25E-36	hypothetical protein
PA4142	-2.22	9.68E-38	probable secretion protein
PA4150	2.32	1.90E-05	probable dehydrogenase E1 component
PA4152	2.02	5.87E-04	probable hydrolase
PA4153	2.06	6.97E-06	2,3-butanediol dehydrogenase
PA4218	-3.85	1.69E-96	probable transporter
PA4219	-3.78	4.91E-79	uncharacterized protein
PA4222	-3.76	1.06E-117	probable ATP-binding component of ABC transporter
PA4341	2.06	1.36E-10	transcriptional regulators
PA4834	2.24	5.17E-04	putative membrane transporter, zinc/cobalt uptake, <i>zrmD</i>
PA4836	2.10	1.28E-06	probable nicotianamine synthase protein (important divalent metal chelator), <i>zrmB</i>
PA4837	2.50	4.41E-18	probable outer membrane protein; TonB-dependent outer membrane protein, <i>zrmA</i>
PA5027	-2.10	2.48E-10	hypothetical protein
PA5096	2.20	9.91E-59	probable binding protein component of ABC transporter
PA5097	2.03	2.19E-36	probable amino acid permease
PA5352	2.78	3.30E-06	uncharacterized protein
PA5383	-7.43	2.38E-20	membrane protein
PA5530	-2.13	5.91E-05	probable MFS dicarboxylate transporter
PA5540	2.16	1.29E-03	carbonate dehydratase
<i>pchA</i>	-4.46	2.20E-181	salicylate biosynthesis isochorismate synthase
<i>pchB</i>	-4.29	6.09E-141	salicylate biosynthesis protein PchB
<i>pchC</i>	-3.78	1.27E-21	pyochelin biosynthetic protein PchC
<i>pchD</i>	-4.26	9.09E-170	pyochelin biosynthesis protein PchD
<i>pchE</i>	-5.37	1.39E-193	dihydroaeruginosic acid synthetase
<i>pchF</i>	-5.11	2.85E-176	pyochelin synthetase
<i>pchG</i>	-4.22	9.83E-172	pyochelin biosynthetic protein PchG
<i>pchH</i>	-4.35	1.58E-124	ABC transporter
<i>phhB</i>	2.11	5.94E-42	pterin-4- α -carbinolamine dehydratase
<i>phhR</i>	-2.97	3.56E-30	transcriptional regulator PhhR
<i>phrS</i>	-2.12	1.82E-08	non-coding RNA gene
<i>phzA2</i>	-4.82	3.89E-48	phenazine biosynthesis protein
<i>phzB2</i>	-4.19	5.38E-121	phenazine biosynthesis protein

<i>phzC2</i>	-4.25	3.09E-38	phenazine biosynthesis protein PhzC
<i>phzG2</i>	-3.53	1.62E-35	probable pyridoxamine 5-phosphate oxidase
<i>piv</i>	-2.18	1.29E-68	protease IV; PvdS-regulated endoprotease
<i>purB</i>	-2.42	5.32E-13	adenylosuccinate lyase
<i>purE</i>	-2.14	3.02E-05	phosphoribosylaminoimidazole carboxylase, catalytic subunit
<i>pyrQ</i>	2.35	2.74E-04	dihydroorotase
<i>rhlA</i>	-2.20	4.45E-64	rhamnosyltransferase chain A
<i>rhlB</i>	-2.36	2.29E-71	rhamnosyltransferase chain B
<i>rplB</i>	-2.02	8.70E-29	50S ribosomal protein L2
<i>rplD</i>	-2.08	7.52E-34	50S ribosomal protein L4
<i>rplF</i>	-2.35	2.67E-42	50S ribosomal protein L6
<i>rplO</i>	-2.12	5.68E-35	50S ribosomal protein L15
<i>rplR</i>	-2.17	1.28E-28	50S ribosomal protein L18
<i>rpsB</i>	-2.07	5.55E-31	30S ribosomal protein S2
<i>rpsE</i>	-2.10	1.52E-33	30S ribosomal protein S5
<i>rpsF</i>	-2.03	1.27E-21	30S ribosomal protein S6
<i>rpsL</i>	-2.06	7.08E-25	30S ribosomal protein S12
<i>rpsM</i>	-2.10	7.35E-30	30S ribosomal protein S13
<i>sbp</i>	-3.45	1.77E-13	sulfate-binding protein precursor
<i>secY</i>	-2.02	1.17E-32	secretion protein SecY

Supplementary Figure 1 *P.aeruginosa* LasR/I circuit model with QQ and QSI both indicated in red. Dashed lines indicate the reactants still remains after the reactions and solid lines indicate the reactants will disappear after the reactions.

