Identification and development of novel antimicrobial therapies

Tan, Sean Yang-Yi

2015

Tan, S. Y.-Y. (2015). Identification and development of novel antimicrobial therapies.
Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/65831>

<https://doi.org/10.32657/10356/65831>

IDENTIFICATION AND DEVELOPMENT OF NOVEL
ANTIMICROBIAL THERAPIES

SEAN TAN YANG YI

SCHOOL OF BIOLOGICAL SCIENCES

2015

Identification and Development of
Novel Antimicrobial Therapies

Sean Tan Yang Yi

Supervisor: Professor Michael Givskov

Co-Supervisor: Professor Yehuda Cohen

SCHOOL OF BIOLOGICAL SCIENCES

NANYANG TECHNOLOGICAL UNIVERSITY

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

2015

Acknowledgements

First, I would like to thank Professor Michael Givskov, for his unwavering support and guidance for me during my PhD candidature. He has always encouraged me to be a creative and independent thinker, and has given me the academic freedom to pursue my research interests. I thank him for his thoughtfulness, care and for upholding the standard of high-quality work. I have benefited greatly from having him as my supervisor.

Special thanks to Professor Yehuda Cohen, who convinced me to choose SCELSE for my PhD studies; a choice that has been instrumental for my personal development. His optimism and inspirational leadership has benefited me in many ways.

I thank Assistant Professor Yang Liang, who as my mentor, guided and taught me the day-to-day skills required by a research professional in the field of microbiology. His enthusiasm and passion for science is astounding, and I am glad to call him a personal friend. I also appreciate highly the help I received from his research group, especially Chua Song Lin, Dr. Liu Yang and Chen Yi Cai.

My thanks go out to my thesis committee advisors: Professors Staffan Kjelleberg and Scott Rice, I cannot ask for any better team than them.

I also thank all my SCELSE colleagues who have helped and accompanied me on my PhD journey: Seth Tan, Shi Ming, Su Chuen Chew, Stan Chan, Clair Liew and Martin Tay. You all have made my PhD life a more enjoyable and easier journey! Special mention goes to Professor Paul Williams for hosting me in Nottingham, as well as Eliza Soh for helping me with my experiments there.

I am grateful to Nanyang Technological University for its generous scholarship support during my PhD education.

I thank my family, personal friends and church friends for their love and support during these years. Last but not least, I thank God for his wisdom and strength, without which this PhD journey may have been impossible.

Table of Contents

ACKNOWLEDGEMENTS.....	III
TABLE OF CONTENTS	III
LIST OF FIGURES	VI
LIST OF TABLES.....	X
ABBREVIATIONS	XII
ABSTRACT	XIV
PUBLICATIONS	15
POSTER PRESENTATIONS	16
PATENT	16
1 INTRODUCTION	16
1.1 THE PROBLEM OF ANTIMICROBIAL RESISTANCE	17
1.2 BIOFILMS	19
1.3 QUORUM SENSING	22
1.4 QUORUM SENSING INHIBITION.....	28
1.5 OBJECTIVES AND THESIS ORGANIZATION	31
2 USING COMPARATIVE GENOMICS TO UNDERSTAND THE DEVELOPMENT OF ANTIBIOTIC RESISTANCE IN ACINETOBACTER BAUMANNII	33
2.1 INTRODUCTION	33
2.2 MATERIALS AND METHODS	35
2.2.1 <i>Bacterial strains and patient background information</i>	35
2.2.2 <i>Antimicrobial susceptibility assay</i>	36
2.2.3 <i>Genome sequencing and assembly</i>	36
2.2.4 <i>Genome comparison</i>	37
2.2.5 <i>Genome annotation</i>	37
2.2.6 <i>SNP comparison and analysis</i>	38
2.2.7 <i>Prediction of antibiotic resistance genes</i>	38
2.2.8 <i>Nucleotide sequence accession numbers</i>	38
2.2.9 <i>iTRAQ-based proteomics analysis</i>	38
2.3 RESULTS	41
2.3.1 <i>General characteristics of the A. baumannii genomes</i>	41

2.3.2	<i>Phylogenetic analysis of A. baumannii strains</i>	42
2.3.3	<i>Antibiotic resistance profile of the A. baumannii 53264 and 48055 strains</i> ...	44
2.3.4	<i>Study of Single Nucleotide Polymorphisms</i>	48
2.3.4.1	SNPs between <i>A. baumannii</i> strains and ACICU	48
2.3.4.2	SNP differences between <i>A. baumannii</i> 53264 and 48055 strains.....	50
2.3.5	<i>Use of proteomics to study the response of PDR A. baumannii 53264 strain to antibiotics</i>	52
2.4	CONCLUSION	55
3	DEVELOPMENT OF A STRUCTURE-BASED VIRTUAL SCREENING PLATFORM FOR THE IDENTIFICATION OF NOVEL LASR INHIBITORS	56
3.1	INTRODUCTION	56
3.2	MATERIAL AND METHODS.....	58
3.2.1	<i>Structure-based virtual screening</i>	58
3.2.2	<i>Protein structure file and ligand database</i>	60
3.2.3	<i>Molecular Docking</i>	60
3.2.4	<i>Selection of QSI Candidates</i>	61
3.2.5	<i>Bacterial strains</i>	62
3.2.6	<i>Growth medium and conditions</i>	62
3.2.7	<i>P. aeruginosa QS inhibition assay</i>	63
3.2.8	<i>E. coli competition assay between QSI compounds and OdDHL</i>	63
3.2.9	<i>Inhibition of the rhl and pqs QS systems in wild-type PAO1 and PAO1 ΔlasR mutant</i>	64
3.2.10	<i>Glass slide biofilm assay for observation of eDNA release</i>	64
3.2.11	<i>iTRAQ-based proteomics analyses for G1</i>	65
3.2.12	<i>Elastase assay</i>	65
3.3	RESULTS	65
3.3.1	<i>Structure-based virtual screening for QSIs</i>	65
3.3.2	<i>Inhibition assay with the P. aeruginosa lasB-gfp(ASV) strain</i>	71
3.3.3	<i>IC₅₀ value comparisons of the five QSI candidates</i>	75
3.3.4	<i>AHL competition assay using the heterologous E. coli lasB-gfp(ASV) strain</i> ..	77
3.3.5	<i>Effect of QSIs on rhl and pqs quorum sensing systems</i>	79
3.3.6	<i>Effect of G1 on the extracellular DNA release in P. aeruginosa biofilms</i>	80
3.3.7	<i>iTRAQ-based quantitative proteomic analysis</i>	81
3.3.8	<i>Effect of G1 on the production of elastase by P. aeruginosa</i>	83
3.4	DISCUSSION.....	85
3.4.1	<i>Comments on structure-based virtual screening</i>	85
3.4.2	<i>Comments on G1 as a QSI</i>	89

3.4.3	<i>Comments on the effects of G1 on the proteome of P. aeruginosa</i>	90
3.5	SUMMARY	92
4	ELUCIDATING THE ANTI-VIRULENCE AND ANTI-BIOFILM EFFECTS OF IBERIN THROUGH A COMPARATIVE SYSTEMS BIOLOGY APPROACH	93
4.1	INTRODUCTION	93
4.2	MATERIAL AND METHODS.....	96
4.2.1	<i>Bacterial strains, plasmids and growth conditions</i>	96
4.2.2	<i>RNA preparation</i>	97
4.2.3	<i>RNA sequencing and data analysis</i>	97
4.2.4	<i>iTRAQ proteomics analyses</i>	98
4.2.5	<i>Construction of the rsmZ-gfpmut3b* and rsmY-gfpmut3b* transcriptional fusions</i>	100
4.2.6	<i>Reporter fusion assay</i>	100
4.2.7	<i>Biofilm assay</i>	101
4.3	RESULTS	102
4.3.1	<i>Determination of the sampling point for RNA-Seq and iTRAQ proteomics</i> ...	102
4.3.2	<i>Comparative analysis of iberin's mode of inhibition by RNA-Sequencing and proteomics</i>	102
4.3.3	<i>Analysis of the inhibition of small regulatory RNAs by iberin</i>	109
4.3.4	<i>Inhibition of pyoverdine expression by iberin</i>	111
4.3.5	<i>Iberin reduces P. aeruginosa biofilm formation in a slide biofilm assay</i>	113
4.4	DISCUSSION.....	115
4.4.1	<i>RNA-Seq and iTRAQ proteomics result with iberin</i>	115
4.4.2	<i>Iberin as an inhibitor of small RNAs</i>	118
4.5	SUMMARY	120
5	CONCLUSION	121
6	REFERENCES	123
	APPENDIX A	143
	APPENDIX B	144
	APPENDIX C	147
	APPENDIX D	148

List of Figures

Figure 1-1. Estimates on the overall societal costs (e.g. deaths, hospital days or illnesses) in the European Union, Thailand and the United States of America.	17
Figure 1-2. Timeline showing the year when an antibiotic was deployed and the year when resistance to that antibiotic was observed.	18
Figure 1-3. Diagram showing the wide-ranging impacts of biofilms on human health and industries.	19
Figure 1-4. Diagram showing the five main stages of biofilm development based on <i>in vitro</i> observations.	20
Figure 1-5. Three possible mechanisms for antibiotic resistance in biofilms.	21
Figure 1-6. Phenotypes known to be controlled by QS in Gram-negative bacteria.	23
Figure 1-7. The three <i>Pseudomonas aeruginosa</i> quorum-sensing systems: <i>lasRI</i> , <i>rhlRI</i> and PQS systems.	25
Figure 1-8. The interplay between the four main quorum-sensing systems found in <i>P. aeruginosa</i>	25
Figure 1-9. An overview of the main QS systems in <i>P. aeruginosa</i> (<i>las</i> , <i>rhl</i> , and PQS) and the Gac/Rsm signal transduction system between the four main quorum-sensing systems found in <i>P. aeruginosa</i>	25
Figure 1-10. (A) The red macroalga <i>Delisea pulchra</i> photographed in the shallows of Cape Banks, Sydney, Australia. (B) Structures of the AHLs (BHL and HHL), and two halogenated furanones produced by <i>D. pulchra</i>	28
Figure 1-11. Scanning confocal laser microscopy images of <i>P. aeruginosa</i> PAO1 biofilms cultured for 3 days in flow cells without furanone or with 10 μ M furanone.	30

Figure 2-1. Overview of the spread of <i>Acinetobacter baumannii</i> in a hospital ward, between patients, hospital staff and equipment..	34
Figure 2-2. Heat map based on a pair-wise distance matrix of whole genome alignment as computed by Progressive Mauve.	42
Figure 2-3. An unrooted phylogenetic tree showing the <i>A. baumannii</i> 53264 and <i>A. baumannii</i> 48055 strains in relation to 42 other <i>A. baumannii</i> strains..	44
Figure 2-4. Venn diagrams showing the number of proteins whose expression was either induced or reduced in the presence of a specific antibiotic..	52
Figure 3-1. An example of a <i>Chromobacterium violaceum</i> bioassay.	56
Figure 3-2. Structure-based virtual screening for new ligands.	57
Figure 3-3. Overview of the Structure-Based Virtual Screening Process	59
Figure 3-4. The chemical structures of the reference ligand, OdDHL, and other known QSIs used as comparisons for the structure-based virtual screening.....	66
Figure 3-5. Structures of five QSI candidates.....	71
Figure 3-6. Dose-responses curves of five QSI candidates when incubated with the <i>P. aeruginosa</i> PAO1 <i>lasB-gfp</i> (ASV) strain..	73
Figure 3-7. Interaction maps between residues within the LasR LBD and five QSI compounds.....	74
Figure 3-8. Crystal structure model of the interactions between residues in the LasR ligand-binding pocket with the autoinducer molecule OdDHL.....	75
Figure 3-9. The half maximal inhibitory concentrations(IC ₅₀) of the five QSI compounds tested.....	76

Figure 3-10. Competition assay results of the following 4 QSIs (C1, F1, F2, H1) when incubated with the <i>E. coli lasB-gfp</i> (ASV) strain and increasing concentrations of OdDHL.....	77
Figure 3-11. The response of the <i>E. coli lasB-gfp</i> (ASV) strain to varying concentrations of G1 and OdDHL. .	78
Figure 3-12. The expression of (a) <i>rhIA-gfp</i> (ASV) and (b) <i>pqsA-gfp</i> (ASV) in <i>P. aeruginosa</i> PAO1 wild-type and a $\Delta lasR$ mutant was measured when treated with 50 μ M of each QSI.....	79
Figure 3-13. Biofilms of <i>gfp</i> -tagged PAO1 grown for 24h either in ABTG medium (A and C) or ABTG medium containing 10 μ M G1 (B and D) were stained with propidium iodide (PI).....	81
Figure 3-14. Effect of G1 on the elastase activity of <i>P. aeruginosa</i> cultures.....	84
Figure 3-15. Model of pyoverdine gene regulation in <i>P. aeruginosa</i>	91
Figure 4-1. Chemical structure of the isothiocyanate compound iberin.....	94
Figure 4-2. Growth and normalized <i>gfp</i> gene expression of the <i>P. aeruginosa</i> PAO1 biosensor strains carrying <i>lasB-gfp</i> in ABTGC medium with 500 μ M iberin.....	102
Figure 4-3. Growth and normalized <i>gfp</i> gene expression of the <i>P. aeruginosa</i> PAO1 biosensor strains carrying <i>rsmY-gfp</i> (A, C) and <i>rsmZ-gfp</i> (B, D) in ABTGC medium containing varying concentrations of iberin.	111
Figure 4-4. A suggested model that links the relationship between the Gac/Rsm system, c-di-GMP and siderophore (e.g. pyoverdine production).	109
Figure 4-5. Growth and normalized <i>pvdA-gfp</i> expression of the $\Delta pvdA$ <i>P. aeruginosa</i> PAO1 mutant strain carrying <i>pvdA-gfp</i> (A, C) and the wild-type <i>P. aeruginosa</i> PAO1 <i>pvdA-gfp</i> biosensor strain in ABTGC medium containing varying concentrations of iberin.....	113

Figure 4-6. Three-dimensional confocal images of 1-day-old miniTn7-gfp-tagged *P. aeruginosa* PAO1 slide biofilms with either ABTGC medium or 500 μ M iberin 114

Figure 4-7. An overview of the the type III secretion system (T3SS) in *P. aeruginosa*.
.....117

List of Tables

Table 2-1. General characteristics of the <i>A. baumannii</i> 53264 and <i>A. baumannii</i> 48055 genomes as obtained from the RAST annotation server (Aziz, et al. 2008).....	41
Table 2-2. Minimum inhibitory concentrations (µg/ml) for the PDR <i>A. baumannii</i> 53264 and XDR <i>A. baumannii</i> 48055 strains, and the antibiotic-sensitive <i>A. baumannii</i> 52082 strain towards the following six antibiotics: tobramycin, colistin, ceftazidime, tetracycline, ciprofloxacin and meropenem.....	45
Table 2-3. Antibiotic resistance profiles of <i>A. baumannii</i> 53264 and <i>A. baumannii</i> 48055 strains	46
Table 2-4. Non-synonymous single nucleotide polymorphisms observed in the <i>ampC</i> , <i>gyrB</i> and <i>parC</i> genes of <i>A. baumannii</i> 53264 and <i>A. baumannii</i> 48055 strains in reference to the <i>A. baumannii</i> ACICU strain.....	49
Table 2-5. List of SNP differences between the XDR <i>A. baumannii</i> 48055 and the PDR <i>A. baumannii</i> 53264 strains..	50
Table 2-6. Proteins, whose expression was down-regulated by all 3 antibiotics.	53
Table 2-7. Proteins, whose expression was up-regulated by all 3 antibiotics.	54
Table 3-1. List of <i>P. aeruginosa</i> and <i>E. coli</i> strains used in the present study.....	62
Table 3-2. Structures and docking scores of reference compounds.	67
Table 3-3. Structures and docking scores of twenty-two top-scoring compounds with the following selection criteria: Molecular weight below 200 Daltons, (docking) rerank score below -60.....	68
Table 3-4. Key residues within the LasR ligand binding pocket having hydrogen bonding interactions with OdDHL and the corresponding 5 QSI molecules.	72

Table 3-5. Proteins whose abundance in the <i>P. aeruginosa</i> PAO1 strain decreased significantly upon 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (G1) addition.....	82
Table 3-6. Structures of C1 , G1 and molecule 404	86
Table 4-1. Characteristics of the bacterial strains and plasmids used in this study.....	96
Table 4-2. List of genes/proteins found to be significantly up-regulated upon iberin treatment.....	103
Table 4-3. List of genes/proteins found to be significantly down-regulated upon iberin treatment.....	105

Abbreviations

2D	2-dimensional
3D	3-dimensional
AFLP	Amplified fragment length polymorphism
AHL	Acyl homoserine lactone
AMR	Antimicrobial resistance
BHL	<i>N</i> -butanoyl homoserine lactone
CLSM	Confocal laser scanning microscopy
DMSO	Dimethyl sulfoxide
eDNA	Extracellular DNA
EPS	Extracellular polymeric substances
GFP	Green fluorescent protein
HTS	High throughput screening
IC ₅₀	Half maximal inhibitory concentration
ICU	Intensive care unit
ITC	Isothiocyanate
iTRAQ	Isobaric tags for relative and absolute quantitation
KEGG	Kyoto encyclopedia of genes and genomes
LBD	Ligand binding domain
MDR	Multi drug resistant
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MVD	Molegro Virtual Docker
OD	Optical density
OdDHL	<i>N</i> -(3-oxododecanoyl)-L-homoserine lactone
PDB	Protein data bank
PDR	Pandrug resistant
PI	Propidium iodide
PMNs	Polymorphonuclear leukocytes
PQS	<i>Pseudomonas</i> quinolone signal

QS	Quorum sensing
QSI	Quorum sensing inhibitor
QSI _S	Quorum sensing inhibitor selector
RAST	Rapid Annotations using Subsystem Technology
RNA-Seq	RNA sequencing
SB-VS	Structure-based virtual screening
SNP	Single nucleotide polymorphism
T3SS	Type III secretion system
WHO	World Health Organization
XDR	Extensively drug resistant

Abstract

Antibiotic resistance in bacteria is a serious problem. Currently, we have seen the emergence of pandrug resistant strains – bacteria that are resistant to all known antibiotic therapies. To make matters worse, bacteria in the environment exist predominantly in the biofilm mode of life which makes them hundreds of times more resistant to antibiotics than their planktonic forms. Antimicrobial therapies have largely ignored the biofilm mode of life, and as such there is a need for novel antimicrobial therapies to address it.

In the first part of my thesis, I describe the use of whole genome sequencing to understand the development of drug resistance in two *Acinetobacter baumannii* strains that were isolated from patients hospitalized in an Intensive Care Unit in Rigshospitalet, Denmark. By using proteomics analyses, we are also able to predict the mechanisms for antibiotic resistance, and suggest possible antimicrobial therapies against these drug-resistant strains.

A recently developed antimicrobial strategy that has proved effective involves the inhibition of bacterial quorum sensing (QS). QS is a phenomenon in which bacteria in a biofilm community communicate intercellularly by way of secretion of signal molecules into their environment. QS is utilized by bacterial populations to coordinate a community response, such as biofilm formation or release of virulence factors. QS inhibitors (QSIs) have been shown to attenuate the virulence of bacteria such as *Pseudomonas aeruginosa* and promote its clearance by the body's immunity. As such, one of my aims is to discover new and effective QSIs against *P. aeruginosa*.

Hence, in the second part of my thesis, I describe how I developed a fast, cost-effective and accurate *in silico* platform for the identification of novel QSIs against the *las* QS system in *P. aeruginosa*. We obtained several lead compounds, and have filed a patent for intellectual property protection of these chemical structures.

Lastly, I describe how we used comparative systems biology tools (RNA-sequencing and proteomics) to shed light on a novel QS inhibition mechanism, the inhibition of small regulatory RNAs, by iberin, a natural compound isolated from horseradish.

Publications

1. **Tan, S. Y.-Y.**, Chua, S.L., Liu, Y., Hoiby, N., Andersen, L.P., Givskov, M., Song, Z. and Yang, L. 2013. Comparative genomic analysis of rapid evolution of an extreme-drug-resistant *Acinetobacter baumannii* clone. *Genome Biology and Evolution* **5**:807-818.
2. Chua, S.L., **Tan, S. Y.-Y.**, Rybtke, M.T., Chen, Y., Rice, S.A., Kjelleberg, S., Tolker-Nielsen, T., Yang, L. and Givskov, M., 2013. Bis-(3'-5')-cyclic dimeric GMP regulates antimicrobial peptide resistance in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **57**: 2066-2075.
3. Teo, J., **Tan, S. Y.-Y.**, Tay, M., Ding, Y., Kjelleberg, S., Givskov, M., Lin, R. T. and Yang, L. 2013. First case of *E anophelis* outbreak in an intensive-care unit. *Lancet* **382**:855-856.
4. **Tan, S. Y.-Y.**, Chua, S. L., Chen, Y., Rice, S. A., Kjelleberg, S., Nielsen, T. E., Yang, L. and Givskov, M. 2013. Identification of five structurally unrelated quorum-sensing inhibitors of *Pseudomonas aeruginosa* from a natural-derivative database. *Antimicrobial Agents and Chemotherapy* **57**:5629-5641.
5. Tan, S. Y.-E., Chew, S. C., **Tan, S. Y.-Y.**, Givskov, M. and Yang, L. 2014. Emerging frontiers in detection and control of bacterial biofilms. *Current Opinion in Biotechnology* **26**:1-6. (Review)
6. **Tan, S. Y.-Y.**, Teo, J. and Yang, L. 2013. *E. anophelis* outbreak in an intensive-care unit - Authors' reply. *The Lancet* 382:2064-2065.
7. Teo, J., **Tan, S. Y.-Y.**, Liu, Y., Tay, M., Ding, Y., Li, Y., Kjelleberg, Y., Givskov, M., Lin, R. T. and Yang, L. 2014. Comparative genomic analysis of malaria mosquito vector-associated novel pathogen *Elizabethkingia anophelis*. *Genome biology and evolution* **6**:1158-1165.
8. **Tan, S. Y.-Y.**, Liu, Y., Chua, S. L., Vejborg, R. M, Jakobsen, T. H, Chew, S. C, Li, Y., Nielsen, T. E., Tolker-Nielsen, T., Yang, L. and Givskov, M. 2014. Comparative systems biology analysis and mode of action of the isothiocyanate compound iberin on *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*. **58**:6648-6659

Poster Presentations

Tan, S.Y., Chua, S.L., Chen, Y., Rice, S.A., Kjelleberg, S., Nielsen, T.E., Yang, L., Givskov, M., 2013. Identification of five structurally unrelated quorum-sensing inhibitors of *Pseudomonas aeruginosa* from a natural-derivative database. Pseudomonas Conference 2013, Lausanne, Switzerland.

Patent

Michael Givskov, Yang Liang, **Sean Yang-Yi Tan**. Novel quorum sensing inhibitors. (National Phase Entry in USA based on PCT Application No: PCT/SG2014/000115)

1 Introduction

1.1 The Problem of Antimicrobial Resistance

According to the World Health Organization (WHO) in 2012, infectious diseases caused by microbes (bacteria, fungi, parasites and viruses) remained the second leading cause of death worldwide. The development of antimicrobial resistance (AMR) is a serious problem; it has allowed the emergence of new diseases, and the re-emergence of older ones. In the report entitled “Antimicrobial Resistance: Global Report on Surveillance 2014”, the WHO discusses the worsening problem of AMR globally (World Health Organization, 2014). An infographic by WHO illustrates the extremely detrimental impact of AMR – in terms of deaths, hospitalization days and overall societal costs in three selected regions (Figure 1-1).

Figure 1-1. Estimates on the overall societal costs (e.g. deaths, hospital days or illnesses) of antimicrobial resistance in the European Union, Thailand and the United States of America. Image taken from “Antimicrobial resistance: global report on surveillance 2014” (World Health Organization, 2014).

In my thesis, I focus exclusively on antimicrobial resistance in bacteria. Bacteria cells have evolved resistance against most, if not all, of the antibiotics used today, thus prompting the question as to whether we are indeed living in a post-antibiotic era (Alanis, 2005) (Figure 1-2).

Figure 1-2. Timeline showing the year when an antibiotic was deployed and the year when resistance to that antibiotic was observed. Names above the timeline show the year when a particular antibiotic was released and conversely, names below the timeline show the year when resistance was developed against a particular antibiotic. Image from Clatworthy et al. (2007).

The emergence of multidrug-resistant (MDR) bacterial strains in hospital settings has been credited with being the main cause of nosocomial infections on a global scale (Nikaido 2009). MDR strains can be defined as bacteria exhibiting resistance to three or more classes of antibiotics. Recently, extensively drug resistant (XDR) and pandrug resistant (PDR) bacterial strains were isolated from hospital infections (Paterson and Doi, 2007).

XDR strains are defined as being resistant to all but one or two categories of antimicrobial agents, and PDR bacterial strains, are defined as being resistant to all antimicrobial agents in all categories (Magiorakos et al., 2012). I have chosen to adopt the terms XDR and PDR as these are harmonized terms that have been adopted globally (Falagas and Karageorgopoulos, 2008; Magiorakos et al., 2012).

Therefore, the antimicrobial strategies used to combat these ‘superbugs’ is of critical importance, and we have to understand their antibiotic resistance mechanisms in order to develop effective next-generation antimicrobial agents. However, bacteria in the environment persist as

biofilms and this greatly impacts the antimicrobial strategies that have to be employed against them. In the next section, I explain what biofilms are and their resistance to antibiotics.

1.2 Biofilms

As mentioned earlier, bacteria in the environment predominantly exist in the biofilm mode of life as opposed to the planktonic, non-sessile mode of life; bacteria in either the planktonic or biofilm form are highly different (Costerton et al., 1978; Costerton et al., 1995). Biofilms have been defined as “structured communities of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface” (Costerton et al., 1999). Living as a biofilm provides advantages to bacteria: increased protection against antibiotics and chemicals, as well as bacteriophages and phagocytic amoeba in the environment (Donlan and Costerton, 2002).

Biofilms have wide-spread beneficial as well as detrimental impacts on our lives, and can be found on teeth as dental plaque, in water treatment systems and on medical implants and catheters (Figure 1-3).

Figure 1-3. Diagram showing the wide-ranging impacts of biofilms on human health and industries. [Image credit: Phil Stewart / Peg Dirckx (1996), Montana State University Center for Biofilm Engineering]

Bacteria in general proceed through several stages in its development from an initially planktonic community into a mature biofilm. Figure 1-4 highlights the main stages in the development of a biofilm based on *in vitro* observations (Stoodley et al., 2002). In the first stage, planktonic bacteria initially attaches to a substratum. Next, irreversible attachment occurs when bacteria start to produce extracellular polymeric substances (EPS) and become encased in this self-produced EPS matrix. In stage three, there is early maturation of the biofilm into microcolonies. The biofilm then continues to grow in size and develops complex architecture as mature mushroom-shaped and pillar-like structures with water-filled spaces (Davies et al., 1998). In the final stage, there is a dispersal of biofilm cells which have reverted back to a planktonic motile state, and these dispersal cells are ready to colonize a new environment.

5 stages of Biofilm Development

Figure 1-4. Diagram showing the five main stages of biofilm development based on *in vitro* observations. (1) Initial reversible attachment, (2) irreversible attachment, (3) early maturation / microcolony formation, (4) late maturation, and (5) dispersion. The panels below show photomicrographs of *P. aeruginosa* cultured in a continuous flow cell system, with images taken at different developmental stages of growth. Image from Don Monroe (Monroe, 2007), Image Credit: D. Davis.

An important discovery was that bacterial biofilm cells may become up to 500 times more resistant to antibiotics than their planktonic counterparts (Costerton et al., 1995; Donlan, 2001). Mechanisms that account for the increased resistance include reduced penetration of antibiotics, production of compounds that annihilate the activity of antibiotics, slow growth of biofilm cells, adaptive stress responses, the formation of persister cell populations, and the creation of altered microenvironments that inhibit antibiotic activity (Stewart, 2002; Stewart and William Costerton, 2001). Figure 1-5 highlights three of these possible reasons for the antibiotic resistance of biofilm cells: slow penetration of antibiotics, resistant phenotypes (persisters) and an altered microenvironment.

Figure 1-5. Three possible mechanisms for antibiotic resistance in biofilms. Image taken from Stewart and William Costerton (2001).

Hence, the problem of rising drug resistance seen in bacteria is compounded by the problem of increased antibiotic resistance afforded to bacteria by existing as biofilms. Pharmaceutical companies have shown little consideration for the biofilm mode of life in their antibiotic development projects, and therefore there is a pressing need for novel antimicrobial strategies that tackle the problems posed by biofilms.

One antimicrobial strategy that has proved effective against bacteria has been to target the communication systems – the quorum sensing (QS) systems, of bacteria. In the next section I will describe QS and QS inhibition.

1.3 Quorum Sensing

Bacterial intercellular communication was first discovered in the bioluminescent marine bacterium *Vibrio fischeri* which possesses the LuxI/LuxR quorum-sensing (QS) system (Nealson et al., 1970; Nealson and Hastings, 1979). Later, it was shown that bacterial cell-cell communication occurs through a phenomenon that has been termed QS in which bacteria secrete autoinducer signal molecules into their surrounding environment (Fuqua et al., 1994).

As cell density increases in a bacterial population, the concentration of secreted autoinducer molecules increases locally. Once the concentration of these signal molecules exceeds a threshold level (at this point the population is said to be ‘quorate’, hence the term ‘quorum’) the population activates a coordinated cellular response such as the production of virulence factors and growth as a biofilm community (Fuqua et al., 1994; Passador et al., 1993; Seed et al., 1995; Williams et al., 1992). QS has been shown to be crucial for many bacterial processes, including virulence, biofilm maturation, motility and EPS production (Davies et al., 1998; de Kievit and Iglewski, 2000; Eberl et al., 1996). Figure 1-6 illustrates some of the phenotypes in Gram-negative bacteria known to be controlled by QS, and highlights the fact that QS controls the expression of multiple virulence factors in *P. aeruginosa*.

The QS system has been proposed as a target for developing next-generation antimicrobial agents. The rationale for interrupting bacterial communication rather than inhibiting growth is because QS inhibitors (QSIs), by targeting non-essential processes, do not promote strong selective pressures for the evolution of resistance mechanisms as compared to the conventional growth-inhibitory compounds (Hentzer and Givskov, 2003). Therefore, QSIs have been termed as anti-pathogenic agents, which attenuate bacterial virulence, rather than being bactericidal or bacteriostatic (Hentzer et al., 2003; reviewed by Rasmussen and Givskov, 2006).

Figure 1-6. Phenotypes known to be controlled by QS in Gram-negative bacteria. The general structure of an acylhomoserine lactone molecule is depicted in the center. Image obtained from Diggle et al. (2007). Image credit: Sporulation picture (top right) from John Heap; fruiting body picture (middle right) from Michiel Voss and Greg Velicer.

QS has been most well-studied in Gram-negative bacteria, and the bacterium *Pseudomonas aeruginosa* has been used as a model organism for studying QS and biofilm biology. In particular, infections caused by MDR strains of *Pseudomonas aeruginosa*, constitutes about a tenth of all nosocomial infections (Hancock and Speert, 2000). This proportion is set to increase due to the continually expanding spectrum of infections associated with this bacterium (Kerr and Snelling, 2009). *P. aeruginosa* is known to cause a range of diseases in immunocompromised patients from eye to pulmonary and urinary tract infections (Iversen et al., 2008). In particular, its infections are known to be frequent and potentially fatal in patients with cystic fibrosis, a genetic disease (Moreau-Marquis et al., 2008).

There are several reasons for the use of *P. aeruginosa* as a model, as reviewed briefly in the book “The Biofilm Mode of Life” (Kjelleberg and Givskov, 2007):

First, the *P. aeruginosa* genome has been completely sequenced (Stover et al., 2000) which makes it suitable for studying genome-wide gene expression with the advent of modern sequencing approaches and “omics” technologies (e.g. transcriptomics and proteomics). An important resource is the Pseudomonas Genome Database (Winsor et al., 2011) which contains annotated *Pseudomonas* genomes and facilitates comparative analysis between *P. aeruginosa* with other bacterial strains.

Secondly, the *Pseudomonas* genus is relatively easy to culture in the lab as it has minimal growth requirements and tolerates a wide range of temperatures from 4 to 42 °C.

Thirdly, *P. aeruginosa* is a good representative of a diverse and ecologically significant group of bacteria. Also, the genomic content and virulence determinants are conserved between environmental and clinical isolates of *P. aeruginosa* (Wolfgang et al., 2003) meaning that knowledge gleaned from studying *P. aeruginosa* from clinical samples can be applied to ecological applications, and vice-versa.

Last but not least, *P. aeruginosa* is responsible for many opportunistic and nosocomial infections (Obritsch et al., 2005). In fact, chronic infections of the lungs by *P. aeruginosa* are the leading cause of death of cystic fibrosis patients (Finnan et al., 2004). Pioneering research showed that *P. aeruginosa* develops mainly as biofilms in the cystic fibrosis lung, and this biofilm mode of life protects *P. aeruginosa* from antibiotics and the host immunity (Høiby and Koch, 1990; Høiby et al., 1977). Furthermore, the sputum from cystic fibrosis lungs was found to contain two kinds of *P. aeruginosa* quorum-sensing molecules with an abundance claimed to reflect laboratory grown biofilms and not planktonic cultures (Singh et al., 2000). This suggests that *P. aeruginosa* exist as biofilms in chronic cystic fibrosis lung infections.

P. aeruginosa has four main QS systems, namely the *las*, *rhl*, *pqs* and the recently discovered integrated quorum sensing (IQS) systems (Lee and Zhang, 2015).

The first two QS systems, LasR-LasI and RhlR-RhlI, are based on the LuxR-LuxI homologues of *Vibrio fischeri* (Fuqua et al., 1994), which makes use of acyl homoserine lactone (AHLs) as signal molecules (Fuqua et al., 2001). The AHL synthases are LasI and RhlI, which produce *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) (Pearson et al., 1994) and *N*-butanoyl homoserine lactone (BHL) respectively (Ochsner and Reiser, 1995). The receptor for

OdDHL is the LasR protein (Gambello and Iglewski, 1991), while the receptor for BHL is the RhlR protein. The *las* and *rhl* QS systems regulate a host of virulence factors such as exoproteases (an example being elastase), siderophores, and toxins (Gambello et al., 1993; Passador et al., 1993).

The third signaling system utilizes another kind of signal molecule, 2-heptyl-3-hydroxy-4-quinolone, that has been termed the *Pseudomonas* quinolone signal (PQS) and is able to affect the expression of Las and Rhl-controlled genes (Pesci et al., 1999). LasR is an attractive target for QS inhibition as LasR exerts control over the other QS circuits (namely Rhl and PQS) within the *P. aeruginosa* hierarchy (Pesci et al., 1997). The *las* and *rhl* systems are at the top and bottom of the hierarchy respectively, while the PQS system intervenes between them. The interplay between the three main QS systems (*las*, *rhl* and PQS) is depicted in Figure 1-7 below.

Figure 1-7. The three *Pseudomonas aeruginosa* quorum-sensing systems: *lasRI*, *rhlRI* and PQS systems. LasI synthesizes OdDHL (squares), RhlI synthesizes BHL (diamonds), which bind to LasR and RhlR respectively. LasR activates the Las regulon and also *mvfR* (also known as *pqsR*) and *pqsH*, which are responsible for the synthesis of 4-hydroxy-2-heptylquinoline (shaded triangles) and PQS (inverted open triangles) respectively. The image was obtained from a review by Sifri (2008).

The fourth signaling system, the IQS system, was discovered recently and this system has been shown to integrate environmental signals with the QS system (Lee et al., 2013). The structure of the IQS signal molecule is 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde. IQS is synthesized by the *ambBCDE* gene cluster, and is a positive regulator of PQS, BHL and virulence factor production. Furthermore, the IQS system is required for *in vivo* pathogenicity of *P. aeruginosa*; this finding explains the sustained virulence of *P. aeruginosa* clinical isolates that have mutated *lasI* and *lasR* genes.

Figure 1-8 depicts the four main QS systems in *P. aeruginosa*.

