The use of quorum quenching enzymes and quorum sensing inhibitors to combat *Pseudomonas aeruginosa* and *Acinetobacter baumannii* biofilm-related infections

Yunhui Zhang

Thesis submitted to obtain the degree of Doctor in Pharmaceutical Sciences

Promotor: Prof. Dr. Tom Coenye

Laboratory of Pharmaceutical Microbiology

2018

COPYRIGHT

The authors and the supervisor give the authorization to consult and copy parts of this manuscript for personal use only. Any other use is limited by the laws of copyright, especially the obligation to refer to the source whenever results from this manuscript are cited.

Ghent, April 2018

Author	Promotor
Yunhui Zhang	Prof. Dr. Tom Coenye

Promoter

Prof. Dr. Tom Coenye

Laboratory of Pharmaceutical Microbiology, Ghent University, Belgium

Members of the examination and reading committee

Prof. Dr. Serge Van Calenbergh (Chairman)

Laboratory of Medicinal Chemistry, Ghent University, Belgium

Prof. Dr. Kevin Braeckmans (Secretary)

Laboratory of General Biochemistry and Physical Pharmacy, Ghent University, Belgium

Dr. Aurélie Crabbé

Laboratory of Pharmaceutical Microbiology, Ghent University, Belgium

Dr. Apr. Heleen Van Acker

Laboratory of Pharmaceutical Microbiology, Ghent University, Belgium

Prof. Dr. Ir. Tom Defoirdt

Center for Microbial Ecology and Technology, Ghent University, Belgium

Prof. Dr. Hans Steenackers

Centre of Microbial and Plant Genetics, University of Leuven, Belgium

ACKNOWLEDGEMENTS

First and foremost, I would like to show my deepest gratitude to my promoter, **Prof. Coenye**, who gave me the opportunity to pursue my PhD at Lab of Pharmaceutical Microbiology. He has provided me with continuous guidance and support throughout my doctoral studies. Without his enlightening instruction, impressive kindness and patience, I could not have completed my thesis. Thanks for his time and great efforts in helping me through the writing of this thesis.

I would like to thank the members of examination and reading committee, **Prof. Van Calenbergh**, **Prof. Braeckmans**, **Prof. Defoirdt**, **Prof. Steenackers**, **Aurélie** and **Heleen**. Thanks a lot for their critical reviews and extremely valuable suggestions to improve this thesis.

Special thanks to all my colleagues in the lab for the past four years. It is my pleasure to work with every one of you. Your kindness and help make my life and work much easier in a foreign country and I really enjoy those days we spent together.

Gilles, thanks for guiding me through first two years of my PhD. Without your help, I cannot get start with the lab work in LPM in a short time. You always have right answers for my questions. I sincerely hope that you could stay longer with us then I could learn more from you. **Aurélie**, I really appreciate your keen and vigorous academic attitude, and many thanks to your suggestions and help for my work. **Heleen**, you are always warm-hearted and ready to help, thanks for your help in RNA extractions. **Andrea**, you are so professional in molecular work, thanks for your time and patience in helping my with the RNA-seq data analysis. **Ilse**, you are also the one who help me a lot when I start my work in LPM and thank you so much. **Frejia** and **Sarah**, also many thanks to all your help, and it is really a nice experience to go fitness with you. **Karl-Jan** and **Eva**, it is nice to start PhD together with you in LPM, you are excellent colleagues to be with! **Lisa**, we are both working on QS and it is really nice to talk with you from time to time. It is also a valuable memory for the conference with you in Spain! **Sanne** and **Annelien**, you both did a great job in LPM, and I am impressed for your passion for dancing as well! **Jasper**, you are always smart and humorous, and it is happy to be colleagues with you. **Charlotte** and **Ian**, you are both sweet colleagues, and I really miss your cute cats! **Frits** and **Sara**, although we don’t have much time to get along, but both of you are kind, nice and

hard-working. Good luck with your PhD study! **Qi**, you seem like my younger sister and it is lucky to have your companion in the lab for these two years. Wish you enjoy the rest time in Belgium!

Rosina, I know you have done a lot work for the lab, and you are always there to help with orders and reimbursements. I also really enjoy the delicious food you prepared for every Christmas party. Special thanks for your help with my reception! **Inne**, I still remember how you guided me to prepare the media on my first day in the lab. Thank you for all your help and encouragement, and don't forget our little talks about your trip to China! **Petra**, you always know where are the things I am looking for, thanks for your help! **Nele**, you are my first "neighbor" to work with in the lab, and you also help me a lot during my first year. **Lisa O**, you are always energetic and helpful, and it's really nice to know you. Sincerely hope that everything goes well with life and work of all my lovely colleagues!

I would like also to thank all my dear friends in Belgium, especially **Ting**, **Han** and **Luyan**, as well as my roommate **Huiyan**. You make my life in Belgium happier and it is a great experience to know you and travel around with you. Hope our friendship can last forever.

Special thanks also go to Chinese Scholarship Council and Ghent University who have funded me to finish my research work.

Last but not least, I would like to express my special thanks to my parents, whose care and support motivate me to move on and make me want to be a better person. I know how hard it is to be separate with me for so many years. I am proud to be your daughter and I will always love you.

Thanks!

Yunhui

TABLE OF CONTENTS

LIST OF ABBREVIATIONS 1

CHAPTER I. INTRODUCTION 3

1. *PSEUDOMONAS AERUGINOSA* AND *ACINETOBACTER BAUMANNII* INFECTIONS 4

1.1 Pathogenicity of *P. aeruginosa* and *A. baumannii* 5
1.2 Antibiotic resistance in *P. aeruginosa* and *A. baumannii* 6
1.3 Biofilm formed by *P. aeruginosa* and *A. baumannii* 7
1.4 Wound infections of *P. aeruginosa* and *A. baumannii* 8
1.5 Novel strategies against *P. aeruginosa* and *A. baumannii* infections 9

2. QUORUM SENSING 10

2.1 General review of quorum sensing 10
2.1.1 LuxI/LuxR-type quorum sensing 10
2.1.2 Peptide-mediated quorum sensing 12
2.1.3 LuxS/AI-2-type quorum sensing 12
2.1.4 Other types of quorum sensing 13
2.2 Quorum sensing in *P. aeruginosa* 13
2.2.1 *las* and *rhl* quorum sensing systems 14
2.2.2 Quinolone signaling 15
2.2.3 The integrated quorum sensing (IQS) system 16
2.2.4 Interconnection of quorum sensing regulatory systems 16
2.2.5 Potential advantages of combining different QS systems 17
2.2.6 Additional regulatory factors of quorum sensing 18
2.2.7 Role of quorum sensing in *P. aeruginosa* virulence and biofilms 21
2.3 Quorum sensing in *A. baumannii* 24
2.3.1 AbaI/AbaR quorum sensing system 25
2.3.2 Role of quorum sensing in *A. baumannii* virulence and biofilms 25

3. QUORUM QUENCHING ENZYMES 27

3.1 Diversity of QQ enzymes 27
3.1.1 AHL-lactonases 28
3.1.2 AHL-acylases 30
3.1.3 AHL-oxidoreductases 31
3.1.4 Physiological roles of QQ enzymes 32
3.2 The use of QQ enzymes to interfere with virulence 33
3.2.1 *In vitro* proof 33
3.2.2 *In vivo* proof 34
3.2.3 Combined use of QQ enzymes 35
3.2.4 Exploring the application of QQ enzymes 37

4. QUORUM SENSING INHIBITORS 38

4.1 Diversity of QSIs 39
4.1.1 Natural QSIs 39

4.1.2 Synthetic QSIs 40
4.1.3 Known drugs as QSIs 41
4.2 QSIs as antivirulence drugs 42
4.2.1 *In vitro* and *in vivo* proof 42
4.2.2 Clinical trials with QSIs 44
4.3 QSIs *vs.* QQ enzymes 44

CHAPTER II. OBJECTIVES 46

CHAPTER III. EXPERIMENTAL WORK 48

Paper I: Pitfalls associated with evaluating enzymatic quorum quenching activity: the case of MomL and its effect on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* biofilms **49**

Paper II: Coumarin reduces virulence and biofilm formation in *Pseudomonas aeruginosa* by affecting quorum sensing, type III secretion and c-di-GMP levels **68**

Paper III: The effects of potential quorum sensing inhibitors on biofilm, motility and virulence of *Acinetobacter baumannii* **99**

CHAPTER IV. BROADER INTERNATIONAL CONTEXT, RELEVANCE, AND FUTURE PERSPECTIVES 111

1. The need for antivirulence drugs 112

2. QS as antivirulence target in infections: promises and challenges 113

3. Plants as abundant sources for natural QSIs 117

4. Methods used in QS inhibition studies 119

5. Comparison of transcriptome studies on QS inhibition in *P. aeruginosa* 121

6. Will QS inhibition lead to resistance? 123

7. Comparison of QQ enzymes and QSIs as antivirulence therapy 125

8. Future perspectives for QS inhibition studies 126

8.1 Future perspectives for QQ enzymes 126
8.2 Future perspectives for QSIs 127
8.3 Future perspectives for QS inhibition therapy in clinic 127