Supplementary Figure 2 Representative growth curves of *P. aeruginosa* incubated with compounds at different concentrations. Shown above are growth curves of (A) G1, (B) AiiA, (C) G1 and AiiA at 32 $\mu\text{g/mL}$, and (D) G1 and AiiA at 16 $\mu\text{g/mL}$ respectively. The compounds didn't show any growth inhibition effect on *P. aeruginosa*. Growth curve was measured using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). Experiments were done in triplicate manner.

Supplementary Figure 3 Dose-dependent curves of compounds with QS reporter strain PAO1-*lasB-gfp*. (A) G1, (B) AiiA, (C) G1 and AiiA at 32 µg/mL, (D) G1 and AiiA at 16 µg/mL. Measurements were done using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). Experiments were done in triplicate manner.

Supplementary Figure 4 Dose-dependent curves of compounds with QS reporter strain PAO1-*pqsA-gfp*. (A) G1, (B) AiiA, and (C) G1 and AiiA at 32 μ g/mL. Measurements were done using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). Experiments were done in triplicate manner.

Supplementary Figure 5 Dose-dependent curves of QS deficient $\Delta lasI\Delta rhII$ double mutant harboring *lasB-gfp* (top) and *rhlA-gfp* (bottom) supplemented with (A) 3-oxo-C12-HSL, (B) 3-oxo-C12-HSL with G1 50 μ M, (C) C4-HSL, and (D) C4-HSL with G1 50 μ M. Experiments were done in triplicate manner, only representative data are shown.

Supplementary Figure 6 Dose-dependent curves of compounds with QS reporter strain PAO1-*rhlA-gfp*. (A) G1, (B) AiiA, and (C) G1 and AiiA at 32 μ g/mL. Measurements were done using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). Experiments were done in triplicate manner.

Supplementary Figure 7 IC_{50} values of compounds and combination therapy for PAO1-*rhlA-gfp*. For the IC_{50} values calculation, it was determined at the time point where inhibition started to occur (between 4-6 hours). Calculation are based on three biological replicates and done using GraphPad Prism 6 software. The IC_{50} values for G1 is $3.65 \pm 0.95 \mu\text{M}$ and $17.79 \pm 1.77 \mu\text{g/ml}$ for AiiA. Addition of $32 \mu\text{g/mL}$ of AiiA to G1 reduced IC_{50} values to $1.38 \pm 0.16 \mu\text{M}$.

Supplementary Figure 8 HPLC analysis of enzymatic analysis of AiiA. The 3-oxo-C12-HSL (OdDHL) hydrolysis activity of AiiA was tested with 500 μ M OdDHL and 30 μ M AiiA in reaction buffer (20 mM Tris-HCl, 150 mM NaCl, pH 8.0). The hydrolyzed product of OdDHL could be observed as the read peak eluted earlier at $t = 500$ s.

Supplementary Figure 9 PAO1-*gfp* expression of compounds benzbromarone, 8-quinolinol sulfate monohydrate (QSM), cetylpyridium chloride (CPC), zinc pyridine (hydroxypyridine-2-thione-zinc, or ZnP), and carbimazole. The compounds didn't show any inhibition of GFP signal and autofluorescence property when tested at their highest working concentration (10 μ M). DMSO 0.1% was used as solvent control. Fluorescence intensity was measured using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland) with excitation wavelength at 485 nm and emission wavelength at 535 nm. Experiments were done in triplicate manner.

Supplementary Figure 10 Dose-dependent curves of metal salts (ZnSO₄ and CoBr₂) with QS reporter strain PAO1-*lasB-gfp*. DMSO 0.1% was used as solvent control. Measurements were done using Tecan Infinite 200 Pro plate reader (Tecan Group Ltd, Männedorf, Switzerland). Experiments were done in triplicate manner, only representative data are shown.

Supplementary Figure 11 Overview of genes that are (A) upregulated and (B) downregulated upon treatment with ZnP based on the gene functional classification.

Supplementary Figure 12 Summary of genes that are (A) upregulated and (B) downregulated upon treatment with CoP based on the gene functional classification.

Supplementary Figure 13 Gel images of transformed evolved strains with *lasB-gfp* (gel A, column 1-6, 12-17), *rhlA-gfp* (gel A, column 7-11, 18-22), and *pqsA-gfp* (gel B). Image was taken with GelDoc XR+ Imager (Bio-Rad, USA).

¹H and ¹³C NMR spectra of compound **1**

¹H and ¹³C NMR spectra of compound 2

¹H and ¹³C NMR spectra of compound 3

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