Figure 1-8. The interplay between the four main quorum-sensing systems found in *P. aeruginosa* with their associated signal molecules, receptors, and downstream virulence factors. Arrows indicate activation, perpendicular lines indicate inhibition. Image adapted from Lee et al. (2015).

Another important system that regulates QS in *P. aeruginosa* is the Gac/Rsm regulatory signal transduction pathway. The two-component GacS/GacA signal transduction system is situated upstream of the *las* and *rhl* QS systems, and can therefore modulate the production of the signal molecules OdDHL and BHL respectively (Reimann et al., 1997). LadS is an activator of GacS through phosphorylation, while RetS inhibits GacS from being phosphorylated. The GacS/GacA system induces the expression of the small regulatory RNAs, RsmY and RsmZ, which binds and sequesters RsmA, such that it is unable to post-transcriptionally inhibit *lasI* and *rhlI* (Kay et al., 2006; Pessi et al., 2001). In other words, when GacS and GacA are not activated, there is less RsmY and RsmZ; this results in higher levels of RsmA and increased LasI and RhlI production. Figure 1-9 shows us an overview of the main QS systems, along with the Gac/Rsm signal transduction pathway.

Figure 1-9. An overview of the main QS systems in *P. aeruginosa* (*las*, *rhl*, and PQS) and the Gac/Rsm signal transduction system. GacS is activated by LadS and repressed by RetS. GacA induces the expression of the small RNAs RsmY and RsmZ which bind and sequester RsmA, which in turn is a post-transcriptional inhibitor of *lasI* and *rhlI*. Arrows and T-bars represent induction and repression respectively. Image adapted from a review by Jakobsen et al (2013).

1.4 Quorum Sensing Inhibition

In the early 1990s, scientists working in the University of New South Wales (UNSW) noticed that the seaweed *Delisea pulchra* (Figure 1-10A) was generally free of surface colonization by bacteria (a process referred to as marine biofouling). This interesting observation led to the discovery that *D. pulchra* was able to produce and secrete secondary metabolites known as halogenated furanones onto its surfaces; these compounds possessed antifouling and antimicrobial properties (de Nys et al., 1993; de Nys et al., 1995). Furanone compounds from *D. pulchra* generally consist of a furan ring structure with a substituted acyl chain at the C-3 position and a bromine substitution at C-4 (Figure 1-10B). Because of their structural similarity to bacterial AHLs, it was hypothesized that halogenated furanones could act as AHL competitors that would interfere with bacterial AHL-based recognition systems.

In 1996, through a collaborative effort between the Technological University of Denmark and UNSW, Michael Givskov and his colleagues showed that these halogenated furanones produced by *D. pulchra* were in fact recognized as signals, able to interfere with the AHL-driven swarming motility behavior of *Serratia liquefaciens* (Givskov et al., 1996). Swimming motility which is not AHL-mediated was not affected. Furthermore, they also found that halogenated furanones were able to reduce the expression of another AHL-regulated phenotype, in this case, the bioluminescence phenotype of *Vibrio fischeri* and *Vibrio harveyi* by 50- to 100-fold.

Figure 1-10. (A) The red macroalga *Delisea pulchra* photographed in the shallows of Cape Banks, Sydney, Australia. Image credit: Rocky de Nys, from the commentary “Surface Warfare in the Sea” (Kjelleberg and Steinberg, 2001); (B) Structures of the AHLs (BHL and HHL), and two halogenated furanones produced by *D. pulchra*. Image taken from Givskov et al. (1996).

Halogenated furanones were found to specifically interact with the LuxR-like receptor proteins, which activate transcription in AHL regulatory systems (Manefield et al., 1999). Knowing that halogenated furanones could interfere with AHL-driven behaviour; these compounds were then investigated for their potential in inhibiting QS.

In 2002, a novel halogenated furanone was found to be able to interfere with QS-controlled gene expression in the *P. aeruginosa* PAO1 strain (Hentzer et al., 2002). This study made use of newly developed bacteria reporter strains that featured a molecular fusion of the *lasB* promoter with an unstable version of the green fluorescent protein (GFP) gene (Andersen et al., 1998). Short peptide sequences were added to the C-terminal end of GFP which allows GFP to be recognized by housekeeping proteases that degrade it. Thus, this unstable GFP variant has a short half-life and enables the *in situ* real-time monitoring of QS gene expression without the need for externally added chemicals or dyes. The *lasB* gene promoter is a target of AHL-bound LasR. Hence, when OdDHL levels increase and the bacterial community begins to ‘quorum sense’, the activation of LasR (as a result of OdDHL binding) will result in the corresponding increase in unstable GFP expression. This was the first study to demonstrate the use of a furanone compound as a QSI and its addition to *P. aeruginosa* interfered with the transcription of QS-controlled genes, reducing the production of virulence factors such as elastase and chitinase. Furanone was also shown to inhibit QS activation in *P. aeruginosa* biofilms grown in continuous flow systems.

As QS was found to control the expression of virulence factors, the next step was to study whether furanones could be used to inhibit QS, and in so doing reduce the virulence of pathogenic bacteria. A seminal study conducted by Hentzer *et al.* found that a synthetic derivative of natural furanones (C-30) was able to inhibit QS in *P. aeruginosa* (Hentzer et al., 2003). Transcriptomics (DNA microarray) analysis revealed that this QSI was able to specifically target QS systems (mainly the *las* and *rhl* systems) and inhibit expression of virulence factors (e.g. elastase, LasA protease, rhamnolipids and phenazines).

When C-30 was added to *P. aeruginosa* biofilms, it caused the biofilm to be more susceptible to tobramycin, an aminoglycoside antibiotic commonly used to treat cystic fibrosis, Figure 1-11. In the absence of furanone, only the cells at the surface of the biofilm were killed,

while in the presence of furanones, tobramycin was effective in killing 90-95% of the cells, even penetrating to the deeper layers of the biofilm.

Figure 1-11. Scanning confocal laser microscopy images of *P. aeruginosa* PAO1 biofilms cultured for 3 days in flow cells without furanone (A, C) or with 10 μ M furanone (B, D). The biofilms were then exposed to 100 μ g/ml tobramycin for 24h and then stained with the LIVE/DEAD *BacLight* Bacterial Viability Kit. Red areas show dead bacteria, while green areas show live bacteria. (C) and (D) show controls without the addition of tobramycin. Image from Hentzer et al. (2003).

Furthermore, in a mouse lung infected with *P. aeruginosa*, it was directly demonstrated that C-30 inhibited the QS systems of *P. aeruginosa* and allowed the biofilm to be cleared by the host immune response. The results of this study are a “proof of concept” demonstrating that chemically blocking bacterial communication functions as an antimicrobial principle.

A later study showed that application of QSIs to *P. aeruginosa* biofilms rendered them more sensitive to treatments with tobramycin and hydrogen peroxide, and they are more easily phagocytosed by polymorphonuclear leukocytes (Bjarnsholt et al., 2005a). Hence QSIs could be used as anti-virulence agents, and in conjunction with conventional antibiotics to treat biofilm bacteria in chronic infections.

QSI compounds are potentially useful in many areas besides its use in treating bacterial infections. They can be potentially applied in waste-water industries that make use of membrane

technology to minimize biofouling, in agriculture to prevent bacteria colonization of crops, and in industries for the prevention of biofilm growth on pipe surfaces and ship hulls (Dobretsov et al., 2009; Zhang and Dong, 2004).

1.5 Objectives and Thesis Organization

The main objective of my research project would be to discover novel antimicrobial strategies and development of tools to identify and study novel QSIs.

In chapter 2, I describe the use of whole genome sequencing and proteomics analysis to understand the development of drug resistance in two *Acinetobacter baumannii* strains that were isolated from trauma patients hospitalized in an Intensive Care Unit in Rigshospitalet, Denmark. In so doing, we are able to suggest possible antimicrobial therapies against these drug-resistant *A. baumannii* strains. This work has been published in the Journal of Genome biology and evolution, entitled “Comparative genomic analysis of rapid evolution of an extreme-drug-resistant *Acinetobacter baumannii* clone” (Tan et al., 2013a).

In chapter 3, I describe how I developed a fast, cost-effective and accurate *in silico* platform for the identification of novel QSIs against the *las* QS system in *P. aeruginosa*. We used the LasR receptor protein of *P. aeruginosa* as the molecular target, and developed a structure-based virtual screening platform to identify novel LasR inhibitors. Several promising QSI compounds were found to be effective and we have filed a patent for these chemical structures. This work has been published in the Journal of Antimicrobial Agents and Chemotherapy, entitled “Identification of five structurally unrelated quorum-sensing inhibitors of *Pseudomonas aeruginosa* from a natural-derivative database” (Tan et al., 2013b).

In chapter 4, I will describe the use of comparative systems biology approaches, namely RNA-sequencing transcriptomics and iTRAQ proteomics, for the identification of the molecular targets of the QSI, iberin. Iberin is a natural compound derived from horseradish. We uncover a novel means of QS mechanism: inhibition of small regulatory RNAs and suggest that comparative systems biology is an effective means of discovering the means of anti-virulence and anti-biofilm effects by QSIs. This work has been published in the Journal of Antimicrobial

Agents and Chemotherapy, entitled “Comparative systems biology analysis and mode of action of the isothiocyanate compound iberin on *Pseudomonas aeruginosa*” (Tan et al., 2014).

2 Using comparative genomics to understand the development of antibiotic resistance in *Acinetobacter baumannii*

2.1 Introduction

The bacterium, *Acinetobacter baumannii*, is an example of a fast-evolving organism which causes healthcare-associated infections (Garnacho-Montero and Amaya-Villar, 2010). In the 1970s, *A. baumannii* was sensitive to most antibiotics, but now several PDR strains have emerged that are resistant to all antibiotics (Howard et al., 2012). On the whole, approximately 2 to 10% of all Gram-negative infections in intensive care units (ICUs) are caused by *A. baumannii* and it is linked to increased mortality of infected patients (Lockhart et al., 2007; Poirel et al., 2003). Figure 2-1 illustrates the mode of entry and spread of *A. baumannii* within the ward, and highlights the interplay between infected patients, healthcare workers and equipment (Dijkshoorn et al., 2007).

There are three main international groups of epidemic *A. baumannii* strains: clone I, clone II and clone III, as determined through ribotyping and amplified fragment length polymorphism (AFLP) genomic fingerprinting approaches (Dijkshoorn et al., 1996; van Dessel et al., 2004). Multilocus sequence typing (MLST) then became the standard for discriminating and classifying bacterial strains, and MLST typing was used extensively for the identification and classification of *A. baumannii* strains (Bartual et al., 2005; Jolley et al., 2012). MLST profiling has shown that populations of *A. baumannii* clinical isolates have a highly homogenous core genome (Diancourt et al., 2010). Nevertheless, fingerprinting-based methods provide limited phylogenetic information. They are unable to discriminate between genetic idiosyncrasies, whether these occur intra-clonally or inter-clonally. We believe that genomic analyses of whole genome sequences are needed for us to have a thorough epidemiological understanding of the development of antibiotic resistance in *A. baumannii*.

Figure 2-1. Overview of the spread of *Acinetobacter baumannii* in a hospital ward, between patients, hospital staff and equipment. Image from Dijkshoorn et al. (2007).

In this chapter, I describe the comparative genomics approach used to analyse the rapid evolution of a colistin-sensitive XDR *A. baumannii* strain into a colistin-resistant PDR strain, all within a short one month interval. Two strains were sequenced: an XDR *A. baumannii* 48055 strain, and a PDR *A. baumannii* 53264 strain, both isolated from two patients admitted to the ICU of Rigshospitalet in Copenhagen, Denmark. These two strains were isolated a month apart and within this one month period, this *A. baumannii* clone had developed from a XDR (colistin-sensitive) into a PDR (colistin-resistant) phenotype.

Using various genomic annotation tools, we identified several possible drug resistance mechanisms present in these *A. baumannii* strains. We also compared the single nucleotide polymorphisms (SNPs) between the *A. baumannii* 53264 and 48055 strains in order to try to understand the genome-level changes involved in the transformation of a XDR strain into a PDR one. Lastly, we used quantitative proteomic analysis to investigate the response of the PDR *A. baumannii* 53264 strain towards three different classes of antibiotics: namely, ceftazidime, colistin and tobramycin. I will close the chapter with some implications of our study on possible antimicrobial therapies that could be effective against PDR *A. baumannii* strains.

2.2 Materials and Methods

2.2.1 Bacterial strains and patient background information

The *A. baumannii* 48055 strain was isolated from a patient with 50% burn trauma, in the ICU of Rigshospitalet, Copenhagen, Denmark, on September 2010. This patient received antibiotic treatment with meropenem (2g intravenously (i.v.), three times daily), ciprofloxacin (600 mg i.v., twice daily) and fucidin (500 mg i.v., three times daily) to prevent Gram-negative bacterial infections and treat *Staphylococcus aureus* bacteraemia. After *A. baumannii* was found in the blood and airway, the treatment was changed to meropenem and colistin (6 million units i.v., once daily) as well as fucidin (500 mg i.v., three times daily). The treatment was later changed to meropenem (2g i.v., three times daily), colistin (2 million units, three times daily) and vancomycin (1 g i.v., twice daily). After several operations to remove the necrosed tissues and skin transplantation, the patient recovered and was sent home.

A month later, the *A. baumannii* 53264 strain was isolated from a patient with 50% burn trauma, in the ICU of Rigshospitalet, Copenhagen, Denmark, on October 2010. This patient received antibiotic treatment with meropenem (1g i.v., three times daily) and ciprofloxacin (400 mg i.v., twice daily) in the beginning, then with fucidin (500 mg orally, three times daily) due to *Staphylococcus epidermidis* bacteraemia. The treatment was changed to ceftazidime (1 g i.v., three times daily) with ciprofloxacin (400 mg i.v., twice daily) and vancomycin (1 g i.v., twice daily) to prevent Gram-negative bacterial infection and treat *Staphylococcus haemolyticus*

bacteraemia. After *A. baumannii* was found in the blood and airway, the treatment was changed to meropenem (1g i.v., three times daily), ciprofloxacin (400 mg i.v., twice daily), colistin (2 million units i.v., three times daily) and colistin inhalation (2 million units, twice daily). After several operations to remove the necrosed skin tissues and skin transplantation, the patient recovered very well and was sent back to the local hospital to continue treatment until complete recovery.

2.2.2 Antimicrobial susceptibility assay

A. baumannii's susceptibility to 18 antimicrobial agents was tested by disc diffusion following the CLSI recommendations using the blood agar plate produced by Statens Serum Institut, Denmark. The *A. baumannii* 53264 and 48055 strains were resistant to all our tested antibiotics including ampicillin, aztreonam, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol, ciprofloxacin, colistin, gentamicin, imipenem, mecillinam, meropenem, penicillin, piperacillin/tazocin, sulfonamide, tigecycline, tobramycin and trimethoprim.

To confirm this result, the minimum inhibitory concentration (MIC) was determined by microdilution for the *A. baumannii* 53264 strain, *A. baumannii* 48055 strain, and an antibiotic-sensitive *A. baumannii* strain 52082 to the following six representative antibiotics: tobramycin (aminoglycoside), colistin (antimicrobial peptide), ceftazidime (cephalosporin), tetracycline, ciprofloxacin (fluoroquinolone) and meropenem (carbapenem). Diluted overnight cultures of bacteria were seeded into wells containing serially diluted antibiotic stocks, from concentrations ranging from 0 µg/ml to 1024 µg/ml.

2.2.3 Genome sequencing and assembly

Whole genome DNA of the *A. baumannii* strains were purified using the Wizard genomic DNA purification kit (Promega) and sequenced by the Beijing Genomics Institute on an Illumina HiSeq2000 platform generating 90 bp long paired-end reads. Reads were mapped against the genome of the *A. baumannii* ACICU strain (Genbank accession number CP000863) using

Novoalign (Novocraft Technologies) (Krawitz et al., 2010). The best assembly result was then assembled by SOAPdenovo (<http://soap.genomics.org.cn/>; version: 1.05) with filtered data. Average insert sizes were 468 nucleotides, and the average genomic coverage depths were 112-116 fold. Pileups of the read alignments were produced by SAMtools release 0.1.7 (Li et al., 2009).

2.2.4 Genome comparison

The *A. baumannii* 53264 and *A. baumannii* 48055 genomes were first compared to the genomes of 11 *A. baumannii* strains available from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. A pair-wise genome content distance matrix was computed using Progressive Mauve (Darling et al., 2010), followed by whole genome alignment. The distance matrix was converted to a heat map using the R heatmap function clustering package (<http://www.r-project.org/>). Progressive Mauve was then used to compare the genomes of these two strains with the genome sequences of 42 other *A. baumannii* strains (their genome sequences were downloaded from NCBI FTP site). First, the Mauve software computed a genome content distance matrix for all 44 *A. baumannii* strains, after which a neighbour-joining algorithm was used to produce a phylogenetic guide tree. Phylogenetic tree diagrams were prepared using the software FigTree ver 1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>).

2.2.5 Genome annotation

For genome annotation, the *A. baumannii* 53264 and *A. baumannii* 48055 sequence files were submitted to the RAST (Rapid Annotations using Subsystem Technology) Server (Aziz et al., 2008) for bacterial genome annotation. Default settings were used. The RAST annotated *A. baumannii* 53264 and *A. baumannii* 48055 genomes are accessible from the RAST server by logging in with the guest account (userID: guest, password: guest) at the web addresses: (<http://rast.nmpdr.org/seedviewer.cgi?page=Organism&organism=470.131>) and (<http://rast.nmpdr.org/seedviewer.cgi?page=Organism&organism=470.130>) respectively.

2.2.6 SNP comparison and analysis

The identification of single nucleotide polymorphisms between the *A. baumannii* 53264 strain, the *A. baumannii* 48055 strain and the annotated *A. baumannii* ACICU strain (NC_010611) was performed using DNASTAR SeqManNGen and analysed using DNASTAR SeqMan Pro software version 10.1.1 (DNASTAR, Inc., Madison, WI, USA). Paired-end reads in FASTQ format were mapped against the respective annotated Genbank template file.

2.2.7 Prediction of antibiotic resistance genes

The *A. baumannii* 53264 and *A. baumannii* 48055 sequences were also submitted to the Antibiotic Resistance Genes Database (Liu and Pop 2009) and the recently described ResFinder database (Zankari et al., 2012), with a 98% threshold for identification of genes involved in antibiotic resistance.

2.2.8 Nucleotide sequence accession numbers

The Whole Genome Shotgun bioproject for *A. baumannii* 53264 has been deposited at DDBJ/EMBL/GenBank under the accession ALPW000000000. The version described in this paper is the first version, ALPW01000000. The Whole Genome Shotgun bioproject for *A. baumannii* 48055 has been deposited at DDBJ/EMBL/GenBank under the accession AOSP000000000. The version described in this paper is the first version, AOSP01000000.

2.2.9 iTRAQ-based proteomics analysis

Isobaric tag for relative and absolute quantitation (iTRAQ)–based proteomic analysis was used to study the changes in protein expression of the *A. baumannii* 53264 strain in response to three antibiotics: colistin, tobramycin and ceftazidime. Proteomics experiments were performed at the Proteomic Core Facility of the Biological Research Center, School of Biological Sciences, Nanyang Technological University, Singapore.

Protein preparation and digestion. *A. baumannii* 53264 was grown in LB until mid-exponential phase (OD 600 nm = 0.5). Sub-lethal concentrations of ceftazidime (32 µg/ml), colistin (32 µg/ml) and tobramycin (64 µg/ml) were added to independent *A. baumannii* 53264 cultures respectively. Control and antibiotic-treated cultures were incubated for 3 hours at 37°C with shaking before harvesting. After harvesting, cell pellets were washed with 1×PBS and resuspended in 2 ml of lysis buffer containing 0.5M TEAB and 0.1M SDS. The cells were ruptured by sonication, and the cell debris was removed by centrifugation at 4 °C at 16000 × g for 15 min. 200 µg of proteins from different growth conditions were dissolved in equal volume of sample buffer (Invitrogen) supplemented with 0.5% 2-mercaptoethanol and denatured by boiling at 95°C for 5 min. 1D gel electrophoresis was carried out using 10% SDS-PAGE for in-gel digestion.

Proteins were first reduced using 5 mM Tris-(2-carboxyethyl) phosphine (TCEP) for 1 h at 60°C, followed by blocking of cysteine residues by 10 mM methyl methanethiosulfate (MMTS) for 30 min at room temperature in the dark. Trypsin was added at a ratio of 1:50 (trypsin/sample). It was then incubated at 37°C overnight. The tryptic peptides were extracted by 50%ACN/5%Acetic Acid from gel for 3 times and were desalted using Sep-Pak C18 cartridges (Waters, Milford, MA) and dried in a SpeedVac (Thermo Electron, Waltham, MA). All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

iTRAQ labeling. The iTRAQ labelling of the tryptic peptides was performed using 4-plex iTRAQ reagent kit (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. 200 µg of peptides from each condition were individually labelled with respective isobaric tags: control sample with 114, ceftazidime-treated sample with 115, colistin-treated sample with 116, and tobramycin-treated sample with 117. After 2 h incubation, the samples were quenched by water, desalted using C18 solid phase extraction cartridge, and then vacuum-centrifuged to dryness. The iTRAQ-labelled peptides were reconstituted in Buffer A (10 mM ammonium acetate, 85% acetonitrile, 0.1% formic acid) and fractionated using ERLIC column (200 x 4.6 mm, 5µm particle size, 200 Å pore size) by HPLC system (Shimadzu, Japan) at a flow rate of 1.0 ml/min using a previously optimized protocol (Hao et al., 2010). The HPLC chromatograms were recorded at 280 nm and fractions were collected online using automated

fraction collector. 20 fractions were collected and concentrated using vacuum centrifuge and reconstituted in 3% ACN with 0.1% formic acid for LC-MS/MS analysis.

LC-MS/MS. The peptides were separated and analyzed on a home-packed nanobore C18 column (15 cm x 75 μ m; Reprosil-Pur C18-AQ, 3 μ m, Dr Maisch, Germany) with a Picofritnanospray tip (New Objectives, Woburn, MA, USA) on a TempoTM nano-MDLC system coupled with a QSTAR® Elite Hybrid LC-MS/MS system (Applied Biosystems). Peptides from each fraction were analysed in triplicate by LC-MS/MS over a gradient of 90 min. The flow rate of the LC system was set to a constant 300 nl/min. Data acquisition in QSTAR Elite was set to positive ion mode using Analyst® QS 2.0 software (Applied Biosystems). MS data was acquired in positive ion mode with a mass range of 300–1600 m/z. Peptides with +2 to +4 charge states were selected for MS/MS. For each MS spectrum, the three most abundant peptides above a five-count threshold were selected for MS/MS and dynamically excluded for 30 s with a mass tolerance of 0.03 Da. Smart information-dependent acquisition was activated with automatic collision energy and automatic MS/MS accumulation. The fragment intensity multiplier was set to 20 and maximum accumulation time was 2 s.

Data analysis. Spectra acquired from the three technical replicates were submitted to ProteinPilot (v3.0.0.0, Applied Biosystems) for peak-list generation, protein identification and quantification. User defined parameters of the Paragon algorithm in ProteinPilot software were configured as follows: (i) Sample Type, iTRAQ2-plex (Peptide Labeled); (ii) Cysteine alkylation, MMTS; (iii) Digestion, Trypsin; (iv) Instrument, QSTAR Elite ESI; (v) Special factors, Urea denaturation; (vi) Species, None; (vii) Specify Processing, Quantitate & Bias Correction; (viii) ID Focus, biological modifications, amino acid substitutions; (ix) Database, *A. baumannii* 53264; (x) Search effort, thorough ID; (xi) Result quality, Unused ProtScore (Conf) >0.05 (10.0%). Default precursor and MS/MS tolerance for QSTAR ESI MS instrument were adopted automatically by the software. For iTRAQ quantitation, the peptide for quantification was automatically selected by Pro Group algorithm to calculate the reporter peak area, error factor (EF) and p-value. The resulting data was auto bias-corrected by build-in ProteinPilot algorithm to get rid of any variations imparted due to the unequal mixing during combining different labelled samples. During bias correction, the software identifies the median average protein ratio and corrects it to unity, and then applies this factor to all quantitation results. A

strict cut-off of unused ProteinScore ≥ 2 , which corresponds to a confidence limit of 99 %, was considered for protein identifications and further analysis.

2.3 Results

2.3.1 General characteristics of the *A. baumannii* genomes

The clinical isolates *A. baumannii* 53264 and *A. baumannii* 48055 were isolated from the ICU of Rigshospitalet at Copenhagen, Denmark. *A. baumannii* 53264 exhibited high resistance to all of the 18 tested antibiotics, including tigecycline and the polymyxin, colistin; thus *A. baumannii* 53264 is classified as a PDR strain. In comparison, *A. baumannii* 48055 is a colistin-sensitive XDR strain that was isolated one month before *A. baumannii* 53264. Both *A. baumannii* strains belong to multilocus sequence type (MLST) ST208, a molecular type previously reported in European clone II (Runnegar et al., 2010).

The general characteristics of the *A. baumannii* 53264 and *A. baumannii* 48055 strains obtained from the RAST server (Aziz et al., 2008) are shown in Table 2-1. For *A. baumannii* 53264, we obtained 130 contigs with a total length of 3,976,592 bp and 3,791 predicted coding sequences. For *A. baumannii* 48055, we obtained 135 contigs with a total length of 4,049,562 bp and 3,858 predicted coding sequences. The average GC% of *A. baumannii* 53264 and *A. baumannii* 48055 is 38.93% and 39.00% respectively.

Table 2-1. General characteristics of the *A. baumannii* 53264 and *A. baumannii* 48055 genomes as obtained from the RAST annotation server (Aziz, et al. 2008).

	<i>A. baumannii</i> 53264	<i>A. baumannii</i> 48055
Characteristic	Value	Value
Genome		
Size (bp)	3,976,592	4,049,562
No. of contigs	130	135
G + C content (%)	38.93 %	39.00%
No. of coding sequences	3791	3858
No. of subsystems	440	440
No. of RNAs	62	63

2.3.2 Phylogenetic analysis of *A. baumannii* strains

The *A. baumannii* 53264 and *A. baumannii* 48055 genomes were first compared to the genomes of eleven *A. baumannii* strains available from the KEGG database. A pair-wise genome content distance matrix was computed using Progressive Mauve (Darling et al., 2010), followed by whole genome alignment. The distance matrix was converted to a heat map (Figure 2-2) using the R statistical package.

Figure 2-2. Heat map based on a pair-wise distance matrix of whole genome alignment as computed by Progressive Mauve. Pair-wise genome alignments were performed using the genomes of *A. baumannii* 53264, *A. baumannii* 48055, and 11 *A. baumannii* clones whose complete sequences were available in the KEGG database. This heat map was created using the R statistical program (<http://www.r-project.org/>) with heatmap clustering methods. Dendrograms across the top and left of the diagram indicate the relatedness of the genomes based on genome conservation, while strain names are listed to the right of the heatmap. Distance values range from 0.0 to 0.3, and correspond to a gradient of colour steps ranging from light blue (lowest distance value) to dark purple (highest distance value).

Pair-wise genomic comparisons (Figure 2-2) shows us that the *A. baumannii* 53264 and *A. baumannii* 48055 genomes had the greatest amount of similarity to each other. The average nucleotide identity (ANI) between these two *A. baumannii* strains and another multidrug resistant strain *A. baumannii* ACICU (Iacono et al., 2008) from European clone II is 94% as determined by Progressive Mauve (Darling et al., 2010).

In order to find out which clonal group the *A. baumannii* 53264 and *A. baumannii* 48055 genomes belonged to, Progressive Mauve was then used to compare the genomes of these two strains with the genome sequences of 42 other *A. baumannii* strains (their genome sequences were downloaded from NCBI FTP site). The phylogenetic tree based on the neighbour-joining algorithm shows that the *A. baumannii* 53264 and *A. baumannii* 48055 strains belong to the group of International Clone II *A. baumannii* strains (Figure 2-3).

Figure 2-3. An unrooted phylogenetic tree showing the *A. baumannii* 53264 and *A. baumannii* 48055 strains in relation to 42 other *A. baumannii* strains. The clonal groups are as follows: International Clone I (red box), Clone II (blue box) and Clone III (green box). Genome sequences of these 42 sequences were downloaded from NCBI FTP site. This phylogenetic tree was produced by pair-wise genome comparisons by Progressive Mauve. The *A. baumannii* 53264 and *A. baumannii* 48055 strains belongs to the group of International Clone II *A. baumannii* strains.

2.3.3 Antibiotic resistance profile of the *A. baumannii* 53264 and 48055 strains

Using disc diffusion antimicrobial susceptibility testing, the *A. baumannii* 53264 and 48055 strains were found to be resistant to all eighteen tested antibiotics, except for *A. baumannii* 48055's sensitivity to colistin. To confirm this result, the MIC profiles were determined for *A. baumannii* 53264, *A. baumannii* 48055, and an antibiotic-sensitive *A. baumannii* 52082 strain to the following six representative antibiotics: tobramycin

(aminoglycoside), colistin (antimicrobial peptide), ceftazidime (cephalosporin), tetracycline, ciprofloxacin (fluoroquinolone) and meropenem (carbapenem); the antibiogram is shown in Table 2-2. The *A. baumannii* 52082 strain was sensitive to all six antibiotics (MIC: 1 – 2 µg/ml). The MIC profiles of the *A. baumannii* 53264 and 48055 strains were similar, differing only in their sensitivity to colistin (MIC: 128 vs 2 µg/ml).

	<i>A. baumannii</i> strain 53264 (PDR)	<i>A. baumannii</i> strain 48055 (XDR)	<i>A. baumannii</i> strain 52082 (sensitive)
	Minimum inhibitory concentrations (µg/ml)		
Tobramycin	256	256	2
Colistin	128	2	2
Ceftazidime	32	64	2
Tetracycline	> 1024	>1024	1
Ciprofloxacin	64	64	1
Meropenem	16	64	1

Table 2-2. Minimum inhibitory concentrations (µg/ml) for the PDR *A. baumannii* 53264 and XDR *A. baumannii* 48055 strains, and the antibiotic-sensitive *A. baumannii* 52082 strain towards the following six antibiotics: tobramycin, colistin, ceftazidime, tetracycline, ciprofloxacin and meropenem.

The *A. baumannii* 53264 and *A. baumannii* 48055 sequences were submitted to both the recently described ResFinder database (Zankari et al., 2012) and the older Antibiotic Resistance Genes Database (ARDB) (Liu and Pop, 2009) to identify genes involved in antibiotic resistance. Table 2-3 shows the genes involved in the resistance of these *A. baumannii* strains to aminoglycosides, beta-lactams, sulphonamides and tetracyclines. Both strains showed a similar resistance profile when submitted to the ResFinder database, hence only one result is shown.

Table 2-3. Antibiotic resistance profiles of *A. baumannii* 53264 and *A. baumannii* 48055 strains

Antibiotic Class	Resistance Gene ^a	NCBI DNA Accession	Description of Gene	Description of Gene Product ^b	Resistance conferred ^b	Source
Aminoglycosides	<i>aac(3)-Ia</i>	X15852	Plasmid R1033 (Tn1696) <i>aacC1</i> gene for gentamicin acetyltransferase-3-I (AAC(3)-I).	Aminoglycoside N-acetyltransferase, which modifies aminoglycosides by acetylation.	astromicin gentamicin sisomicin	Plasmid R1033, from <i>P. aeruginosa</i>
	<i>aac(6')-Iaf</i>	AB462903	<i>Pseudomonas aeruginosa</i> DNA, class 1 integron In123, complete sequence.		amikacin dibekacin isepamicin netilmicin sisomicin tobramycin	<i>Pseudomonas aeruginosa</i>
	<i>aac(6')-II</i>	Z54241	<i>C. freundii</i> int and <i>aac(6')-I</i> genes. 6'-N-aminoglycoside acetyltransferase.			<i>Citrobacter freundii</i>
	<i>aph(3')-Ia</i>	V00359	Transposon Tn903.	Aminoglycoside O-phosphotransferase, which modifies aminoglycosides by phosphorylation.	gentamicin kanamycin lividomycin neomycin paromomycin ribostamycin	<i>Escherichia coli</i>
	<i>aph(3')-Ic</i>	X62115	<i>K. pneumoniae</i> plasmid pBWH77 <i>aphA7</i> gene for neomycin phosphotransferase.			<i>Klebsiella pneumoniae</i>
	<i>aph(3')-VIa</i>	X07753	<i>Acinetobacter baumannii</i> <i>aph A-6</i> gene.		amikacin butirosin gentamicin isepamicin kanamycin neomycin paromomycin ribostamycin	<i>Acinetobacter baumannii</i>
	<i>strA/aph(3'')-Ib</i> <i>strB/aph(6)-Id</i>	M96392	<i>Erwinia amylovora</i> plasmid pEa34 transposon Tn5393 streptomycin phosphotransferase (<i>strA</i>) and streptomycin phosphotransferase (<i>strB</i>) genes.		streptomycin	<i>Erwinia amylovora</i>
Beta-lactam	<i>bla_{OXA-23}</i>	HQ700358	<i>Acinetobacter baumannii</i> isolate AB210 AbaR4-type multiple antibiotic resistance island, complete sequence.	Class D beta-lactamase.	carbapenems	<i>Acinetobacter baumannii</i>

Table 2-3 – continues overleaf

Table 2-3 – continued. Antibiotic resistance profiles of <i>A. baumannii</i> 53264 and <i>A. baumannii</i> 48055 strains						
Antibiotic Class	Resistance Gene ^a	NCBI DNA Accession	Description of Gene	Description of Gene Product ^b	Resistance conferred ^b	Source
Beta-lactam	<i>blaTEM-1/RblaTEM-1</i>	AF188200	<i>Escherichia coli</i> beta-lactamase variant <i>TEM-1D</i> (<i>blaTEM-1D</i>) gene.	Class A beta-lactamase. This enzyme breaks the beta-lactam antibiotic ring open and deactivates the molecule's antibacterial properties.	cephalosporin penicillin	<i>Escherichia coli</i>
Fluoroquinolones	No resistance genes found.					
Fosfomycin	No resistance genes found.					
Fusidic Acid	No resistance genes found.					
MLS – Macrolide – Lincosamide – Streptogramin B	No resistance genes found.					
Phenicol	No resistance genes found.					
Rifampicin	No resistance genes found.					
Sulfonamide	<i>sul1</i>	AY224185	<i>Escherichia coli</i> isolate Ec1484R sulphonamide resistance protein (<i>sul1</i>) gene.	Sulfonamide-resistant dihydropteroate synthase, which cannot be inhibited by sulfonamide.	sulfonamide	<i>Escherichia coli</i>
	<i>sul2</i>	FM179941	<i>Pasteurella multocida</i> pCCK1900 plasmid, isolate 1900.			<i>Pasteurella multocida</i>
	<i>sul3</i>	AB281182	<i>Pseudomonas aeruginosa</i> <i>sul3</i> gene for dihydropteroatesynthetase.			<i>Pseudomonas aeruginosa</i>
Tetracycline	<i>tet(B)</i>	AP000342	<i>Shigella flexneri</i> 2b plasmid R100 DNA, complete sequence.	Major facilitator superfamily transporter, tetracycline efflux pump.	tetracycline	Plasmid R100 from <i>S. flexneri</i>
Trimethoprim	No resistance genes found.					
Glycopeptide	No resistance genes found.					

^aResistance genes identified with greater than 98.00% sequence identity by ResFinder (Zankari, et al. 2012).

^bAntibiotic resistance conferred is based on Antibiotic Resistance Genes Database (ARDB) (Liu and Pop 2009).

Antibiotic resistance genes present in the *A. baumannii* 53264 and 48055 genomes are similar to those found in a wide variety of other bacteria. *A. baumannii* acquires its multi-antibiotic resistance phenotype through the acquisition of mobile genetic elements, for example plasmids and transposons (Fournier et al., 2006). From Table 2-3, we note that the *A. baumannii* strains possess the antibiotic resistance genes, *aac(6')-Iaf* and *sul3*, which are similar to genes present in *P. aeruginosa*. *P. aeruginosa* and *A. baumannii* are the two most prevalent non-fermentative bacteria isolated from hospital patients (Karlowsky et al., 2003) and can be assumed to be in close contact with each other, for example in an infection site, thus allowing gene transfer. A striking example of this is transfer of an extended-spectrum β -lactamase (ESBL) integron (*bla_{VEB-1}*) from *P. aeruginosa* to *A. baumannii* in a hospital setting (Poirel et al., 2003).