CHAPTER V. SUMMARY 128

REFERENCES 134

LIST OF ABBREVIATIONS

3-OH-C12-HSL	N-(3-hydroxydodecanoyl)- homoserine lactone
3-oxo-C12-HSL	N-3-oxo-dodecanoyl-L-homoserine lactone
6-CABA	2-Amino-6-chlorobenzoic acid
Agr	Accessory gene regulator
AHL	N-acyl-homoserine lactone
AI	Autoinducer
AIP	Autoinducing peptide
ATP	Adenosine triphosphate
C4-HSL	N-butyryl-L-homoserine lactone
cAMP	Cyclic adenosine monophosphate
c-di-GMP	Cyclic diguanylate
CFU	Colony forming unit
CIP	Ciprofloxacin
CRISPR	Clustered regularly interspaced short palindromic repeats
CST	Colistin
CTB	Celltiter-Blue
CV	Crystal violet
DGC	Diguanylate cyclase
DMSO	Dimethyl sulfoxide
DSF	Diffusible signal factor
eDNA	Extracellular deoxyribonucleic acid
EHEC	Enterohemorrhagic *E. Coli*
FDR	False discovery rate
GLM	Generalized linear model
GTP	Guanosine triphosphate
HCN	Hydrogen cyanide
HHQ	2-heptyl-4-quinolone
HPLC	High-performance liquid chromatography
ICU	Intensive care unit
IPTG	Isopropyl-β-D-thiogalactopyranoside
IQS	Integrated quorum sensing, 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde
LB	Luria-Bertani medium
MBL	Metallo-β-lactamase
mBTL	Meta-bromo-thiolactone
MDR	Multidrug resistance
MEM	Meropenem
MH	Mueller-Hinton broth
MIC	Minimum inhibitory concentration

NADP	Nicotinamide adenine dinucleotide phosphate
NP	Nanoparticles
OD	Optical density
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PLL	PTE-like lactonases
PMNs	Polymorphonuclear leukocytes
PON	Paraoxonase
ppGpp	Guanosine tetraphosphate
PQS	The *Pseudomonas* quinolone signal, 2-heptyl-3-hydroxy-4-quinolone
PS	Physiological saline
PTE	Phosphotriesterase
QQ	Quorum quenching
QS	Quorum sensing
QSI	Quorum sensing inhibitor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Quantitative real-time polymerase chain reaction
SAM	S-adenosylmethionine
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
T3SS	Type III secretion system
TCA	Tricarboxylic acid
TOB	Tobramycin
TSA	Tryptic soy agar
VAP	Ventilator-associated pneumonia
WHO	World health organization
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER I. INTRODUCTION

1. *PSEUDOMONAS AERUGINOSA* AND *ACINETOBACTER BAUMANNII* INFECTIONS

Despite decades of intensive antimicrobial development, bacterial infections remain an important cause of morbidity and mortality in hospital. Meanwhile, the increasing bacterial resistance to conventional antibiotics further poses a considerable health threat worldwide. *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are two Gram-negative pathogens which are frequently found in clinical samples. These nosocomial pathogens affect severely ill patients with compromised immune systems, and cause a wide spectrum of infections in wounds, urinary tract, bloodstream and lung. *P. aeruginosa* has long been recognized as a pathogen with notable virulence, while *A. baumannii* is less virulent, it is also increasingly associated with numerous outbreaks of infections, especially in the intensive care units (ICUs). Types of possible P. aeruginosa and A. baumannii infections are listed in Table 1.1.

Table 1.1 Types of infections can be caused by *P. aeruginosa* and *A. baumannii*.

Pathogens	**Infections**
P. aeruginosa	Respiratory tract infection: ventilator-associated pneumonia, cystic fibrosis
	Central nervous system infection: meningitis
	Ear infection: chronic otitis media, malignant otitis externa
	Eye infection: keratitis, endophthalmitis
	Skeletal system infection
	Gastrointestinal tract infection
	Urinary tract infection
	Skin and soft tissue infection: burns and chronic wound infection
	Blood stream infection: bacteremia, sepsis
	Endocarditis
A. baumannii	Respiratory tract infection: pneumonia
	Blood stream infection: bacteremia, sepsis
	Central nervous system infection: meningitis.
	Wound and surgical site infections
	Urinary tract infection

Both of these pathogens are noted for their genetic potential to carry and transfer diverse antibiotic resistance mechanisms, leading to limited therapeutic options in the clinic. According to the global priority list of antibiotic-resistant bacteria released by

the World Health Organization (WHO) in 2017 [1], carbapenem-resistant *A. baumannii* is on top of the list, followed by carbapenem-resistant *P. aeruginosa*, both with critical priority (Table 1.2). Given the rapid emergency of resistant *P. aeruginosa* and *A. baumannii* strains, discovery of alternative treatment strategies for these pathogens has become an urgent priority.

Table 1.2 Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics [1] .

Priority	Pathogen
Priority 1: CRITICAL	
	Acinetobacter baumannii, carbapenem-resistant
	Pseudomonas aeruginosa, carbapenem-resistant
	Enterobacteriaceae, carbapenem-resistant, 3rd generation cephalosporin-resistant
Priority 2: HIGH	
	Enterococcus faecium, vancomycin-resistant
	Staphylococcus aureus, methicillin-resistant, vancomycin intermediate and resistant
	Helicobacter pylori, clarithromycin-resistant
	Campylobacter, fluoroquinolone-resistant
	Salmonella spp., fluoroquinolone-resistant
	Neisseria gonorrhoeae, 3rd generation cephalosporin-resistant, fluoroquinolone-resistant
Priority 3: MEDIUM	
	Streptococcus pneumoniae, penicillin-non-susceptible
	Haemophilus influenzae, ampicillin-resistant
	Shigella spp., fluoroquinolone-resistant

1.1 Pathogenicity of *P. aeruginosa* and *A. baumannii*

As a successful opportunistic pathogen that takes advantage of a host with a weak immune system, *P. aeruginosa* shows high adaptability and can survive under nutrient-poor conditions and different stresses. The pathogenesis of *P. aeruginosa* is mediated by various virulence factors such as adhesins, proteases, secreted toxins and pigments which help *P. aeruginosa* to colonize and escape the host immune systems [2, 3]. The production of several virulence factors is closely related to the complex quorum sensing (QS) regulatory network. Type III secretion system (T3SS), a protein appendage found in several Gram-negative bacteria, is also a major determinant of virulence, which injects toxins directly into host cells during infections. However, the pathogenicity of *P. aeruginosa* varies from strain to strain, and

differential sets of pathogenicity factors and mechanisms are found in different isolates.

A. baumannii has emerged as a highly troublesome pathogen in last decades, and similar to *P. aeruginosa* it can cause various types of infections, and spread quickly during hospital outbreaks. A number of virulence factors have been identified in *A. baumannii* including secretion systems, surface glycoconjugates and iron acquisition systems [4-6]. However, discernible toxins or molecular determinants which can account for the virulence potential of a particular *A. baumannii* strain are often absent, and a “persist and resist” strategy contributes to the success of *A. baumannii* [6]. The most striking fact about *A. baumannii* is its ability to acquire antibiotic resistance and the globally rapid emergence of multidrug resistance (MDR) strains.

1.2 Antibiotic resistance in *P. aeruginosa* and *A. baumannii*

P. aeruginosa and *A. baumannii* strains are resistant to many antimicrobials through inherent structural or functional characteristics, and they can also acquire resistance easily by mutations or acquisition of genetic material [7]. Different types of *β*-lactamases have been identified in both *P. aeruginosa* and *A. baumannii*, such as AmpC cephalosporinases and metallo-*β*-lactamases [8]. Aminoglycoside-modifying enzymes are also commonly found in these two pathogens, attributing to the resistance to aminoglycosides [8]. Other important resistance mechanisms shared by *P. aeruginosa* and *A. baumannii* include mutations in porins and overexpression of efflux pumps. The accumulation of multiple resistance mechanisms leads to MDR strains which are resistant to almost all available antibiotics. Antibiotics cause a selective pressure by killing susceptible bacteria, allowing antibiotic-resistant bacteria to survive and multiply and leading to the emergence of MDR strains. These MDR strains can spread quickly resulting in prolonged outbreaks. As carbapenem-resistant *P. aeruginosa* and *A. baumannii* strains are increasing in the hospital worldwide, only a few treatment options are still available. Colistin has been regarded as the last-resort antibiotic to treat *P. aeruginosa* and *A. baumannii* MDR strains, but resistance against colistin was also described and might spread worldwide [9, 10].

1.3 Biofilm formed by *P. aeruginosa* and *A. baumannii*

Biofilms are structures formed by surface-attached bacteria embedded in an extracellular polymeric matrix consisting of proteins, nucleic acids and polysaccharides. Chronic infections such as pneumonia in cystic fibrosis patients, chronic wounds and implant- and catheter-associated infections are closely associated with biofilm formation [11]. Bacteria within biofilms are shielded from antibiotic treatment and host immune defense system. An altered microenvironment existing in biofilms with gradients of nutrients, pH and oxygen, and gene expression in biofilm cells is significantly distinct from those in planktonic cells. In addition, a relatively large population of persister cells, which are able to survive antibiotic treatment and resume their growth, can be found in biofilms [12, 13]. *P. aeruginosa* is frequently involved in biofilm-associated infections and has been used as a model organism to study biofilms in Gram-negative bacteria. The whole process of biofilm formation includes cell attachment, microcolony formation, maturation, expansion and dissemination (Figure 1.1) [14]. Rhamnolipids, secreted polysaccharides like Pel, Psl and alginate, and extracellular DNA (eDNA) play an important role in maintaining the structure of the biofilm and matrix in *P. aeruginosa* [14]. *A. baumannii* clinical isolates also possess a strong ability to form biofilms [15], both in tissues and on the surfaces of medical devices. A number of genes have been reported to be involved in adhesion and biofilm development of *A. baumannii* as reviewed by Longo *et al*. [16].