Regarding beta-lactam resistance genes, our PDR *A. baumannii* strains carry the *bla_{OXA-23}* and the *bla_{TEM-1}* gene. The *bla_{OXA-23}* gene that confers imipenem resistance was first observed in Scotland (Scaife et al., 1995) but was later observed even in China (Zhou et al., 2007), in Bulgaria (Stoeva et al., 2008), in Brazil (Carvalho et al., 2009) and eventually world-wide (Mugnier et al., 2010). The *bla_{OXA-23}* gene encodes a Class D beta-lactamase that confers resistance against carbapenems (e.g. imipenem and meropenem) as well as ceftazidime (Mugnier et al., 2010). The *bla_{TEM-1}* gene encodes a Class A beta-lactamase that is found in 90% of ampicillin-resistant *E. coli* strains (Livermore, 1995). Because the *bla_{TEM-1}* gene is plasmid-borne and utilizes transposon-mediated transfer, this gene spreads easily among bacteria and has been observed in *Enterobacteriaceae*, *P. aeruginosa*, and *Haemophilus influenza* (Bradford, 2001).

2.3.4 Study of Single Nucleotide Polymorphisms

2.3.4.1 SNPs between *A. baumannii* strains and ACICU

We then studied the non-synonymous single nucleotide polymorphisms (SNPs) between the *A. baumannii* ACICU strain (Iacono et al., 2008) and our PDR *A. baumannii* 53264 and XDR *A. baumannii* 48055 strains. Non-synonymous refers to base changes which result in an amino acid change. Table 2-4 highlights the SNPs in three particular genes: *ampC*, *gyrB*, and *parC*.

	AB 53264 vs. ACICU		AB 48055 vs. ACICU	
	DNA Change	Protein Change	DNA Change	Protein Change
SNPs in <i>ampC</i> gene	c.31->T	S11fs		
	c.217C>A	R73S	c.217C>A	R73S
	c.427C>A	Q143K	c.427C>A	Q143K
	c.827T>C	F276S	c.827T>C	F276S
	c.828->G	F276fs	c.828->G	F276fs
	c.911G>A	S304N	c.911G>A	S304N
	c.1001A>C	N334T	c.1001A>C	N334T
SNPs in <i>gyrB</i> gene	c.1114G>A	D372N	c.1114G>A	D372N
	c.1738A>G	Y580H	c.1738A>G	Y580H
SNPs in <i>parC</i> gene	c.251C>T	S84L	c.251C>T	S84L
	c.623G>A	G208E	c.623G>A	G208E
	c.1982T>C	V661A	c.1982T>C	V661A

Table 2-4. Non-synonymous single nucleotide polymorphisms observed in the *ampC*, *gyrB* and *parC* genes of *A. baumannii* 53264 and *A. baumannii* 48055 strains in reference to the *A. baumannii* ACICU strain. Abbreviations: fs (frameshift).

Overproduction of the AmpC cephalosporinase by *A. baumannii* isolates has been shown to be important in conferring high levels of resistance to beta-lactam antibiotics (i.e. ceftazidime) (Corvec et al., 2003). Point mutations within the *ampC* gene promoter and attenuator regions have been shown to result in the hyperproduction of AmpC in *E. coli* strains (Nelson and Elisha, 1999). In our case, the SNPs detected were within the *ampC* gene coding sequence. Molecular evolution of beta-lactamases confers extended substrate specificity, thus improving bacterial inactivation of a wider range of antibiotics (Nukaga et al., 1995). In *P. aeruginosa*, point mutations within the *ampC* gene resulted in increased beta-lactamase activity of AmpC which resulted in increased resistance to ceftazidime (Tam et al., 2007). The *parC* gene encodes subunit A of topoisomerase IV, while the *gyrB* gene encodes DNA gyrase B. These products are the target for inhibition by quinolone-based antibiotics and mutations within the *parC* and *gyrB* genes confer resistance to quinolones (Eaves et al., 2004; Vila et al., 2007; Yoshida et al., 1991).

The SNPs detected in the two *A. baumannii* strains are identical, except for an additional thymine inserted at position 31 of the *ampC* gene of *A. baumannii* strain 53264. The *A. baumannii* 53264 strain was isolated about a month after the *A. baumannii* 48055 strain was isolated, which would explain the additional time for mutation. This also lends support to the fact that the PDR *A. baumannii* 53264 strain has rapidly evolved from the XDR *A. baumannii* 48055 strain in as short as a one month period.

2.3.4.2 SNP differences between *A. baumannii* 53264 and 48055 strains

We then analysed the SNP differences between the PDR *A. baumannii* 53264 and XDR *A. baumannii* 48055 strains in order to find out the reasons behind the evolution of colistin resistance. There were 61 non-synonymous (coding change) SNPs detected between the two strains (Table 2-5).

Table 2-5. List of SNP differences between the XDR *A. baumannii* 48055 and the PDR *A. baumannii* 53264 strains. The list shows differences in DNA and protein sequence that occur in *A. baumannii* 48055 strain, with regards to the *A. baumannii* 53264 genome sequence.

#	Feature Name	DNA Change	Protein Change
1	LSU ribosomal protein L34p	c.136A>-	.45fs
2	Biosynthetic Aromatic amino acid	c.536G>T	P179H
3	Transcriptional regulator, LysR family	c.[847G>T]+[847G>G]	Q283K, Q283Q
4	FIG000988: Predicted permease	c.122T>G	V41G
5	FIG000906: Predicted Permease	c.[866G>T]+[866G>G]	C289C, C289F
6	FIG000906: Predicted Permease	c.[869C>T]+[869C>C]	S290S, S290F
7	FIG000906: Predicted Permease	c.[874A>T]+[874A>A]	I292I, I292F
8	Acetoacetyl-CoA synthetase (EC	c.[1213C>C]+[1213C>A]	G405G, G405C
9	FIG00350520: hypothetical protein	c.266T>A	F89Y
10	FIG022199: FAD-binding protein	c.1206C>A	N402K
11	Transcriptional regulator, LysR family	c.762C>A	F254L
12	FIG00350303: hypothetical protein	c.[1502T>T]+[1502T>G]	V501G, V501V
13	L-carnitinedehydratase/bile acid-inducible	c.[530T>T]+[530T>G]	V177G, V177V
14	FIG00350277: hypothetical protein	c.2311A>T	F771I
15	CmaU	c.[27T>T]+[27T>G]	F9L, F9F
16	CmaU	c.32T>C	V11A
17	CmaU	c.34C>A	P12T
18	4Fe-4S ferredoxin, iron-sulfur binding	c.62C>A	A21D
19	FIG00350535: hypothetical protein	c.[56T>T]+[56T>G]	V19G, V19V
20	CELL SURFACE PROTEIN	c.[571A>G]+[571A>A]	T191T, T191A
21	FIG00352920: hypothetical protein	c.[163G>G]+[163G>A]	E55K, E55E
22	FIG00352920: hypothetical protein	c.[176T>T]+[176T>C]	V59A, V59V
23	FIG00351830: hypothetical protein	c.[278G>G]+[278G>C]	G93A, G93G
24	putative hemolysin	c.[50G>T]+[50G>G]	C17C, C17F
25	Phenylacetic acid degradation protein	c.1004T>G	N335T
26	Two-component hybrid sensor and regulator	c.1561C>T	H521Y
27	5'-nucleotidase (EC 3.1.3.5)	c.[526A>T]+[526A>A]	F176I, F176F
28	Transcriptional regulator, AraC family	c.322A>C	F108V
29	Transcriptional regulator, AraC family	c.313A>C	F105V
30	Xylonatedehydratase (EC 4.2.1.82)	c.419G>C	G140A
31	Phenylalanine-specific permease	c.[203C>C]+[203C>A]	C68C, C68F
32	Alcohol dehydrogenase (EC 1.1.1.1)	c.440G>A	G147E
33	Alcohol dehydrogenase (EC 1.1.1.1)	c.443G>T	G148V
34	RNA polymerase sigma factor RpoH	c.107G>A	G36E
35	Sulfatepermease	c.[829A>C]+[829A>A]	C277G, C277C
36	FIG00351986: hypothetical protein	c.4G>T	P2T

Table 2-5 continues.

Table 2-5 continued.

#	Feature Name	DNA Change	Protein Change
35	Sulfatepermease	c.[829A>C]+[829A>A]	C277G, C277C
36	FIG00351986: hypothetical protein	c.4G>T	P2T
37	FIG00349989: hypothetical protein	c.[10C>T]+[10C>C]	V4M, V4V
38	RarD protein	c.689T>G	E230A
39	RarD protein	c.677A>C	F226C
40	RarD protein	c.674A>C	V225G
41	Glycerate kinase (EC 2.7.1.31)	c.571C>G	P191A
42	Transcriptional regulator, TetR family	c.3C>A	L1F
43	Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3)	c.1325C>A	G442V
44	FIG00352445: hypothetical protein	c.[1485T>T]+[1485T>A]	Q495Q, Q495H
45	FIG00352445: hypothetical protein	c.[1200T>T]+[1200T>A]	Q400Q, Q400H
46	Cell division protein FtsJ / Ribosomal	c.473G>A	A158V
47	Putative hemagglutinin/hemolysin-related protein	c.[592T>T]+[592T>C]	T198T, T198A
48	Putative hemagglutinin/hemolysin-related protein	c.[589T>T]+[589T>G]	I197I, I197L
49	Putative hemagglutinin/hemolysin-related protein	c.[436C>T]+[436C>C]	V146I, V146V
50	Putative hemagglutinin/hemolysin-related protein	c.[205T>T]+[205T>C]	I69I, I69V
51	Sensory histidine kinase QseC	c.680A>G	V227A
52	Sensory histidine kinase QseC	c.623G>A	P208L
53	Putative stomatin/prohibitin-family	c.[172G>T]+[172G>G]	V58V, V58L
54	Hypothetical protein; putative signal peptide	c.407G>T	A136D
55	FIG00351726: hypothetical protein	c.[947A>C]+[947A>A]	V316G, V316V
56	N-carbamoylputrescineamidase (3.5.1.53)	c.388->T	I130fs
57	FIG00350819: hypothetical protein	c.79C>A	A27S
58	Sodium-dependent transporter	c.618C>A	M206I
59	FIG00350872: hypothetical protein	c.713G>T	T238K
60	Histone acetyltransferase HPA2	c.[122T>T]+[122T>C]	Q41Q, Q41R
61	Histone acetyltransferase HPA2	c.[118T>T]+[118T>C]	T40T, T40A

From Table 2-5, we noticed two SNPs occurring within the histidine kinase sensor *qseC* gene; QseC is a highly conserved regulator of virulence that responds to both bacteria signals and host cell factors (Clarke et al., 2006). Inhibition of QseC markedly reduced the virulence of *Salmonella enterica* serovar Typhimurium (Rasko et al., 2008). Transcriptomic analysis revealed a role for QseC in the antimicrobial peptide (i.e. polymyxins) and oxidative stress resistance responses (Karavolos et al., 2008).

As these mutations occurred within a one month period, these 61 SNP changes indicate the fast evolution of the *A. baumannii* strains. Colistin resistance has been shown to result from a complete loss of lipopolysaccharide (LPS) production by deletion of LpxA, LpxB and LpxD (Moffatt et al., 2010) or modifications of LPS through mutations in the pmrAB two-component system (Beceiro et al., 2011). PDR strains are defined by their resistance to colistin; however, we were not able to detect any SNP changes that would confer resistance to colistin. Hence, we

decided to use iTRAQ-based quantitative proteomics to study the proteome changes in the PDR *A. baumannii* 53264 strain in response to antibiotics.

2.3.5 Use of proteomics to study the response of PDR *A. baumannii* 53264 strain to antibiotics

We then studied the stress response of the *A. baumannii* 53264 strain to treatment by three different classes of antibiotics (colistin, ceftazidime and tobramycin) in order to understand the stress response of this PDR *A. baumannii* clone to various antibiotics. Figure 2-4 shows the number of proteins whose expression was induced or reduced in the presence of a specific antibiotic (ceftazidime, colistin or tobramycin).

Figure 2-4. Venn diagrams showing the number of proteins whose expression was either induced or reduced in the presence of a specific antibiotic. The Venn diagram on the left shows the number of proteins whose expression was induced in the presence of a specific antibiotic. The Venn diagram on the right shows the number of proteins whose expression was reduced in the presence of a specific antibiotic.

We defined induced proteins as those whose abundance was increased by at least 2-fold versus the control (without antibiotic addition). Conversely, reduced proteins were defined as those whose abundance was decreased by at least 2-fold versus the control. Overlapping regions

of the Venn diagrams show the number of proteins whose expression was found to be commonly induced (or reduced) by one or more antibiotics.

There was more commonality between the genes induced (or reduced) in the presence of ceftazidime and colistin as compared to those induced (or reduced) by tobramycin. This indicates that the resistance mechanisms of *A. baumannii* 53264 strain to ceftazidime and colistin have more in common than its resistance mechanism to tobramycin. A different set of proteins may be required for its resistance to tobramycin. This could be due to the fact that ceftazidime and colistin targets the bacterial cell wall (Hayes and Orr, 1983) and cell membrane (Falagas et al., 2005) respectively, whereas tobramycin targets the 30S ribosomal subunit (Walter et al., 1999).

Next, we wanted to study the common set of genes which were induced or reduced by all three antimicrobials, as this would indicate a core set of genes essential to the stress response of PDR *A. baumannii* 53264 to antibiotics. Table 2-6 lists four proteins which were found to be commonly down-regulated (by at least 2-fold) by *A. baumannii* 53264 in all three antibiotic treatments, while Table 2-7 shows the proteins found to be up-regulated (by at least 2-fold) by *A. baumannii* 53264 in the presence of all 3 antibiotics.

Accession No.	Name	Function	No. of matched Peptides (95%)	%Cov (95%)	Treatment with:		
					Cef	Col	Tob
gi 183211243	Succinylargininedihydrolase	Arginine and proline metabolism	31	68.9	0.45	0.43	0.43
gi 183209017	Dihydroorotase	Pyrimidine biosynthesis	7	68.6	0.44	0.10	0.47
gi 183210937	Long-chain fatty acid transport protein	Membrane transport of long-chain fatty acids	19	60.04	0.35	0.37	0.32
gi 183211425	Putative porin	Membrane transport	7	62.35	0.30	0.17	0.49

Table 2-6. Proteins, whose expression was down-regulated by all 3 antibiotics. Abbreviations: Cef: ceftazidime, Col: colistin and Tob: tobramycin. Percent coverage (%Cov) refers to the percent of the residues in each protein sequence that has been identified at 95% confidence level. Numbers in the Cef, Col and Tob columns refer to the fold-change of the protein abundance as compared to the control sample (without antibiotics).

Accession no.	Name	Function	No. of matched Peptides (95%)	%Cov (95)	Treatment with:		
					Cef	Col	Tob
gi 183211233	Glycyl-tRNA synthetase, beta subunit	Synthesis of glycyl-tRNA	12	11.76	34.99	5.40	4.37
gi 183210389	Prephenate dehydratase	Biosynthesis of Phe, Tyr, Trp	4	6.233	12.47	2.29	5.20
gi 183208218	DNA-directed RNA polymerase, beta' subunit/160 kD subunit	Transcription	60	20.47	9.46	3.10	4.25
gi 183211192	50S ribosomal protein L3	Translation	8	28.97	8.63	12.71	5.55
gi 183208264	Translation initiation factor 2 (IF-2; GTPase)	Translation	17	13.68	6.49	2.42	2.03
gi 183210439	30S ribosomal protein S2	Translation	28	39.6	6.43	3.98	3.16
gi 183208618	Ribonucleotide reductase, alpha subunit	DNA synthesis	26	17.69	5.86	2.47	2.56
gi 183208515	putative 50S ribosomal protein L20	Translation	13	15.97	5.45	2.73	2.68
gi 183208426	Histidyl-tRNA synthetase	Synthesis of histidyl-tRNA	3	13.02	4.33	4.02	2.73
gi 183211186	30S ribosomal subunit protein S3	Translation	16	16	3.84	14.45	7.59
gi 183211272	50S ribosomal protein L19	Translation	9	16.39	3.73	2.91	2.27
gi 183211383	predicted NAD/FAD-binding protein	Binding of NAD and FAD	10	22.69	3.47	2.86	2.83
gi 183211175	30S ribosomal protein S5	Translation	9	32.73	2.31	4.33	2.11
gi 183211189	50S ribosomal protein L2	Translation	5	20.66	2.03	4.09	2.11
gi 183210808	hypothetical protein ACICU_02894	Unknown	3	3.324	2.01	4.53	2.70

Table 2-7. Proteins, whose expression was up-regulated by all 3 antibiotics. Abbreviations: Cef: ceftazidime, Col: colistin and Tob: tobramycin. Percent coverage (%Cov) refers to the percent of the residues in each protein sequence that has been identified at 95% confidence level. Numbers in the Cef, Col and Tob columns refer to the fold-change of the protein abundance as compared to the control sample (without antibiotics).

From Table 2-6, we note the decreased expression of a putative porin (gi|183211425). This outer membrane protein (OMP) was found to be at least 2-fold under expressed in all 3 antibiotic treatments, with the colistin treatment causing a more than 5-fold decrease in expression of this porin. OMPs are involved in the uptake of antibiotics into the bacterial cell. For example, the OMP OprD of *P. aeruginosa* has been shown to be important in the uptake of positively-charged peptides, and carbapenem antibiotics (Nikaido, 2003). Also, the expression of the outer membrane protein OmpW of *A. baumannii* was found to be reduced in a colistin-resistant strain (Vila et al., 2007). Hence, down-regulation of porin expression may reduce colistin uptake by the *A. baumannii* 53264 strain and explain its resistance towards colistin.

2.4 Conclusion

In this chapter, we used a combination of whole genome sequencing and iTRAQ proteomics to study two *Acinetobacter baumannii* strains – one of which exhibited extensive drug resistance (XDR *A. baumannii* 48055 strain), while the other exhibited pandrug resistance (PDR *A. baumannii* 53264 strain). Both strains were isolated from patients admitted to the ICU of Rigshospitalet, Denmark, for suffering burn trauma.

Our genomic analysis results showed that these *A. baumannii* strains belonged to the group of International Clone II strains, with greatest similarity to ACICU. These strains were highly similar to each other, leading us to believe that they developed from the same clonal population.

Colistin resistance emerged within a short one month interval between these two strains – which may be explained by single nucleotide polymorphisms detected in the *ampC*, *gyrB* and *parC* genes of the colistin-resistant *A. baumannii* 53264 strain.

Lastly, iTRAQ proteomics showed that the production of a putative porin was down-regulated in the PDR *A. baumannii* 53264 strain in response to the antibiotics ceftazidime, tobramycin and colistin. Down-regulation of this porin may be the cause for this strains's resistance against colistin. As such, this suggests potential novel antimicrobial strategies that aim to induce porin expression in drug-resistant *A. baumannii* strains, and in so doing, increase the efficiency of polymyxin-based (e.g. colistin) and other types of antibiotic treatment against these PDR strains.

3 Development of a structure-based virtual screening platform for the identification of novel LasR inhibitors

3.1 Introduction

Several methods have been used for the identification of novel QSIs. The conventional method for the discovery of QSIs has been through the use of biosensors. One simple system uses the *Chromobacterium violaceum* CV026 strain which produces the purple pigment, violacein, upon the addition of AHL (McClellan et al., 1997). Disks soaked with test compounds would then be placed onto an agar lawn confluent with *C. violaceum*. An effective QSI that is non-growth inhibitory would create a colourless zone around the disk, as violacein production is inhibited (Figure 3-1) but would not kill the bacterial cells within this zone of QS inhibition.

Figure 3-1. An example of a *Chromobacterium violaceum* bioassay. In this case, compound 2 may (colourless zone) be hypothesized to be a potential QSI candidate. Compound 8 (transparent zone) on the other hand would not be selected as it inhibits bacterial growth. Image adapted from Adonizio et al. (2006).

However, the *C. violaceum* CV026-based-screen is not very quantitative and has low-throughput. As such, cell-based biosensors were created. One notable example would be the QSI selector (QSIS) systems (Rasmussen et al., 2005a) which comprise fusion of QS-regulated promoters to genes encoding lethal products. As such, QSI compounds would rescue the QSIS cells from death.

Other simpler biosensors involve fusion of QS-regulated promoters to the *lux*, β -galactosidase or *gfp* gene. The development of cell-based biosensors has allowed the use of high throughput screening for QSI discovery. Recently, a high-throughput method has been developed to screen a library of 200,000 compounds for QS agonist and antagonist activities leading to the discovery of several interesting lead compounds (Müh et al., 2006).

In contrast to the conventional lab-based screens, some have utilized a computer-based approach to drug screening known as structure-based virtual screening (SB-VS). SB-VS can be defined as a method to computationally screen large compound libraries for molecules that bind targets of known structure, and then test experimentally those predicted to bind well (Shoichet, 2004). Recent successes of this approach include: inhibitors against the apoptosis regulator Bcl-2, Hsp90, G-protein coupled receptors and metalloenzymes (reviewed by Ghosh et al., 2006).

A simplified overview of the SB-VS process, which uses molecular docking software for *in silico* identification of potential lead compounds is shown in Figure 3-2.

Figure 3-2. Structure-based virtual screening for new ligands. Test compounds (shapes shown on the top right) are docked into the structure of a target receptor (in blue) by an automated computer docking program. Each compound is then scored and the top-scoring hits are then tested through *in vitro* experimental assays. Image taken from Shoichet (2004).

With the recent availability of crystal structures of bacteria QS receptor proteins such as LasR of *Pseudomonas aeruginosa* (Bottomley et al., 2007) and TraR of *Agrobacterium tumefaciens* (Vannini et al., 2002), SB-VS has become a viable option for QSI discovery.

Recently, a SB-VS approach was used in the search for novel inhibitors of the LasR protein of *P. aeruginosa* (Yang et al., 2009b). A total of 147 compounds from the SuperNatural (Dunkel et al., 2006) and SuperDrug databases (Goede et al., 2005) were selected for the screening through a rational approach, based on 2-dimensional (2D) structure similarity to known QSIs. These compounds were then subjected to molecular docking against the ligand-binding domain of LasR and the top-scoring “hits” were then tested for biological activity. Several recognized drugs, such as salicylic acid, were identified as QSIs.

In this chapter, I will describe the development of this SB-VS approach to the identification and validation of novel inhibitors of LasR. The method is largely based on previously described work (Yang et al., 2009b), with a few improvements, notably the use of DG-AMMOS (Lagorce et al., 2009) for the conversion of a two-dimensional compound library into three-dimensional form for molecular docking. Using this newly developed SB-VS system, we identified five candidate compounds with the ability to inhibit LasR QS, with one compound, 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (G1) exhibiting the highest specificity for LasR. Proteomics was used to study the effects of G1 on *P. aeruginosa*, and was found to reduce the expression of several virulence factors. Hence, we present this method as a cost-effective and fast means of identifying novel inhibitors.

3.2 Material and Methods

3.2.1 Structure-based virtual screening

The process workflow for the structure-based virtual screening is largely based on the methods used previously (Yang et al., 2009b), with the major difference being the usage of DG-AMMOS for the conversion of the entire compound library into 3-dimensional (3D) structures instead of just a selection of structurally-related ones. Figure 3-3 overleaf shows an overview of the process.

Figure 3-3. Overview of the Structure-Based Virtual Screening Process we used in our study.

3.2.2 Protein structure file and ligand database

The X-ray crystal structure of the *P. aeruginosa* LasR ligand-binding domain (LBD) bound to OdDHL, its natural ligand, was downloaded from the Protein Data Bank (PDB) website (ID: 2UV0) and used for SB-VS (Bottomley et al., 2007). For the SB-VS, the structures of 3040 ligands derived from natural sources, from TimTec's (TimTec LLC, Newark, DE; <http://www.timtec.net>) Natural Derivatives Library were downloaded from Timtec's website (<http://www.timtec.net/NDL-3000-Natural-Derivatives-Library.html>). These structures were in 2D coordinate structure-data file (SDF) format, hence, an automated program, DG-AMMOS (Lagorce et al., 2009) was used to convert the entire library of 2D chemical structures into 3D conformations based on distance geometry and automated molecular mechanics optimization. Thus, the entire library of compounds from TimTec's Natural Derivatives Library, 3040 in all, was converted to 3D structures and used for the subsequent molecular docking procedures.

2D structures of eight reference compounds (OdDHL, patulin, salicylic acid, 3-oxo-C12-2-aminophenol, furanone C30, 4-Nitropyridine-*N*-oxide, nifuroxazide and chlorzoxazone) were drawn manually in MarvinSketch ver 5.9.0 (ChemAxon Ltd., Hungary) and then saved as 2D SDF files. The SDF files were merged into a single SDF file using OpenBabel ver. 2.3.1 (OpenEye Scientific Software) and like-wise converted to 3D structures through the use of DG-AMMOS.

3.2.3 Molecular Docking

The Molegro Virtual Docker (MVD) Ver 5.0.0 program (Molegro Aps., Denmark) was used for the automated docking procedure (Thomsen and Christensen, 2006). MVD uses the MOLDOCK algorithm which was able to correctly identify the binding mode of ligands with 87% accuracy which was higher than that of the modern docking programs GLIDE (Friesner et al., 2004) and Surflex (Jain, 2003). From the LasR PDB structure file, only the E monomer ligand binding domain in a complex with the OdDHL ligand, OHN 1169 [E], was imported into the MVD workspace. Water molecules were not imported, and default parameters were used for the import process.

For the docking process, a docking template was first generated based on the interaction of the native OddHL ligand with the LasR LBD. This template included steric, hydrogen donor, hydrogen acceptor and ring contributions. Template docking was then carried out using Ligand Evaluator for scoring of the poses and Moldock Optimizer for the search algorithm. The docking was constrained to a sphere of 9Å radius centered on the OddHL ligand and its corresponding cavity. For molecular docking, 3040 ligands from the Natural Derivative Library and eight reference ligands (OddHL reference ligand along with 7 known QSIs) were used. Each compound was docked for 10 iterations in order to obtain an energy-minimized protein-ligand conformation. One pose was generated for each run, and the resulting poses were ranked. The parameters used for import of molecules and docking in MVD are specified in Appendix A, Table A. The program LIGPLOT ver 4.5.3 (Wallace et al., 1995) was used to map the interactions between QSI lead compounds and the residues within the LasR protein LBD. PyMOL ver 1.4 (Schrodinger, LLC) was used for preparation of 3D images.

3.2.4 Selection of QSI Candidates

Compounds from the TimTec Natural Derivative Library were docked against the LasR LBD in MVD and subsequently ranked according to their rerank score, molecular weight in Daltons, and the calculated ligand efficiency (computed as Rerank Score divided by the number of heavy atoms in a compound). In order to select for small molecule QSI candidates, only compounds having a molecular weight less than 200 Daltons and a rerank score below -60 were selected. The rerank score value of -60 was used as a cut-off because the reference QSIs that were docked in MVD generally had a score below -60. A total of 22 QSI candidates were selected, and were purchased from TimTec, Inc. (TimTec LLC, Newark, DE; <http://www.timtec.net>). Compounds were shipped in glass vials in powder form, and these were dissolved in dimethyl sulfoxide (DMSO) prior to *in vitro* experiments.

3.2.5 Bacterial strains

To determine the QSI activity of the compounds selected from the virtual screening, the following *P. aeruginosa* and *E. coli* monitor strains were used (Table 3-1).

Table 3-1. List of *P. aeruginosa* and *E. coli* strains used in the present study.

Species	Name/Genotype	Description ^a	Reference ^b
<i>P. aeruginosa</i>	PAO1	Wild-type	(Hentzer et al., 2002)
	PAO1-gfp	GFP-tagged wild-type	(Yang et al., 2007)
	PAO1/ <i>lasB</i> -gfp	Gm ^r ; PAO1 containing <i>lasB</i> -gfp (ASV) reporter fusion	(Hentzer et al., 2002)
	PAO1/ <i>rhlA</i> -gfp	Gm ^r ; PAO1-ATCC, <i>rhlA</i> -gfp (ASV) reporter fusion	(Yang et al., 2009b)
	PAO1/ <i>pqsA</i> -gfp	Gm ^r ; PAO1-ATCC, <i>pqsA</i> -gfp(ASV) reporter fusion	(Yang et al., 2007)
	PAO1 Δ <i>lasR</i>	PAO1 <i>lasR</i> mutant	(Hentzer et al., 2003)
	PAO1 Δ <i>lasR</i> / <i>rhlA</i> -gfp	Gm ^r /Carb ^r ; PAO1 <i>lasR</i> mutant, <i>rhlA</i> -gfp(ASV) reporter fusion	(Yang et al., 2009b)
	PAO1 Δ <i>lasR</i> / <i>pqsA</i> -gfp	Gm ^r /Carb ^r ; PAO1 <i>lasR</i> mutant, <i>rhlA</i> -gfp(ASV) reporter fusion	(Yang et al., 2009b)
<i>E. coli</i>	MT102	Wild-type	(Hentzer et al., 2002)
	MT102/ <i>lasB</i> -gfp	MT102 containing <i>lasB</i> -gfp(ASV) reporter fusion	(Hentzer et al., 2002)

^aDescription of the strains' antibiotic resistance. Gm^r: gentamycin resistance. Carb^r: carbenicillin resistance.

^bReferences describing the sources of the respective strains.

These monitor strains have their respective promoters fused to an unstable GFP protein that has a C-terminal oligopeptide extension containing the amino acids ASV; this causes the GFP protein to be more susceptible to degradation by housekeeping proteases and therefore have a short half-life. As such, unstable *gfp* (ASV) allows for monitoring of temporal QS-regulated gene expression (Andersen et al., 1998). For proteomic analysis, we used the sequenced *P. aeruginosa* PAO1 wild-type strain obtained from the *Pseudomonas* Genetic Stock Center (www.pseudomonas.med.ecu.edu; PAO0001).

3.2.6 Growth medium and conditions

The bacteria strains were grown in either ABTGC or Luria-Bertani (LB) medium.

ABTGC media: AB minimal medium (Clark and Maaloe, 1967) containing 2.5 mg/L thiamine, supplemented with 0.2% (wt/vol) glucose and 0.2% (wt/vol) Casamino Acids. LB media: 1.0% tryptone, 0.5% yeast extract and 1.0% NaCl adjusted to pH7.0.

Overnight cultures were grown for 16 h at 37°C and shaken at 180 rpm. Selective media were supplemented with ampicillin (100 mg liter⁻¹) or gentamicin (60 mg liter⁻¹) where appropriate.

3.2.7 *P. aeruginosa* QS inhibition assay

Test compounds were dissolved in 100% DMSO and mixed with ABTGC media, after which they were added to the first column of wells of a 96-well microtitre plate (Nunc) to give a final concentration of 100 µM in a final volume of 200 µl. 100 µL of ABTGC medium was then added to the remaining wells in the plate and serial two-fold dilutions of the inhibitors were done by adding 100 µL of the preceding inhibitor-containing well to the subsequent one. The final column was left without inhibitor as a control.

Next, an overnight culture of *P. aeruginosa lasB-gfp* (ASV) strain, grown in LB medium at 37°C with shaking, was diluted to an optical density (OD) at 450 nm of 0.2, and 100 µl of bacterial suspension was added to each well of the microtitre plate. Hence, inhibitor concentrations ranged from 50 µM to 0.78125 µM across the plate, in a volume of 200 µl. The microtitre plate was incubated at 37°C in a Tecan Infinite 200 Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland). GFP fluorescence (excitation at 485 nm, emission at 535 nm) and cell density (OD₆₀₀) measurements were collected at 15 min intervals for at least 14 h. The *P. aeruginosa* Rhl and Pqs inhibition assays were performed in a similar manner to the LasR inhibition assay.

3.2.8 *E. coli* competition assay between QSI compounds and OdDHL

The *E. coli lasB-gfp* (ASV) reporter strain was used for the competition assay, which was performed in a similar manner to the *P. aeruginosa* LasR inhibition assay. OdDHL and the QSI to be studied were added to the wells of a microtitre plate containing ABTGC medium. Wells without OdDHL and/or QSI were included as controls. An overnight culture of *E. coli lasB-gfp* (ASV) strain, grown in LB medium at 37°C with shaking, was diluted to an OD₄₅₀ of 0.2, and

100 µl of bacterial suspension was added to each well of the microtitre plate. Hence, the plate contained OdDHL at concentrations ranging from 20 nM to 320 nM, and QSI at concentrations ranging from 3.125 µM to 50 µM (including control wells without OdDHL, QSI or both). GFP and OD₆₀₀ readings were obtained as described above for the *P. aeruginosa* LasR inhibition assay.

3.2.9 Inhibition of the rhl and pqs QS systems in wild-type PAO1 and PAO1 $\Delta lasR$ mutant

Wild-type *P. aeruginosa* PAO1 strains harbouring either the *rhlA-gfp* (ASV) reporter or the *pqsA-gfp* (ASV) reporter, and a *P. aeruginosa* PAO1 $\Delta lasR$ mutant harbouring either the *rhlA-gfp* (ASV) reporter or the *pqsA-gfp* (ASV) reporter were used for this experiment (Refer to Table 2-1 for strain information). Overnight cultures of these four strains were diluted 100-fold in ABTGC media within 96-well microtitre plates to a final volume of 200 µL per well. Each of the five QSIs was added in triplicate to a final concentration of 50 µM. 0.5% DMSO was used as a negative control. The microtitre plate was incubated at 37°C in a Tecan Infinite 200 Pro plate reader where GFP and OD₆₀₀ readings were measured at 15 min intervals.

3.2.10 Glass slide biofilm assay for observation of eDNA release

The glass slide biofilm assay was performed as previously reported (Liu et al., 2010). Briefly, *gfp*-tagged *P. aeruginosa* PAO1 biofilms were cultivated in 50 ml BD falcon tubes containing 15 ml ABTG medium. A sterile 24 mm x 60 mm glass cover slide was inserted into each falcon tube for supporting biofilm growth. G1 (10 µM) was added into the biofilm medium to examine its impact on *P. aeruginosa* PAO1 biofilm formation. In another tube, DMSO was added alone to the medium as control. Biofilms were incubated at 37°C without shaking. 2 µM propidium iodide (Sigma-Aldrich) was added to biofilm cultures to stain extracellular DNA (eDNA) for 5 min after 24-hour growth. After that, biofilm attached glass slides were observed by confocal laser scanning microscopy (CLSM).

3.2.11 iTRAQ-based proteomics analyses for G1

Isobaric tag for relative and absolute quantitation (iTRAQ)–based proteomic analysis was used to study the changes in protein expression of the *P. aeruginosa* PAO1 strain in response to the addition of 25 μ M of G1. Proteomics experiments were performed at the Proteomic Core Facility of the Biological Research Center, School of Biological Sciences, Nanyang Technological University, Singapore. A full description of the proteomics workflow is included as Appendix B.

3.2.12 Elastase assay

P. aeruginosa PAO1 wild-type and an elastase negative *lasIrhII* mutant were cultivated in LB medium overnight at 37°C with shaking. Overnight cultures were 1:100 diluted to 5 ml ABTGC medium and incubator at 37°C with shaking. Compound G1 was supplemented into the PAO1 cultures at final concentrations of 0, 50 and 100 μ M respectively. After 2h incubation, 0.8 mL culture supernatants were sampled by centrifugation (18000g, 4min) and filtration through 0.2 micron filters. Elastase activity of *P. aeruginosa* culture supernatants was measured by using the EnzChekElastase Assay Kit (Invitrogen), which uses BODIPY-FL-labeled DQ elastin conjugate as the substrate of elastase. The BODIPY-FL-labeled DQ elastins conjugate when cleaved by elastase enzyme, yields highly fluorescent fragments. Fluorescence was recorded every 6 min for 180 min by using a Tecan Infinite 200 Pro plate reader (excitation at 490 nm, emission at 520 nm).

3.3 Results

3.3.1 Structure-based virtual screening for QSIs

Molecular docking was first performed using the reference ligand OdDHL and several known LasR inhibitors, against the ligand binding domain of LasR (PDB ID: 2UV0) in MVD. These compounds and their structures are shown in Figure 3-4 overleaf.

Figure 3-4. The chemical structures of the reference ligand, OddHL, and other known QS inhibitors used as comparisons for the structure-based virtual screening. The references for each compound are in superscript. (A) 3-Oxo-C12-HSL (OddHL) (Pearson et al., 1994); (B) Patulin (Rasmussen et al., 2005b); (C) Salicylic acid (Yang et al., 2009b); (D) 3-Oxo-C12-(2-aminophenol) (Smith et al., 2003); (E) Furanone C30 (Hentzer et al., 2003); (F) 4-Nitropyridine-*N*-oxide (Rasmussen et al., 2005a); (G) Nifuroxazide (Yang et al., 2009b); and (H) Chlorzoxazone (Yang et al., 2009b).

The docking scores of these reference compounds (Table 3-2) provide a comparison for the selection of potential QSI candidates from the library compound screening. Ligand efficiency is calculated by dividing rerank score by the number of heavy atoms present in a molecule. In this way, it helps us to rank how ‘efficient’ a ligand is at docking, rather than simply having large molecular weight compounds dominate the rerank score, due to their higher propensity for forming more interactions with the ligand binding pocket. Based on the results of the docking of these reference compounds, an arbitrary cut-off of -60 rerank score was used for selection of potential inhibitor compounds.

Table 3-2. Structures and docking scores of the reference compounds.

IUPAC Name	Structure	Molecular Weight	Rerank Score	Ligand Efficiency
3-Oxo-C12-HSL (OdDHL)		297.39	-81.2886	-3.87089
Patulin		154.12	-62.0131	-5.63755
Salicylic acid		137.113	-63.5007	-6.35007
3-Oxo-C12-(2-aminophenol)		305.412	-77.6409	-3.52913
Furanone C30		252.868	-62.0532	-6.89479
4-Nitropyridine- <i>N</i> -oxide		140.097	-63.2568	-6.32568
Nifuroxazide		275.217	-20.4001	-1.02001
Chlorzoxazone		168.557	-72.5769	-6.5979

Next, a 3D structural database containing 3,040 structures of compounds from TimTec's Natural Derivatives Library was created using DG-AMMOS (Lagorce et al., 2009) after which it was docked against the ligand binding domain of LasR. Twenty-two compounds having a rerank score below -60 and having a molecular weight less than 200 Da were selected as QSI candidates. These 22 structures with their associated molecular weight, docking rerank score and ligand efficiency score are shown in Table 3-3. The cut-off value for the rerank score was set as -60 because most of the reference QSIs that were docked earlier had scores below that value (Table 3-2). The molecular weight cut-off value of 200 Da was arbitrarily determined in order to select for the most effective small molecule inhibitors that can easily penetrate the bacteria cell. Thus, the 22 compounds selected were obtained and tested for its inhibition *in vitro*.

Table 3-3. Structures and docking scores of twenty-two top-scoring compounds with the following selection criteria: Molecular weights below 200 Daltons, (docking) rerank score below -60.

ID ^a	IUPAC Name	Structure	Molecular Weight	Rerank Score	Ligand Efficiency ^b
A1	7-aminodecanoic acid		186.271	-64.26	-4.94
B1	2-prop-2-enyloxy-2H-3,4,5,6-tetrahydropyran		137.156	-62.71	-6.27
C1	6-hydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one		136.092	-64.15	-6.41
D1	methyl 3-(3,5-dioxo-2H,4H-1,2,4-triazin-6-yl)propanoate		199.164	-69.15	-4.94
E1	xanthen-9-one		196.201	-74.45	-4.96

F1	2-amino-3-(3-fluorophenyl)propanoic acid		182.172	-70.69	-5.44
G1	5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one		151.106	-69.93	-6.36
H1	2-amino-3-hydroxy-3-phenylpropanoic acid		180.181	-67.92	-5.22
A2	(2R,6R)-2,6-diaminoheptanedioic acid		188.181	-68.80	-5.29
B2	4-[1-hydroxy-2-(methylamino)ethyl]benzene-1,2-diol, chloride		183.204	-60.05	-4.62
C2	3-acetyl-2-oxo-3-hydrobenzoxazole		177.157	-61.49	-4.73
D2	2-(5-hydroxyindol-3-yl)acetic acid		185.136	-65.42	-4.67
E2	methyl (2S,4R)-4-hydroxypyrrolidine-2-carboxylate, chloride		145.156	-71.23	-7.12

F2	indole-3-carboxylic acid		159.141	-69.33	-5.78
G2	5-hydroxybenzo[d][1,3-oxathiolan-2-one		168.17	-71.13	-6.47
H2	3,4-dihydroisoquinoline-6,7-diol		163.173	-72.14	-6.01
A3	methyl 3,5-dihydroxybenzoate		162.099	-67.71	-5.64
B3	6-methoxy-3,4-dihydroisoquinolin-7-ol		177.2	-74.23	-5.71
C3	6,7-dihydroimidazo[5,4-c]pyridine-6-carboxylic acid		163.133	-75.50	-6.29
D3	5-methyl-1-(2-methylpropyl)-1,3-dihydropyrimidine-2,4-dione		182.22	-60.08	-4.62
E3	2-(purin-6-ylamino)acetic acid		190.139	-77.05	-5.51

F3	methyl 2-oxopyran-3-carboxylate		154.12	-68.61	-6.24
----	---------------------------------	---	--------	--------	-------

^aCompound IDs are arbitrarily named based on the well number in the shipping plate.

^bLigand efficiency is computed as rerank score divided by the number of heavy atoms present in the compound.

3.3.2 Inhibition assay with the *P. aeruginosa lasB-gfp*(ASV) strain

In the preliminary screen, the 22 selected QSI candidates were screened for their ability to inhibit QS-controlled green fluorescent protein (GFP) expression in the *P. aeruginosa lasB-gfp* (ASV) strain. Elastase (encoded by the *lasB* gene) is a virulence factor that is controlled by LasR and therefore a good indicator for LasR activity (Pesci et al., 1997).

Five compounds, code-named C1, F1, G1, H1 and F2, (Figure 3-5) were found to inhibit LasR-controlled GFP expression in a dose-dependent manner without affecting cell growth. For ease of identification, each compound was designated a short compound identification code based on its well position in the shipment in place of its standard IUPAC name.

C1	F1	G1	H1	F2
6-hydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one	2-amino-3-(3-fluorophenyl)propanoic acid	5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one	2-amino-3-hydroxy-3-phenylpropanoic acid	indole-3-carboxylic acid

Figure 3-5. Structures of five QSI candidates. Five QSIs: 6-hydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (C1), 2-amino-3-(3-fluorophenyl)propanoic acid (F1), 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (G1), 2-amino-3-hydroxy-3-phenylpropanoic acid (H1) and indole-3-carboxylic acid (F2).

The dose-response curves of these five QSI candidates, C1, F1, G1, H1 and F2, when cultured with the *P. aeruginosa* PAO1 *lasB-gfp* (ASV) strain are shown in Figure 3-6. GFP expression, which was measured in relative fluorescence units, was normalized by dividing the GFP value by the corresponding OD₆₀₀ value measured at that time-point. 'Control' refers to the PAO1 *lasB-gfp* (ASV) strain grown without the presence of QSI, and as expected, it had the highest GFP per OD values. For these five compounds, dose-dependent inhibition of *lasB-gfp* expression was observed, i.e. the higher the concentration of QSI that was present, the greater the inhibition of *gfp* expression.

In order to map the interactions between the five QSI compounds and the residues within the LasR protein ligand-binding site, the program LIGPLOT ver 4.5.3 (Figure 3-7) (Wallace et al., 1995) was used. This program provides a 2D map showing the hydrogen-bonding and hydrophobic interactions between atoms in the ligand and that of the binding partner. PyMOL was also used for 3D representations of these interaction maps (Appendix C). Table 3-4 summarizes the interactions between our five QSI compounds and residues within the LasR ligand binding domain (LBD).

Table 3-4. Key residues within the LasR ligand binding pocket having hydrogen bonding interactions with OdDHL and the corresponding 5 QSI molecules.

Compound	Residues within the LasR Ligand Binding Pocket having H-bonding interactions with ligand
OdDHL	Tyr 56, Trp 60, Asp 73, Ser 129
C1	Thr 75, Tyr 93
F1	Thr 75, Tyr 93
G1	Trp 60, Thr 75, Tyr 93
H1	Tyr 56, Thr 75, Ser 129
F2	Tyr 56, Ser 129

Figure 3-6. Dose-responses curves of (A) 6-hydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (**C1**); (B) 2-amino-3-(3-fluorophenyl)propanoic acid (**F1**); (C) 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (**G1**); (D) 2-amino-3-hydroxy-3-phenylpropanoic acid (**H1**); and (E) indole-3-carboxylic acid (**F2**) when incubated with the *P. aeruginosa* PAO1 *lasB-gfp*(ASV) strain. The legend shows the concentrations of the respective QSI used. The experiments were performed in triplicate; each figure shows a representative experiment.

Figure 3-7. Interaction maps between residues within the LasR LBD and the following compounds: (A) the native acyl homoserine lactone ligand, OdDHL; (B) 6-hydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (**C1**); (C) 2-amino-3-(3-fluorophenyl) propanoic acid (**F1**); (D) 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (**G1**); (E) 2-amino-3-hydroxy-3-phenylpropanoic acid (**H1**); and (F) indole-3-carboxylic acid (**F2**).

In a recent study, the LasR LBD was crystallized with OdDHL and other AHL agonists (Zou and Nair, 2009) which showed that LasR and OdDHL appeared to interact at residues Tyr56, Trp60, Arg61, Asp73 and Ser129 (Figure 3-8). Those results agree with the residues identified by LIGPLOT as determined in this study (Table 3-4).

Figure 3-8. Crystal structure model of the interactions between residues in the LasR ligand-binding pocket with the autoinducer molecule OdDHL. The wire mesh shows the electron density map. Image adapted from Zou and Nair (2009).

3.3.3 IC₅₀ value comparisons of the five QSI candidates

The slope of the curve for each QSI was calculated based on its respective dose-response curves (from Figure 3-6) and plotted against the log inhibitor concentration. The slope is indicative of the biosynthesis rate of GFP due to AHL induction. The half maximal inhibitory concentrations (IC₅₀) of the five QSI candidates were calculated using the Graphpad Prism 6 software package (GraphPad Software Inc., CA, USA). The IC₅₀ values of the five QSIs were mainly in the low micromolar range, with two compounds (C1 and G1) having values in the high nanomolar range (Figure 3-9).

G1 had the lowest IC₅₀ value of 0.64 μ M meaning that this inhibitor was able to inhibit *lasB-gfp* expression with high efficiency. However, these compounds were not necessarily interacting directly with LasR, and therefore we used the heterologous *E. coli* strain containing the *lasB-gfp* (ASV) reporter to test for LasR-specific inhibition.

Figure 3-9. The half maximal inhibitory concentration (IC_{50}) of (A) 6-hydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (**C1**); (B) 2-amino-3-(3-fluorophenyl) propanoic acid (**F1**); (C) 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (**G1**); (D) 2-amino-3-hydroxy-3-phenylpropanoic acid (**H1**); and (E) indole-3-carboxylic acid (**F2**). The minimum concentration of inhibitor was arbitrarily designated by the Graphpad PRISM software to have a $\log$ [inhibitor] value of -10 ($\log$) μM , as the logarithm of zero is undefined.

3.3.4 AHL competition assay using the heterologous *E. coli lasB-gfp* (ASV) strain.

The five QSI candidates were tested for their specificity in inhibiting the LasR receptor by incubating these QSIs with the *E. coli lasB-gfp* (ASV) reporter strain. LasR is under the control of the Lac promoter and is constitutively expressed, and only LasR can activate *gfp* expression in this system. *P. aeruginosa*-based QS systems do not exist in *E. coli*, so exogenous OdDHL has to be added in order to activate *lasB-gfp* expression. Hence this heterologous system provides a way to eliminate the contribution of QSI interaction with higher levels of control.

Of these five QSI candidates, only one compound, G1, was found to specifically inhibit LasR activity in the competition assay. The other four compounds did not show specific inhibition in the *E. coli lasB-gfp* (ASV) inhibition assay (Figure 3-10).

Figure 3-10. Competition assay results of the following 4 QSIs when incubated with the *E. coli lasB-gfp* (ASV) strain and increasing concentrations of OdDHL. (A) 6-hydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (**C1**); (B) 2-amino-3-(3-fluorophenyl)propanoic acid (**F1**); (C) indole-3-carboxylic acid (**F2**), and (D) 2-amino-3-hydroxy-3-phenylpropanoic acid (**H1**). The legend refers to the concentrations of the respective QSI used.

Figure 3-11 shows the relative GFP fluorescence of the *E. coli lasB-gfp* (ASV) strain in response to varying concentrations of G1 and OdDHL.

Figure 3-11. The response of the *E. coli lasB-gfp* (ASV) strain to varying concentrations of 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (G1) and OdDHL. Relative fluorescence is normalized through dividing GFP values by OD₆₀₀ values.

From Figure 3-11, we see that increasing levels of OdDHL increase *lasB-gfp* expression, while increasing levels of the QSI G1 decrease it. The highest amount of relative fluorescence was observed for the condition of no G1 and 320 nM OdDHL (value = 1340.4) and the lowest amount of relative fluorescence for the condition of 100 μM G1 and no OdDHL (value = 438.1). The relative fluorescence values for the condition of 320 nM OdDHL with 100 μM G1, and the condition without OdDHL or G1, are 711.5 and 627.6 respectively. In the presence of 320 nM OdDHL, we have 46.9% inhibition by 100 μM G1 (as compared to the control without G1), while in the absence of OdDHL, we have only 30.2% inhibition by 100 μM G1 (as compared to the control without G1).

The results also show that even in the presence of 320 nM OdDHL, 1.56 μM of G1 was sufficient in inhibiting OdDHL-LasR induction of *gfp* expression. If the OdDHL concentration were to be increased further, an out-competition of G1 by OdDHL will eventually be seen, where lower levels of G1 would be ineffective in inhibiting *gfp* expression and only higher levels of G1 would be able to inhibit *gfp* expression. The half maximal effective concentration (EC₅₀) of

OdDHL for LasR activation has been previously determined to be 10 nM (Geske et al., 2007), therefore the range of OdDHL concentrations used for this assay is considered relatively high.

3.3.5 Effect of QSIs on *rhl* and *pqs* quorum sensing systems

In order to address the problem of the specificity of our compounds, our five QSI compounds were tested to see if they had any effect on the *rhl* and *pqs* systems. The five QSIs were tested against a PAO1 wild-type and a PAO1 $\Delta lasR$ mutant harbouring either the *rhlA-gfp* (ASV) bioreporter or the *pqsA-gfp* (ASV) bioreporter. The *rhlA* system is dependent on the RhlR/I system. By doing so, we were able to determine if a QSI is able to inhibit the other two QS systems (i.e. *rhl* and *pqs*) in a LasR-dependent/independent manner (Figure 3-12).

Figure 3-12. The expression of (a) *rhlA-gfp* (ASV) and (b) *pqsA-gfp*(ASV) in *P. aeruginosa* PAO1 wild-type and a $\Delta lasR$ mutant was measured when treated with 50 μ M of each QSI. Results are the average relative fluorescence values (GFP readings divided by OD₆₀₀ values) from a single time point measurement corresponding to maximal induction of the reporters. Averages and standard deviation are from triplicate experiments.

G1 was found to inhibit *rhlA-gfp* expression in the wild-type PAO1 strain (23.1% inhibition) and also in the *lasR* mutant (46.5% inhibition) (Figure 3-12 A). The *P. aeruginosa* *rhl* QS system uses a signal molecule BHL, which is structurally similar to the OdDHL of the *las* QS system, to regulate gene expression. Hence, it is likely that G1 as an inhibitor of LasR could also inhibit RhlR in the absence of LasR. Our results suggest that G1 has a higher binding specificity to LasR than RhlR.

G1 was observed to strongly inhibit *pqsA-gfp* expression in the wild-type PAO1 (57.5% inhibition) and lesser in the *lasR* mutant (24.4% inhibition) (Figure 3-12 B). Because the *pqs* QS system is positively regulated by the *las* system (McGrath et al., 2006), inhibition of the *las* system by G1 would result in the down-regulation of the *pqs* expression. This shows that G1 inhibits the *las* QS system specifically, and inhibition of *pqs* is through a LasR-dependent mechanism.

Interestingly, F1 was able to inhibit both the *rhl* and *pqs* systems in a LasR-independent manner. F1 inhibited *rhlA-gfp* expression in the wild-type PAO1 by 61.7%, and in the *lasR* mutant showed an inhibition of 63.1% (Fig. 3-12A). F1 was also found to inhibit *pqsA-gfp* expression by 25.4% in the wild-type and 39.4% in the *lasR* mutant (Fig. 3-12 B). Our results suggest that F1 has a higher binding specificity to RhlR than LasR.

3.3.6 Effect of G1 on the extracellular DNA release in *P. aeruginosa* biofilms

The *pqs* QS system regulates the release of eDNA, which is an important structural component for *P. aeruginosa* biofilms (Allesen-Holm et al., 2006; Yang et al., 2007). Using a slide biofilm assay, G1 was tested for its ability to reduce eDNA release in *P. aeruginosa* biofilms. Propidium iodide (PI) staining was used to stain and visualize eDNA. PI can stain both eDNA and dead cells, however, eDNA appears as string-like structures rather than circular structures indicative of dead cells. A large amount of eDNA was observed in the substratum of *P. aeruginosa* PAO1 biofilms cultivated in ABTG medium (Figure 3-13 A&C) while less eDNA was observed in PAO1 biofilms cultivated in ABTG medium containing 10 μ M G1 (Figure 3-13 B&D). This indicates that G1 was able to reduce eDNA release in *P. aeruginosa*.

Figure 3-13. Biofilms of *gfp*-tagged PAO1 grown for 24h either in ABTG medium (A and C) or ABTG medium containing 10 μ M G1 (B and D) were stained with propidium iodide (PI). Images visualizing cells (green) and extracellular DNA (appearing red) were acquired by CLSM. Scale bar is 10 μ m in length.

3.3.7 iTRAQ-based quantitative proteomic analysis

In order to study the proteins whose expression was down-regulated in *P. aeruginosa* PAO1 as a result of G1 addition, iTRAQ was used as the labelling strategy for comparative quantitative proteomic analysis (performed with a False Discovery Rate below 1%).

The following cut-offs were used for protein identification: Unused Protein Score of at least 2 (i.e. 99% confidence of identification) and having more than 1 peptide identified. Using these cut-offs, 2258 proteins were identified. Using a p-value (115:114) cut-off of 0.05, 46 proteins were found to be significantly affected by G1; the abundance of 19 proteins was up-regulated, while the abundance of 27 proteins was down-regulated. In our study, up-regulation was defined as an abundance (115:114 score) of at least 1.5 (~ 0.6 fold increase), and down-regulation defined as an abundance value (115:114 score) below 0.66 (~ 0.6 fold decrease).

Table 3-5 shows the 27 proteins whose abundance was significantly decreased in the G1-treated *P. aeruginosa* PAO1 strain versus the control PAO1 strain without G1 addition.

Table 3-5. Proteins whose abundance in the *P. aeruginosa* PAO1 strain decreased significantly upon 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one (G1) addition. Significance was defined as a 115:114 abundance <0.66, p-value 115:114 <0.05).

PA No.	Gene name	Description of Product ^a	Peptides (95%)	Coverage (95%)	115:114 ^b	p-value 115:114	Reference ^c
PA3862	<i>dauB</i>	NAD(P)H-dependent anabolic L-arginine dehydrogenase, DauB	12	58.41	0.23	2.91E-03	(Nouwens et al., 2003)
PA4175	<i>piv/prpL</i>	protease IV	16	35.93	0.29	9.87E-03	
PA5100	<i>hutU</i>	Urocanate hydratase [Pseudomonas aeruginosa MPAO1/P2]	13	34.88	0.39	3.80E-04	
PA3922	-	conserved hypothetical protein	10	29.23	0.42	4.90E-03	(Rasmussen et al., 2005b)
PA3919	-	conserved hypothetical protein	18	41.47	0.43	8.84E-03	
PA2300	<i>chiC</i>	chitinase	16	36.85	0.44	3.65E-05	
PA1372	-	hypothetical protein PA1372	12	24.47	0.45	4.52E-03	(Nouwens et al., 2003)
PA0572	-	hypothetical protein PA0572	12	20.26	0.46	8.03E-03	
PA0792	<i>prpD</i>	propionate catabolic protein PrpD	31	51.21	0.47	2.98E-04	
PA0400	<i>metBmetC</i>	probable cystathionine gamma-lyase	76	62.94	0.49	2.97E-02	(Stintzi et al., 2006)
PA5213	<i>gcvP1</i>	glycine cleavage system protein P1	9	15.66	0.52	2.54E-02	
PA2951	<i>etfA</i>	electron transfer flavoprotein alpha-subunit	87	65.37	0.52	3.87E-02	
PA0586	-	conserved hypothetical protein	9	20.89	0.53	3.21E-02	(Whiteley et al., 1999)
PA2399	<i>pvdD</i>	pyoverdine synthetase D	126	45.55	0.54	2.49E-05	
PA3924	-	probable medium-chain acyl-CoA ligase	10	32.32	0.54	1.24E-02	
PA2290	<i>gcd</i>	glucose dehydrogenase	24	35.74	0.59	4.22E-03	(Wagner et al., 2003)
PA2424	<i>pvdL</i>	pyoverdine chromophore synthetasePvdL	368	50.25	0.60	0.00E+00	
PA3148	<i>wbpI</i>	UDP-N-acetylglucosamine 2-epimerase WbpI	53	65.54	0.60	5.85E-03	
PA2302	<i>ambE</i>	AmbE. Involved in L-2-amino-4-methoxy-trans-3-butenic acid (AMB) biosynthesis	49	31.45	0.61	4.46E-07	(Whiteley et al., 1999)
PA2402	-	probable non-ribosomal peptide synthetase	273	41.58	0.62	2.46E-10	
PA0852	-	chitin-binding protein CbpD precursor	16	36.76	0.62	1.41E-02	
PA3083	<i>pepN</i>	aminopeptidase N	50	36.27	0.63	1.72E-02	(Nouwens et al., 2003)

Table 3-5 continues overleaf.

Table 3-5 continued.

PA No.	Gene name	Description of Product ^a	Peptides (95%)	Coverage (95%)	115:114 ^b	p-value	Reference ^c
-	-	putative ClpA/B protease ATP binding subunit [<i>Pseudomonas aeruginosa</i> MPAO1/P2]	30	36.05	0.63	2.90E-03	
PA0588	-	conserved hypothetical protein	49	41.25	0.64	4.74E-04	(Wagner et al., 2003)
PA2445	<i>gcvP2</i>	glycine cleavage system protein P2	143	51.51	0.64	9.94E-04	
PA0399	-	cystathionine beta-synthase	64	68.71	0.64	4.28E-03	(Wagner et al., 2003)
PA5172	-	ornithine carbamoyltransferase, catabolic	47	44.94	0.65	7.22E-03	

^aDescription is obtained from the *Pseudomonas* Genome Database (Winsor et al., 2011) (<http://www.pseudomonas.org>)

^b115:114 refers to the ratio of the protein's abundance in the **G1**-treated sample (115) compared to the untreated control (114).

^cReferences showing that gene has been shown to be related to QS.

3.3.8 Effect of G1 on the production of elastase by *P. aeruginosa*

The metalloprotease elastase B is a *las* QS system regulated virulence factor produced and excreted by *P. aeruginosa* (Pearson et al., 1997). QSIs that inhibit the *las* QS system should be able to inhibit the production of elastase B. A standard enzymatic assay was used to test whether G1 could inhibit the elastase activity of *P. aeruginosa* cultures. The result showed that addition of G1 to *P. aeruginosa* PAO1 cultures at 50 and 100 μ M could almost abolish elastase production within a 2 hour cultivation period (Figure 3-14), matching the levels of a *P. aeruginosa* PAO1 *lasIrhII* mutant. This *P. aeruginosa* PAO1 *lasIrhII* mutant is deficient in quorum-sensing and used as a negative control. The amount of elastase produced by *P. aeruginosa* upon exposure to G1 was similar to this negative control, which indicates that G1 was efficient in inhibiting elastase production.

Figure 3-14. Effect of G1 on the elastase activity of *P. aeruginosa* cultures. Elastase activity of *P. aeruginosa* culture supernatants was measured by using The EnzChekElastase Assay Kit (Invitrogen). Fluorescence was recorded every 6 min for 180 min by using a Tecan Infinite 200 Pro plate reader (excitation at 490 nm, emission at 520 nm). The *P. aeruginosa* PAO1 *lasI rhII* strain served as a negative control.

3.4 Discussion

3.4.1 Comments on structure-based virtual screening

The SB-VS method is a relatively new concept in the drug discovery process. It has served well in identifying several QSI candidates (Yang et al., 2009b; Zhu et al., 2012), alongside other frequently used screening methods such as various QS inhibition assays and high throughput techniques. It not only accelerates the drug screening process, but also contributes to cost-efficiency in drug discovery as only compounds identified to be potential drugs are procured for laboratory testing (Lyne, 2002).

In this study, from a total of 3,040 compounds tested, 22 potential lead compounds were identified, and five compounds were found to inhibit QS as indicated by reduced GFP expression in the *P. aeruginosa lasB-gfp* (ASV) reporter strain in a dose-dependent manner. Out of these five compounds, only one compound, G1, was able to inhibit GFP expression in the *E. coli* system.

It is not clear why the other four QSIs (C1, F1, H1 and F2) were able to inhibit GFP expression in the *P. aeruginosa lasB-gfp* system, but not in the *E. coli lasB-gfp* system. This is puzzling because they were identified by molecular docking against the LasR ligand-binding domain and were predicted to have specific H-bonding interactions with residues within the LasR ligand binding pocket. Therefore, these compounds should in theory, specifically target LasR. This suggests two different scenarios: (1) these four QSIs do not bind specifically to LasR and instead inhibit *gfp* expression indirectly in the *P. aeruginosa* reporter strain through other pathways that affect the *las* QS pathway, or (2) *E. coli* has molecular machinery that is able to either degrade or selectively pump out these compounds of the cell, such that the intracellular concentration of QSI never reaches a level that allows for competition with OdDHL for the LasR binding pocket.

These results suggest that the structure of G1 may be important for its ability to interact with and inhibit LasR specifically. If we were to compare the structure of G1 and C1 (Table 3-6), we see that their structures are very similar, the difference being the presence of an additional imino group on G1. Hence, it is interesting why G1, but not C1, was able to inhibit *lasB-gfp* (ASV) in the *E. coli* system, despite their structural similarity. In fact, there are other compounds

similar to G1 that may have been missed. A structure subgroup search for compounds within the Natural Derivatives Library found the molecule 404 (Table 3-6) to be similar in size and structure to G1. However, molecule 404 had a docking Rerank score of -5.03, and was therefore excluded by the -60 cut-off score we used.

Table 3-6. Structures of **C1**, **G1** and molecule **404**

C1	G1	404
6-hydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one	5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one	purine-2,6-diamine

As such, the cut-off of -60 that was used may have been too stringent, and we may have missed hits by using that cut-off value. Hence the idea should be to use a cut-off for excluding low-scoring compounds, but not use it for identifying top-scoring hits; a larger starting pool of molecules would then be tested for *in vitro* inhibition efficacy, and this may result in a better hit rate.

In a previous study (Yang et al. 2009), 147 compounds were screened using MVD and 6 top-scoring hits were identified and tested for QSI activity. Out of these six, three were found to have dose-dependent inhibition of QS-related gene expression and associated phenotypes. This is similar to the results obtained in the present study and support the use of DG-AMMOS for *in silico* screening. One advantage of using DG-AMMOS, rather than relying on structural similarity to the ligand of interest, e.g. OdDHL, is the avoidance of rational bias in the screening process, and therefore allows the detection of lead compounds that may not be able to be identified rationally. Hence, it is possible that DG-AMMOS can be extended to the conversion of larger compound libraries (e.g. 10,000 compound libraries, combinatorial chemistry libraries) and we may be able to discover new compounds that may have little structural similarity to QSIs or AHLs, yet possess QSI properties. Presumably, this is in part based on the 3D structure of the compound and its ability to enter the cell, followed by displacement or prevention of AHL binding to LasR.

SB-VS has been used extensively in the pharmaceutical industry, famous examples being Relenza, an anti-influenza drug that targets sialidase (Von Itzstein et al., 1993), and Viracept, a human immunodeficiency virus protease inhibitor (Kaldor et al., 1997). In recent years, SB-VS approaches have also been used in the search for novel QSIs, and here are a few recent examples:

- Discovery of hamamelitannin, a natural compound from *Hamamelis virginiana* (witch hazel) that inhibits QS in drug-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* (Kiran et al., 2008). In a rat model, this compound was able to prevent *Staphylococcal* device-associated infections. Hence it could be beneficial in the treatment of methicillin-resistant *S. aureus* / *S. epidermidis* (MRSA/MRSE).
- Identification of novel AI-2 inhibitors of *Vibrio harveyi* by SB-VS with the crystal structure of LuxP (Li et al., 2008). The AI-2 QS system is present in both Gram-positive and Gram-negative bacteria.
- Discovery of 7-(1-bromoethyl)-3,3-dimethyl-bicyclo[4.1.0]heptan-2-one from the *Melia dubia* bark extract which was able to inhibit the QS regulator SdiA present in uropathogenic *E. coli* (UPEC). The compound was able to reduce the haemolytic and biofilm forming ability of UPEC (Ravichandiran et al., 2012).
- Screening of 1,920 natural compounds through SB-VS against the LasR and RhIR receptor proteins revealed five compounds (rosmarinic acid, naringin, chlorogenic acid, morin and mangiferin) with potential QSI properties. These compounds inhibited biofilm formation while four were found to be effective in inhibiting the production of protease, elastase and hemolysin (Annapoorani et al., 2012).
- Screening of 800,000+ compounds from the Chembridge library through a pharmacophore-based approach for compounds similar to OdDHL revealed 5 inducers and 3 inhibitors of LasR (Skovstrup et al., 2013).

Hit rate can be defined as the percentage of lead compounds identified from the total pool of compounds that were tested. In the study by Doman (2002), they used both high-throughput screening (HTS) and SB-VS to search for inhibitors of the enzyme protein tyrosine phosphatase-1B (PTP-1B), a target for treatment of Type II diabetes (Doman et al., 2002). Using HTS, they

screened 400,000 compounds and found 85 hits with an IC_{50} below 100 μ M (hit rate = 0.021%). In contrast, using SB-VS, they tested 365 compounds and found 127 hits with an IC_{50} below 100 μ M (hit rate = 34.8%). This may suggest that SB-VS provides a much higher hit rate than HTS, but this is not always the case as hit rates of SB-VS projects vary greatly. Compared to the 35% hit rate against PTP-1B, SB-VS had only a 5% rate against the enzyme AmpC β -lactamase (Powers et al., 2002).

Hence, one major limitation of SB-VS is the problem of false-positives and false-negatives predicted by the docking software. However, with the development of newer and better algorithms, the problem of false hits may be minimized. Also the aim may not be to eliminate false-positives entirely, but to reduce it to a tolerable level, reason being that false-positives may lead to the discovery of novel molecular interactions. As such, the cut-off of a -60 Rerank Score that was used in our study for identifying potential QSI candidates might have been too stringent, and a higher value (i.e. less negative) should have been used instead so as to increase the number of potential hits. However, using a less stringent cut-off would increase the number of false-positives, and this trade-off between the number of potential leads and the number of false-positives must be considered for all SB-VS studies.

On the whole, SB-VS methods provide a faster and cheaper alternative to HTS approaches for several reasons. Firstly, if the search strategy in SB-VS is restricted to commercially available compound libraries, the lead compounds identified through SB-VS can be purchased easily and one does not need to undertake a costly and tedious chemical synthesis process. Secondly, SB-VS can be used to dock known drugs or natural plant derivatives, which would be likely to have lower toxicity than compounds synthesized through combinatorial chemistry. Thus, compounds identified through screening of known drugs/natural product libraries can avoid failure in the *in vitro* and *in vivo* testing stages due to toxicity. Lastly, SB-VS is able to first narrow down the list of compounds to be tested before proceeding with actual *in vitro* tests for efficacy, and this would greatly reduce costs as compared to conventional HTS methods where all compounds have to be tested.

3.4.2 Comments on G1 as a QSI

The compound G1 was shown to have dose-dependent inhibition of *lasB-gfp* expression in both the *P. aeruginosa* and the *E. coli* backgrounds, indicating its specificity for the LasR protein. Among the five QSI candidates, it had the lowest IC₅₀ of 0.64 μ M.

G1 was also able to delay the induction of the *rhl* QS system but not repress it. Eventually, the levels of *rhl* gene expression increase to match the levels seen in the *P. aeruginosa* that had no addition of G1. This suggests that G1 specifically inhibits the *las* QS system but not the *rhl* QS system. But since it does have some effect on the *rhl* QS system, perhaps it either has very weak binding affinity or allosteric effects. This may be due to the presence of the homoserine lactone ring present in both the LasR ligand, OdDHL, and the RhlR ligand, BHL. Studies have shown that the lactone ring is important for interaction with the LasR binding pocket (Geske et al., 2007). Presumably, a RhlR structure would be very useful to help understand the differences in the binding pockets and hence the binding of compounds like G1. However, the crystal structure of RhlR is unavailable, so homology modelling may be used to generate a putative structure for docking studies.

Besides the *rhl* system, G1 was also found to repress the PQS system. Previous studies have shown that the *las* QS system positively regulates the *pqs* QS system (McGrath et al., 2006) and the results of our study suggest that G1 represses expression of the PQS system through inhibition of the *las* QS system. The *pqs* QS system regulates release of extracellular DNA (eDNA), which is an important structural component for *P. aeruginosa* biofilms (Allesen-Holm et al., 2006; Yang et al., 2007). As such, we found that G1 was able to reduce the amount of eDNA being released by *P. aeruginosa*. Previous studies have shown that eDNA is essential for biofilm formation (Whitchurch et al., 2002) and further investigation is necessary to study the potential of G1 in the prevention of biofilm formation.

3.4.3 Comments on the effects of G1 on the proteome of *P. aeruginosa*

The abundances of 27 proteins were found to be significantly decreased upon the addition of G1. Of these 27 proteins whose abundance was down-regulated in the presence of iberin (shown in Table 3-5), ten had been previously found to be QS-regulated: protease IV, chitinase, hypothetical protein PA0572, pyoverdine synthetase D, pyoverdine chromophore synthetase PvdL, AmbE, probable non-ribosomal peptide synthetase, chitin-binding protein CbpD precursor, conserved hypothetical protein PA0588 and cystathionine beta-synthase.

In a previous study, proteomic analysis of the extracellular proteins regulated by the *las* and *rhl* encoded systems in *P. aeruginosa* was performed (Nouwens et al., 2003). In that study, they found the expression of protease IV (PA4175) to be significantly down-regulated in a *las* mutant, however, the amount of down-regulation was not quantified. In our study, the abundance of protease IV (PA4175) was found to be reduced by 2.1 fold versus the untreated control. Protease IV is an extracellular protease that causes tissue damage in *P. aeruginosa* infections (Engel et al., 1998), hence reducing the expression of this virulence factor may attenuate *P. aeruginosa* virulence. Garlic extract and 4-NPO were also found to reduce the expression of protease IV by -6.9 and -20.7 fold respectively (Rasmussen et al., 2005a).

Protease IV, also known as PrpL, is regulated by PvdS, which is an alternative sigma factor that regulates genes involved in siderophore biosynthesis genes (Wilderman et al., 2001). *pvdS* gene expression is regulated by the iron-sensing Fur repressor protein, such that pyoverdine is produced only in iron-limiting conditions (Cunliffe et al., 1995; Leoni et al., 1996). Figure 3-15 illustrates this iron-based regulation of pyoverdine gene transcription. Therefore, G1 may act through the interaction with the Fur protein to inhibit PvdS-regulated induction of pyoverdine synthesis genes.

Figure 3-15. Model of pyoverdine gene regulation in *P. aeruginosa*. When iron levels are high, transcription of *pvdS* is blocked because of the Fur-Fe(II) complexes binding to the Fur boxes overlapping the *pvdS* promoter. However, when iron levels are low, Fur is released from binding to the Fur box, which allows transcription of *pvdS* by RNA polymerase. ISB: iron starvation box. cRNAP: core RNA polymerase. Image taken from Ambrosi et al. (2002).