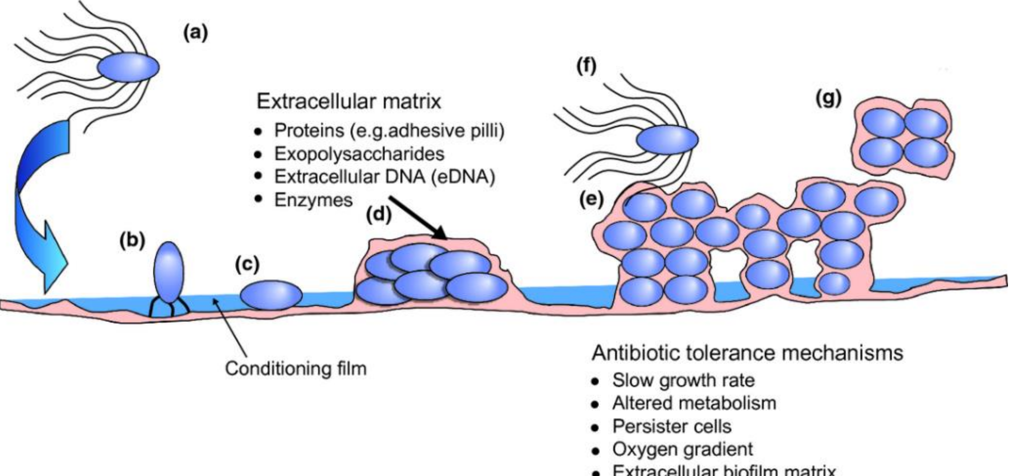

Figure 1.1 Biofilm formation and characteristics. The different stages of biofilm formation from free-swimming cells (a), attachment to the surface (b,c), formation of microcolonies (d), formation of a

mature three-dimensional biofilm architecture (e), and dispersion (f,g). (Modified from Römling *et al.*, 2012 [13])

1.4 Wound infections caused by *P. aeruginosa* and *A. baumannii*

Acute and chronic wound infections affect millions of people worldwide and represent a considerable healthcare burden [17]. Surgical or traumatic wounds and burns are typical acute wounds. Chronic wounds are those detained in the normal wound healing process, such as diabetic, arterial, venous and pressure ulcers. Although several factors contribute to wound healing, bacterial infections can significantly delay this process. Chronic wounds can be colonized by a wide range of bacteria and fungi, including *P. aeruginosa* and *A. baumannii* [18, 19]. *A. baumannii* wound infections have been reported with increasing incidences of outbreaks among victims of combat injuries and natural disasters [20]. It is also an infrequent cause of skin and soft tissue infections in intensive care unit patients [21]. *P. aeruginosa* is among the most common bacteria isolated from chronic wounds [22]; it also causes infections in burn wounds, leading to rapid clinical deterioration, systemic spread, and in some cases death within days or weeks [23].

Biofilms are prevalent especially in chronic wounds and are often composed of diverse bacterial species. Fungi like *Candida albicans* and the bacterial pathogen *S. aureus* are common species in wound infections. Biofilms formed by *C. albicans* and *S. aureus* revealed a unique architecture in which *S. aureus* is associated with the hyphae of *C. albicans* [24]. The simultaneous infection with *S. aureus* and *P. aeruginosa* can significantly impair wound closure as compared to monospecies biofilms in mouse and rabbit wound healing models [25, 26]. Polymicrobial infections involving *P. aeruginosa* and *A. baumannii* have also been frequently reported [27-29]. A better understanding of the precise mechanisms by which bacterial biofilms delay repair processes as well as optimizing methods for biofilm detection and prevention may enhance opportunities for chronic wounds healing.

Wound biofilms raise difficult problems in clinical diagnosis and treatment. Debridement has been studied as an approach of wound cleansing which facilitates the wound healing by reducing wound biofilms [30]. Maggot debridement therapy (MDT), in which live and 'medical-grade' fly larvae (*Lucilla sericata*) are applied to the

patient's wounds to achieve debridement, disinfection and wound healing has recently received considerable attention [31, 32]. MDT mainly helps wound healing by debridement of necrotic tissue by both mechanical and biochemical means; a mixture of proteolytic enzymes can be secreted by the maggots and lyse nonviable tissue [33]. However, QS-regulated virulence factors of *P. aeruginosa* are toxic to the maggots, leading to the failure of MDT treatment in wound infections with *P. aeruginosa* [34].

1.5 Novel strategies against *P. aeruginosa* and *A. baumannii* infections

To cope with the growing threat of resistant pathogens, several novel strategies have been proposed and are currently widely studied. Except for new antibiotics, anti-virulence agents are considered as promising ways to disarm *P. aeruginosa* and *A. baumannii* during infections without interfering with bacterial viability and/or exterting a selective stress like conventional antibiotics. Inhibitors against specific virulence factors such as elastase, T3SS and lectins in *P. aeruginosa* have been reported, however, one such inhibitor alone may not be able to block the virulence of *P. aeruginosa* [35]. Iron chelation is also frequently discussed for both *A. baumannii* and *P. aeruginosa* [36, 37]. For each approach, many questions remain to be answered before application will be possible. In the following part of this thesis, we will focus on the QS regulation system, one of the most extensively studied anti-virulence targets in many bacteria including *P. aeruginosa* and *A. baumannii*.

2. QUORUM SENSING

2.1 General review of quorum sensing

Research in QS started with the study of the growth-dependent bioluminescence in the marine bacteria *Vibrio fischeri* and *Vibrio harveyi* in 1970s [38, 39]. A specific substance (autoinducer) was assumed to accumulate and induce the synthesis of the luminescence system components in this "autoinduction" phenomenon [39], as bioluminescence is only observed at high cell population density. Autoinducers in both species were identified later as *N*-acyl-homoserine lactones (AHLs) [40, 41], which now have been recognized as the dominant signal molecules in QS of many Gram-negative bacteria. A typical QS cascade includes synthesis of signal molecules, signal detection and transduction, and transcriptional gene regulation. When the threshold concentration of autoinducers is exceeded, bacteria can alter their gene expression and behavior correspondingly on a population-wide scale [42-44]. QS synchronizes processes such as symbiosis, conjugation, biofilm formation, motility, virulence [45], as well as the production of public goods (secreted compounds that might provide more benefit at higher population densities) [46].

So far, a collection of different chemical signals is known to be used in different QS systems (Figure 1.2), and more than one signal or pathway can be found in individual species of bacteria where they form a hierarchical QS network. Here, several typical QS systems are introduced briefly.

2.1.1 LuxI/LuxR-type quorum sensing

As mentioned above, AHLs are the most common autoinducers in many Gram-negative bacteria. Their typical structure contains a core *N*-acylated homoserine-lactone ring and an acyl chain (4-18 carbons) with an occasional hydroxy or olefinic double bond modification at the C3 position (Figure 1.2) [47]. Responsible for the production of AHLs, LuxI-type synthases can be found in hundreds of Gram-negative bacterial species [48]. In most cases, they synthesize AHLs from S-adenosylmethionine (SAM) and acyl carrier protein which participates in fatty acid biosynthesis [49]. Short-chain AHLs like *N*-butyryl-L-homoserine lactone (C4-HSL)

can freely diffuse out of cells, whereas AHLs with longer fatty acid chains may also require transporters to be released from cells [50]. The concentration of AHLs increases along with the growth of the cell population. Binding of LuxR-type receptors only occurs when an intracellular AHL concentration threshold is reached, and then the LuxR-AHL complexes bind (or release) DNA promoter elements and regulate transcription of QS controlled genes [51]. An acyl binding pocket at the N-terminal domain of LuxR protein precisely accommodates the acyl chain of its cognate AHL signal, which ensures the specificity of the LuxR-AHL interaction [52]. The expression of LuxI can also be enhanced by the binding of AHL and LuxR, forming a positive regulatory feedback loop in these QS systems [53].

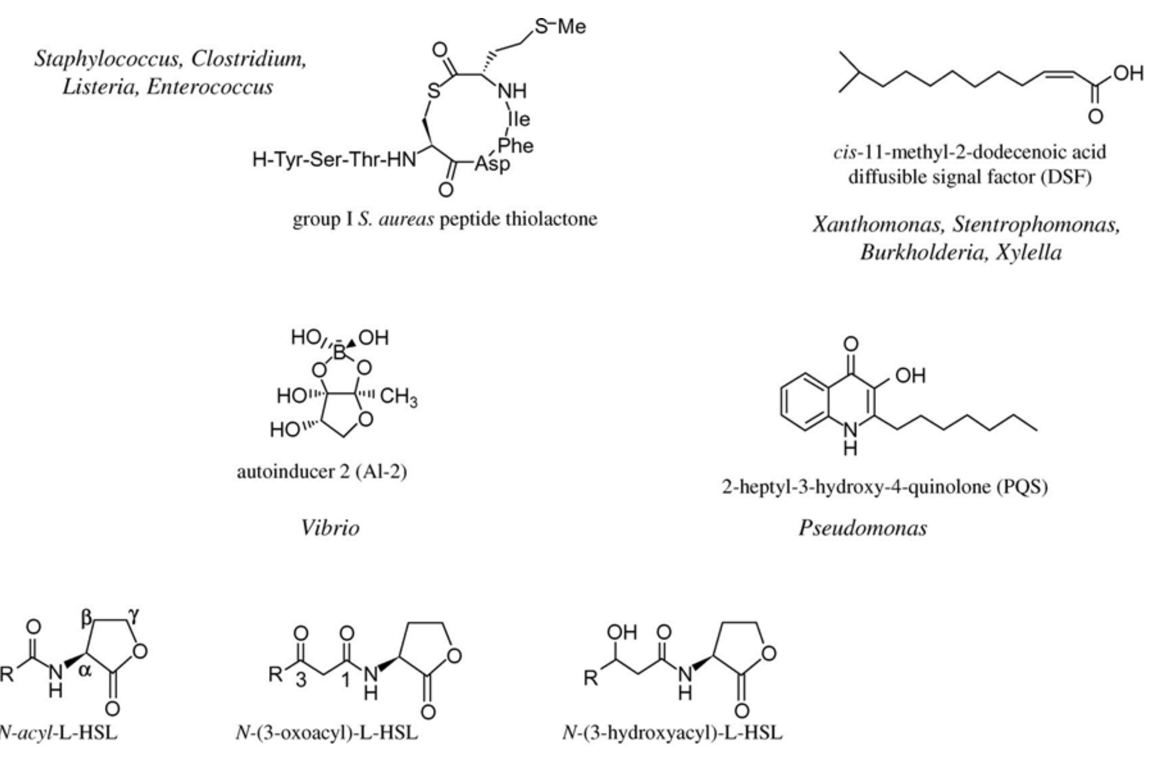

Figure 1.2 The structures of several common QS signal molecules. (Modified from Atkinson *et al.*, 2009 [44])

First identified in *V. fisheri* [54], LuxI/LuxR-type QS has also been reported and widely investigated in *Agrobacterium tumefaciens* (TraI/R) [55], *Chromobacterium violaceum* (CviI/R) [56], *Erwinia carotovora* (ExpI/R, CarI/R) [57, 58], *Burkholderia cenocepacia* (CepI/R) [59] and *Yersinia enterocolitica* (YenI/R) [60], as well as in

pathogens *P. aeruginosa* and *A. baumannii* which will be discussed in detail in the following part. Orphan LuxR-type receptors without an associated synthase are also frequently identified, such as TrlR in *A. tumefaciens*, QscR in *P. aeruginosa* and SdiA in *Salmonella enterica* and *Escherichia coli* [61]. These orphan receptors participate in the regulation of existing QS network, and could potentially respond to external environmental stimuli such as plant compounds that can be detected by TrlR in *A. tumefaciens* [61].

2.1.2 Peptide-mediated quorum sensing

Unlike Gram-negative bacteria, Gram-positive bacteria employ auto inducing peptides (AIPs) ranging from 5 to 17 amino acids in length as QS signals. AIPs are cleaved or modified after ribosomal synthesis, and ATP-binding cassette transporters are involved in their secretion. Sensor kinases belonging to typical two-component adaptive response systems recognize AIPs, and the activity of the response regulator is influenced through a phosphorylation cascade [62, 63].

Peptide-mediated QS was first reported in *Lactococcus lactis* and *Streptococcus pneumoniae* [64, 65]. The accessory gene regulator (Agr) system in the human pathogen *Staphylococcus aureus* has also intensively been studied [66, 67]; it positively regulates the expression of many toxins and degradative exoenzymes, and negatively regulates the expression of several colonization factors during infections.

2.1.3 LuxS/AI-2-type quorum sensing

A furanosyl borate diester [68], designated autoinducer-2 (AI-2), was first reported to co-regulate density-dependent luminescence by a Gram-positive-like two-component phospho-relay system in *V. harveyi* [69, 70]. Different from AHLs and oligopeptides, AI-2 is produced by many Gram-negative and Gram-positive bacteria with identical biosynthetic pathway, and might act as a universal signal in interspecies communication [69]. LuxS is the key enzyme involved in the synthesis of the linear form of AI-2, 4,5-dihydroxy-2,3-pentanedione (DPD). However, since LuxS is also an enzyme in an important central metabolic pathway, the activated methyl cycle (AMC), it is equally possible that in some species AI-2 is merely a metabolic by-product [71]. AI-2 regulates biofilm formation, motility and other virulence traits among *Bacillus*

cereus, *E. coli*, *Haemophilus influenza* and *S. aureus* [71], and it can also influence the biofilm formation of non-AI-2-producing *P. aeruginosa* [72].

2.1.4 Other types of quorum sensing

It seems common that a single bacterial species uses multiple autoinducers, even from different types. In the complex network of *V. harveyi*, a third autoinducer CAI-1 (a long-chain amino ketone) was identified [73], which forms a parallel QS circuits with AHL and AI-2 type QS [42].

The molecule 2-heptyl-3-hydroxy-4-quinolone, termed PQS, has been found to be a specific signal in the *P. aeruginosa* QS cascade [74] which will be discussed in the following section.

'Diffusible signal factor' (DSF) is a designation used for a collection of cis-2-unsaturated fatty acids with differing chain length and branching pattern. It influences the virulence of a number of plant and human pathogenic bacteria including *Xanthomonas* spp., *B. cenocepacia* and *P. aeruginosa* [75].

Other less prevalent QS signals have also been reported, e.g. pyrone in *Photorhabdus luminescens* [76] and AI-3 (of which the structure has not yet been identified) in Enterohemorrhagic *E. coli* (EHEC) [77].

2.2 Quorum sensing in *P. aeruginosa*

As a notorious opportunistic human pathogen which causes severe and persistent infections in immune-compromised patients, one of the major factors responsible for the successful invasive behavior and persistence of *P. aeruginosa* is its high adaptability under different conditions. This adaptability is related to its complex regulatory signaling networks involving four connected major QS systems (Figure 1.3) as well as multiple known QS regulators. Approximately 5~10% genes in the genome of *P. aeruginosa* can be influenced positively or negatively by QS [78, 79].

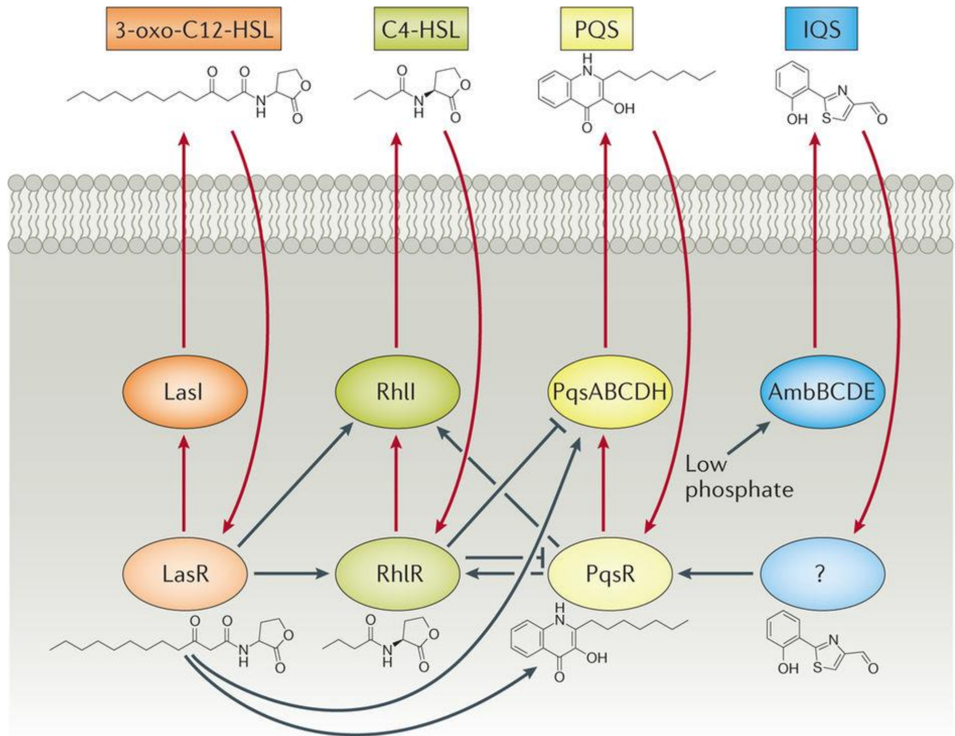

Figure 1.3 QS network in *P. aeruginosa*. [80]

2.2.1 *las* and *rhl* quorum sensing systems

The AHL-based QS systems in *P. aeruginosa* have been intensively studied. In the first identified *las* system, LasI directs the synthesis of *N*-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL). The LuxR homolog LasR acts as the transcriptional regulator which was initially identified to positively regulate the expression of elastase LasB [81-83]. The *las*R gene is also responsible for regulating the production of alkaline protease and exotoxin A as a global virulence gene regulator [84]. 3-oxo-C12-HSL binding is necessary for LasR multimerization and LasR functions as a multimer in *P. aeruginosa* [85]. The MexAB-OprD efflux system is involved in active efflux of 3-oxo-C12-HSL from cells [86].

C4-HSL was later identified as a second AHL produced by *P. aeruginosa* [87]. Its receptor and synthase are RhlR and RhlI; they are encoded by the rhamnolipid synthase gene cluster *rhlABR* and *rhlI*, respectively [88, 89]. Compared to the wild type, *rhlR* and *rhlI* mutants showed defects in the production of elastase, LasA protease, rhamnolipid, and pyocyanin [89]. When regulating rhamnolipid biosynthesis,

RhlR binds to the promoter of *rhlAB* both in presence or absence of C4-HSL [90], but transcription can only be activated in presence of C4-HSL, whereas RhlR alone acts to repress transcription [90]. Besides the dual activator and repressor role of RhlR, a recent study also showed the possibility for RhlR to function in the absence of C4-HSL, and crucial RhlR-regulated virulence factors can be expressed in the absence of RhlI [91], which indicates the possible existence of an alternative RhlR ligand besides C4-HSL.

Transcriptome analyses of *las* and *rhl* mutant revealed "*las*-specific genes", "*rhl*-specific genes" and also genes of which the expression is controlled by both AHLs [78, 92, 93]. The molecular basis for the specific responses depends on the conserved palindromic sequences termed *las*-*rhl* boxes in target promoters binding to either one or both of the regulators [79].