In support of this idea, the abundance of two pyoverdine synthetases were found to be significantly reduced: pyoverdine synthetase D (*pvdD*|PA2399) by -0.89 fold and pyoverdine chromophore synthetase (*pvdL*|PA2424) by -0.73 fold. Pyoverdine is a siderophore that is required for iron acquisition, and siderophore-mediated signalling regulates the expression of several virulence factors (Lamont et al., 2002). In a recent study by Taguchi *et al.* (2009), a *pvdL* mutant of *Pseudomonas syringae* pv. tabaci 6605 exhibited reduced virulence on host tobacco plants. The production of EPS and AHL was reduced and this *pvdL* mutant was less tolerant to antibiotic (chloramphenicol and spectinomycin) treatment. Further testing would be required to find out if G1 could affect the resistance of *P. aeruginosa* to antibiotics.

In the proteomics result for G1, elastase (*lasB*|PA3724) which is known to be induced by the *las* system was not identified as down-regulated. In another study, garlic extract and 4-NPO reduced the expression of elastase by -6.8 and -22.6 fold respectively (Rasmussen et al., 2005a). Other QSIs such as patulin and penicillic acid also reduced elastase expression by -7 and -12 fold respectively, and in the *P. aeruginosa* PAO1 *lasR* mutant, elastase expression was decreased -13 fold (Rasmussen et al., 2005b). Hence, it was rather surprising not to find elastase on the list of

down-regulated proteins. We believe that this is because elastase is secreted extracellularly, and the low intracellular levels were below the detection limit of proteomics. Nevertheless, we found by using an enzymatic assay that elastase production was significantly reduced with the addition of G1, matching the levels of a *P. aeruginosa* PAO1 *lasI rhlI* mutant.

3.5 Summary

In summary, this study has shown that structure-based virtual screening is a viable and effective means for the discovery of novel QS inhibitors. From a library of 3,040 natural compounds, 22 compounds met selection criteria and were tested for biological activity. Five of these compounds were found to have dose-dependent inhibition of the *las* QS system, while only one compound, G1, was able to inhibit LasR specifically. G1 was able to down-regulate the activity of the *pqs* system (through inhibition of the *las* QS system) but not the *rhl* QS system. The secretion of eDNA, an important component of the EPS matrix, was also found to be reduced by G1. iTRAQ-based proteomics analysis revealed that G1 was able to reduce the expression of several QS-regulated virulence factors in *P. aeruginosa* such as protease IV and pyoverdine synthetases. The results of an elastase assay also confirmed that G1 was able to reduce the production of elastase by *P. aeruginosa*.

These results emphasize the application of SB-VS for the discovery of target-specific inhibitors, and future work includes the extension of this method to new molecular targets. We also aim to work with chemists to develop new QSI compounds, and to perform quantitative structure-activity relationship (QSAR) studies to aid in our knowledge of rational drug design.

4 Elucidating the anti-virulence and anti-biofilm effects of iberin through a comparative systems biology approach

4.1 Introduction

It is a generally well-known and accepted fact that the food we eat affects our health. This extends even to chronic disease conditions, such as cancer, where certain foods or compounds isolated from food, have been shown to have preventive or alleviative effects. Food, which includes plants and herbs, has been a major source of our medicine for generations, notwithstanding the rising popularity of ‘alternative’ medicine and traditional remedies. Certain health-promoting foods (such as seaweed) have antimicrobial and QS-inhibitory activity (Givskov et al., 1996). This has led us to believe that certain diets may in fact provide health benefits, and have prophylactic effects on bacteria-caused ailments (Givskov, 2012).

One of the best recognized foods with medicinal properties is garlic, which has antibacterial, antifungal and antiprotozoal activity (Block, 2010). Garlic extract has been shown to block QS in *P. aeruginosa*, which increases the susceptibility of these biofilms to antibiotics. Furthermore, in an *in vivo* mouse model, garlic extract promotes bacterial clearance by polymorphonuclear leukocytes (PMNs) through the decreased synthesis of rhamnolipids which normally kills PMNs upon contact (Bjarnsholt et al., 2005b; Jensen et al., 2007).

Ajoene is just one of several QSIs found in crushed garlic extract. Through DNA microarray transcriptomics, ajoene was found to be highly specific in its inhibition – only 11 genes were found to be down-regulated, with 10 being QS-regulated. Several of these QS-regulated genes code for virulence factors such as rhamnolipids, chitinase, lectin and LasA protease. Also, ajoene was shown to work synergistically with antibiotics and host immune cells to promote *P. aeruginosa* clearance in a biofilm model and a mouse model of pulmonary infection respectively (Jakobsen et al., 2012a). Based on a comparison to another study which investigated the role of the RNA-binding protein, Hfq, on *P. aeruginosa* QS (Sonnleitner et al., 2003), the Hfq protein or RsmY RNA was suggested as the possible targets of ajoene.

Two other findings also support the idea that ajoene’s effect is mediated through small regulatory RNAs. Firstly, the GFP fluorescence of the *lasB-gfp* translational reporter fusion was

reduced by ajoene, but the effect on *lasB* transcription, as determined by RT-PCR and DNA microarray, was only minor. This suggests that ajoene's effect on *lasB* expression is on the post-transcriptional level, mediated through small regulatory RNAs such as RsmY. Secondly, ajoene had no effect on the gene expression of the *lasI* and *rhlI* AHL synthases as reflected in the transcriptomics result. This indicates that ajoene inhibits RhlI post-transcriptionally, possibly mediated through targeting of either Hfq or RsmY which both bind to RsmA, a negative regulator of RhlI messenger translation (Jakobsen et al., 2012a; Sorger-Domenigg et al., 2007).

On the back of these successes, Michael Givskov and his team went on to screen 69 food products (e.g. Camembert cheese, liquorice root candy), fruits (e.g. kiwi fruit, strawberry) and plants (e.g. lemongrass, celery) for their ability to inhibit *P. aeruginosa* QS. Among these foods, iberin, which the authors identified as the QSI component of horseradish (*Armoracia rusticana*), had the greatest QS-inhibitory effect and was studied for its QSI properties (Jakobsen et al., 2012b).

Iberin contains the isothiocyanate ($-N=C=S$) chemical group and is therefore classified as an isothiocyanate (ITC) compound (Figure 4-1). ITCs, which can be found in cruciferous vegetables, are bioactive compounds that have come into the research spotlight for their ability in preventing certain cancers (Conaway et al., 2002). Examples of such cancer-preventive ITCs are: sulforaphane against colon cancer (Gamet-Payraastre et al., 2000), allyl isothiocyanate against prostate cancer (Xiao et al., 2003), and phenethyl isothiocyanate against cancer cells (Trachootham et al., 2006). Furthermore, ITCs are also known for their antimicrobial activity (Dufour et al., 2012; Lin et al., 2000).

Figure 4-1. Chemical structure of the isothiocyanate compound iberin.

In the previous study, iberin was found to inhibit expression of the *lasB-gfp* and *rhlA-gfp* reporter fusions in a dose-dependent manner. A competition experiment showed that iberin was able to block the interaction of BHL with its cognate receptor RhlR, but not the interaction between OdDHL and LasR. Iberin was also shown to inhibit rhamnolipid production by *P. aeruginosa*, and inhibited *lasB* and *rhlA* gene expression on a transcriptional level, as evidenced by real-time PCR. Through DNA microarray analysis of the *P. aeruginosa* transcriptome, iberin was found to down-regulate 49 QS-controlled genes including several recognized virulence factor genes such as *lasB*, *rhlAB*, *chiC*, *lecA*, *pivA* and *phz*. Iberin was also tested in a mouse silicone implant model, but no significant improvement in bacterial clearances versus the placebo was observed, as iberin could have been efficiently exported out of the bacterial cells through the activation of efflux pumps (MexEF-OprN transporter) (Hentzer et al., 2003; Jakobsen et al., 2012b).

These results suggest that iberin inhibits both the *las* and *rhl* QS systems, but the mechanism of inhibition is generally unclear. It was speculated that iberin inhibited BHL through structural similarity to AHL; however, iberin bears only little similarity. We wondered if iberin was inhibiting rhamnolipid synthesis (RhlI synthase) through interacting with small regulatory RNAs (such as RsmY), a question introduced by the ajoene study. Therefore, we decided to elucidate the underlying mechanisms of iberin's inhibition of QS in *P. aeruginosa*.

In this chapter, I will describe the comparative systems biology approach undertaken for this study. Here, we use RNA sequencing (RNA-Seq) transcriptomics and isobaric tags for relative and absolute quantitation (iTRAQ) proteomics approaches to find out the various genes and proteins affected by iberin treatment. I also show that iberin inhibits the expression of the small regulatory RNAs *rsmY* and *rsmZ*, which in turn regulate levels of pyoverdine production. Next, I describe my proposal that iberin targets the Gac/Rsm network in *P. aeruginosa* and show that iberin effectively inhibits *P. aeruginosa* biofilm formation. Lastly, I discuss iberin's potential as a QSI with a novel mode of action and how the use of systems biology analyses provides insight for the development of anti-pathogenic drugs.

4.2 Material and Methods

4.2.1 Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 4-1.

Table 4-1. Characteristics of the bacterial strains and plasmids used in this study.

Strain(s) or plasmid	Relevant characteristic(s)	Reference
<i>Bacterial strains</i>		
PAO1	Prototypic wild-type strain	(Holloway and Morgan, 1986)
PAO1 $\Delta pvdA$	Isogenic <i>pvdA</i> mutant of PAO1	(Yang et al., 2009a)
PAO1 miniTn7- <i>gfp</i>	PAO1 carrying the miniTn7- <i>gfp</i> that constitutively expresses <i>gfp</i>	(Yang et al., 2009a)
PAO1 <i>lasB</i> - <i>gfp</i>	PAO1 carrying the <i>lasB</i> - <i>gfp</i> fusion reporter	(Hentzer et al., 2002)
PAO1 <i>rsmZ</i> - <i>gfp</i>	PAO1 carrying the <i>rsmZ</i> - <i>gfp</i> fusion reporter	(Chua et al., 2014)
PAO1 <i>rsmY</i> - <i>gfp</i>	PAO1 carrying the <i>rsmY</i> - <i>gfp</i> fusion reporter	(Chua et al., 2014)
PAO1 <i>pvdA</i> - <i>gfp</i>	PAO1 carrying the <i>pvdA</i> - <i>gfp</i> fusion reporter	(Yang et al., 2009a)

The *P. aeruginosa* PAO1 strain (Holloway and Morgan, 1986) was used for all experiments. For marker selection in *P. aeruginosa*, 30 μg gentamicin mL^{-1} , 50 μg tetracycline mL^{-1} , and 200 μg carbenicillin mL^{-1} were used, as appropriate. Batch cultivation of *P. aeruginosa* was carried out at 37°C in ABT minimal medium (Clark and Maaloe, 1967) supplemented with 0.25% (wt/vol) glucose and 0.25% (wt/vol) casamino acids (referred to as ABTGC medium). *P. aeruginosa* cells were harvested at OD₆₀₀ of 0.5 (mid-log phase) for both RNA-Seq and iTRAQ proteomic analyses.

A PAO1 suspension (OD₆₀₀ = 0.01) was added to a 24-well plate either with (500 μM) or without iberin. ABTGC media was used for culturing bacteria, and each well had a final volume of 1 mL. After reaching an OD₆₀₀ of 0.5 (approximately after 3.5 hours of incubation), cultures were mixed immediately with 2 volumes of RNeasy Protect® Bacteria Reagent (Qiagen). After 5 mins incubation at room temperature, samples were centrifuged at 7000 $\times$ g for 5 min at 4°C, the supernatant was removed and the pellets were stored at -80°C.

4.2.2 RNA preparation

Total RNA was extracted with RNeasy Protect Bacteria Mini Kit with on-column DNase digestion, according to the manufacturer's instructions (Qiagen). The integrity of total RNA and DNA contamination was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies) and Qubit® 2.0 Fluorometer (Invitrogen). The 16S, 23S and 5S rRNA were removed by using the Ribo-Zero™ Magnetic Kit (Bacteria) (Epicentre).

4.2.3 RNA sequencing and data analysis

Gene expression analysis (two biological replicates) was conducted by Illumina RNA sequencing (RNA-Seq technology). The rRNA-depleted RNA was fragmented to 200-300 bp fragments, then first and second strand cDNA were synthesized, followed by end repair and adapter ligation. After 12 cycles of PCR enrichment, the quality of the libraries was assessed through the use of the Bioanalyzer (Agilent Technologies, USA). The libraries were sequenced using the Illumina HiSeq2000 platform with a paired-end protocol and read lengths of 100 nucleotides.

The RNA-Seq datasets are available in the NCBI GEO Short Read Archives: SRP028308 and SRR950389. The sequence reads were assembled and analysed in the RNA-Seq and expression analysis application of CLC genomics Workbench 6.0 (CLC Bio, Aarhus, Denmark). The PAO1 genome (<http://www.ncbi.nlm.nih.gov/nucore/110645304>) was utilized as the reference genome for the assembly. The following criteria were used to filter the unique sequence reads: minimum length fraction of 0.9, minimum similarity fraction of 0.8, and maximum number of two mismatches. Data were normalized by calculating the reads per kilobase per million mapped reads (total reads/mapped reads in millions x gene length in kb) for each gene and annotated with PseudoCAP (<http://www.geneontology.org/GO.current.annotations.shtml>). T-test was performed on transformed data (0.5 was added to each number to deal with zero counts) to identify the genes with significant changes in expression (p-value < 0.05, Fold change > 5.0 or < -5.0) between the control and the iberin-treated samples.

4.2.4 iTRAQ proteomics analyses

The iTRAQ proteomics experiment at the Proteomic Core Facility of the Biological Research Center, School of Biological Sciences, Nanyang Technological University, Singapore, and carried out as per our previous study (Chua et al., 2013).

Protein preparation and digestion. *P. aeruginosa* PAO1 was grown in ABTG medium with and without 500 μ M iberin to an OD₆₀₀ value of 0.5 at 37 °C with shaking, after which the cells were harvested. After harvesting, the cell pellet was washed with 1X PBS and resuspended in 2 ml of lysis buffer containing 0.5M triethyl ammonium bicarbonate (TEAB), 0.1M SDS and protease inhibitor cocktails (Sigma-Aldrich). The cells were ruptured by sonication, and the cell debris was removed by centrifugation at 4 °C at 16000 $\times$ g for 15 min. Three biological replicates for each growth conditions were pooled together and 200 μ g of proteins from each growth condition were dissolved in equal volume of sample buffer (Invitrogen) supplemented with 0.5% 2-mercaptoethanol and denatured by boiling at 95 °C for 5 min. 1D gel electrophoresis was carried out using 10% SDS-PAGE for in-gel digestion.

Proteins were first reduced in 5 mM Tris-(2-carboxyethyl) phosphine (TCEP) for 1 h at 60 °C, followed by blocking cysteine residues in 10 mM methyl methanethiosulfate (MMTS) for 30 min at room temperature in the dark. Trypsin was added at a ratio of 1:50 (trypsin/sample). It was then incubated at 37 °C overnight. The tryptic peptides were extracted by 50% ACN/5% Acetic Acid from gel three times and were desalted using Sep-Pak C18 cartridges (Waters, Milford, MA) and dried in a SpeedVac (Thermo Electron, Waltham, MA). All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

iTRAQ labeling. The iTRAQ labeling of the tryptic peptides was performed using 4-plex iTRAQ reagent kit (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. 200 μ g peptides from each condition were individually labeled with respective isobaric tags (control sample with 114, iberin-treated sample with 115), followed by two hours of incubation, then quenched by water, desalted using C18 solid phase extraction cartridge, and then vacuum centrifuged to dryness. The iTRAQ labeled peptides were reconstituted in Buffer A (10 mM ammonium acetate, 85% acetonitrile, 0.1% formic acid) and fractionated using ERLIC column (200 x 4.6 mm, 5 μ m particle size, 200 Å pore size) by HPLC system (Shimadzu, Japan)

at flow rate of 1.0 ml/min using our previously optimized protocol (Hao et al., 2010). The HPLC chromatograms were recorded at 280 nm and fractions were collected online using automated fraction collector. Twenty fractions were collected and concentrated using vacuum centrifuge and reconstituted in 3% ACN with 0.1% formic acid for LC-MS/MS analysis.

LC-MS/MS. The peptides were separated and analyzed on a home-packed nanobore C18 column (15 cm x 75 μ m; Reprosil-Pur C18-AQ, 3 μ m, Dr Maisch, Germany) with a Picofrit nanospray tip (New Objectives, Woburn, MA, USA) on a TempoTM nano-MDLC system coupled with a QSTAR[®] Elite Hybrid LC-MS/MS system (Applied Biosystems). Peptides from each fraction were analyzed in triplicate by LC-MS/MS over a gradient of 90 min. The flow rate of the LC system was set to a constant 300 nl/min. Data acquisition in QSTAR Elite was set to positive ion mode using Analyst[®] QS 2.0 software (Applied Biosystems). MS data was acquired in positive ion mode with a mass range of 300–1600 m/z. Peptides with +2 to +4 charge states were selected for MS/MS. For each MS spectrum, the three most abundant peptides above a five-count threshold were selected for MS/MS and dynamically excluded for 30 s with a mass tolerance of 0.03 Da. Smart information-dependent acquisition was activated with automatic collision energy and automatic MS/MS accumulation. The fragment intensity multiplier was set to 20 and maximum accumulation time was 2 s.

Data analysis. Spectra acquired from the three technical replicates were submitted to ProteinPilot (v3.0.0.0, Applied Biosystems) for peak-list generation, protein identification and quantification. User defined parameters of the Paragon algorithm in ProteinPilot software were configured as follows: (i) Sample Type, iTRAQ 2-plex (Peptide Labeled); (ii) Cysteine alkylation, MMTS; (iii) Digestion, Trypsin; (iv) Instrument, QSTAR Elite ESI; (v) Special factors, Urea denaturation; (vi) Species, None; (vii) Specify Processing, Quantitate & Bias Correction; (viii) ID Focus, biological modifications, amino acid substitutions; (ix) Database, *Pseudomonas aeruginosa* PAO1 PAO1-UW; (x) Search effort, thorough ID; (xi) Result quality, Unused ProtScore (Conf) >0.05 (10.0%). Default precursor and MS/MS tolerance for QSTAR ESI MS instrument were adopted automatically by the software. For iTRAQ quantitation, the peptide for quantification was automatically selected by Pro Group algorithm to calculate the reporter peak area, error factor (EF) and p-value. The resulting data was auto bias-corrected by build-in ProteinPilot algorithm to get rid of any variations imparted due to the unequal mixing

during combining different labelled samples. During bias correction, the software identifies the median average protein ratio and corrects it to unity, and then applies this factor to all quantitation results.

The following cut-offs were used for protein identification: an Unused Protein Score of at least 2 (i.e. 99% confidence of identification) and having more than 1 peptide identified; in this way, a total of 1948 proteins were identified. Using a p-value cut-off of 0.05, and a fold change of at least +2.0 (for up-regulation) and -2.0 (for down-regulation), the abundances of 119 proteins were found to be significantly affected by iberin; the abundance of 27 proteins was up-regulated, while the abundance of 92 proteins was down-regulated (shown in Tables 4-2 and 4-3 respectively).

4.2.5 Construction of the *rsmZ-gfpmut3b and *rsmY-gfpmut3b** transcriptional fusions**

The *rsmZ-gfpmut3b** and *rsmY-gfpmut3b**-based monitor plasmids pRV59_1 and pRV60_1 were constructed by subcloning the *rsmZ* and *rsmY* promoter regions of pMP220*rsmZ-lacZ* and pMP220*rsmY-lacZ* (Bordi et al., 2010) into pMH305 (Rybtko et al., 2012), a pUCP22NotI-based expression vector carrying a promoterless *gfpmut3b** gene (pMH305 carries the RBSII-*gfp*(Mut3)-T₀-T₁ fragment of pJBA25 (Moller et al., 1998) in the NotI site (Rybtko et al., 2012)). The promoter regions were excised by digestion with *KpnI* and *EcoRI* and inserted into *KpnI/EcoRI*-digested pMH305, generating *rsmZ-gfpmut3b** and *rsmY-gfpmut3b** transcriptional fusions. The ligation mixtures were transformed into *E.coli* K-12 and selected on selective plates containing 100 µg ampicillin mL⁻¹. The plasmid constructs were verified by restriction analysis and subsequently moved into *P. aeruginosa* by electroporation and plating on 30 µg gentamicin mL⁻¹ (Choi et al., 2006).

4.2.6 Reporter fusion assay

The growth and *gfp* expression of the *P. aeruginosa* strains containing the *lasB-gfp*, *rsmY-gfp* or *rsmZ-gfp* biosensors in the presence of iberin was monitored by using a VICTOR X

Multilabel Plate Reader (Perkin Elmer). These strains were cultivated in 96-well microtiter plates with ABTGC medium with different concentrations of iberin at 34 °C without shaking. OD₄₅₀ (or OD₆₀₀ in the case of the *lasB-gfp* strain) and GFP fluorescence (in relative fluorescence units, RFU, with excitation and emission wavelengths of 485 and 535 nm respectively) were measured every 30 mins until the culture reached stationary growth phase.

The growth, *gfp* expression and pyoverdine production of the PAO1 *pvdA-gfp* and PAO1 $\Delta pvdA$ mutant strains in the presence of iberin was measured using an Infinite 200 Pro Series Plate Reader (Tecan). These strains were cultivated in 96-well microtiter plates with ABTGC medium with different concentrations of iberin at 37°C without shaking. OD₆₀₀ readings, GFP fluorescence (in relative fluorescence units, RFU, with excitation and emission wavelengths of 485 and 535 nm respectively) and pyoverdine fluorescence (in relative fluorescence units, RFU, with excitation and emission wavelengths of 398 and 460 nm respectively (Imperi et al., 2009)) were measured every 30 mins until the culture reached stationary growth phase.

4.2.7 Biofilm assay

Overnight cultures of PAO1 were diluted 100-fold in 50 ml BD Falcon™ tubes containing 10ml of ABTGC medium with 500 µM Iberin. No Iberin was added in the controls. Sterilized coverslips were placed into the medium and incubated at 37°C under static conditions for the cultivation of surface-attached biofilm at the air-liquid interface. Images of 1 day old biofilms were captured using confocal microscopy at 63x magnification (ZEISS LSM780 Confocal System), and analysed using the Imaris software package (Bitplane, AG).

4.3 Results

4.3.1 Determination of the sampling point for RNA-Seq and iTRAQ proteomics

Prior to the extraction of samples for either proteomics or RNA-Seq analysis, we optimized the appropriate time point for harvesting the cells by using the *P. aeruginosa* PAO1 strain containing the *lasB-gfp* reporter, which can monitor the onset of QS induction of gene expression (Figure 4-2). Based on this assay, we chose the sampling time-point in the mid-log phase of growth (i.e. $OD_{600} = 0.5$, approximately after 3.5 hours of incubation). Hence, for the actual RNA-Seq and iTRAQ proteomics experiments, we cultured *P. aeruginosa* PAO1 in the presence or absence of 500 μ M iberin, and harvested the samples for subsequent analyses when the cultures reached an OD_{600} of 0.5.

Figure 4-2. Growth and normalized *gfp* gene expression (RFU divided by OD_{600}) of the *P. aeruginosa* PAO1 biosensor strains carrying *lasB-gfp* in ABTGC medium with 500 μ M iberin. Every 60 minutes, growth and GFP fluorescence were measured using OD_{600} values and relative fluorescence units (RFU) respectively. Experiments were performed in triplicate. Arrow indicates sampling point for RNA-sequencing.

4.3.2 Comparative analysis of iberin's mode of inhibition by RNA-Sequencing and proteomics

In order to study the mechanisms behind iberin's inhibition of QS, we first wanted to find out the changes in gene expression and protein abundance of *P. aeruginosa* PAO1 strain in the

presence of media containing 500 μ M iberin. To do so, we decided to use RNA-Sequencing and iTRAQ proteomics to monitor changes in gene expression and protein abundance respectively.

The genes and proteins whose expression or abundance were significantly increased or decreased in the presence of 500 μ M iberin are summarized in Table 4-2 (up-regulated genes/proteins) and Table 4-3 (down-regulated genes/proteins) respectively.

Table 4-2. List of genes/proteins found to be significantly up-regulated upon iberin treatment. Up-regulation was defined as greater than 5-fold increase in the RNA-Seq result, or a greater than 2-fold increase in the iTRAQ proteomics result. Only genes/proteins showing a significant difference in expression from the control (i.e. p-value < 0.05) were selected.

PA No.	Gene	QS ^a	Description of Product	RNA-sequencing (500 μ M iberin)		iTRAQ Proteomics (500 μ M iberin)	
				Fold Change	P Value	Fold Change	P Value
PA0140	<i>ahpF</i>		Alkyl hydroperoxide reductase subunit F			4.85	<0.01
PA0182			Probable short-chain dehydrogenase	7.6	0.04		
PA0185			Probable permease of ABC transporter	8.9	0.04		
PA0201			Hypothetical protein	25.9	0.01		
PA0202			Probable amidase	20.7	0.03		
PA0283	<i>sbp</i>		Sulfate-binding protein precursor	26.0	0.01		
PA0284			Hypothetical protein	32.9	0.04		
PA0565			Conserved hypothetical protein			2.91	<0.01
PA0849	<i>trxB2</i>		Thioredoxin reductase 2			2.94	0.01
PA0865	<i>hpd</i>		4-hydroxyphenylpyruvate dioxygenase	42.2	0.04	2.75	<0.01
PA0878			Hypothetical protein	7.9	0.04		
PA1240			Probable enoyl-CoA hydratase/isomerase			3.45	<0.01
PA1260			Amino acid ABC transporter periplasmic binding protein			4.49	<0.01
PA1285			Probable transcriptional regulator	5.2	0.05		
PA1310	<i>phnW</i>		2-aminoethylphosphonate:pyruvate aminotransferase	19.3	0.05		
PA1332			Hypothetical protein	17.7	0.05		
PA1334			Probable oxidoreductase	74.7	0.04	5.74	<0.01
PA1493	<i>cysP</i>		Sulfate-binding protein of ABC transporter	8.2	<0.01		
PA1999	<i>dhcA</i>		DhcA, dehydrocarnitine CoA transferase, subunit A	9.1	0.01		
PA2000	<i>dhcB</i>		DhcB, dehydrocarnitine CoA transferase, subunit B	9.5	<0.01		
PA2008	<i>fahA</i>		Fumarylacetoacetase	54.8	0.03		
PA2009	<i>hmgA</i>		Homogentisate 1,2-dioxygenase	62.0	0.02		
PA2197			Conserved hypothetical protein			2.07	<0.01
PA2247	<i>bkdA1</i>		2-oxoisovalerate dehydrogenase (alpha subunit)			2.71	<0.01

Table 4-2 continues overleaf

Table 4-2 continued.

PA No.	Gene	QS ^a	Description of Product	RNA-sequencing (500 μ M iberin)		iTRAQ Proteomics (500 μ M iberin)	
				Fold Change	P Value	Fold Change	P Value
PA2248	<i>bkdA2</i>		2-oxoisovalerate dehydrogenase (beta subunit)			2.98	<0.01
PA2249	<i>bkdB</i>		Branched-chain alpha-keto acid dehydrogenase (lipoamide component)			2.64	<0.01
PA2277	<i>arsR</i>		ArsR protein	15.3	0.05		
PA2278	<i>arsB</i>		ArsB protein	9.6	0.02		
PA2310			Hypothetical protein	20.5	0.03		
PA2311			Hypothetical protein	51.8	0.05		
PA2312			Probable transcriptional regulator	29.6	<0.01		
PA2327		X	Probable permease of ABC transporter	6.6	0.05		
PA2359			Probable transcriptional regulator	34.5	0.03		
PA2444	<i>glyA2</i>		Serine hydroxymethyltransferase	37.4	0.05		
PA2445	<i>gcvP2</i>		Glycine cleavage system protein P2			3.67	<0.01
PA2483			Conserved hypothetical protein			3.30	<0.01
PA2490			Conserved hypothetical protein	16.4	0.05		
PA2491	<i>mexS</i>		MexS	34.1	0.03	5.08	0.01
PA2493	<i>mexE</i>		Resistance-Nodulation-Cell Division (RND) multidrug efflux membrane fusion protein MexE precursor			5.69	<0.01
PA2494	<i>mexF</i>		Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter MexF	129.4	0.04	5.69	<0.01
PA2495	<i>oprN</i>		Multidrug efflux outer membrane protein OprN precursor	102.3	0.03	5.34	<0.01
PA2535			probable oxidoreductase			3.02	<0.01
PA2575			Hypothetical protein	28.5	0.02	4.01	<0.01
PA2579	<i>kynA</i>		L-Tryptophan:oxygen 2,3-oxidoreductase (deacylizing) KynA	5.3	0.05		
PA2580			Conserved hypothetical protein	26.1	0.01	4.58	0.01
PA2594		X	Conserved hypothetical protein	9.0	0.02		
PA2599			Conserved hypothetical protein	12.1	0.05		
PA2600			Hypothetical protein	21.0	<0.01		
PA2610			Conserved hypothetical protein	5.2	0.03		
PA2758			Probable transcriptional regulator	63.7	0.02		
PA2759			Hypothetical protein	179.3	0.01		
PA2767			Probable enoyl-CoA hydratase/isomerase	5.8	0.03		
PA2786			Hypothetical protein	8.6	0.05		
PA2812			Probable ATP-binding component of ABC transporter	20.4	0.03		
PA2813			Probable glutathione S-transferase	32.7	0.04		
PA2844			Conserved hypothetical protein	8.3	0.04		
PA2845			Hypothetical protein	91.2	0.04		
PA2931	<i>cifR</i>		CifR	10.4	0.03		
PA2932	<i>morB</i>		Morphinone reductase	45.7	0.03		
PA3035			Probable glutathione S-transferase	8.0	<0.01		
PA3126	<i>ibpA</i>		heat-shock protein IbpA			4.37	<0.01
PA3222			Hypothetical protein	5.4	0.04		
PA3229			Hypothetical protein	311.5	0.04		
PA3230			Conserved hypothetical protein	69.5	0.01		

Table 4-2 continues overleaf

Table 4-2 continued.

PA No.	Gene	QS ^a	Description of Product	RNA-sequencing (500 μ M iberin)		iTRAQ Proteomics (500 μ M iberin)	
				Fold Change	P Value	Fold Change	P Value
PA3938			Probable periplasmic taurine-binding protein precursor	24.5	0.05		
PA3446			Conserved hypothetical protein	63.5	0.05		
PA3450			Probable antioxidant protein	39.4	0.01		
PA3931			Conserved hypothetical protein	44.7	0.05		
PA4166			Probable acetyltransferase	77.6	0.02		
PA4167	<i>dkgB</i>		Probable oxidoreductase	112.0	<0.01	4.93	<0.01
PA4173			Conserved hypothetical protein	12.4	0.01		
PA4236	<i>katA</i>		Catalase			4.01	<0.01
PA4354			Conserved hypothetical protein				
PA4355	<i>pyeM</i>		PyeM	37.3	<0.01		
PA4356	<i>xenB</i>		Xenobiotic reductase			4.28	<0.01
PA4385	<i>groEL</i>		GroEL protein	6.4	0.04	2.48	<0.01
PA4386	<i>groES</i>		GroES protein	6.4	0.03		
PA4387			Conserved hypothetical protein	8.0	0.05		
PA4613	<i>katB</i>		Catalase			5.10	<0.01
PA4623			Hypothetical protein	727.4	0.01		
PA4973	<i>thiC</i>		Thiamin biosynthesis protein ThiC			2.58	<0.01

^aAn 'X' in the box indicates that the gene/protein is regulated by quorum sensing (Hentzer et al., 2003), in reference to the QS-regulated genes/proteins as determined previously.

Table 4-3. List of genes/proteins found to be significantly down-regulated upon iberin treatment. Down-regulation was defined as greater than 5-fold decrease in the RNA-Seq result, or a greater than 2-fold decrease in the iTRAQ proteomics result. Only genes/proteins showing a significant difference in expression from the control (i.e. p-value < 0.05) were selected.

PA No.	Gene	QS ^a	Description of Product	RNA-seq (500 μ M iberin)		iTRAQ Proteomics (500 μ M iberin)	
				Fold Change	P Value	Fold Change	P Value
PA0059	<i>osmC</i>	X	Osmotically inducible protein OsmC			-2.91	<0.01
PA0081	<i>fha1</i>		Fha1			-2.25	<0.01
PA0084	<i>tssC1</i>		TssC1			-2.01	<0.01
PA0088	<i>tssF1</i>		TssF1			-2.13	<0.01
PA0122	<i>rahU</i>	X	RahU	-32.8	0.05	-5.50	<0.01
PA0176	<i>aer2</i>		Aerotaxis transducer Aer2			-3.67	<0.01
PA0180	<i>cttP</i>		Chemotactic transducer for trichloroethylene [positive chemotaxis], CttP			-2.10	<0.01
PA0523	<i>norC</i>		Nitric-oxide reductase subunit C	-10.9	0.04		
PA0575			Conserved hypothetical protein			-2.18	<0.01

Table 4-3 continues overleaf.

Table 4-3 continued.

PA No.	Gene	QS ^a	Description of Product	RNA-seq (500 μ M iberin)		iTRAQ Proteomics (500 μ M iberin)	
				Fold Change	P Value	Fold Change	P Value
PA0586			Conserved hypothetical protein			-2.17	<0.01
PA0587			Conserved hypothetical protein			-2.38	<0.01
PA0704			Probable amidase			-5.21	<0.01
PA0707	toxR		Transcriptional regulator ToxR	-5.4	0.02		
PA0852	cbpD	X	Chitin-binding protein CbpD precursor	-22.0	0.04	-4.92	<0.01
PA1001	phnA	X	Anthranilate synthase component I	-7.2	0.01		
PA1027			Probable aldehyde dehydrogenase			-3.22	<0.01
PA1127			Probable oxidoreductase			-2.09	<0.01
PA1130	rhlC	X	Rhamnosyltransferase 2	-11.7	0.04		
PA1131		X	Probable major facilitator superfamily (MFS) transporter	-8.9	0.05		
PA1134			Hypothetical protein	-6.0	0.01		
PA1214			Hypothetical protein	-8.2	0.03		
PA1215			Hypothetical protein	-7.7	0.02		
PA1216			Hypothetical protein	-9.0	0.04		
PA1220			Hypothetical protein	-5.4	0.03		
PA1221			Hypothetical protein	-6.8	0.04		
PA1245			Hypothetical protein PA1245			-5.46	<0.01
PA1246	aprD	X	Alkaline protease secretion protein AprD			-2.43	<0.01
PA1247	aprE	X	Alkaline protease secretion protein AprE			-2.99	<0.01
PA1248	aprF	X	Alkaline protease secretion outer membrane protein AprF precursor			-3.84	<0.01
PA1249	aprA	X	Alkaline metalloproteinase precursor			-3.99	<0.01
PA1300			Probable sigma-70 factor, ECF subfamily	-5.5	0.03		
PA1318	cyoB		Cytochrome o ubiquinol oxidase subunit I			-2.15	<0.01
PA1323		X	Hypothetical protein	-6.9	0.03		
PA1324		X	Hypothetical protein	-7.7	0.05	-3.96	<0.01
PA1327			Probable protease			-3.80	<0.01
PA1408			Hypothetical protein PA1408			-2.31	<0.01
PA1529	ligA		DNA ligase			-2.03	<0.01
PA1559			Hypothetical protein	-7.3	0.03		
PA1560			Hypothetical protein	-8.9	0.02		
PA1705	pcrG		Regulator in type III secretion	-7.3	0.03		
PA1706	pcrV		Type III secretion protein PcrV	-6.1	0.03		
PA1708	popB		Translocator protein PopB	-6.7	0.03		
PA1710	exsC		ExsC, exoenzyme S synthesis protein C precursor.	-6.6	0.01		
PA1730			Conserved hypothetical protein			-3.06	<0.01
PA1871	lasA	X	LasA protease precursor	-25.5	0.02		
PA1875		X	Probable outer membrane protein precursor	-5.1	0.04		
PA1906			Hypothetical protein	-20.4	0.01		
PA1912	femI		ECF sigma factor, FemI	-5.1	0.01		
PA1914			Conserved hypothetical protein	-14.0	0.02		
PA1927	metE		5-methyltetrahydropteroyltryglutamate-homocysteine S-methyltransferase			-3.19	<0.01
PA1930			Probable chemotaxis transducer			-2.66	<0.01
PA2067			Probable hydrolase	-16.4	0.04		

Table 4-3 continues overleaf.

Table 4-3 continued.

PA No.	Gene	QS ^a	Description of Product	RNA-seq (500 μ M iberin)		iTRAQ Proteomics (500 μ M iberin)	
				Fold Change	P Value	Fold Change	P Value
PA2068		X	Probable major facilitator superfamily (MFS) transporter	-37.5	0.02		
PA2069		X	Probable carbamoyl transferase	-107.8	0.02	-5.97	<0.01
PA2072			Conserved hypothetical protein			-2.48	<0.01
PA2144	glgP		Glycogen phosphorylase			-2.55	<0.01
PA2151			Conserved hypothetical protein			-3.18	<0.01
PA2152			Probable trehalose synthase			-4.32	<0.01
PA2153	glgB		1,4- α -glucan branching enzyme			-3.44	<0.01
PA2159			Conserved hypothetical protein	-5.4	0.04		
PA2160			Probable glycosyl hydrolase			-4.85	<0.01
PA2162			Probable glycosyl hydrolase			-3.89	<0.01
PA2163			Hypothetical protein PA2163			-3.32	0.03
PA2164			Probable glycosyl hydrolase			-2.15	0.01
PA2167			Hypothetical protein	-5.3	0.02		
PA2169			Hypothetical protein	-6.7	0.03		
PA2170			Hypothetical protein	-9.1	0.04		
PA2177			Probable sensor/response regulator hybrid			-2.35	<0.01
PA2193	hcnA	X	Hydrogen cyanide synthase HcnA	-27.7	0.05		
PA2194	hcnB	X	Hydrogen cyanide synthase HcnB	-15.9	0.01		
PA2195	hcnC	X	Hydrogen cyanide synthase HcnC	-12.6	<0.01		
PA2261			Probable 2-ketogluconate kinase			-3.08	0.04
PA2300	chiC	X	Chitinase	-29.9	0.03	-5.71	<0.01
PA2305	ambB	X	AmbB			-2.30	<0.01
PA2322			Gluconate permease	-11.4	0.02		
PA2345			Conserved hypothetical protein			-2.60	<0.01
PA2363			Hypothetical protein PA2363			-2.41	0.01
PA2371			Probable ClpA/B-type protease			-2.78	<0.01
PA2377			Hypothetical protein	-8.5	0.05		
PA2384			Hypothetical protein	-7.4	<0.01		
PA2414		X	L-sorbose dehydrogenase	-10.5	0.02		
PA2415		X	Hypothetical protein	-8.6	0.02		
PA2416	treA		Periplasmic trehalase precursor			-2.79	0.04
PA2427			Hypothetical protein	-6.9	0.01		
PA2448			Hypothetical protein PA2448			-3.16	<0.01
PA2566		X	Conserved hypothetical protein	-5.9	0.04		
PA2570	lecA	X	LecA	-38.9	0.03		
PA2573			Probable chemotaxis transducer			-2.94	<0.01
PA2587	pqsH	X	Probable FAD-dependent monooxygenase			-2.19	<0.01
PA2588		X	Probable transcriptional regulator	-8.9	0.02		
PA2717	cpo		Chloroperoxidase precursor			-2.88	0.01
PA2788			Probable chemotaxis transducer	-5.8	0.03	-2.64	<0.01
PA2815	fadE		Probable acyl-CoA dehydrogenase			-2.51	<0.01
PA2920			Probable chemotaxis transducer			-2.03	<0.01
PA3040			Conserved hypothetical protein			-2.15	0.03
PA3118	leuB		3-isopropylmalate dehydrogenase			-2.15	<0.01
PA3155	wbpE		UDP-2-acetamido-2-dideoxy-d-ribo-hex-3-uluronic acid transaminase, wbpE			-2.03	<0.01
PA3305.1	phrS		PhrS	-6.5	0.05		

Table 4-3 continues overleaf.

Table 4-3 continued.

PA No.	Gene	QS ^a	Description of Product	RNA-seq (500 μ M iberin)		iTRAQ Proteomics (500 μ M iberin)	
				Fold Change	P Value	Fold Change	P Value
PA3326	clpP2	X	ClpP2	-12.7	0.04	-4.97	0.01
PA3327	fabH2	X	Probable non-ribosomal peptide synthetase	-8.1	0.02	-4.69	<0.01
PA3328	fabH2	X	Probable FAD-dependent monooxygenase	-27.0	0.01	-4.93	<0.01
PA3329	fabH2	X	Hypothetical protein	-11.4	0.04	-5.28	<0.01
PA3330	fabH2	X	Probable short chain dehydrogenase	-31.5	0.03		
PA3331	fabH2	X	Cytochrome P450	-15.9	0.03	-4.61	<0.01
PA3332	fabH2	X	Conserved hypothetical protein	-19.5	0.03	-3.07	<0.01
PA3333	fabH2	X	3-oxoacyl-[acyl-carrier-protein] synthase III	-19.0	0.03		
PA3334		X	Probable acyl carrier protein	-14.0	0.02		
PA3346			probable two-component response regulator			-2.35	<0.01
PA3361	lecB	X	Fucose-binding lectin PA-IIL	-35.3	0.02		
PA3408	hasR		Heme uptake outer membrane receptor HasR precursor			-2.71	<0.01
PA3418	ldh		Leucine dehydrogenase			-2.42	<0.01
PA3431			Conserved hypothetical protein	-6.1	0.05		
PA3460			Probable acetyltransferase			-3.61	<0.01
PA3461			Conserved hypothetical protein			-3.91	<0.01
PA3477	rhIR	X	Transcriptional regulator RhIR	-5.7	<0.01		
PA3478	rhIB	X	Rhamnosyltransferase chain B	-27.6	<0.01	-5.47	<0.01
PA3479	rhIA	X	Rhamnosyltransferase chain A	-110.4	<0.01		
PA3520		X	Hypothetical protein				
PA3554	arnA		ArnA			-2.56	<0.01
PA3613			Hypothetical protein PA3613			-2.14	<0.01
PA3621.1	rsmZ		Regulatory RNA RsmZ	-4.0	0.04		
PA3691		X	Hypothetical protein PA3691			-3.43	<0.01
PA3692	lptF	X	Lipotoxon F, LptF			-2.23	<0.01
PA3724	lasB	X	Elastase LasB	-124.4	0.02		
PA3734			Hypothetical protein	-5.6	0.05		
PA3923			Hypothetical protein			-2.07	<0.01
PA3974	ladS		Lost Adherence Sensor, LadS			-2.03	0.01
PA4078			Probable nonribosomal peptide synthetase			-3.60	<0.01
PA4112			Probable sensor/response regulator hybrid			-2.82	<0.01
PA4141		X	Hypothetical protein	-144.7	0.02		
PA4142		X	Probable secretion protein	-43.3	0.01	-2.15	0.03
PA4143			Probable toxin transporter	-20.8	0.02	-5.39	<0.01
PA4144			Probable outer membrane protein precursor	-16.2	<0.01		
PA4156			Probable TonB-dependent receptor	-11.5	<0.01		
PA4159	fepB		Ferrienterobactin-binding periplasmic protein precursor FepB	-9.7	0.02		
PA4160	fepD		Ferric enterobactin transport protein FepD	-5.0	0.03		
PA4175	piv	X	Protease IV	-24.3	<0.01	-2.56	<0.01
PA4207	mexI		Probable Resistance-Nodulation-Cell Division (RND) efflux transporter			-2.94	<0.01
PA4209	phzM	X	Probable phenazine-specific methyltransferase	-25.1	0.02		
PA4213	phzD1		Phenazine biosynthesis protein PhzD	-71.8	0.05		
PA4217	phzS	X	Flavin-containing monooxygenase			-4.69	0.02
PA4228	pchD		Pyochelin biosynthesis protein PchD			-2.92	<0.01

Table 4-3 continues overleaf.

Table 4-3 continued.

PA No.	Gene	QS ^a	Description of Product	RNA-seq (500 μ M iberin)		iTRAQ Proteomics (500 μ M iberin)	
				Fold Change	P Value	Fold Change	P Value
PA4230	pchB		Salicylate biosynthesis protein PchB			-3.15	<0.01
PA4362			Hypothetical protein PA4362			-2.37	<0.01
PA4471			Hypothetical protein	-16.0	0.04		
PA4570			Hypothetical protein	-5.7	0.01		
PA4624			Hypothetical protein PA4624			-2.62	<0.01
PA4785			Probable acyl-CoA thiolase			-2.21	<0.01
PA4835			Hypothetical protein PA4835			-2.58	<0.01
PA4836			Hypothetical protein PA4836			-2.33	<0.01
PA5058	phaC2	X	Poly(3-hydroxyalkanoic acid) synthase 2			-4.42	<0.01
PA5061			Conserved hypothetical protein			-4.40	0.01
PA5113			Hypothetical protein			-4.40	0.01
PA5171	arcA		Arginine deiminase			-2.23	<0.01
PA5213	gcvP1		Glycine cleavage system protein P1			-3.27	<0.01
PA5220		X	Hypothetical protein	-8.5	0.01		

^aAn 'X' in the box indicates that the gene/protein is regulated by quorum sensing (Hentzer et al., 2003), in reference to the QS-regulated genes/proteins as determined previously.

For the RNA-Seq result, 62 genes had increased expression (more than 5-fold) and 79 genes had decreased expression (more than 5-fold) in the presence of iberin. In the iTRAQ proteomics result, 27 proteins had increased abundance (more than 2-fold) and 92 proteins had decreased abundance (more than 2-fold). For these results in Table 4-2 and 4-3, we had determined an arbitrary cut-off of +5.0 and -5.0 fold change for up-regulation and down-regulation of gene expression respectively in the RNA-Seq result. Nevertheless, we have included in Appendix D all the genes in the RNA-Seq result with more than a +2.0 or -2.0 fold change. The complete lists of the 145 up-regulated and 258 down-regulated genes in the RNA-Seq result are presented in Appendix D as Supplementary Tables D-1 and D-2 respectively.

4.3.3 Analysis of the inhibition of small regulatory RNAs by iberin

One valuable advantage of the RNA-Seq method as compared to the DNA microarray method used previously (Jakobsen et al., 2012b) is that the former is able to monitor the expression of small regulatory RNAs. Our RNA-Seq result showed that the expression of two small regulatory RNAs, encoded by the *phrS* (PA3305.1) and *rsmZ* (PA3621.1) genes, were

repressed by iberin treatment by 6.5 fold and 4.0 fold respectively as compared to the untreated control (Table 4-3, highlighted in bold font). PhrS and RsmZ have been reported to positively regulate alkylquinolone-based QS and AHL-based QS systems respectively (Kay et al., 2006; Sonnleitner et al., 2011). A $\Delta rsmY\Delta rsmZ$ mutant was shown to have deficient synthesis of the exoproducts hydrogen cyanide, pyocyanin, elastase, chitinase, and chitin-binding protein (Kay et al., 2006). This phenotype agrees with our RNA-Seq results showing that iberin inhibited the transcription of genes involved in the production of those exoproducts. Hence we wanted to confirm whether iberin inhibited *rsmY/rsmZ* expression.

In order to test the inhibitory effects of iberin on *rsmY* and *rsmZ* expression, PAO1 strains containing *rsmY-gfp* or *rsmZ-gfp* plasmids were cultured in the presence of varying concentrations of iberin. Our results showed that iberin inhibited the expression of *rsmY* and *rsmZ* in a dose-dependent manner (Figure 4-3); however, high levels of iberin were slightly growth-inhibitory.

Figure 4-3. Growth and normalized *gfp* gene expression (RFU divided by OD₄₅₀) of the *P. aeruginosa* PAO1 biosensor strains carrying *rsmY-gfp* (A, C) and *rsmZ-gfp* (B, D) in ABTGC medium containing varying concentrations of iberin. Every 30 minutes, growth and *gfp* fluorescence were measured using OD₄₅₀ values and relative fluorescence units (RFU) respectively. Experiments were performed in triplicate; only representative ones are shown.

4.3.4 Inhibition of pyoverdine expression by iberin

A study by Frangipani *et al.* (2014) has shown that RsmY and RsmZ sequester RsmA; a situation that allows production of the siderophores, pyochelin and pyoverdine (Frangipani *et al.*, 2014). Figure 4-4 illustrates the possible link between the Gac/Rsm system and siderophore production as suggested by their study. A reduction of RsmY and RsmZ levels by iberin, would in theory lead to higher amounts of RsmA, lower c-di-GMP levels, and reduced siderophore production (e.g. pyoverdine and pyochelin).

Figure 4-4. A suggested model that links the relationship between the Gac/Rsm system, c-di-GMP and siderophore (e.g. pyoverdine production). Image adapted from paper by Frangipani et al. (2014).

Pyoverdine is important for development of the characteristic mushroom like structures of *P. aeruginosa* biofilms (Yang et al., 2009a) and is also believed to play a role in its virulence, for example in the production of the virulence factor pyocyanin (Meyer et al., 1996). Pyoverdine is also known to be activated by the *las* QS system (Stintzi et al., 1998).

Hence we were interested to find out whether iberin could affect pyoverdine production. *P. aeruginosa* PAO1 wild-type strain containing the *pvdA-gfp* reporter was cultured in varying concentrations of iberin. Iberin was able to inhibit the expression of the *pvdA-gfp* gene and the production of pyoverdine (Fig. 4-5B and 4-5C); indicating that inhibition of pyoverdine synthesis occurs on a transcriptional level, mediated through released RsmA protein, which is usually sequestered by the small regulatory RNA molecules. This is supported by our RNA-Seq result, in which the expression of the pyoverdine biosynthesis genes *pvdA*, *pvdP*, *pvdG* and *pvdS* genes were reduced by -3.2, -3.6, -3.9 and -3.6 fold respectively (Appendix D, Supplementary Table D-2).

Figure 4-5. Growth (A), normalized *gfp* expression (B), and normalized pyoverdine production (C) of the *P. aeruginosa* PAO1 biosensor strain carrying *pvdA-gfp* in ABTGC medium containing varying concentrations of iberin. Every 30 minutes, growth, *gfp* fluorescence, and pyoverdine production were measured using OD₆₀₀ values and relative fluorescence units (RFU) respectively. Normalization was calculated as RFU divided by OD₆₀₀. Experiments were performed in triplicate; only representative ones are shown.

4.3.5 Iberin reduces *P. aeruginosa* biofilm formation in a slide biofilm assay

The exopolysaccharides, Pel and Psl, along with alginate are important components of the extracellular matrix of *P. aeruginosa* biofilms (Ryder et al., 2007). It has been shown that both the *pel* and *psl* polysaccharide genes are post-transcriptionally regulated by RsmY and RsmZ (Goodman et al., 2004; Kay et al., 2006). As such, we performed a slide biofilm assay in order to determine if iberin was able to reduce biofilm formation by *P. aeruginosa*, through the down-regulation of *pel* and *psl*.

Figure 4-6 shows that the sample treated with 500 μM iberin had significantly less biofilm formation versus the control sample in ABTGC. This suggests that iberin may be used not only as an anti-pathogenic agent but also as an anti-biofilm agent.

Figure 4-6. Three-dimensional confocal images of 1-day-old miniTn7-gfp-tagged *P. aeruginosa* PAO1 slide biofilms with either ABTGC medium (control, top panel) or 500 μM iberin (bottom panel). Images were obtained using confocal microscopy at 63x magnification (ZEISS LSM780 Confocal System), and analysed using the Imaris software package (Bitplane, AG). Only a representative image of three replicates is shown.

4.4 Discussion

4.4.1 RNA-Seq and iTRAQ proteomics result with iberin

Iberin was able to affect the expression of genes regulated by QS, with reference to the list of QS-regulated genes in Hentzer's study (Hentzer et al., 2003); these are marked with an 'X' in Tables 4-2 and 4-3. Among the genes/proteins that had significantly increased expression/abundance in the presence of iberin, only 3% of the up-regulated genes (2 of 62) in the RNA-Seq result were QS-regulated, which supported the previous view of iberin acting as a QSI and not a QS-activator. None of the 27 up-regulated proteins in the iTRAQ proteomics result was related to QS control. Among the genes/proteins that had significantly decreased expression/abundance in the presence of iberin, 49% of the down-regulated genes (39 of 79) in the RNA-Seq result and 27% of the down-regulated proteins (25 of 92) in the iTRAQ proteomics result were QS-regulated.

Surprisingly, the groups of genes and proteins up or down-regulated by iberin as identified by either RNA-Seq or iTRAQ proteomic analysis do not share much in common. Only 9 of the 81 unique significantly up-regulated genes/proteins (11%) and 16 of the 157 unique significantly down-regulated genes/proteins (10%) were detected by both methods. Among the 16 down-regulated genes/proteins, 14 (i.e. 88%) were subject to QS control, important examples being chitinase (*chiC*), protease IV (*piv*) and rhamnosyl transferase chain B (*rhlB*). This finding suggests the strong inhibition of these particular QS-regulated virulence factors by iberin on both the transcriptional and post-transcriptional/translational level.

Suppression of many known QS-regulated genes (Hentzer et al., 2003) was only observed by the RNA-Seq and not by means of the iTRAQ proteomics analysis, and vice versa (Table 4-3). For example, the decreased expression of genes encoding hydrogen cyanide synthase (*hcnA*/PA2193, *hcnB*/PA2194 and *hcnC*/PA2195), *lecA* and *lecB* (PA2570/PA3361), rhamnosyltransferase chain A (*rhlA*/PA3479) and elastase (*lasB*/PA3724) was mainly reflected in the RNA-Seq result but not in the proteomics result (Table 4-3). This indicates that expression of these virulence factors were strongly inhibited by iberin on the transcriptional level which would also be expected to manifest as a reduction in the protein content. However, the missing correlation to the proteomic analysis suggests that these proteins were below the detection limit

of the iTRAQ method. In fact, our iTRAQ protocol only allowed for detection (with 99% confidence) of a total of 1948 proteins. Elastase (LasB), was only detected in the RNA-Seq (-124.4 fold decrease) and not in the proteomics analysis, which agrees with our previous paper in which iTRAQ proteomics was used to study the effects of the QSI, 5-imino-4,6-dihydro-3H-1,2,3-triazolo[5,4-d]pyrimidin-7-one, on *P. aeruginosa* (Tan et al., 2013b). In that study, elastase was also not detected through proteomics. Since only the cells and not the surrounding medium were subjected to iTRAQ analysis, this extracellular enzyme was likely not to be present in high enough amounts intracellularly to be detected. We put forward that this explanation is also valid for Lectin (LecA, LecB).

Our results also show that iberin was able to inhibit the expression of multiple components of the highly complex type III secretion system (T3SS) of *P. aeruginosa*, a system which is important in pathogenesis (Hauser, 2009). Figure 4-7 illustrates the various genes, components and transcriptional control of the T3SS in *P. aeruginosa*.

Gene expression of the effector proteins ExoT and ExoS toxins known to impair phagocytosis by host immune cells (Frithz-Lindsten et al., 1997; Garrity-Ryan et al., 2000) were reduced by -2.0 and -3.0 fold in the RNA-Seq result (Appendix D, Supplementary Table D-2). Also, expression of the genes for the T3SS regulatory proteins ExsB, ExsD, ExsC and ExsE (Yahr and Wolfgang, 2006) were reduced by -3.5, -6.6, -3.2, and -4.4 fold respectively. Expression of the genes for needle components PscC and PscG were reduced -2.1 and -2.6 respectively. Lastly, gene expression of the translocator protein PopB (Schoehn et al., 2003), was decreased -6.7 fold in the RNA-Seq result.

Figure 4-7. An overview of the type III secretion system (T3SS) in *P. aeruginosa*. (a) Genes involved in *P. aeruginosa* T3SS. (b) Five components of the T3SS: effector proteins, chaperone proteins, translocation proteins, regulatory proteins and needle complex and other proteins. (c) When secretion is turned off, *ExsE* binds *ExsC*, which results in *ExsA-ExsD* binding and no transcription of T3SS genes. When secretion is activated, *ExsE* is secreted from the cell, which results in *ExsC-ExsD* binding, and the freeing of *ExsA* to promote the transcription of T3SS genes. Image taken from Hauser et al. (2009).

In the previous study by Jakobsen *et al.*, they showed that iberin was able to compete effectively with BHL for binding to RhIR, with the expression of *rhlR*, *rhlB* and *rhlA* reduced by -5.3, -42.1 and -59.0 fold respectively upon the addition of 64 µg/ml iberin to PAO1 as determined by DNA microarray (Jakobsen *et al.*, 2012b). Also, iberin inhibited rhamnolipid production in a concentration-dependent manner, with a complete inhibition at a concentration of 200 µM iberin. Similarly, our results showed that the expression of the *rhlR*, *rhlB* and *rhlA* genes were significantly decreased in the RNA-Seq result by -5.7, -27.6 and -110.4 fold respectively. Our iTRAQ result only showed RhIB being significantly down-regulated (-5.47 fold).

In the previous study, the expression of *lasB* was found to be decreased by 89.8 fold upon the addition of 64 µg/ml iberin to PAO1 as determined by DNA microarray. However, they also showed that iberin was unable to competitively inhibit OdDHL driven expression of the *lasB-gfp* fusion when present in an *E. coli* background (Jakobsen *et al.*, 2012b). In the present study, neither the transcriptomic nor proteomic analysis showed iberin inhibiting the expression of the major QS regulator, LasR. These findings taken together strongly suggest that iberin is not targeting LasR; neither does it compete with OdDHL for binding to LasR. Despite this, the *lasB* gene, whose expression is positively regulated by LasR, was found to be reduced by 124.4 fold in the RNA-Seq result. Thus, comparison of the mRNA and corresponding protein content does not reveal the mechanisms by which iberin inhibit the *las* QS-controlled gene expression.

4.4.2 Iberin as an inhibitor of small RNAs

The expression of the GacA-dependent small RNAs, *rsmY* and *rsmZ*, is essential for AHL-mediated QS and virulence in *P. aeruginosa* (Kay *et al.*, 2006). Previous works in elucidating QS inhibitory mechanisms have already reported that the QS inhibitor, azithromycin, inhibits the expression of *rsmY* and *rsmZ* transcription through the PA0588-PA0584 gene cluster (Perez-Martinez and Haas, 2011). However, our transcriptomic data did not show that iberin affected the expression of the PA0588-PA0584 gene cluster, which suggests that iberin uses a different mechanism from azithromycin to inhibit the expression of *rsmY* and *rsmZ*.

Our proteomics result shows that iberin led to a 2-fold decrease in the abundance of the Lost Adherence Sensor, LadS (PA3974). LadS is a sensor kinase that phosphorylates GacS, which in turn is responsible for activating GacA and the expression of *rsmY* and *rsmZ* (Ventre et al., 2006). This result suggests that iberin inhibits the expression of LadS post-transcriptionally, which in turn leads to reduced activation of GacS, thus reducing RsmY and RsmZ levels.

In addition to the RsmY and RsmZ, PhrS was also reported to be an important small regulatory RNA that can stimulate synthesis of the *Pseudomonas* quinolone signal via activating PqsR (Sonnleitner et al., 2011). PQS is well known to regulate synthesis of the virulence factors pyocyanin and rhamnolipid and release of extracellular DNA (Diggle et al., 2006; Yang et al., 2007). In our RNA-Seq result, we see that iberin reduced the expression of *phrS* by -6.5 fold. Also, the expression of the *pqs* operon (PA0996-PA1001) was reduced by 3 to 4 fold and the phenazine biosynthesis genes (*phnA* and *phnB*) by 7.2 and 4.8 fold respectively (Appendix D, Supplementary Table D-2). As such, iberin has an inhibitory effect on the expression of the *pqs* QS system and phenazine biosynthesis genes.

4.5 Summary

Our study shows that both of the systems biology methods used (i.e. RNA-sequencing and proteomics) complement each other and provide a thorough overview of the impact of iberin on *P. aeruginosa*. RNA sequencing-based transcriptomics showed that iberin inhibits the expression of the GacA-dependent small regulatory RNAs RsmY and RsmZ; this was verified by using *gfp*-based transcriptional reporter fusions comprising the *rsmY* or *rsmZ* promoter regions. iTRAQ proteomics showed that iberin reduces the abundance of the LadS protein, an activator of GacS. Taken together, the findings suggest that iberin's mode of QS inhibition is through down-regulation of the Gac/Rsm QS network which in turn leads to repression of QS-regulated virulence factors such as pyoverdine, chitinase and protease IV.

Lastly, as expected from the observed repression of small regulatory RNA synthesis, we also show that iberin effectively reduces biofilm formation. This suggests that small regulatory RNAs might serve as potential targets for the future development of therapies against pathogens that use QS for controlling virulence factor expression and assume the biofilm mode of growth in the process of causing disease.

5 Conclusion

During the course of my PhD research, I have used next-generation sequencing and system biology approaches to study extreme antibiotic resistance, identified several novel QSIs, and also discovered that small regulatory RNAs can be a potential target for anti-pathogenic agents. These findings may lay the foundation for future antimicrobial therapies.

Firstly, I used a combination of whole genome sequencing and proteomics in order to study two *Acinetobacter baumannii* strains – one showed extensive drug resistance, while the other showed pandrug resistance. Colistin resistance emerged within a short one month interval between these two strains; this may be explained by the non-synonymous single nucleotide polymorphisms detected in the *ampC*, *gyrB* and *parC* genes of the colistin-resistant *A. baumannii* 53064 strain. iTRAQ proteomics showed that the production of a putative porin was up-regulated in this PDR *A. baumannii* 53064 strain in response to the antibiotics ceftazidime, tobramycin and colistin. As such, this suggests potential novel antimicrobial strategies revolving round the inhibition of porin expression in *A. baumannii* strains.

Secondly, I showed that structure-based virtual screening is indeed a fast and cost-effective means for the discovery of novel QSIs. By screening a library of 3,040 natural compounds, five small molecule compounds were found to have dose-dependent inhibition of the *las* QS system. One compound, in particular, G1, was able to inhibit LasR specifically. G1 was able to down-regulate the activity of the *pqs* QS system, and reduced the secretion of extracellular DNA, an important component of the EPS matrix. Proteomics experiments also showed that G1 was able to reduce the expression of several QS-regulated virulence factors in *P. aeruginosa*. As such, these results support the application of SB-VS for the discovery of novel antimicrobial candidates.

Lastly, RNA-sequencing and proteomics in combination with systems biology approaches, were used to uncover a novel QS inhibitory mechanism of iberin, a natural compound derived from horseradish. We found that iberin was able to reduce virulence factor expression through the inhibition of the small regulatory RNAs RsmY and RsmZ. The global effects on virulence factor expression exerted by modulating the amounts of RsmY and RsmZ indicates that small RNAs are a potential antimicrobial target.

As such, I believe that many of the recently developed tools available to us today such as genomics, *in silico* screening, transcriptomics and proteomics, can and should be used in a concerted fashion in the quest for novel antimicrobial strategies in the war on bacteria.

6 References

- Adonizio, A.L., Downum, K., Bennett, B.C., Mathee, K., 2006. Anti-quorum sensing activity of medicinal plants in southern Florida. *Journal of Ethnopharmacology* 105, 427-435.
- Alanis, A.J., 2005. Resistance to antibiotics: are we in the post-antibiotic era? *Archives of Medical Research* 36, 697-705.
- Allesen-Holm, M., Barken, K.B., Yang, L., Klausen, M., Webb, J.S., Kjelleberg, S., Molin, S., Givskov, M., Tolker-Nielsen, T., 2006. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Molecular Microbiology* 59, 1114-1128.
- Ambrosi, C., Leoni, L., Visca, P. 2002. Different responses of pyoverdine genes to autoinduction in *Pseudomonas aeruginosa* and the group *Pseudomonas fluorescens-Pseudomonas putida*. *Applied and Environmental Microbiology* 68, 4122-4126.
- Andersen, J.B., Sternberg, C., Poulsen, L.K., Bjørn, S.P., Givskov, M., Molin, S., 1998. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Applied and Environmental Microbiology* 64, 2240-2246.
- Annapoorani, A., Umamageswaran, V., Parameswari, R., Pandian, S.K., Ravi, A.V., 2012. Computational discovery of putative quorum sensing inhibitors against LasR and RhIR receptor proteins of *Pseudomonas aeruginosa*. *Journal of Computer Aided Molecular Design* 26, 1067-1077.
- Aziz, R., Bartels, D., Best, A., DeJongh, M., Disz, T., Edwards, R., Formsma, K., Gerdes, S., Glass, E., Kubal, M., 2008. The RAST Server: rapid annotations using subsystems technology. *BMC genomics* 9, 75.
- Bartual, S.G., Seifert, H., Hippler, C., Luzon, M.A., Wisplinghoff, H., Rodriguez-Valera, F., 2005. Development of a multilocus sequence typing scheme for characterization of clinical isolates of *Acinetobacter baumannii*. *Journal of Clinical Microbiology* 43, 4382-4390.
- Beceiro, A., Llobet, E., Aranda, J., Bengoechea, J.A., Doumith, M., Hornsey, M., Dhanji, H., Chart, H., Bou, G., Livermore, D.M., 2011. Phosphoethanolamine modification of lipid A in colistin-resistant variants of *Acinetobacter baumannii* mediated by the pmrAB two-component regulatory system. *Antimicrobial Agents and Chemotherapy* 55, 3370-3379.

Bjarnsholt, T., Jensen, P.Ø., Burmølle, M., Hentzer, M., Haagensen, J.A.J., Hougen, H.P., Calum, H., Madsen, K.G., Moser, C., Molin, S., 2005a. *Pseudomonas aeruginosa* tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent. *Microbiology* 151, 373-383.

Bjarnsholt, T., Jensen, P.O., Rasmussen, T.B., Christophersen, L., Calum, H., Hentzer, M., Hougen, H.P., Rygaard, J., Moser, C., Eberl, L., Hoiby, N., Givskov, M., 2005b. Garlic blocks quorum sensing and promotes rapid clearing of pulmonary *Pseudomonas aeruginosa* infections. *Microbiology* 151, 3873-3880.

Block, E., 2010. Garlic and Other Alliums. The Lore and the Science Publisher. Royal Society of Chemistry, Cambridge, UK.

Bordi, C., Lamy, M.C., Ventre, I., Termine, E., Hachani, A., Fillet, S., Roche, B., Bleves, S., Mejean, V., Lazdunski, A., Filloux, A., 2010. Regulatory RNAs and the HptB/RetS signalling pathways fine-tune *Pseudomonas aeruginosa* pathogenesis. *Molecular Microbiology* 76, 1427-1443.

Bottomley, M.J., Muraglia, E., Bazzo, R., Carfi, A., 2007. Molecular insights into quorum sensing in the human pathogen *Pseudomonas aeruginosa* from the structure of the virulence regulator LasR bound to its autoinducer. *Journal of Biological Chemistry* 282, 13592-13600.

Bradford, P.A., 2001. Extended-spectrum β -lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews* 14, 933-951.

Carvalho, K.R., Carvalho-Assef, A.P.D.A., Peirano, G., Santos, L.C.G.d., Pereira, M.J.F., Asensi, M.D., 2009. Dissemination of multidrug-resistant *Acinetobacter baumannii* genotypes carrying *bla*_{OXA-23} collected from hospitals in Rio de Janeiro, Brazil. *International journal of antimicrobial agents* 34, 25-28.

Choi, K.H., Kumar, A., Schweizer, H.P., 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. *Journal of Microbiological Methods* 64, 391-397.

Chua, S.L., Tan, S.Y., Rybtke, M.T., Chen, Y., Rice, S.A., Kjelleberg, S., Tolker-Nielsen, T., Yang, L., Givskov, M., 2013. Bis-(3'-5')-cyclic dimeric GMP regulates antimicrobial peptide resistance in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* 57, 2066-2075.

Chua, S.L., Liu, Y., Yam, J.K.H., Chen, Y., Vejborg, R.M.V., Tan, B.G.C., Kjelleberg, S., Tolker-Nielsen, T., Givskov, M., Yang, L., 2014. Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles. *Nature Communications* 5.

Clark, D.J., Maaloe, O., 1967. DNA replication and the division cycle in *Escherichia coli*. *Journal of Molecular Biology* 23, 99-112.

Clarke, M.B., Hughes, D.T., Zhu, C., Boedeker, E.C., Sperandio, V., 2006. The QseC sensor kinase: a bacterial adrenergic receptor. *Proceedings of the National Academy of Sciences* 103, 10420-10425.

Clatworthy, A.E., Pierson, E., Hung, D.T., 2007. Targeting virulence: a new paradigm for antimicrobial therapy. *Nature Chemical Biology* 3, 541-548.

Conaway, C.C., Yang, Y.M., Chung, F.L., 2002. Isothiocyanates as cancer chemopreventive agents: their biological activities and metabolism in rodents and humans. *Current Drug Metabolism* 3, 233-255.

Corvec, S., Caroff, N., Espaze, E., Giraudeau, C., Drugeon, H., Reynaud, A., 2003. AmpC cephalosporinase hyperproduction in *Acinetobacter baumannii* clinical strains. *Journal of Antimicrobial Chemotherapy* 52, 629-635.

Costerton, J.W., Geesey, G.G., Cheng, K.J., 1978. How bacteria stick. *Scientific American* 238, 86-95.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., Lappin-Scott, H.M., 1995. Microbial biofilms. *Annual Reviews in Microbiology* 49, 711-745.

Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial biofilms: a common cause of persistent infections. *Science* 284, 1318-1322.

Cunliffe, H.E., Merriman, T.R., Lamont, I.L., 1995. Cloning and characterization of *pvdS*, a gene required for pyoverdine synthesis in *Pseudomonas aeruginosa*: PvdS is probably an alternative sigma factor. *Journal of Bacteriology* 177, 2744-2750.

Darling, A.E., Mau, B., Perna, N.T., 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLOS ONE* 5, e11147.

Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J., Greenberg, E., 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280, 295-298.

de Kievit, T.R., Iglewski, B.H., 2000. Bacterial quorum sensing in pathogenic relationships. *Infection and Immunity* 68, 4839-4849.

de Nys, R., Wright, A.D., König, G.M., Sticher, O., 1993. New halogenated furanones from the marine alga *delisea pulchra* (cf. *fimbriata*). *Tetrahedron* 49, 11213-11220.

de Nys, R., Steinberg, P., Willemsen, P., Dworjanyn, S., Gabelish, C., King, R., 1995. Broad spectrum effects of secondary metabolites from the red alga *Delisea pulchra* in antifouling assays. *Biofouling* 8, 259-271.

Diancourt, L., Passet, V., Nemec, A., Dijkshoorn, L., Brisse, S., 2010. The Population Structure of *Acinetobacter baumannii*: Expanding Multiresistant Clones from an Ancestral Susceptible Genetic Pool. *PLOS ONE* 5, e10034.

Diggle, S.P., Cornelis, P., Williams, P., Camara, M., 2006. 4-quinolone signalling in *Pseudomonas aeruginosa*: old molecules, new perspectives. *International journal of medical microbiology : International Journal of Medical Microbiology* 296, 83-91.

Diggle, S.P., Crusz, S.A., Cámara, M., 2007. Quorum sensing. *Current biology: CB* 17, R907.

Dijkshoorn, L., Aucken, H., Gerner-Smidt, P., Janssen, P., Kaufmann, M.E., Garaizar, J., Ursing, J., Pitt, T.L., 1996. Comparison of outbreak and nonoutbreak *Acinetobacter baumannii* strains by genotypic and phenotypic methods. *Journal of Clinical Microbiology* 34, 1519-1525.

Dijkshoorn, L., Nemec, A., Seifert, H., 2007. An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nature Reviews Microbiology* 5, 939-951.

Dobretsov, S., Teplitski, M., Paul, V., 2009. Mini-review: quorum sensing in the marine environment and its relationship to biofouling. *Biofouling* 25, 413-427.