2.2.2 Quinolone signaling

The *Pseudomonas* quinolone signal (PQS), 2-heptyl-3-hydroxy-4-quinolone, was identified as a third signal in QS system of *Pseudomonas aeruginosa* [74]. 4-quinolones are secondary metabolites known for their potent antibiotic activity [75]. PQS synthesis is controlled by the adjacent *pqsABCDE* and *phnAB* operons, as well as by *pqsH* located separately on the chromosome [94]. These genes are also responsible for the synthesis of other alkyl-quinolones (AQs) [95]. *phnAB* genes encode an anthranilate synthase which provide primary precursors for PQS synthesis [95]. PqsA is an anthranilate-coenzyme A ligase and catalyzes the first step in PQS synthesis [96]. The resulting anthranilate-coenzyme A and a β-ketodecanoate are condensed to form 2-heptyl-4-quinolone (HHQ) via PqsBCD. As the direct precursor of PQS, HHQ can be converted to PQS by the mono-oxygenase PqsH [97, 98]. HHQ has been regarded as a messenger molecule in this pathway, and it can also bind to the PQS receptor PqsR (also known as MvfR) in a less potent way compared to PQS [99]. PqsR regulates the transcription of *pqsA-E* and *phnAB* [97], leading to more production of HHQ and PQS and forming a positive feedback loop. PqsE is not involved in the production of PQS but acts as an effector in response to PQS [100], and its role in virulence and interaction between other QS systems has been discussed in the review of Sams *et al*. [100]. However, it is noteworthy that PQS

seems dispensable for virulence since *pqsH* mutation does not attenuate virulence in mice [99], while mutants of *pqsA*, *pqsR* and *pqsE* showed reduced virulence [101].

2.2.3 The integrated quorum sensing (IQS) system

In 2013, a new cell-cell communication signal was identified and named as integrated QS (IQS) for its role in integrating the QS network and stress response mechanisms [102]. IQS is 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde, and its synthesis is controlled by the gene cluster *ambBCDE* which is conserved in various *P. aeruginosa*. Disruption of IQS biosynthesis reduces PQS and C4-HSL production and attenuates bacterial virulence in different animal models [102]. Intriguingly, IQS production is tightly controlled by *las* under normal culture conditions but is also activated by phosphate limitation, a common stress factor encountered during infections, and IQS was demonstrated to be able to partially take over the functions of the central *las* system [102]. Therefore, the IQS system was considered to connect the *las* system and phosphate-stress response mechanism to the *pqs* and *rhl* regulatory systems. More work is still needed to identify the IQS receptor and to elucidate the detailed mechanisms of interplay between *las*, *rhl* and *pqs* systems as well as the phosphate regulons.

2.2.4 Interconnection of quorum sensing regulatory systems

The *las*, *rhl, pqs*, and IQS systems form a sophisticated and interconnected QS network in *P. aeruginosa*, which enables the organism to efficiently regulate the production of virulence factors and secondary metabolites. Though more and more missing parts have been added to the QS puzzle of *P. aeruginosa*, there always seems more to be discovered.

The interrelation of the two AHL QS systems has been explored shortly after they were discovered. Pesci *et al*. and Latifi *et al*. reported in the same year that *rhlR* transcription was positively regulated by LasR/3-oxo-C12-HSL [103, 104]. 3-oxo-C12-HSL can block C4-HSL from binding to RhlR, indicating the existence of post-translational control of RhlR by *las* [103]. However, RhlR has also been reported to induce the transcription of *las*I in a LasR mutant, which means that the *rhl* system may take over the deficient *las* system under certain conditions [105].

The synthesis and bioactivity of PQS are tightly connected to both *las* and *rhl* systems [74], and the PQS system seems to play an indispensable and multifunctional role in the QS network of *P. aeruginosa*. Specifically, LasR regulates the expression of PqsH, thus controling the conversion of HHQ to PQS [94, 97]. LasR also activates PqsR transcription by binding to its promoter, and further influences the PQS production controlled by the *pqsA-E* operon [106, 107]. In addition, the transcription of PqsR is repressed by RhlR [106]. PQS production is dependent on the ratio of 3-oxo-C12-HSL to C4-HSL, allowing a precise regulatory balance between these three QS systems [106, 107]. In turn, PQS induces the expression of *rhlI*, thus regulating the *rhl* system [106]. PQS and *rhl* system have also been reported to be linked by PqsE, since PQS regulates the expression of *pqsE*, and PqsE could enhance the ability of RhlR and C4-HSL to positively regulate gene expression [108]. Additionally, PqsR was reported to contribute to the induction of both RhlR and LasR, challenging the current well-known QS model in which the *las* system is placed at the top of the QS hierarchy [109].

The IQS system also emphasizes the variable roles of these QS systems under different conditions, especially during infection. The interactions between QS systems are also highly dependent on the stage of growth. For instance, positive regulation of *pqsR* by LasR happens during exponential stage, but *pqsR* becomes LasR independent in the absence of LasR at later stage of growth [106]. Considering that compensations or partial replacement have also been reported between different QS systems [102, 105], the hierarchy in QS networks seems quite flexible to changes.

2.2.5 Potential advantages of combining different QS systems

A complex network combining different types of QS systems in *V. harveyi* and *P. aeruginosa* might help them to conteract the possible bias in a changing environment. If QS-regulated gene expression would be based on the detection of only one signal molecule, then bias could be simply achieved by inactivation of this signal [110]. For bacteria only used one AHL-type QS system, their QS system might be easily disturbed since the AHL molecule might be unstable at high pH or degraded by other quorum quenching enzymes producing organisms, resulting in the failure of infections. However, combination of different QS systems may allow replacement or complementation by other QS systems. In *P. aeruginosa*, the IQS system can

partially take over the functions of *las* system under phosphate limitation [102]. Additionally, in clinical LasR variant isolates, virulence factors usually considered to be LasR dependent can be produced through other means, such as via RhlR directly, or through activation of RhlR via quinolone signaling molecules [111]. Therefore, in bacteria with multiple QS systems, the effect due to the interference of one signal molecule may be minimized, and a functional QS network can be retained as far as possible under different conditions.

2.2.6 Additional regulatory factors of quorum sensing

As a global regulatory system, QS itself is embedded in an even more complex network of global regulation (Figure 1.4) [79]. Many QS-controlled genes seem not simply triggered by the accumulation of signal but also require additional factors [79]. Interactions between QS and other regulatory systems have been reported, and several well-known ones are discussed below.

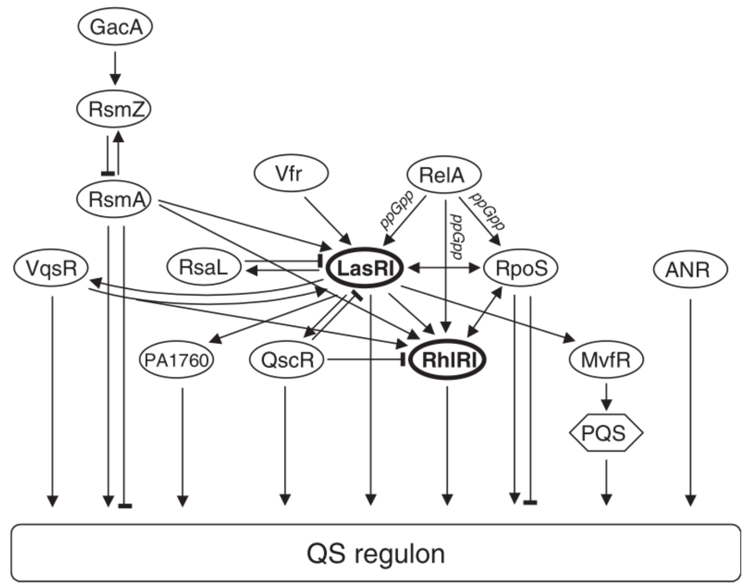

Figure 1.4 The interconnection of QS with other regulatory networks. [79]

2.2.5.1 Transcriptional regulators

The pivotal transcriptional regulator RsaL has been reported to repress *lasI* expression by binding to the promoter of *lasI* [112, 113] and is involved in maintaining 3-oxo-C12-HSL homeostasis. RsaL affects transcript level of many genes either by repressing *lasI* or interacting with their promoters directly [114]. A positive QS

modulator VqsM can also bind to the *lasI* promoter region [115, 116]. A *vqsM* mutant showed repressed type III secretion and reduced virulence in a mouse model, however its biofilm formation and resistance to several antibiotics were increased [115], indicating the multifaceted regulatory mechanisms of this regulator. AlgR2 (AlgQ) is also a global regulator modulating the levels of GTP (guanosine triphosphate), ppGpp (guanosine tetraphosphate), alginate and polyphosphate [117]. It negatively regulates the expression of *lasR* and *rhlR* by binding to their promoters [118]. MvaT is a repressor of AHL QS, and it was considered to be required for the appropriate timing of expression of the QS regulon [94]. The *mvaT* mutant exhibits increased and premature expression of QS-regulated genes [119]. Another transcriptional regulator AmpR positively regulates *rhlR* expression and negatively regulates *lasI/R* expression [120]. It is also involved in the positive regulation of AmpC and PoxB *β*-lactamase [120]. A TetR-like transcriptional repressor PA3699 was shown to repress the *lasR* promoter and the production of LasR-dependent virulence factors [121].

The two-component regulatory system BfmS/R has been reported to influence *rhl* system [122]. Deletion of the regulatory sensor *bfmS* leads to the activation of *bfmR* and decreases the expression of *rhlR* [122]. The response regulator CzcR in the two-component regulatory system CzcRS (related to metal response) was found to interact with QS. Full expression of *lasI* and *rhlI* is impaired in the absence of CzcR, and CzcR is important for the expression of pyocyanin, LasB elastase and rhamnolipids [123]. The most extensively studied two-component regulatory system modulating QS is GacAS which positively control AHL production [124]. It affects QS post-transcriptionally through the small regulatory RNAs RsmY and RsmZ, and the RNA binding protein RsmA [125-127].