Doman, T.N., McGovern, S.L., Witherbee, B.J., Kasten, T.P., Kurumbail, R., Stallings, W.C., Connolly, D.T., Shoichet, B.K., 2002. Molecular docking and high-throughput screening for novel inhibitors of protein tyrosine phosphatase-1B. *Journal of Medicinal Chemistry* 45, 2213-2221.

Donlan, R.M., 2001. Biofilms and device-associated infections. *Emerging infectious diseases* 7, 277-281.

Donlan, R.M., Costerton, J.W., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews* 15, 167-193.

Dufour, V., Alazzam, B., Ermel, G., Thepaut, M., Rossero, A., Tresse, O., Baysse, C., 2012. Antimicrobial activities of isothiocyanates against *Campylobacter jejuni* isolates. *Frontiers in Cellular and Infection Microbiology* 2, 53.

Dunkel, M., Fullbeck, M., Neumann, S., Preissner, R., 2006. SuperNatural: a searchable database of available natural compounds. *Nucleic Acids Research* 34, D678-D683.

Eaves, D.J., Randall, L., Gray, D.T., Buckley, A., Woodward, M.J., White, A.P., Piddock, L.J., 2004. Prevalence of mutations within the quinolone resistance-determining region of *gyrA*, *gyrB*, *parC*, and *parE* and association with antibiotic resistance in quinolone-resistant *Salmonella enterica*. *Antimicrobial Agents and Chemotherapy* 48, 4012-4015.

Eberl, L., Winson, M.K., Sternberg, C., Stewart, G.S., Christiansen, G., Chhabra, S.R., Bycroft, B., Williams, P., Molin, S., Givskov, M., 1996. Involvement of N-acyl-L-homoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. *Molecular Microbiology* 20:127-136.

Engel, L.S., Hill, J.M., Caballero, A.R., Green, L.C., O'Callaghan, R.J., 1998. Protease IV, a unique extracellular protease and virulence factor from *Pseudomonas aeruginosa*. *Journal of Biological Chemistry* 273, 16792-16797.

Falagas, M.E., Kasiakou, S.K., Saravolatz, L.D., 2005. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clinical Infectious Diseases* 40, 1333.

Falagas, M.E., Karageorgopoulos, D.E., 2008. Pandrug Resistance (PDR), Extensive Drug Resistance (XDR), and Multidrug Resistance (MDR) among Gram-Negative Bacilli: Need for International Harmonization in Terminology. *Clinical Infectious Diseases* 46, 1121-1122.

Finnan, S., Morrissey, J.P., O'gara, F., Boyd, E.F., 2004. Genome diversity of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients and the hospital environment. *Journal of Clinical Microbiology* 42, 5783-5792.

Fournier, P.E., Richet, H., Weinstein, R.A., 2006. The epidemiology and control of *Acinetobacter baumannii* in health care facilities. *Clinical Infectious Diseases* 42, 692-699.

Frangipani, E., Visaggio, D., Heeb, S., Kaefer, V., Camara, M., Visca, P., Imperi, F., 2014. The Gac/Rsm and cyclic-di-GMP signalling networks coordinately regulate iron uptake in *Pseudomonas aeruginosa*. *Environmental Microbiology* 16, 676-688.

Friesner, R.A., Banks, J.L., Murphy, R.B., Halgren, T.A., Klicic, J.J., Daniel, T., Repasky, M.P., Knoll, E.H., Shelley, M., Perry, J.K., 2004. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *Journal of Medicinal Chemistry* 47, 1739-1749.

Frithz-Lindsten, E., Du, Y., Rosqvist, R., Forsberg, A., 1997. Intracellular targeting of exoenzyme S of *Pseudomonas aeruginosa* via type III-dependent translocation induces phagocytosis resistance, cytotoxicity and disruption of actin microfilaments. *Molecular Microbiology* 25, 1125-1139.

Fuqua, W.C., Winans, S.C., Greenberg, E.P., 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *Journal of Bacteriology* 176, 269-275.

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

Gambello, M.J., Iglewski, B.H., 1991. Cloning and characterization of the *Pseudomonas aeruginosa* lasR gene, a transcriptional activator of elastase expression. *Journal of Bacteriology* 173, 3000-3009.

Gambello, M.J., Kaye, S., Iglewski, B.H., 1993. LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (apr) and an enhancer of exotoxin A expression. *Infection and Immunity* 61, 1180-1184.

Gamet-Payraastre, L., Li, P., Lumeau, S., Cassar, G., Dupont, M.A., Chevolleau, S., Gasc, N., Tulliez, J., Terce, F., 2000. Sulfuraphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. *Cancer Research* 60, 1426-1433.

Garnacho-Montero, J., Amaya-Villar, R., 2010. Multiresistant *Acinetobacter baumannii* infections: epidemiology and management. *Current Opinion in Infectious Diseases* 23, 332-339.

Garrity-Ryan, L., Kazmierczak, B., Kowal, R., Comolli, J., Hauser, A., Engel, J.N., 2000. The arginine finger domain of ExoT contributes to actin cytoskeleton disruption and inhibition of internalization of *Pseudomonas aeruginosa* by epithelial cells and macrophages. *Infection and Immunity* 68, 7100-7113.

Geske, G.D., O'Neill, J.C., Miller, D.M., Mattmann, M.E., Blackwell, H.E., 2007. Modulation of bacterial quorum sensing with synthetic ligands: systematic evaluation of N-acylated homoserine lactones in multiple species and new insights into their mechanisms of action. *Journal of the American Chemical Society* 129, 13613-13625.

Ghosh, S., Nie, A., An, J., Huang, Z., 2006. Structure-based virtual screening of chemical libraries for drug discovery. *Current Opinion in Chemical Biology* 10, 194-202.

Givskov, M., de Nys, R., Manefield, M., Gram, L., Maximilien, R., Eberl, L., Molin, S., Steinberg, P.D., Kjelleberg, S., 1996. Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. *Journal of Bacteriology* 178, 6618-6622.

Givskov, M., 2012. Beyond nutrition: health-promoting foods by quorum-sensing inhibition. *Future Microbiology* 7, 1025-1028.

Goede, A., Dunkel, M., Mester, N., Frommel, C., Preissner, R., 2005. SuperDrug: a conformational drug database. *Bioinformatics* 21, 1751-1753.

Goodman, A.L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R.S., Lory, S., 2004. A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Developmental Cell* 7, 745-754.

Hancock, R.E., Speert, D.P., 2000. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. *Drug Resistance Updates : Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy* 3, 247-255.

Hao, P., Guo, T., Li, X., Adav, S.S., Yang, J., Wei, M., Sze, S.K., 2010. Novel application of electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) in shotgun proteomics: comprehensive profiling of rat kidney proteome. *Journal of Proteome Research* 9, 3520-3526.

Hauser, A.R., 2009. The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nature Reviews Microbiology* 7, 654-665.

Hayes, M.V., Orr, D.C., 1983. Mode of action of ceftazidime: affinity for the penicillin-binding proteins of *Escherichia coli* K12, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* 12, 119-126.

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

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

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

Høiby, N., Flensburg, E.W., Beck, B., Friis, B., Jacobsen, S.V., Jacobsen, L., 1977. *Pseudomonas aeruginosa* infection in cystic fibrosis. Diagnostic and prognostic significance of *Pseudomonas aeruginosa* precipitins determined by means of crossed immunoelectrophoresis. *Scandinavian Journal of Respiratory Diseases* 58, 65.

Høiby, N., Koch, C., 1990. *Pseudomonas aeruginosa* infection in cystic fibrosis and its management. *Thorax* 45, 881-884.

Holloway, B.W., Morgan, A.F., 1986. Genome Organization in *Pseudomonas*. *Annual Review of Microbiology* 40, 79-105.

Howard, A., O'Donoghue, M., Feeney, A., Sleator, R.D., 2012. *Acinetobacter baumannii*: an emerging opportunistic pathogen. *Virulence* 3, 243-250.

Iacono, M., Villa, L., Fortini, D., Bordoni, R., Imperi, F., Bonnal, R.J.P., Sicheritz-Ponten, T., De Bellis, G., Visca, P., Cassone, A., 2008. Whole-genome pyrosequencing of an epidemic multidrug-resistant *Acinetobacter baumannii* strain belonging to the European clone II group. *Antimicrobial Agents and Chemotherapy* 52, 2616-2625.

Imperi, F., Tiburzi, F., Visca, P., 2009. Molecular basis of pyoverdine siderophore recycling in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Science USA* 106, 20440-20445.

Iversen, B.G., Brantsaeter, A.B., Aavitsland, P., 2008. Nationwide study of invasive *Pseudomonas aeruginosa* infection in Norway: importance of underlying disease. *The Journal of Infection* 57, 139-146.

Jain, A.N., 2003. Surflex: fully automatic flexible molecular docking using a molecular similarity-based search engine. *Journal of Medicinal Chemistry* 46, 499-511.

Jakobsen, T.H., van Gennip, M., Phipps, R.K., Shanmugham, M.S., Christensen, L.D., Alhede, M., Skindersoe, M.E., Rasmussen, T.B., Friedrich, K., Uthe, F., 2012a. Ajoene, a sulfur-rich molecule from garlic, inhibits genes controlled by quorum sensing. *Antimicrobial agents and chemotherapy* 56, 2314-2325.

Jakobsen, T.H., Bragason, S.K., Phipps, R.K., Christensen, L.D., van Gennip, M., Alhede, M., Skindersoe, M., Larsen, T.O., Høiby, N., Bjarnsholt, T., 2012b. Food as a source for quorum sensing inhibitors: iberin from horseradish revealed as a quorum sensing inhibitor of *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology* 78, 2410-2421.

Jakobsen, T.H., Bjarnsholt, T., Jensen, P.Ø., Givskov, M., Høiby, N., 2013. Targeting quorum sensing in *Pseudomonas aeruginosa* biofilms: current and emerging inhibitors. *Future Microbiology* 8, 901-921.

Jensen, P.Ø., Bjarnsholt, T., Phipps, R., Rasmussen, T.B., Calum, H., Christoffersen, L., Moser, C., Williams, P., Pressler, T., Givskov, M., 2007. Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by *Pseudomonas aeruginosa*. *Microbiology* 153, 1329-1338.

Jolley, K.A., Bliss, C.M., Bennett, J.S., Bratcher, H.B., Brehony, C., Colles, F.M., Wimalaratna, H., Harrison, O.B., Sheppard, S.K., Cody, A.J., Maiden, M.C., 2012. Ribosomal multilocus sequence typing: universal characterization of bacteria from domain to strain. *Microbiology* 158, 1005-1015.

Kaldor, S.W., Kalish, V.J., Davies, J.F., Shetty, B.V., Fritz, J.E., Appelt, K., Burgess, J.A., Campanale, K.M., Chirgadze, N.Y., Clawson, D.K., 1997. Viracept (nelfinavir mesylate, AG1343): a potent, orally bioavailable inhibitor of HIV-1 protease. *Journal of Medicinal Chemistry* 40, 3979-3985.

Karavolos, M., Spencer, H., Bulmer, D., Thompson, A., Winzer, K., Williams, P., Hinton, J., Khan, C.A., 2008. Adrenaline modulates the global transcriptional profile of *Salmonella* revealing a role in the antimicrobial peptide and oxidative stress resistance responses. *BMC Genomics* 9, 458.

Karlowsky, J.A., Draghi, D.C., Jones, M.E., Thornsberry, C., Friedland, I.R., Sahm, D.F., 2003. Surveillance for antimicrobial susceptibility among clinical isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* from hospitalized patients in the United States, 1998 to 2001. *Antimicrobial Agents and Chemotherapy* 47, 1681-1688.

Kay, E., Humair, B., Denervaud, V., Riedel, K., Spahr, S., Eberl, L., Valverde, C., Haas, D., 2006. Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. *Journal of Bacteriology* 188, 6026-6033.

Kerr, K.G., Snelling, A.M., 2009. *Pseudomonas aeruginosa*: a formidable and ever-present adversary. *The Journal of Hospital Infection* 73, 338-344.

Kiran, M.D., Adikesavan, N.V., Cirioni, O., Giacometti, A., Silvestri, C., Scalise, G., Ghiselli, R., Saba, V., Orlando, F., Shoham, M., 2008. Discovery of a quorum-sensing inhibitor of drug-resistant staphylococcal infections by structure-based virtual screening. *Molecular Pharmacology* 73, 1578-1586.

Kjelleberg, S., Steinberg, P., 2001. Surface warfare in the sea. *Microbiology Today* 28, 134-135.

Kjelleberg, S., Givskov, M., 2007. The biofilm mode of life : mechanisms and adaptations / edited by Staffan Kjelleberg and Michael Givskov. Wymondham : Horizon Bioscience, c2007.

Krawitz, P., Rodelsperger, C., Jager, M., Jostins, L., Bauer, S., Robinson, P.N., 2010. Microindel detection in short-read sequence data. *Bioinformatics* 26, 722-729.

Lagorce, D., Pencheva, T., Villoutreix, B.O., Miteva, M.A., 2009. DG-AMMOS: A New tool to generate 3D conformation of small molecules using Distance Geometry and Automated Molecular Mechanics Optimization for in silico Screening. *BMC Chemical Biology* 9, 6.

Lamont, I.L., Beare, P.A., Ochsner, U., Vasil, A.I., Vasil, M.L., 2002. Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Science USA* 99, 7072-7077.

Lee, J., Wu, J., Deng, Y., Wang, J., Wang, C., Wang, J., Chang, C., Dong, Y., Williams, P., Zhang, L.-H., 2013. A cell-cell communication signal integrates quorum sensing and stress response. *Nature Chemical Biology* 9, 339-343.

Lee, J., Zhang, L.H., 2015. The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein & Cell* 6, 26-41.

Leoni, L., Ciervo, A., Orsi, N., Visca, P., 1996. Iron-regulated transcription of the *pvdA* gene in *Pseudomonas aeruginosa*: effect of Fur and PvdS on promoter activity. *Journal of Bacteriology* 178, 2299-2313.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25, 2078-2079.

Li, M., Ni, N., Chou, H.T., Lu, C.D., Tai, P.C., Wang, B., 2008. Structure-based discovery and experimental verification of novel AI-2 quorum sensing inhibitors against *Vibrio harveyi*. *ChemMedChem* 3, 1242-1249.

Lin, C.M., Preston, J.F., 3rd, Wei, C.I., 2000. Antibacterial mechanism of allyl isothiocyanate. *Journal of Food Protection* 63, 727-734.

Liu, B., Pop, M., 2009. ARDB—antibiotic resistance genes database. *Nucleic acids research* 37, D443-D447.

Liu, Y., Yang, L., Molin, S., 2010. Synergistic activities of an efflux pump inhibitor and iron chelators against *Pseudomonas aeruginosa* growth and biofilm formation. *Antimicrobial Agents and Chemotherapy* 54, 3960-3963.

Livermore, D.M., 1995. beta-Lactamases in laboratory and clinical resistance. *Clinical Microbiology Reviews* 8, 557-584.

Lockhart, S.R., Abramson, M.A., Beekmann, S.E., Gallagher, G., Riedel, S., Diekema, D.J., Quinn, J.P., Doern, G.V., 2007. Antimicrobial resistance among Gram-negative bacilli causing infections in intensive care unit patients in the United States between 1993 and 2004. *Journal of Clinical Microbiology* 45, 3352-3359.

Lyne, P.D., 2002. Structure-based virtual screening: an overview. *Drug Discovery Today* 7, 1047-1055.

Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T., Monnet, D.L., 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection* 18, 268-281.

- Manefield, M., de Nys, R., Naresh, K., Roger, R., Givskov, M., Peter, S., Kjelleberg, S., 1999. Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. *Microbiology* 145, 283-291.
- McClellan, K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., Daykin, M., Lamb, J.H., Swift, S., Bycroft, B.W., 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology* 143, 3703-3711.
- McGrath, S., Wade, D.S., Pesci, E.C., 2006. Dueling quorum sensing systems in *Pseudomonas aeruginosa* control the production of the Pseudomonas quinolone signal (PQS). *FEMS Microbiol Lett* 230, 27-34.
- Meyer, J.M., Neely, A., Stintzi, A., Georges, C., Holder, I.A., 1996. Pyoverdinin is essential for virulence of *Pseudomonas aeruginosa*. *Infection and Immunity* 64, 518-523.
- Moffatt, J.H., Harper, M., Harrison, P., Hale, J.D.F., Vinogradov, E., Seemann, T., Henry, R., Crane, B., Michael, F.S., Cox, A.D., 2010. Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrobial Agents and Chemotherapy* 54, 4971-4977.
- Moller, S., Sternberg, C., Andersen, J.B., Christensen, B.B., Ramos, J.L., Givskov, M., Molin, S., 1998. In situ gene expression in mixed-culture biofilms: evidence of metabolic interactions between community members. *Applied and Environmental Microbiology* 64, 721-732.
- Monroe, D., 2007. Looking for chinks in the armor of bacterial biofilms. *PLoS Biology* 5, e307.
- Moreau-Marquis, S., Stanton, B.A., O'Toole, G.A., 2008. *Pseudomonas aeruginosa* biofilm formation in the cystic fibrosis airway. *Pulmonary Pharmacology & Therapeutics* 21, 595-599.
- Mugnier, P.D., Poirel, L., Naas, T., Nordmann, P., 2010. Worldwide Dissemination of the blaOXA-23 Carbapenemase Gene of *Acinetobacter baumannii*. *Emerging infectious diseases* 16, 35.
- Müh, U., Schuster, M., Heim, R., Singh, A., Olson, E.R., Greenberg, E.P., 2006. Novel *Pseudomonas aeruginosa* quorum-sensing inhibitors identified in an ultra-high-throughput screen. *Antimicrobial Agents Chemotherapy* 50, 3674-3679.

Nealson, K.H., Platt, T., Hastings, J.W., 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. *Journal of Bacteriology* 104:313-322.

Nealson, K.H., Hastings, J.W., 1979. Bacterial bioluminescence: its control and ecological significance. *Microbiological reviews* 43:496-518.

Nelson, E., Elisha, B.G., 1999. Molecular basis of AmpC hyperproduction in clinical isolates of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* 43, 957-959.

Nikaido, H., 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews* 67, 593-656.

Nouwens, A.S., Beatson, S.A., Whitchurch, C.B., Walsh, B.J., Schweizer, H.P., Mattick, J.S., Cordwell, S.J., 2003. Proteome analysis of extracellular proteins regulated by the *las* and *rhl* quorum sensing systems in *Pseudomonas aeruginosa* PAO1. *Microbiology* 149, 1311-1322.

Nukaga, M., Haruta, S., Tanimoto, K., Kogure, K., Taniguchi, K., Tamaki, M., Sawai, T., 1995. Molecular evolution of a class C-lactamase extending its substrate specificity. *Journal of Biological Chemistry* 270, 5729-5735.

Obritsch, M.D., Fish, D.N., MacLaren, R., Jung, R., 2005. Nosocomial Infections Due to Multidrug-Resistant *Pseudomonas aeruginosa*: Epidemiology and Treatment Options. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy* 25, 1353-1364.

Ochsner, U.A., Reiser, J., 1995. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences* 92, 6424-6428.

Passador, L., Cook, J.M., Gambello, M.J., Rust, L., Iglewski, B.H., 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. *Science* 260, 1127.

Paterson, D.L., Doi, Y., 2007. A step closer to extreme drug resistance (XDR) in gram-negative bacilli. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 45, 1179-1181.

Pearson, J.P., Gray, K.M., Passador, L., Tucker, K.D., Eberhard, A., Iglewski, B.H., Greenberg, E., 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proceedings of the National Academy of Sciences USA* 91, 197-201.

Pearson, J.P., Pesci, E.C., Iglewski, B.H., 1997. Roles of *Pseudomonas aeruginosa* las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *Journal of Bacteriology* 179, 5756-5767.

Perez-Martinez, I., Haas, D., 2011. Azithromycin inhibits expression of the GacA-dependent small RNAs RsmY and RsmZ in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* 55, 3399-3405.

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

Pesci, E.C., Milbank, J.B.J., Pearson, J.P., McKnight, S., Kende, A.S., Greenberg, E.P., Iglewski, B.H., 1999. Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Science USA* 96, 11229-11234.

Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M.T., Camara, M., Haas, D., Williams, P., 2001. The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in *Pseudomonas aeruginosa*. *Journal of Bacteriology* 183, 6676-6683.

Poirel, L., Menuteau, O., Agoli, N., Cattoen, C., Nordmann, P., 2003. Outbreak of extended-spectrum β -lactamase VEB-1-producing isolates of *Acinetobacter baumannii* in a French hospital. *Journal of Clinical Microbiology* 41, 3542-3547.

Powers, R.A., Morandi, F., Shoichet, B.K., 2002. Structure-based discovery of a novel, noncovalent inhibitor of AmpC β -lactamase. *Structure* 10, 1013-1023.

Rasko, D.A., Moreira, C.G., Li, D.R., Reading, N.C., Ritchie, J.M., Waldor, M.K., Williams, N., Taussig, R., Wei, S., Roth, M., 2008. Targeting QseC signaling and virulence for antibiotic development. *Science Signalling* 321, 1078.

Rasmussen, T.B., Bjarnsholt, T., Skindersoe, M.E., Hentzer, M., Kristoffersen, P., Kte, M., Nielsen, J., Eberl, L., Givskov, M., 2005a. Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. *Journal of Bacteriology* 187, 1799-1814.

Rasmussen, T.B., Skindersoe, M.E., Bjarnsholt, T., Phipps, R.K., Christensen, K.B., Jensen, P.O., Andersen, J.B., Koch, B., Larsen, T.O., Hentzer, M., 2005b. Identity and effects of quorum-sensing inhibitors produced by *Penicillium* species. *Microbiology* 151, 1325-1340.

Rasmussen, T.B., Givskov, M., 2006. Quorum-sensing inhibitors as anti-pathogenic drugs. *International Journal of Medical Microbiology* 296, 149-161.

Ravichandiran, V., Shanmugam, K., Anupama, K., Thomas, S., Princy, A., 2012. Structure-based virtual screening for plant-derived SdiA-selective ligands as potential antivirulent agents against uropathogenic *Escherichia coli*. *European Journal of Medicinal Chemistry* 48, 200-205.

Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A., Haas, D., 1997. The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Molecular Microbiology* 24, 309-319.

Runnegar, N., Sidjabat, H., Goh, H.M., Nimmo, G.R., Schembri, M.A., Paterson, D.L., 2010. Molecular epidemiology of multidrug-resistant *Acinetobacter baumannii* in a single institution over a 10-year period. *J Clin Microbiol* 48, 4051-4056.

Rybtke, M.T., Borlee, B.R., Murakami, K., Irie, Y., Hentzer, M., Nielsen, T.E., Givskov, M., Parsek, M.R., Tolker-Nielsen, T., 2012. Fluorescence-based reporter for gauging cyclic di-GMP levels in *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology* 78, 5060-5069.

Ryder, C., Byrd, M., Wozniak, D.J., 2007. Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Current Opinion in Microbiology* 10, 644-648.

Scaife, W., Young, H.-K., Paton, R.H., Amyes, S.G., 1995. Transferable imipenem-resistance in *Acinetobacter* species from a clinical source. *Journal of Antimicrobial Chemotherapy* 36, 585-587.

Schoehn, G., Di Guilmi, A.M., Lemaire, D., Attree, I., Weissenhorn, W., Dessen, A., 2003. Oligomerization of type III secretion proteins PopB and PopD precedes pore formation in *Pseudomonas*. *EMBO Journal* 22, 4957-4967.

Seed, P. C., Passador, L., Iglewski, B. H., 1995. Activation of the *Pseudomonas aeruginosa* lasI gene by LasR and the *Pseudomonas* autoinducer PAI: an autoinduction regulatory hierarchy. *Journal of Bacteriology* 177(3), 654-659.

Shoichet, B.K., 2004. Virtual screening of chemical libraries. *Nature* 432, 862-865.

Sifri, C.D., 2008. Quorum sensing: bacteria talk sense. *Clinical Infectious Diseases* 47, 1070-1076.

Singh, P.K., Schaefer, A.L., Parsek, M.R., Moninger, T.O., Welsh, M.J., Greenberg, E., 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* 407, 762-764.

Skovstrup, S., Qument, L., Thordal, S., Hansen, T., Jakobsen, T.H., Harmsen, M., Tolker-Nielsen, T., Nielsen, T.E., Givskov, M., Taboureau, O., 2013. Identification of LasR Ligands through a Virtual Screening Approach. *ChemMedChem* 8, 157-163.

Smith, K.M., Bu, Y., Suga, H., 2003. Library Screening for Synthetic Agonists and Antagonists of a *Pseudomonas aeruginosa* Autoinducer. *Chemistry and Biology* 10, 563-571.

Sonnleitner, E., Hagens, S., Rosenau, F., Wilhelm, S., Habel, A., Jager, K.E., Blasi, U., 2003. Reduced virulence of a hfq mutant of *Pseudomonas aeruginosa* O1. *Microbial pathogenesis* 35, 217-228.

Sonnleitner, E., Gonzalez, N., Sorger-Domenigg, T., Heeb, S., Richter, A.S., Backofen, R., Williams, P., Huttenhofer, A., Haas, D., Blasi, U., 2011. The small RNA PhrS stimulates synthesis of the *Pseudomonas aeruginosa* quinolone signal. *Molecular Microbiology* 80, 868-885.

Sorger-Domenigg, T., Sonnleitner, E., Kaberdin, V.R., Blasi, U., 2007. Distinct and overlapping binding sites of *Pseudomonas aeruginosa* Hfq and RsmA proteins on the non-coding RNA RsmY. *Biochemical and Biophysical Research Communications* 352, 769-773.

Stewart, P.S., William Costerton, J., 2001. Antibiotic resistance of bacteria in biofilms. *The Lancet* 358, 135-138.

Stewart, P.S., 2002. Mechanisms of antibiotic resistance in bacterial biofilms. *International Journal of Medical Microbiology* 292, 107-113.

Stintzi, A., Evans, K., Meyer, J.M., Poole, K., 1998. Quorum-sensing and siderophore biosynthesis in *Pseudomonas aeruginosa*: lasR/lasI mutants exhibit reduced pyoverdine biosynthesis. *FEMS Microbiology Letters* 166, 341-345.

Stintzi, A., Evans, K., Meyer, J., Poole, K., 2006. Quorum-sensing and siderophore biosynthesis in *Pseudomonas aeruginosa*: lasR/lasI mutants exhibit reduced pyoverdine biosynthesis. *FEMS Microbiol Lett* 166, 341-345.

Stoeva, T., Higgins, P., Bojkova, K., Seifert, H., 2008. Clonal spread of carbapenem-resistant OXA-23-positive *Acinetobacter baumannii* in a Bulgarian university hospital. *Clinical Microbiology and Infection* 14, 723-727.

Stoodley, P., Sauer, K., Davies, D., Costerton, J., 2002. Biofilms as complex differentiated communities. *Annual Reviews in Microbiology* 56, 187-209.

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

Taguchi, F., Suzuki, T., Inagaki, Y., Toyoda, K., Shiraishi, T., Ichinose, Y., 2010. The siderophore pyoverdine of *Pseudomonas syringae* pv. *tabaci* 6605 is an intrinsic virulence factor in host tobacco infection. *Journal of Bacteriology* 192, 117-126.

Tam, V., Schilling, A., LaRocco, M., Gentry, L., Lolans, K., Quinn, J., Garey, K., 2007. Prevalence of AmpC over-expression in bloodstream isolates of *Pseudomonas aeruginosa*. *Clinical microbiology and infection* 13, 413-418.

Tan, S.Y., Chua, S.L., Liu, Y., Hoiby, N., Andersen, L.P., Givskov, M., Song, Z., Yang, L., 2013a. Comparative genomic analysis of rapid evolution of an extreme-drug-resistant *Acinetobacter baumannii* clone. *Genome Biology and Evolution* 5, 807-818.

Tan, S.Y., Chua, S.L., Chen, Y., Rice, S.A., Kjelleberg, S., Nielsen, T.E., Yang, L., Givskov, M., 2013b. Identification of five structurally unrelated quorum-sensing inhibitors of *Pseudomonas aeruginosa* from a natural-derivative database. *Antimicrobial Agents and Chemotherapy* 57, 5629-5641.

Tan, S. Y., Liu, Y., Chua, S. L., Vejborg, R. M., Jakobsen, T. H, Chew, S. C, Li, Y., Nielsen, T. E., Tolker-Nielsen, T., Yang, L. and Givskov, M. 2014. Comparative systems biology analysis and mode of action of the isothiocyanate compound iberin on *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*. 58, 6648-6659.

Thomsen, R., Christensen, M.H., 2006. MolDock: a new technique for high-accuracy molecular docking. *Journal of Medicinal Chemistry* 49, 3315-3321.

Trachootham, D., Zhou, Y., Zhang, H., Demizu, Y., Chen, Z., Pelicano, H., Chiao, P.J., Achanta, G., Arlinghaus, R.B., Liu, J., Huang, P., 2006. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate. *Cancer Cell* 10, 241-252.

van Dessel, H., Dijkshoorn, L., van der Reijden, T., Bakker, N., Paauw, A., van den Broek, P., Verhoef, J., Brisse, S., 2004. Identification of a new geographically widespread multiresistant *Acinetobacter baumannii* clone from European hospitals. *Research in Microbiology* 155, 105-112.

Vannini, A., Volpari, C., Gargioli, C., Muraglia, E., Cortese, R., De Francesco, R., Neddermann, P., Di Marco, S., 2002. The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA. *EMBO Journal* 21, 4393-4401.

Ventre, I., Goodman, A.L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., Bleves, S., Lazdunski, A., Lory, S., Filloux, A., 2006. Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proceedings of the National Academy of Sciences USA* 103, 171-176.

Vila, J., Martí, S., Sánchez-Céspedes, J., 2007. Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy* 59, 1210-1215.

Von Itzstein, M., Wu, W.Y., Kok, G.B., Pegg, M.S., Dyason, J.C., Jin, B., Van Phan, T., Smythe, M.L., White, H.F., Oliver, S.W., 1993. Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* 363, 418-423.

Wagner, V.E., Bushnell, D., Passador, L., Brooks, A.I., Iglewski, B.H., 2003. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *Journal of Bacteriology* 185, 2080-2095.

Wallace, A.C., Laskowski, R.A., Thornton, J.M., 1995. LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. *Protein Engineering* 8, 127-134.

Walter, F., Vicens, Q., Westhof, E., 1999. Aminoglycoside–RNA interactions. *Current Opinion in Chemical Biology* 3, 694-704.

Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P.C., Mattick, J.S., 2002. Extracellular DNA required for bacterial biofilm formation. *Science* 295, 1487-1487.

Whiteley, M., Lee, K.M., Greenberg, E., 1999. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences USA* 96, 13904-13909.

Wilderman, P.J., Vasil, A.I., Johnson, Z., Wilson, M.J., Cunliffe, H.E., Lamont, I.L., Vasil, M.L., 2001. Characterization of an endoprotease (PrpL) encoded by a PvdS-regulated gene in *Pseudomonas aeruginosa*. *Infection and Immunity* 69, 5385-5394.

Williams, P., Bainton, N.J., Swift, S., Chhabra, S.R., Winson, M.K., Stewart, G.S., Salmond, G.P., Bycroft, B.W. 1992. Small molecule-mediated density-dependent control of gene expression in prokaryotes: bioluminescence and the biosynthesis of carbapenem antibiotics. *FEMS Microbiol Lett* 100:161-167.

Winsor, G.L., Lam, D.K.W., Fleming, L., Lo, R., Whiteside, M.D., Nancy, Y.Y., Hancock, R.E.W., Brinkman, F.S.L., 2011. *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. *Nucleic Acids Research* 39, D596-D600.

Wolfgang, M.C., Kulasekara, B.R., Liang, X., Boyd, D., Wu, K., Yang, Q., Miyada, C.G., Lory, S., 2003. Conservation of genome content and virulence determinants among clinical and environmental isolates of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences USA* 100, 8484-8489.

World Health Organization, 2014. Antimicrobial resistance: global report on surveillance 2014.

Xiao, D., Srivastava, S.K., Lew, K.L., Zeng, Y., Hershberger, P., Johnson, C.S., Trump, D.L., Singh, S.V., 2003. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G2/M arrest and inducing apoptosis. *Carcinogenesis* 24, 891-897.

Yahr, T.L., Wolfgang, M.C., 2006. Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system. *Molecular Microbiology* 62, 631-640.

Yang, L., Barken, K.B., Skindersoe, M.E., Christensen, A.B., Givskov, M., Tolker-Nielsen, T., 2007. Effects of iron on DNA release and biofilm development by *Pseudomonas aeruginosa*. *Microbiology* 153, 1318-1328.

Yang, L., Nilsson, M., Gjermansen, M., Givskov, M., Tolker-Nielsen, T., 2009a. Pyoverdine and PQS mediated subpopulation interactions involved in *Pseudomonas aeruginosa* biofilm formation. *Molecular Microbiology* 74, 1380-1392.

Yang, L., Rybtke, M.T., Jakobsen, T.H., Hentzer, M., Bjarnsholt, T., Givskov, M., Tolker-Nielsen, T., 2009b. Computer-aided identification of recognized drugs as *Pseudomonas aeruginosa* quorum-sensing inhibitors. *Antimicrobial Agents and Chemotherapy* 53, 2432-2443.

Yoshida, H., Bogaki, M., Nakamura, M., Yamanaka, L.M., Nakamura, S., 1991. Quinolone resistance-determining region in the DNA gyrase *gyrB* gene of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* 35, 1647-1650.

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F.M., Larsen, M.V., 2012. Identification of acquired antimicrobial resistance genes. *Journal of Antimicrobial Chemotherapy* 67, 2640-2644.

Zhang, L.H., Dong, Y.H., 2004. Quorum sensing and signal interference: diverse implications. *Molecular Microbiology* 53, 1563-1571.

Zhou, H., Pi, B.-R., Yang, Q., Yu, Y.-S., Chen, Y.-G., Li, L.-J., Zheng, S.-S., 2007. Dissemination of imipenem-resistant *Acinetobacter baumannii* strains carrying the ISAba1–blaOXA-23 genes in a Chinese hospital. *Journal of Medical Microbiology* 56, 1076-1080.

Zhu, P., Peng, H., Ni, N., Wang, B., Li, M., 2012. Novel AI-2 quorum sensing inhibitors in *Vibrio harveyi* identified through structure-based virtual screening. *Bioorganic & Medicinal Chemistry Letters* 22, 6413-6417.

Zou, Y., Nair, S.K., 2009. Molecular Basis for the Recognition of Structurally Distinct Autoinducer Mimics by the *Pseudomonas aeruginosa* LasR Quorum-Sensing Signaling Receptor. *Chemistry and Biology* 16, 961-970.

Appendix A

Table A. Parameters used for the import of molecules, template creation and docking in Molegro Virtual Docker.

Import Molecules	
Preparation	
Assign bonds	If missing
Assign bond orders and hybridization	If missing
Create explicit hydrogens	If missing
Assign charges (calculated by MVD)	Always
Detect flexible torsions in ligands	Always
Assign Tripos atom types	If missing
Template docking wizard	
Similarity measure	(Radius, strength, count)
Steric	1.8, 0.5, 21
Hydrogen donor	1.8, 1, 3
Hydrogen acceptor	1.8, 1, 4
Ring	1.8, 1, 5
Docking wizard	
Template docking: Docking using Ligand Template	
Scoring strength	-500.00
Energy grid resolution (Å)	0.20
Scoring function	
Score	Ligand evaluator
Steric interactions	Yes
Torsional interactions	Yes
Binding site	
Center (x,y,z)	24.07, 18.19, 77.44
Radius	9
Search algorithm	
Algorithm	MolDock Optimizer
Number of runs	10
Parameter settings	
Constrain poses to cavity	Yes
Population size	50
Max Iterations	2000
Scaling factor	0.50
Crossover rate	0.90
Offspring scheme	Scheme 1
Termination scheme	Variance-based
Pose clustering	
Multiple poses	Return one pose for each run
Tabu clustering	Yes
RMSD threshold	2.00
RMSD calculation	By atom ID (fast)
Energy penalty	100.00

Appendix B

Appendix B describes the material and methods used in the iTRAQ proteomics experiment in which we studied the effect of 25 μ M G1 on *P. aeruginosa*. Proteomics experiments were performed at the Proteomic Core Facility of the Biological Research Center, School of Biological Sciences, Nanyang Technological University, Singapore. Methods referred to here come from Prof. Newman Sze's prior work (Hao et al., 2010).

iTRAQ-based Proteomics Analysis

Protein preparation and digestion. The *P. aeruginosa* PAO1 strain was grown in ABTG media. Sub-lethal concentrations of **G1** (25 μ M) were added to independent *P. aeruginosa* PAO1 cultures respectively. Cultures were grown in LB medium at 37°C with shaking until late log phase (OD 600 nm = 1.0) before harvesting. After harvesting, cell pellets were washed with 1×PBS and resuspended in 2 ml of lysis buffer containing 0.5M TEAB and 0.1M SDS. The cells were ruptured by sonication, and the cell debris was removed by centrifugation at 4 °C at 16000 $\times$ g for 15 min. 200 μ g of proteins from different growth conditions were dissolved in equal volume of sample buffer (Invitrogen) supplemented with 0.5% 2-mercaptoethanol and denatured by boiling at 95°C for 5 min. 1D gel electrophoresis was carried out using 10% SDS-PAGE for in-gel digestion.