The cyclic adenosine monophosphate (cAMP) receptor protein Vfr has long been known as a QS regulator [128]. Vfr regulates a specific subset of virulence genes depending on cAMP levels, but its activation of the *las*R promoter is cAMP-independent [129]. Vfr can also regulate *rhlR* transcription directly by binding to *rhlR* promoter region and exerts a positive effect on *rhlR* transcription[130].

QscR is an orphan LuxR-type regulator in *P. aeruginosa* which lacks a cognate synthase [131]. QscR can form heterodimers with LasR and RhlR at low AHL density

and by doing so inactivates LasR and RhlR [132]. A *qsc*R mutant is hyper-virulent to *Drosophila melanogaster* [131]. QscR directly controls a specific regulon that overlaps with the LasR- and RhlR-dependent regulons using 3-oxo-C12-HSL [133], and it can also respond to multiple AHLs, sugguesting its potential role in interspecies signaling [134]. Another transcriptional regulator VqsR is similar to a LuxR-type protein but without the AHL-binding domain. A *vqs*R mutant displays reduced production of quorum signals and virulence factors [135]. VqsR itself can be activated by 3-oxo-C12-HSL [79], and expression of *vqsR* is positively affected by PQS signaling [136]. Another study showed that VqsR regulates *las* and *rhl* systems by direct inhibition of QscR [137].

Sigma factors are important transcriptional regulators since they confer promoter recognition specificity to the RNA polymerase and are essential for transcription initiation [138]. Two sigma factors have been found to be involved in the regulation of QS. RpoN negatively controls the *las* and *rhl* systems probably through its effect on other QS regulators such as GacA and Vfr [139]. Another stress and stationary-phase sigma factor RpoS represses the expression of *rhlI* [140].

2.2.5.2 Post-transcriptional regulators

As mentioned above, the RNA-binding protein RsmA represses AHL production at the post-transcriptional level through small regulatory RNAs (RsmY and RsmZ) under the control of GacAS two-component system [126]. RsmY and RsmZ act jointly to antagonize the activity of RsmA and affect the level of free RsmA. GacA controls the expression of RsmY and RsmZ and therefore acts as a positive regulator of QS [126]. RsmA has been shown to play an important role during murine infection by influencing colonization, virulence, persistence, and pulmonary inflammation [141].

Another regulator DksA decreases *rhlI* transcription when overexpressed [142] and regulates the translation of *lasB* and *rhlAB* [143]. In *E. coli*, *dksA* has been suggested to be required for *rpoS* induction by the nutrient stress signal ppGpp [144], and the inhibition of *rhlI* by DksA in *P. aeruginosa* is therefore assumed to be an indirect effect by affecting the expression of *rpoS* [143].

A conserved flavin adenine dinucleotide-binding protein involved in tRNA modification, GidA, affects RhlR at post-transcriptional level [145]. It also affects *rhl*R-

dependent QS phenotypes such as LasA, pyocyanin, and rhamnolipid production [145]. The precise molecular mechanism of GidA is yet to be elucidated.

The *pqs* system is also regulated by several small RNAs. ReaL was recently identified to be negatively regulated by LasR and positively impacts the synthesis of PQS by a post-transcriptional effect on *pqs*C [146]. This is the first report about a small RNA involved in *las*-*pqs* connections. A *reaL* mutant is less virulent in a *Galleria mellonella* infection model, and is also affected in pyocyanin production, biofilm and swarming activity [146]. Another small RNA PhrS is an activator of PqsR synthesis, and its expression requires the oxygen-responsive regulator ANR in low oxygen conditions. Thus PhrS can be considered as a regulatory link between oxygen availability and QS [147].

2.2.5.3 Summary

Except for those QS regulators discussed above, there are also other modulators related to QS. For example, the *ppk* gene encodes a polyphosphate kinase responsible for the synthesis of inorganic polyphosphate from ATP, which positively influences QS and is essential for the virulence of *P. aeruginosa* [148]. In this even more complex network of QS regulators, their roles seem to be overlapping and interconnected, and in some cases highly dependent on growth stage and environmental factors. For most of these QS regulators, more detailed mechanisms behind their QS activation or inhibition effects are yet to be discovered.

2.2.7 Role of quorum sensing in *P. aeruginosa* virulence and biofilms

As discussed above, the absence of one or more functional genes in a particular QS system often results in significantly reduced virulence of *P. aeruginosa* in different animal models, indicating the important role of completely functional QS in *P. aeruginosa* pathogenicity. Typical virulence factors known to be regulated by QS include elastase [149], alkaline protease [84], exotoxin A [84], hydrogen cyanide [150], pyocyanin [151], rhamnolipid [88], lectins [152], catalase and superoxide dismutase [153].

Among these virulence factors, alkaline protease and two elastases, LasA and LasB, are important secreted proteases in *P. aeruginosa* which can cause tissue damage,

degrade immunoglobulins and fibrin and even help *P. aeruginosa* to avoid immune detection during infections [154, 155]. Expression of *las*A and *las*B are under the regulation of the *las* and *rhl* systems [149, 156]. Another secreted protein exotoxin A inactivates elongation factor 2, leading to inhibition of protein synthesis and cell death [157]. It represses host immune response and helps with bacterial invasion [158, 159]. The production of exotoxin A and alkaline protease are activated by LasR [84].

The biosurfactant rhamnolipid is important in the invasion and colonization of *P. aeruginosa* in infections, protecting the bacteria against polymorphonuclear leukocytes (PMNs) [160]. It also plays a major role in swarming activity [161] and maintenance of biofilm structure [162-164]. RhlR acts as a direct activator of *rhlAB* transcription which is responsible for rhamnolipid synthesis [165]. Besides, rhamnolipid production was also reported to be affected by the *pqs* system and other regulators such as RsaL and Vfr [165].

The blue-green pigment pyocyanin causes oxidative stress to the host, induces apoptosis in neutrophils, and inactivates host catalase and mitochondrial electron transport [166]. Two *phzABCDEFG* operons, together with *phzH*, *phzM* and *phzS* genes are responsible for the synthesis of pyocyanin [167]. Mutations in gene involved in the *las*, *rhl* and PQS systems lead to the loss of pyocyanin production [94]. The global regulator GacA has also been reported to influence pyocyanin production via affecting the *las* and *rhl* systems [124].

Another virulence factor, hydrogen cyanide (HCN), produced by *P. aeruginosa* is often considered as a biomarker for *P. aeruginosa* lung infections [168]. The fast killing of *Caenorhabditis elegans* by *P. aeruginosa* is dependent on HCN production [169]. The expression of HCN synthase gene *hcnABC* relies on a synergistic action between LasR, RhlR and the anaerobic regulator ANR [150].

Iron chelation is essential for bacteria during infection since iron availability is low in host environment due to the sequestration by host iron binding proteins, and many aspects of *P. aeruginosa* behavior, including intercellular communication and biofilm formation, are influenced by the concentration of free iron [170]. Two important siderophores, pyoverdine and pyochelin, are produced by *P. aeruginosa* to acquire necessary iron for growth, and pyoverdine also acts as signal molecules triggering other virulence factors such as the protease PrpL and exotoxin A [171]. Pyoverdine

biosynthesis is controlled by the *las* system [172], and further evidence showed that *pqs* system is important for both pyoverdine and pyochelin production [173]. In addition, the QS regulator VqsR is required for the full expression of the pyoverdine and pyochelin biosynthetic locus [174].

The type IV pili of *P. aeruginosa* are considered as virulence factors because of their role in adherence and colonization on mucosal surfaces[175], and they are important for biofilm formation since cells lacking type IV pili are unable to form microcolonies [176]. Twitching activity of *P. aeruginosa* also depends on type IV pili [177]. Both the *las* and *rhl* systems are required for normal twitching activity, and the *rhl* system is required for infection by pilus-specific phages and adherence to human bronchial epithelial cells [178].

Biofilms produced by *P. aeruginosa* are the main cause for persistent infections [179]. They are formed by attached bacteria embedded in a matrix of polysaccharide, DNA and proteins. Bacteria in biofilms are shielded from host defense and more tolerant to antibiotics, increasing the difficulties in treating infections. The links between biofilm formation and QS in *P. aeruginosa* were first reported by Davies *et al.*; a *lasI* mutant forms flat and undifferentiated biofilms compared to those of the wild-type [180]. Since then several aspects of *P. aeruginosa* biofilm formation have been reported to be affected by QS. As mentioned, QS-regulated rhamnolipids are involved in microcolony formation, maintenance of open channels, mushroom cap formation and detachment of cells from biofilms [181]. Bacterial migration in early and late stages is related to pili, flagella and rhamnolipids, and depends on nutritional conditions [181]. QS is essential for the transcription of the *pel* operon encoding a glucose-rich matrix exopolysaccharide present in the biofilm matrix [182]. The release of eDNA in *P. aeruginosa* also can be induced by QS; this eDNA is an important matrix component and provides the structural stability to biofilms [183]. Additionally, iron uptake and denitrification connected to QS can influence biofilm formation under specific environmental conditions [181]. However, different results about the importance of QS in *P. aeruginosa* biofilm formation have also been reported depending on the study and experimental conditions [184]. Therefore, although several links between QS and biofilm have been established, it is still challenging to understand the full involvement of QS in biofilm formation, since both QS and biofilms are highly impacted by the surrounding environment as well as other types of signaling.

Bis-(3'-5')-cyclic-dimeric-GMP (c-di-GMP) is an intracellular secondary messenger in many bacterial species that impacts fundamental bacterial behaviors and is important for the lifestyle switch between motile and biofilm cells [185]. Both QS and c-di-GMP regulate virulence and biofilm formation, and it has been shown that the two signaling pathways may be linked in several species [186, 187]. In *P. aeruginosa*, the interactions between c-di-GMP signaling and QS are just beginning to be understood. The tyrosine phosphatase TpbA was shown to inhibit the activity ofTpbB, an enzyme involved in c-di-GMP synthesis, to reduce c-di-GMP levels and biofilm formation, and TpbA is positively regulated by the Las QS system [188]. This suggests that QS negatively influences c-di-GMP production in *P. aeruginosa*. However, more details such as the concentration of c-di-GMP and the role QS plays in controlling this has not yet been studied. Elucidating the interactions between QS and c-di-GMP might help us to better understand their roles in controlling biofilm and virulence in *P. aeruginosa*.

In summary, QS regulates multiple virulence factors in *P. aeruginosa*, and influences biofilm formation through multiple mechanisms. QS has been shown to be critical for pathogenesis of *P. aeruginosa* in lung and burn infections (reviewed by Rumbaugh *et al*. [189]). Though a comprehensive understanding of the exact roles of QS in the distinct types and stages of infections still needs to be worked on, the currently available evidence suggests a great promise for novel therapeutics aimed at inhibiting the complex QS network of *P. aeruginosa*.

2.3 Quorum sensing in *A. baumannii*

Compared to the complicated QS network in *P. aeruginosa*, the known QS system in pathogen *A. baumannii* is simple and straightforward. However, QS in *A. baumannii* and genes under QS regulation are not as well-understood as in *P. aeruginosa*. So far only one LuxI-type AHL synthase and its cognate receptor were identified in *A. baumannii* [190]. In this part, we will discuss the current knowledge of the existing QS in *A. baumanni* and its role in the virulence of *A. baumannii*.

2.3.1 AbaI/AbaR quorum sensing system

The first AHL synthase in *A. baumannii* strain M2 was identified and named as AbaI in 2008 [190]. The AbaI protein showed high similarity to members of the LuxI family, and genome sequencing of other *A. baumannii* strains indicated that AbaI might be the sole AHL synthase in *A. baumannii* [191]. Based on the genome sequence of *A. baumannii* LMG 17989, coding regions of the cognate receptor gene *abaR* and the open reading frame of unknown function (A1S_0110) adjacent to *abaI* are separated by 930 bp [190]. The primary AHL signal detected in strain M2 was *N*-(3-hydroxydodecanoyl)-HSL (3-OH-C12-HSL), but minor amounts of at least five other AHLs were also identified which might be due to the acetyl transferases [190]. The *abaI* mutant failed to produce detectable AHL and the biofilm formation was reduced by approximately 40% [190]. Homologues of the AbaI/AbaR were identified in other *Acinetobacter* species [192]. Interestingly, different AHL profiles of *Acinetobacter* strains have been reported [193] and nosocomial strains tend to produce long chain AHLs (C10~C16) compared to soil isolates [194]. Whether the diversity of AHLs in strains from different origins indicates a role for QS in virulence has not been clearly revealed yet. AHL produced by *A. baumannii* strain AB 14 was observed to affect the cell wall of *S. aureus* and induce apoptosis in different cancer cell line [195], addressing the possible function of AHL in *A. baumannii* in interactions with other bacterial pathogens and infected mammalian hosts.

2.3.2 Role of quorum sensing in *A. baumannii* virulence and biofilms

A. baumannii has become one of the most successful pathogens responsible for hospital-acquired nosocomial infections in the last decades. Multi-drug resistance and persistence in the healthcare environment are major challenges in *A. baumannii* treatment. The virulence factors contributing to *A. baumannii* pathogenesis include capsular polysaccharides, lipopolysaccharides, phospholipases, outer membrane vesicles, metal acquisition systems, biofilm formation and protein secretion systems [5]. The established relationship between QS and several virulence factors in *A. baumannii* are discussed below.

A. baumannii can adhere and form biofilms on both biotic and abiotic surfaces, leading to severe infections as well as to colonization on hospital equipment and

indwelling medical devices. Pili-mediated *A. baumannii* adherence to epithelial cells has been considered as the initial step for colonization and subsequent host infection [196]. As mentioned above, a QS mutant showed reduced *in vivo* biofilm formation in *A. baumannii* strain M2 [190]. The AHL synthase in *A. baumannii* LMG 17978 showed higher expression in biofilm cells when compared to planktonic cells [197], suggesting a role of QS in *A. baumannii* biofilm formation. QS is hypothesized to affect pili formation which is important for biofilm formation, but the mechanism behind this remains unclear. One study showed that non-native AHL can enhance the expression of the chaperone-usher secretion system involved in pili synthesis in *A. baumannii*, and this expression is necessary for twitching motility [198]. These results confirm the importance of QS during attachment and development of biofilms.

The notable antibiotic resistance of *A. baumannii* relies on several mechanisms involving acquirement of *β*-lactamases, up-regulation of multidrug efflux pumps and modification of aminoglycosides. Recent research showed that 3-OH-C12-HSL may be involved in regulating the expression of several drug-resistance genes responsible for *β*-lactamase production and efflux pump activity in *A. baumannii* [199], directly connecting QS with antibiotic resistance in *A. baumannii*.

Catalase and superoxide dismutase (SOD) are two important antioxidant enzymes to help pathogens to deal with reactive oxygen species (ROS) in infection. The production of these two enzymes has been reported to be affected by QS in *A. baumannii* in a study on the interaction between *P. aeruginosa* and *A. baumannii* [200]. The QS mutant of *A. baumannii* showed lower survival when exposed to H_2O_2 compared to the wild type strain [200]. Interestingly, the quorum quenching (QQ) enzyme AidA in non-motile *A. baumannii* strains also seems to be involved in this process as downregulation of AidA was observed under ROS stress, which might lead to higher activity of the QS system in *A. baumannii* [201].

As a global transcriptional regulation system, it is reasonable to assume that QS controls more virulence-related genes in *A. baumannii* besides those discussed above. Comprehensive transcriptome studies may shed new light on QS and virulence of *A. baumannii*, providing better approaches to combat this pathogen.

3. QUORUM QUENCHING ENZYMES

Generally, bacterial QS circuits can be blocked by targeting the biosynthesis, accumulation and detection of the signal molecules. QQ enzymes represent a powerful weapon against QS by directly degrading or modifying signal molecules. In this way, signal molecules produced by bacteria will not accumulate to the threshold level to activate the QS system and QS-controlled virulence genes downstream. The first QQ enzyme, AiiA was identified in a *Bacillus* sp. strain in 2000 [202], and since then, dozens of QQ enzymes have been found in a wide range of bacteria and even in eukaryotes. QQ enzymes acting towards AHL have been intensively studied, and other QQ enzymes targeting at AI-2 or PQS have also been described [203, 204]. In this section, we will focus on different types of AHL-degrading QQ enzymes, as well as the current progress concerning their possible application in controlling infections of *P. aeruginosa*, *A. baumannii* and other Gram-negative pathogens.

3.1 Diversity of QQ enzymes

Although many QQ enzymes degrading AHLs have been reported so far, most of these enzymes can be categorized into two major types based on their cleavage sites, i.e. AHL-lactonases and AHL-acylases. AHL-lactonases can hydrolyze the lactone bond leading to an inactive acyl-homoserine molecule, while AHL-acylases act on the amide linkage between the fatty acid chain and homoserine lactone ring (Figure 1.5). AHL-lactonases can degrade a broad range of AHLs, but AHL-acylases typically show high substrate specificity based on the length of the acyl chains.

AHL lactonase

AHL acylase

R_1 O N H O O

R_2 COOH + H_2N HSL

Figure 1.5 Hydrolytic cleavage of AHLs by AHL lactonases and AHL acylases. (Modified from Fetzner, 2015 [205])

3.1.1 AHL-lactonases

3.1.1.1 Metallo-*β*-lactamase (MBL) superfamily

Most known AHL-lactonases belong to the MBL superfamily, and other lactonases have also been found in the phosphotriesterase (PTE) family, α/*β* hydrolase family and GDSL hydrolase family [49]. The first identified AHL-lactonase AiiA represents a typical lactonase from the MBL superfamily with a characteristic metallo-binding HXHXDH motif which has also been identified in other AHL-lactonases belonging to this family. Homologues of AiiA were then discovered in many *Bacillus* species [206], as well as in other genera including *Agrobacterium* [207], *Rhodococcus* [208, 209], *Arthrobacter* [210], and *Chryseobacterium* [211] (reviewed by Tang *et al*. [49] and Fetzner [205]).

Among these lactonases, MomL, which is produced by *Muricauda olearia* strain Th120, represents a type of AHL-lactonases that shows relatively low identity (less than 30%) in amino acid sequence with AiiA and other MBL-type AHL-lactonases [212]. Similar lactonases with high identity to MomL were reported later in other marine bacteria from the *Bacteroidetes* phylum, such as Aii20J from *Tenacibaculum* sp. 20J [213] and FiaL from *Flaviramulus ichthyoenteri* Th78^{T} [214]. MomL, Aii20J and FiaL, as well as several predicted AHL-lactonases in *Bacteroidetes* phylum formed a specific marine lactonase cluster (Figure 1.6). Both MomL and Aii20J can degrade unsubstituted and substituted AHLs of different acyl chain length, with a slight preference for long-chain AHLs, and both showed high thermostability [212, 213]. A recently reported MBL-type AHL-lactonase QsdS from a *Sphingomonas ursincola* isolate recovered from an industrial cooling water system also clustered with MomL-type lactonases [215].

Another AHL-lactonase sub-cluster in the MBL superfamily is represented by AidC from *Chryseobacterium*, showing less than 20% amino acid identity with AiiA [211]. AidC has been reported to show a higher k_{cat}/K_m value (10^6 $M^{-1}s^{-1}$) compared to other QQ enzymes including AiiA and MomL (10^4~10^5 $M^{-1}s^{-1}$), indicating it might be more efficient at low AHL concentrations [194]. Another MBL with k_{cat}/K_m value of 10^6 $M^{-1}s^{-1}$ was GcL from the thermophilic *Geobacillus caldoxylosilyticus* [216].