Proteins were first reduced using 5 mM Tris-(2-carboxyethyl) phosphine (TCEP) for 1 h at 60 °C, followed by blocking of cysteine residues by 10 mM methyl methanethiosulfate (MMTS) for 30 min at room temperature in the dark. Trypsin was added at a ratio of 1:50 (trypsin/sample). It was then incubated at 37 °C overnight. The tryptic peptides were extracted by 50% ACN/5% Acetic Acid from gel for 3 times and were desalted using Sep-Pak C18 cartridges (Waters, Milford, MA) and dried in a SpeedVac (Thermo Electron, Waltham, MA). All chemicals were purchased from Sigma-Aldrich unless stated otherwise.

iTRAQ labeling. The iTRAQ labeling of the tryptic peptides was performed using 4-plex iTRAQ reagent kit (Applied Biosystems, Foster City, CA), according to the manufacturers protocol. 200 μ g of peptides from each condition were individually labeled with respective isobaric tags: control sample with 114, and the **G1**-treated sample with 115. After 2 h incubation,

the samples were quenched by water, desalted using C18 solid phase extraction cartridge, and then vacuum-centrifuged to dryness. The iTRAQ-labeled peptides were reconstituted in Buffer A (10 mM ammonium acetate, 85% acetonitrile, 0.1% formic acid) and fractionated using ERLIC column (200 x 4.6 mm, 5µm particle size, 200 Å pore size) by HPLC system (Shimadzu, Japan) at a flow rate of 1.0 ml/min using a previously optimized protocol(1). The HPLC chromatograms were recorded at 280 nm and fractions were collected online using automated fraction collector. 20 fractions were collected and concentrated using vacuum centrifuge and reconstituted in 3% ACN with 0.1% formic acid for LC-MS/MS analysis.

LC-MS/MS. The peptides were separated and analyzed on a home-packed nanobore C18 column (15 cm x 75 µm; Reprosil-Pur C18-AQ, 3 µm, Dr Maisch, Germany) with a Picofritnanospray tip (New Objectives, Woburn, MA, USA) on a TempoTM nano-MDLC system coupled with a QSTAR® Elite Hybrid LC-MS/MS system (Applied Biosystems). Peptides from each fraction were analyzed in triplicate by LC-MS/MS over a gradient of 90 min. The flow rate of the LC system was set to a constant 300 nl/min. Data acquisition in QSTAR Elite was set to positive ion mode using Analyst® QS 2.0 software (Applied Biosystems). MS data was acquired in positive ion mode with a mass range of 300–1600 m/z. Peptides with +2 to +4 charge states were selected for MS/MS. For each MS spectrum, the three most abundant peptides above a five-count threshold were selected for MS/MS and dynamically excluded for 30 s with a mass tolerance of 0.03 Da. Smart information-dependent acquisition was activated with automatic collision energy and automatic MS/MS accumulation. The fragment intensity multiplier was set to 20 and maximum accumulation time was 2s.

Data analysis. Spectra acquired from the three technical replicates were submitted to ProteinPilot (v3.0.0.0, Applied Biosystems) for peak-list generation, protein identification and quantification. User defined parameters of the Paragon algorithm in ProteinPilot software were configured as follows: (i) Sample Type, iTRAQ2-plex (Peptide Labeled); (ii) Cysteine alkylation, MMTS; (iii) Digestion, Trypsin; (iv) Instrument, QSTAR Elite ESI; (v) Special factors, Urea denaturation; (vi) Species, None; (vii) Specify Processing, Quantitate & Bias Correction; (viii) ID Focus, biological modifications, amino acid substitutions; (ix) Database, *P. aeruginosa* PAO1; (x) Search effort, thorough ID; (xi) Result quality, Unused ProtScore(Conf) >0.05 (10.0%). Default precursor and MS/MS tolerance for QSTAR ESI MS instrument were

adopted automatically by the software. For iTRAQ quantitation, the peptide for quantification was automatically selected by Pro Group algorithm to calculate the reporter peak area, error factor (EF) and p-value. The resulting data was auto bias-corrected by build-in ProteinPilot algorithm to get rid of any variations imparted due to the unequal mixing during combining different labeled samples. During bias correction, the software identifies the median average protein ratio and corrects it to unity, and then applies this factor to all quantitation results. A strict cutoff of unused ProteinScore ≥ 2 , which corresponds to a confidence limit of 99 %, was considered for protein identifications and further analysis.

Appendix C

Figure C-1. Three-dimensional representations of interactions between LasR ligand-binding pocket residues interaction with the five QSI compounds: (a) C1, (b) F1, (c) F2, (d) G1 and (e) H1 respectively. Images were prepared using LIGPLOT+ version 1.4.4 and PYMOL.

Appendix D

Appendix D contains the two lists of genes whose expression were either greater than 2-fold increased or decreased in *P. aeruginosa* PAO1 upon treatment with 500 μ M iberin. Supplementary table D-1 contains the 145 up-regulated genes, whereas supplementary table D-2 contains the 258 down-regulated genes.

Supplementary Table D-1. List of genes in the RNA-Seq result that were found to be significantly up-regulated in *P. aeruginosa* upon 500 μ M iberin treatment. Up-regulation was defined as greater than 2-fold increase in the RNA-Seq result. Only genes showing a significant difference in expression from the control (i.e. p-value < 0.05) were selected.

PA No.	Gene Name	QS ^a	Description	Fold Change	P value
PA0119			Probable dicarboxylate transporter	2.2	0.03
PA0132			Beta-alanine--pyruvate transaminase	2.9	0.05
PA0133			Probable transcriptional regulator	4.2	0.01
PA0161			Hypothetical protein	4.7	0.01
PA0181			Probable transcriptional regulator	2.2	0.03
PA0182			Probable short-chain dehydrogenase	7.6	0.04
PA0185			Probable permease of ABC transporter	8.9	0.04
PA0201			Hypothetical protein	25.9	0.01
PA0202			Probable amidase	20.7	0.03
PA0234			Hypothetical protein	2.5	0.03
PA0283	<i>sbp</i>		Sulfate-binding protein precursor	26.0	0.01
PA0284			Hypothetical protein	32.9	0.04
PA0450			Probable phosphate transporter	2.1	0.05
PA0485			Conserved hypothetical protein	2.1	0.04
PA0539			Hypothetical protein	2.1	0.04
PA0709			Hypothetical protein	2.3	0.01
PA0865	<i>hpd</i>		4-hydroxyphenylpyruvate dioxygenase	42.2	0.04
PA0878			Hypothetical protein	7.9	0.04
PA0879			Probable acyl-CoA dehydrogenase	4.1	0.05
PA0905	<i>rsmA</i>		RsmA, regulator of secondary metabolites	3.6	0.01
PA1223			Probable transcriptional regulator	2.7	0.04
PA1226			Probable transcriptional regulator	2.9	0.01
PA1253			Probable semialdehyde dehydrogenase	2.3	0.02
PA1284			Probable acyl-CoA dehydrogenase	2.2	0.01
PA1285			Probable transcriptional regulator	5.2	0.05
PA1310	<i>phnW</i>		2-aminoethylphosphonate:pyruvate aminotransferase	19.3	0.05
PA1332			Hypothetical protein	17.7	0.05
PA1334			Probable oxidoreductase	74.7	0.04

PA1493	<i>cysP</i>	Sulfate-binding protein of ABC transporter	8.2	<0.01
PA1569		Probable major facilitator superfamily (MFS) transporter	4.7	0.03
PA1570		Probable transcriptional regulator	2.7	0.05
PA1602		Probable oxidoreductase	2.4	<0.01
PA1621		Probable hydrolase	2.8	0.04
PA1738		Probable transcriptional regulator	2.6	0.01
PA1742		Probable amidotransferase	3.1	<0.01
PA1756	<i>cysH</i>	3-phosphoadenosine-5-phosphosulfate reductase	3.4	0.04
PA1936		Hypothetical protein	2.1	0.02
PA1999	<i>dhcA</i>	Probable CoA transferase, subunit A	9.1	0.01
PA2000	<i>dhcB</i>	Probable CoA transferase, subunit B	9.5	<0.01
PA2001	<i>atoB</i>	Acetyl-CoA acetyltransferase	5.0	0.02
PA2008	<i>fahA</i>	Fumarylacetoacetase	54.8	0.03
PA2009	<i>hmgA</i>	Homogentisate 1,2-dioxygenase	62.0	0.02
PA2047		Probable transcriptional regulator	2.1	0.04
PA2086		Probable epoxide hydrolase	3.7	0.05
PA2087		Hypothetical protein	2.4	0.05
PA2090		Hypothetical protein	2.2	0.02
PA2095		Hypothetical protein	4.4	0.03
PA2220		Probable transcriptional regulator	2.9	0.03
PA2247	<i>bkdA1</i>	2-oxoisovalerate dehydrogenase (alpha subunit)	2.2	0.04
PA2250	<i>lpdV</i>	Lipoamide dehydrogenase-Val	2.5	0.04
PA2275		Probable alcohol dehydrogenase (Zn-dependent)	3.4	0.04
PA2277	<i>arsR</i>	ArsR protein	15.3	0.05
PA2278	<i>arsB</i>	ArsB protein	9.6	0.02
PA2310		Hypothetical protein	20.5	0.03
PA2311		Hypothetical protein	51.8	0.05
PA2312		Probable transcriptional regulator	29.6	<0.01
PA2326		Hypothetical protein	3.0	0.01
PA2327	x	Probable permease of ABC transporter	6.6	0.05
PA2359		Probable transcriptional regulator	34.5	0.03
PA2444	<i>glyA2</i>	Serine hydroxymethyltransferase	37.4	0.05
PA2481		Hypothetical protein	2.0	0.01
PA2483		Conserved hypothetical protein	3.7	0.03
PA2484		Conserved hypothetical protein	2.1	<0.01
PA2490		Conserved hypothetical protein	16.4	0.05
PA2491		Probable oxidoreductase	34.1	0.03
PA2494	<i>mexF</i>	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter MexF	129.4	0.04
PA2495	<i>oprN</i>	Multidrug efflux outer membrane protein OprN precursor	102.3	0.03
PA2535		Probable oxidoreductase	4.8	0.05
PA2575		Hypothetical protein	28.5	0.02
PA2576		Hypothetical protein	2.3	0.01

PA2578			Probable acetyltransferase	2.7	0.02
PA2579	<i>kynA</i>		L-Tryptophan:oxygen 2,3-oxidoreductase (decyclizing) KynA	5.3	0.05
PA2580			Conserved hypothetical protein	26.1	0.01
PA2593	<i>qteE</i>	x	Hypothetical protein	2.1	<0.01
PA2594		x	Conserved hypothetical protein	9.0	0.02
PA2599			Conserved hypothetical protein	12.1	0.05
PA2600			Hypothetical protein	21.0	<0.01
PA2610			Conserved hypothetical protein	5.2	0.03
PA2636			Hypothetical protein	2.8	0.02
PA2641	<i>nuoF</i>		NADH dehydrogenase I chain F	2.4	0.02
PA2642	<i>nuoG</i>		NADH dehydrogenase I chain G	2.4	<0.01
PA2643	<i>nuoH</i>		NADH dehydrogenase I chain H	2.3	0.02
PA2645	<i>nuoJ</i>		NADH dehydrogenase I chain J	2.4	0.01
PA2647	<i>nuoL</i>		NADH dehydrogenase I chain L	2.1	0.01
PA2648	<i>nuoM</i>		NADH dehydrogenase I chain M	2.2	0.03
PA2649	<i>nuoN</i>		NADH dehydrogenase I chain N	2.1	0.05
PA2665			Probable transcriptional regulator	2.9	0.04
PA2711			Probable periplasmic spermidine/putrescine-binding protein	2.3	0.01
PA2714			Probable molybdopterin oxidoreductase	3.2	<0.01
PA2715			Probable ferredoxin	2.4	0.01
PA2758			Probable transcriptional regulator	63.7	0.02
PA2759			Hypothetical protein	179.3	0.01
PA2766			Probable transcriptional regulator	2.7	0.04
PA2767			Probable enoyl-CoA hydratase/isomerase	5.8	0.03
PA2768			Hypothetical protein	2.5	0.04
PA2786			Hypothetical protein	8.6	0.05
PA2812			Probable ATP-binding component of ABC transporter	20.4	0.03
PA2813			Probable glutathione S-transferase	32.7	0.04
PA2844			Conserved hypothetical protein	8.3	0.04
PA2845			Hypothetical protein	91.2	0.04
PA2846			Probable transcriptional regulator	2.9	0.03
PA2881			Probable two-component response regulator	4.3	0.03
PA2930			Probable transcriptional regulator	2.7	0.05
PA2931	<i>cifR</i>		CifR	10.4	0.03
PA2932	<i>morB</i>		Morphinone reductase	45.7	0.03
PA3035			Probable glutathione S-transferase	8.0	<0.01
PA3176	<i>gltS</i>		Glutamate/sodium ion symporter, GltS	2.2	0.02
PA3222			Hypothetical protein	5.4	0.04
PA3229			Hypothetical protein	311.5	0.04
PA3230			Conserved hypothetical protein	69.5	0.01
PA3424			Hypothetical protein	2.2	0.05
PA3446			Conserved hypothetical protein	63.5	0.05
PA3450			Probable antioxidant protein	39.4	0.01

PA3630		Probable transcriptional regulator	3.7	0.03
PA3663		Hypothetical protein	2.1	0.02
PA3893		Conserved hypothetical protein	2.5	0.05
PA3927		Probable transcriptional regulator	3.1	0.02
PA3931		Conserved hypothetical protein	44.7	0.05
PA3938		Probable periplasmic taurine-binding protein precursor	24.5	0.05
PA3971		Hypothetical protein	2.2	<0.01
PA4166		Probable acetyltransferase	77.6	0.02
PA4167		Probable oxidoreductase	112.0	<0.01
PA4173		Conserved hypothetical protein	12.4	0.01
PA4288		Probable transcriptional regulator	3.9	0.02
PA4355		Probable major facilitator superfamily (MFS) transporter	37.3	<0.01
PA4385	<i>groEL</i>	GroEL protein	6.4	0.04
PA4386	<i>groES</i>	GroES protein	6.4	0.03
PA4387		Conserved hypothetical protein	8.0	0.05
PA4442	<i>cysN</i>	ATP sulfurylase GTP-binding subunit/APS kinase	4.5	0.04
PA4443	<i>cysD</i>	ATP sulfurylase small subunit	3.8	<0.01
PA4475		Conserved hypothetical protein	2.4	0.05
PA4623		Hypothetical protein	727.4	0.01
PA4630		Hypothetical protein	3.2	0.03
PA4918		Hypothetical protein	3.0	0.01
PA4919	<i>pncBI</i>	Nicotinate phosphoribosyltransferase	2.8	0.02
PA4920	<i>nadE</i>	NH ₃ -dependent NAD synthetase	2.1	0.02
PA4921		Hypothetical protein	2.4	0.02
PA4980		Probable enoyl-CoA hydratase/isomerase	3.9	0.02
PA4985		Hypothetical protein	2.1	0.03
PA5103		Hypothetical protein	2.4	0.04
PA5105	<i>hutC</i>	Histidine utilization repressor HutC	2.1	0.02
PA5106		Conserved hypothetical protein	2.2	0.02
PA5246		Conserved hypothetical protein	2.2	0.02
PA5429	<i>aspA</i>	Aspartate ammonia-lyase	4.6	<0.01
PA5446		Hypothetical protein	2.8	0.02

^aAn 'X' in the box indicates that the gene/protein is regulated by quorum sensing (Hentzer et al., 2003), in reference to the QS-regulated genes/proteins as determined previously.

Supplementary Table D-2. List of genes in the RNA-Seq result that were found to be significantly down-regulated in *P. aeruginosa* upon 500 μ M iberin treatment. Down-regulation was defined as greater than 2-fold increase in the RNA-Seq result. Only genes showing a significant difference in expression from the control (i.e. p-value < 0.05) were selected.

PA No.	Gene Name	QS ^a	Description	Fold Change	P value
PA0027		x	Hypothetical protein	-3.3	0.04
PA0028			Hypothetical protein	-3.3	0.04
PA0029			Probable sulfate transporter	-2.0	0.03
PA0039			Hypothetical protein	-2.5	0.05
PA0044	<i>exoT</i>		Exoenzyme T	-2.0	0.02
PA0122	<i>rahU</i>	x	RahU	-32.8	0.05
PA0123			Probable transcriptional regulator	-4.6	0.05
PA0143	<i>nuh</i>	x	Purine nucleosidase Nuh	-3.8	0.05
PA0179			Probable two-component response regulator	-3.1	0.03
PA0200			Hypothetical protein	-4.6	0.04
PA0208	<i>mdcA</i>		Malonate decarboxylase alpha subunit	-3.1	0.01
PA0211	<i>mdcD</i>		Malonate decarboxylase beta subunit	-3.6	0.02
PA0212	<i>mdcE</i>		Malonate decarboxylase gamma subunit	-3.2	0.03
PA0346			Hypothetical protein	-3.6	0.03
PA0460			Hypothetical protein	-2.3	0.05
PA0471			Probable transmembrane sensor	-3.1	0.02
PA0472			Probable sigma-70 factor	-3.6	0.03
PA0519	<i>nirS</i>		Nitrite reductase precursor	-2.2	0.04
PA0523	<i>norC</i>		Nitric-oxide reductase subunit C	-10.9	0.04
PA0524	<i>norB</i>		Nitric-oxide reductase subunit B	-3.9	<0.01
PA0526			Hypothetical protein	-4.7	0.05
PA0572		x	Hypothetical protein	-3.7	<0.01
PA0619			Probable bacteriophage protein	-2.5	0.03
PA0623			Probable bacteriophage protein	-2.6	0.04
PA0625			Hypothetical protein	-2.6	0.05
PA0626			Hypothetical protein	-2.5	0.01
PA0629			Conserved hypothetical protein	-2.4	0.04
PA0633			Hypothetical protein	-2.4	<0.01
PA0634			Hypothetical protein	-2.5	0.05
PA0638			Probable bacteriophage protein	-2.4	0.01
PA0639			Conserved hypothetical protein	-2.2	0.04
PA0640			Probable bacteriophage protein	-2.2	0.05
PA0707	<i>toxR</i>		Transcriptional regulator ToxR	-5.4	0.02
PA0718			Hypothetical protein of bacteriophage Pf1	-3.1	0.05
PA0722			Hypothetical protein of bacteriophage Pf1	-2.3	0.04
PA0795	<i>prpC</i>		Citrate synthase 2	-2.4	0.05

PA0796	<i>prpB</i>		Carboxyphosphoenolpyruvate phosphonmutase	-3.9	0.05
PA0807	<i>ampDh3</i>		AmpDh3	-2.4	0.02
PA0852	<i>cbpD</i>	x	Chitin-binding protein CbpD precursor	-22.0	0.04
PA0958	<i>oprD</i>		Basic amino acid, basic peptide and imipenem outer membrane porin OprD precursor	-2.0	0.01
PA0996	<i>pqsA</i>	x	Probable coenzyme A ligase	-3.1	0.05
PA0997	<i>pqsB</i>	x	PqsB	-4.0	0.05
PA1000	<i>pqsE</i>	x	Quinolone signal response protein	-4.4	0.05
PA1001	<i>phnA</i>	x	Anthranilate synthase component I	-7.2	0.01
PA1002	<i>phnB</i>	x	Anthranilate synthase component II	-4.8	0.02
PA1003	<i>mvfR</i>	x	Transcriptional regulator MvfR	-2.5	0.03
PA1092	<i>fliC</i>		Flagellin type B	-2.1	<0.01
PA1127			Probable oxidoreductase	-3.6	0.05
PA1129			Probable fosfomycin resistance protein	-3.9	0.02
PA1130	<i>rhlC</i>	x	Rhamnosyltransferase 2	-11.7	0.04
PA1131		x	Probable major facilitator superfamily (MFS) transporter	-8.9	0.05
PA1134			Hypothetical protein	-6.0	0.01
PA1196		x	Probable transcriptional regulator	-2.4	0.02
PA1212			Probable major facilitator superfamily (MFS) transporter	-3.3	<0.01
PA1214			Hypothetical protein	-8.2	0.03
PA1215			Hypothetical protein	-7.7	0.02
PA1216			Hypothetical protein	-9.0	0.04
PA1217			Probable 2-isopropylmalate synthase	-4.4	0.03
PA1219			Hypothetical protein	-2.9	0.05
PA1220			Hypothetical protein	-5.4	0.03
PA1221			Hypothetical protein	-6.8	0.04
PA1245			Hypothetical protein	-2.2	0.05
PA1289			Hypothetical protein	-4.0	0.01
PA1300			Probable sigma-70 factor, ECF subfamily	-5.5	0.03
PA1301			Probable transmembrane sensor	-3.7	0.04
PA1323		x	Hypothetical protein	-6.9	0.03
PA1324		x	Hypothetical protein	-7.7	0.05
PA1516			Hypothetical protein	-2.0	0.04
PA1518			Conserved hypothetical protein	-2.1	0.03
PA1519			Probable transporter	-2.2	0.01
PA1559			Hypothetical protein	-7.3	0.03
PA1560			Hypothetical protein	-8.9	0.02
PA1579			Hypothetical protein	-2.2	0.02
PA1673			Hypothetical protein	-3.1	0.01
PA1697			ATP synthase in type III secretion system	-2.3	0.02
PA1705	<i>pcrG</i>		Regulator in type III secretion	-7.3	0.03
PA1706	<i>pcrV</i>		Type III secretion protein PcrV	-6.1	0.03
PA1708			Translocator protein PopB	-6.7	0.03

PA1710	<i>exsC</i>		ExsC	-6.6	0.01
PA1711	<i>exsE</i>		ExsE	-4.4	0.01
PA1712	<i>exsB</i>		Exoenzyme S synthesis protein B	-4.5	0.04
PA1714	<i>exsD</i>		ExsD	-3.2	<0.01
PA1716	<i>pscC</i>		Type III secretion outer membrane protein PscC precursor	-2.1	0.01
PA1720	<i>pscG</i>		Type III export protein PscG	-2.6	0.03
PA1774	<i>cfrX</i>		CfrX protein	-3.8	0.03
PA1775	<i>cmpX</i>		Conserved cytoplasmic membrane protein, CmpX protein	-3.6	0.03
PA1871	<i>lasA</i>	x	LasA protease precursor	-25.5	0.02
PA1875		x	Probable outer membrane protein precursor	-5.1	0.04
PA1878			Hypothetical protein	-4.2	0.02
PA1891		x	Hypothetical protein	-2.5	0.04
PA1894			Hypothetical protein	-3.0	0.03
PA1898	<i>qscR</i>		Quorum-sensing control repressor	-3.0	0.02
PA1906			Hypothetical protein	-20.4	0.01
PA1911	<i>femR</i>		Probable transmembrane sensor	-3.2	0.03
PA1912	<i>femI</i>		Probable sigma-70 factor, ECF subfamily	-5.1	0.01
PA1914		x	Conserved hypothetical protein	-14.0	0.02
PA1934			Hypothetical protein	-2.1	0.02
PA2015	<i>liuA</i>		Putative isovaleryl-CoA dehydrogenase	-2.4	0.04
PA2016	<i>liuR</i>		Regulator of liu genes	-2.3	0.04
PA2018			Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter	-2.2	<0.01
PA2020			Probable transcriptional regulator	-2.1	0.03
PA2064	<i>pcoB</i>		Copper resistance Protein B precursor	-2.2	0.02
PA2067			Probable hydrolase	-16.4	0.04
PA2068		x	Probable major facilitator superfamily (MFS) transporter	-37.5	0.02
PA2069		x	Probable carbamoyl transferase	-107.8	0.02
PA2070		x	Hypothetical protein	-2.6	<0.01
PA2076		x	Probable transcriptional regulator	-2.1	0.05
PA2081	<i>kynB</i>		Kynurenine formamidase, KynB	-2.1	0.01
PA2119			Alcohol dehydrogenase (Zn-dependent)	-2.1	0.05
PA2140			Probable metallothionein	-3.3	0.03
PA2149			Hypothetical protein	-4.5	0.02
PA2150			Conserved hypothetical protein	-3.3	0.04
PA2151			Conserved hypothetical protein	-2.3	0.04
PA2154			Conserved hypothetical protein	-3.2	0.02
PA2157			Hypothetical protein	-2.5	<0.01
PA2159			Conserved hypothetical protein	-5.4	0.04
PA2160			Probable glycosyl hydrolase	-3.6	0.02
PA2162			Probable glycosyl hydrolase	-3.7	0.02
PA2163			Hypothetical protein	-4.3	0.02
PA2164			Probable glycosyl hydrolase	-2.9	0.02

PA2165			Probable glycogen synthase	-3.4	<0.01
PA2167			Hypothetical protein	-5.3	0.02
PA2168			Hypothetical protein	-4.6	0.05
PA2169			Hypothetical protein	-6.7	0.03
PA2170			Hypothetical protein	-9.1	0.04
PA2175			Hypothetical protein	-2.4	0.01
PA2179			Hypothetical protein	-2.4	0.03
PA2193	<i>hcnA</i>	x	Hydrogen cyanide synthase HcnA	-27.7	0.05
PA2194	<i>hcnB</i>	x	Hydrogen cyanide synthase HcnB	-15.9	0.01
PA2195	<i>hcnC</i>	x	Hydrogen cyanide synthase HcnC	-12.6	<0.01
PA2300	<i>chiC</i>	x	Chitinase	-29.9	0.03
PA2320	<i>gntR</i>		Transcriptional regulator GntR	-3.2	0.01
PA2322			Gluconate permease	-11.4	0.02
PA2323			Probable glyceraldehyde-3-phosphate dehydrogenase	-3.7	0.01
PA2377			Hypothetical protein	-8.5	0.05
PA2383			Probable transcriptional regulator	-3.4	<0.01
PA2384			Hypothetical protein	-7.4	<0.01
PA2386	<i>pvdA</i>		L-ornithine N5-oxygenase	-3.2	0.01
PA2392	<i>pvdP</i>		PvdP	-3.6	0.03
PA2393			Probable dipeptidase precursor	-4.3	0.01
PA2403			Hypothetical protein	-2.9	0.01
PA2414		x	L-sorbose dehydrogenase	-10.5	0.02
PA2415		x	Hypothetical protein	-8.6	0.02
PA2425	<i>pvdG</i>		PvdG	-3.9	0.04
PA2426	<i>pvdS</i>		Sigma factor PvdS	-3.6	0.03
PA2427			Hypothetical protein	-6.9	0.01
PA2448			Hypothetical protein	-3.1	<0.01
PA2467	<i>foxR</i>		Anti-sigma factor FoxR	-2.1	0.03
PA2468	<i>foxI</i>		ECF sigma factor FoxI	-2.7	0.03
PA2564		x	Hypothetical protein	-4.0	0.04
PA2565			Hypothetical protein	-4.4	0.01
PA2566		x	Conserved hypothetical protein	-5.9	0.04
PA2570	<i>lecA</i>	x	LecA	-38.9	0.03
PA2587	<i>pqsH</i>	x	Probable FAD-dependent monooxygenase	-3.8	0.02
PA2588		x	Probable transcriptional regulator	-8.9	0.02
PA2589			Hypothetical protein	-4.4	0.03
PA2591		x	Probable transcriptional regulator	-3.9	0.01
PA2686	<i>pfeR</i>		Two-component response regulator PfeR	-2.2	0.03
PA2753		x	Hypothetical protein	-2.3	0.04
PA2788			Probable chemotaxis transducer	-5.8	0.03
PA2862	<i>lipA</i>		Lactonizing lipase precursor	-2.7	0.01
PA3023			Conserved hypothetical protein	-2.3	<0.01
PA3094			Probable transcriptional regulator	-2.9	0.03

PA3103	<i>xcpR</i>		General secretion pathway protein E	-2.2	0.02
PA3190			Probable binding protein component of ABC sugar transporter	-2.3	0.01
PA3195	<i>gapA</i>		Glyceraldehyde 3-phosphate dehydrogenase	-2.3	0.05
PA3274		x	Hypothetical protein	-3.7	0.01
PA3283			Conserved hypothetical protein	-3.6	0.04
PA3284			Hypothetical protein	-3.0	<0.01
PA3305.1	<i>phrS</i>		PhrS	-6.5	0.05
PA3326		x	Probable Clp-family ATP-dependent protease	-12.7	0.04
PA3327		x	Probable non-ribosomal peptide synthetase	-8.1	0.02
PA3328		x	Probable FAD-dependent monooxygenase	-27.0	0.01
PA3329		x	Hypothetical protein	-11.4	0.04
PA3330		x	Probable short chain dehydrogenase	-31.5	0.03
PA3331		x	Cytochrome P450	-15.9	0.03
PA3332		x	Conserved hypothetical protein	-19.5	0.03
PA3333	<i>fabH2</i>	x	3-oxoacyl-[acyl-carrier-protein] synthase III	-19.0	0.03
PA3334		x	Probable acyl carrier protein	-14.0	0.02
PA3337	<i>rfaD</i>		ADP-L-glycero-D-mannoheptose 6-epimerase	-3.3	0.04
PA3361	<i>lecB</i>	x	Fucose-binding lectin PA-III	-35.3	0.02
PA3362			Hypothetical protein	-4.7	0.01
PA3407	<i>hasAp</i>		Heme acquisition protein HasAp	-2.7	0.01
PA3408	<i>hasR</i>		Haem uptake outer membrane receptor HasR precursor	-2.2	0.03
PA3431			Conserved hypothetical protein	-6.1	0.05
PA3452	<i>mqaA</i>		Malate:quinone oxidoreductase	-2.4	0.03
PA3459			Probable glutamine amidotransferase	-3.1	0.01
PA3460			Probable acetyltransferase	-3.7	0.05
PA3461			Conserved hypothetical protein	-4.0	0.04
PA3477	<i>rhlR</i>	x	Transcriptional regulator RhlR	-5.7	<0.01
PA3478	<i>rhlB</i>	x	Rhamnosyltransferase chain B	-27.6	<0.01
PA3479	<i>rhlA</i>	x	Rhamnosyltransferase chain A	-110.4	<0.01
PA3510			Hypothetical protein	-2.1	0.05
PA3530			Conserved hypothetical protein	-4.8	0.02
PA3535			Probable serine protease	-2.9	0.02
PA3554	<i>arnA</i>		ArnA	-3.2	0.03
PA3555	<i>arnD</i>		ArnD	-3.0	0.05
PA3557	<i>arnE</i>		ArnE	-2.9	<0.01
PA3558	<i>arnF</i>		ArnF	-3.5	0.03
PA3559			Probable nucleotide sugar dehydrogenase	-3.2	0.03
PA3621.1	<i>rsmZ</i>		Regulatory RNA RsmZ	-4.0	0.04
PA3622	<i>rpoS</i>		Sigma factor RpoS	-2.0	<0.01
PA3724	<i>lasB</i>	x	Elastase lasB	-124.4	0.02
PA3734			Hypothetical protein	-5.6	0.05
PA3808			Conserved hypothetical protein	-2.1	0.03

PA3809	<i>fdx2</i>		Ferredoxin [2Fe-2S]	-2.6	0.05
PA3841	<i>exoS</i>		Exoenzyme S	-3.0	0.05
PA3842			Probable chaperone	-3.9	0.02
PA3843			Hypothetical protein	-2.0	0.04
PA3899			Probable sigma-70 factor, ECF subfamily	-3.7	0.01
PA3906		x	Hypothetical protein	-3.5	0.01
PA3908		x	Hypothetical protein	-3.3	0.02
PA4078			Probable nonribosomal peptide synthetase	-3.4	0.02
PA4116	<i>bphO</i>		Heme oxygenase, BphO	-2.2	0.02
PA4117	<i>bphP</i>		Bacterial phytochrome, BphP	-3.0	0.03
PA4138	<i>tyrS</i>		Tyrosyl-tRNA synthetase	-2.3	0.04
PA4141		x	Hypothetical protein	-144.7	0.02
PA4142		x	Probable secretion protein	-43.3	0.01
PA4143			Probable toxin transporter	-20.8	0.02
PA4144			Probable outer membrane protein precursor	-16.2	<0.01
PA4156			Probable TonB-dependent receptor	-11.5	<0.01
PA4158	<i>fepC</i>		Ferric enterobactin transport protein FepC Ferrienterobactin-binding periplasmic protein precursor	-4.0	0.03
PA4159	<i>fepB</i>		FepB	-9.7	0.02
PA4160	<i>fepD</i>		Ferric enterobactin transport protein FepD	-5.0	0.03
PA4175	<i>piv</i>	x	Protease IV	-24.3	<0.01
PA4209	<i>phzM</i>	x	Probable phenazine-specific methyltransferase	-25.1	0.02
PA4213	<i>phzD1</i>		Phenazine biosynthesis protein PhzD	-71.8	0.05
PA4217	<i>phzS</i>		Flavin-containing monooxygenase	-3.4	0.04
PA4227	<i>pchR</i>		Transcriptional regulator PchR	-2.2	0.02
PA4294			Hypothetical protein	-2.9	<0.01
PA4338			Hypothetical protein	-2.1	0.03
PA4377			Hypothetical protein	-3.5	0.03
PA4394			Conserved hypothetical protein	-3.2	0.01
PA4470	<i>fumC1</i>		Fumarate hydratase	-3.9	0.01
PA4471			Hypothetical protein	-16.0	0.04
PA4570			Hypothetical protein	-5.7	0.01
PA4585	<i>rtcA</i>		RNA 3-terminal phosphate cyclase	-2.1	0.01
PA4586			Hypothetical protein	-2.7	<0.01
PA4587	<i>ccpR</i>		Cytochrome c551 peroxidase precursor	-2.9	0.05
PA4588	<i>gdhA</i>		Glutamate dehydrogenase	-4.9	0.01
PA4590	<i>pra</i>	x	Protein activator	-4.6	0.02
PA4675			Probable TonB-dependent receptor	-3.5	0.02
PA4774			Hypothetical protein	-2.3	0.05
PA4775			Hypothetical protein	-2.1	0.01
PA4813	<i>lipC</i>		Lipase LipC	-2.1	0.05
PA4847	<i>accB</i>		Biotin carboxyl carrier protein (BCCP)	-2.3	0.05
PA4925			Conserved hypothetical protein	-2.5	0.04

PA5111	<i>gloA3</i>		Lactoylglutathione lyase	-2.1	0.02
PA5150			Probable short-chain dehydrogenase	-3.2	0.02
PA5163	<i>rmlA</i>		Glucose-1-phosphate thymidyltransferase	-2.6	0.02
PA5164	<i>rmlC</i>		dTDP-4-dehydrorhamnose 3,5-epimerase	-2.4	0.02
PA5172	<i>arcB</i>		Ornithine carbamoyltransferase, catabolic	-2.8	0.01
PA5220		x	Hypothetical protein	-8.5	0.01
PA5232		x	Conserved hypothetical protein	-2.5	0.04
PA5424			Conserved hypothetical protein	-3.0	0.05
PA5475			Hypothetical protein	-3.6	0.02
PA5526			Hypothetical protein	-2.0	0.02
PA5531	<i>tonB1</i>		TonB protein	-2.1	0.01

^aAn 'X' in the box indicates that the gene/protein is regulated by quorum sensing (Hentzer et al., 2003), in reference to the QS-regulated genes/proteins as determined previously.