Besides GcL, two other novel AHL-lactonases in the MBL family have been described, AiiT from the thermophilic bacterium *Thermaerobacter marianensis* [217] and AidP from *Planococcus* sp. recovered from Antarctica [218], and both of them showed high similarity to AhlS in *Solibacillus silvestris* [219]. These psychro- or thermo-tolerant MBL-type AHL-lactonases identified from bacteria in extreme environments extend our knowledge about QQ enzymes and might be important for applications under specific conditions.

3.1.1.2 PTE-like lactonases (PLLs)

PTEs are known for their role in organophosphate degradation, while PLLs can proficiently hydrolyze various lactones, especially AHLs, with much lower promiscuous PTE activities. PPH in *Mycobacterium tuberculosis*, AhlA in *Rhodococcus erythropolis* and *Sso*Pox in *Sulfolobus solfataricus* were the first identified PLLs [220]. *Sac*Pox and *Sis*Lac are PLLs from species belonging to the archeael genus in *Sulfolobus* [221, 222]. Interestingly, many PLLs are identified in thermophilic bacteria or archaea, such as *Sso*Pox, GkL in *Geobacillus kaustophilus* [223] and *Vmo*Lac in *Vulcanisaeta moutnovskia* [224]. The biotechnological properties of a *Sso*Pox variant has been investigated recently [225], and it showed resistance to diverse harsh conditions such as heating, exposure to organic solvents, sterilization and immobilization. In addition, it was active at sub-zero temperature and resistant against bacterial secreted materials, making this *Sso*Pox variant an attractive candidate for industrial and medical applications.

3.1.1.3 *α/β* hydrolase family

The known AHL-lactonases in the *α/β* hydrolase family include AiiM in *Microbacterium testaceum* [226], AidH in *Ochrobactrum* sp. [227] and DhlR in *Sinorhizobium* sp. [228]. The newly reported AHL-lactonase AidA in clinical strains of *A. baumannii* also belongs to the *α/β* hydrolase family [201].

3.1.1.4 Other lactonases

Except for the three categories discussed above, QsdH from a *Pseudoalteromonas byunsanensis* strain was identified as the only AHL-lactonase in the GDSL hydrolase

family [229]. PON1, PON2 and PON3 are mammalian paraoxonases which can degrade various AHLs [230, 231]. Recently, a metagenome-derived AHL-lactonase HqiA has been classified in a new family related to the cysteine hydrolase group [232], indicating that there might be other types of AHL-lactonases beyond our current knowledge.

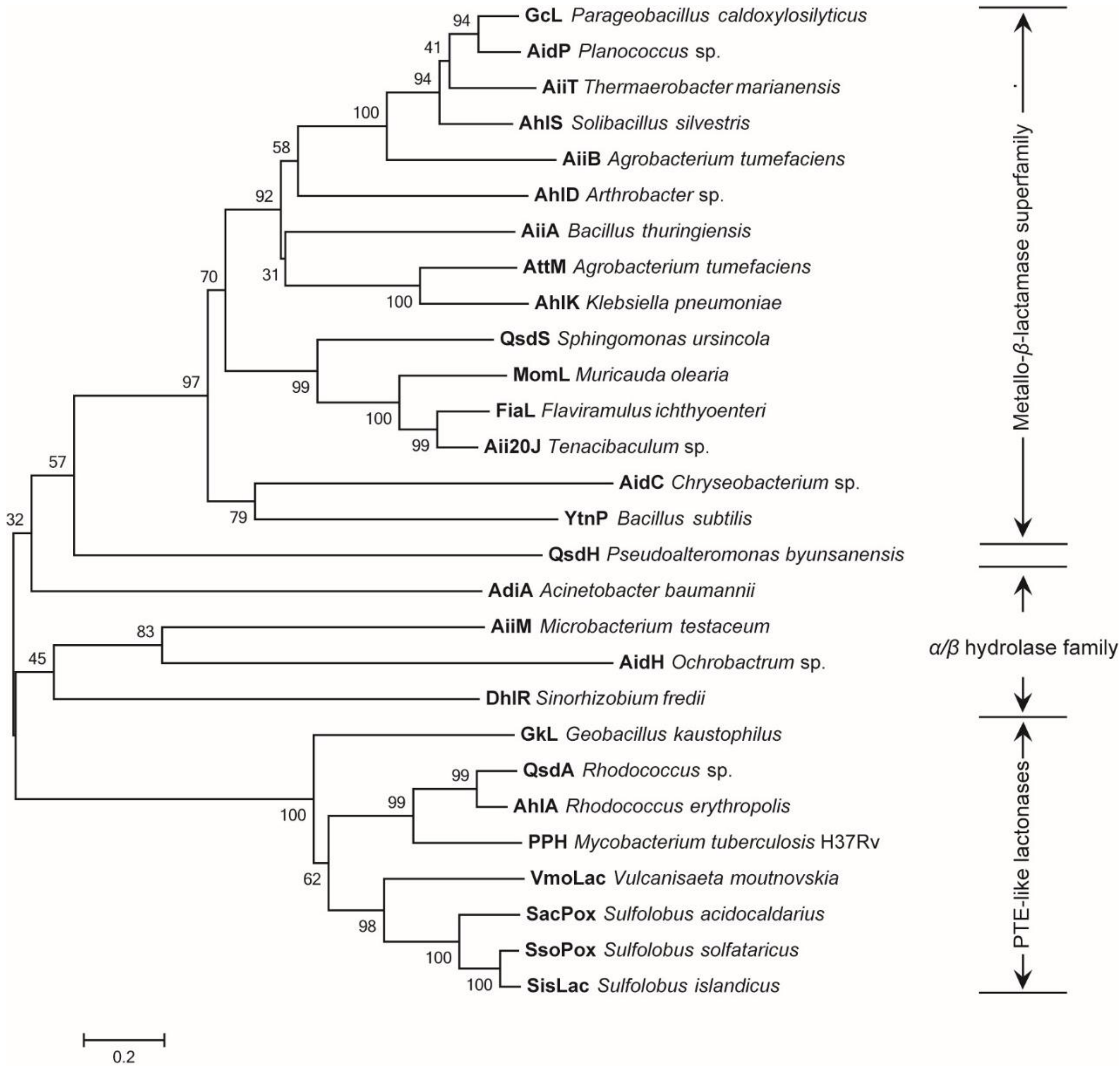

Figure 1.6 Phylogenetic tree from the known protein sequences of bacterial AHL-lactonases. The phylogenetic tree was constructed with the ClustalW program in the MEGA6 (1000 bootstrap replicates). Scale bar, 0.1 substitutions per amino acid position.

3.1.2 AHL-acylases

Most of the known AHL-acylases belong to the N-terminal nucleophile-hydrolase superfamily, and these AHL-acylases group in four clusters: aculeacin A acylase, penicillin G acylase, penicillin V acylase and AmiE amidase family [233] (Figure 1.7).

AHL-acylases from *α/β* hydrolase family have also been reported [234]. The first isolated and characterized AHL-acylase was AiiD from *Ralstonia* sp. [235], and by now AHL-acylases have also been identified from various species in the genera *Pseudomonas*, *Acinetobacter*, *Agrobacterium* and *Streptomyces.* (reviewed by Tang *et al*. [49] and Fetzner [205]). Unlike AHL-lactonases, AHL-acylases show distinct substrate specificities and many of them are active only towards long chain AHLs [233].

Interestingly, cross-reactivity between AHL-acylases and penicillin acylases has been observed [236]; AhlD from *Streptomyces* sp. [237], HacB from *P. aeruginosa* [237] and PmrA from *Pseudoalteromonas flavipulchra* [238] showed activity on either penicillin G, penicillin V or ampicillin. Recently, a novel AHL-acylase MacQ from *Acidovorax* sp. further confirmed the possible bifunctional role of some AHL-acylases which can degrade both various AHLs and *β*-lactams [239, 240]. The MacQ-expressing *E.coli* strain showed 2- to 16-fold higher minimum inhibitory concentration (MIC) values toward several *β*-lactam antibiotics (penicillin G, ampicillin, amoxicillin, carbenicillin, cephalexin, and cefadroxil) than the wild-type [239].

In *P. aeruginosa*, three AHL-acylases have been reported: PvdQ [241], QuiD [242] and HacB. These three AHL-acylases affect the AHL levels and virulence factor production in *P. aeruginosa* [242, 243], but the detailed mechanisms about how they interact with the complicated QS network in *P. aeruginosa* are not clear yet. The production of PvdQ was considered to be induced by iron limitation, since this acylase is also involved in the biosynthesis of pyoverdine [244]. QuiP is responsible for the utilization of long-chain AHLs as carbon source, while HacB is assumed to play a main role in controlling AHL accumulation [243].

3.1.3 AHL-oxidoreductases

AHL-oxidoreductases are a few enzymes which can inactivate AHLs by reduction or oxidation activity. *Rhodococcus erythropolis* W2 [245] and *Burkholderia* sp. strain GG4 [246] showed the ability to reduce 3-oxo-AHLs to the corresponding 3-OH-AHLs, but the relevant genes have not yet been identified. BpiB09, an NADP-dependent reductase which derived from a metagenome library, was also capable of inactivating 3-oxo-C12-HSL in *P. aeruginosa* [247]. CYP102A1, a widely studied cytochrome

P450 from *Bacillus megaterium*, can oxidise AHLs at the ω-1, ω-2, and ω-3 carbons of the acyl chain, and the oxidation products are much less active than the corresponding AHLs [248].

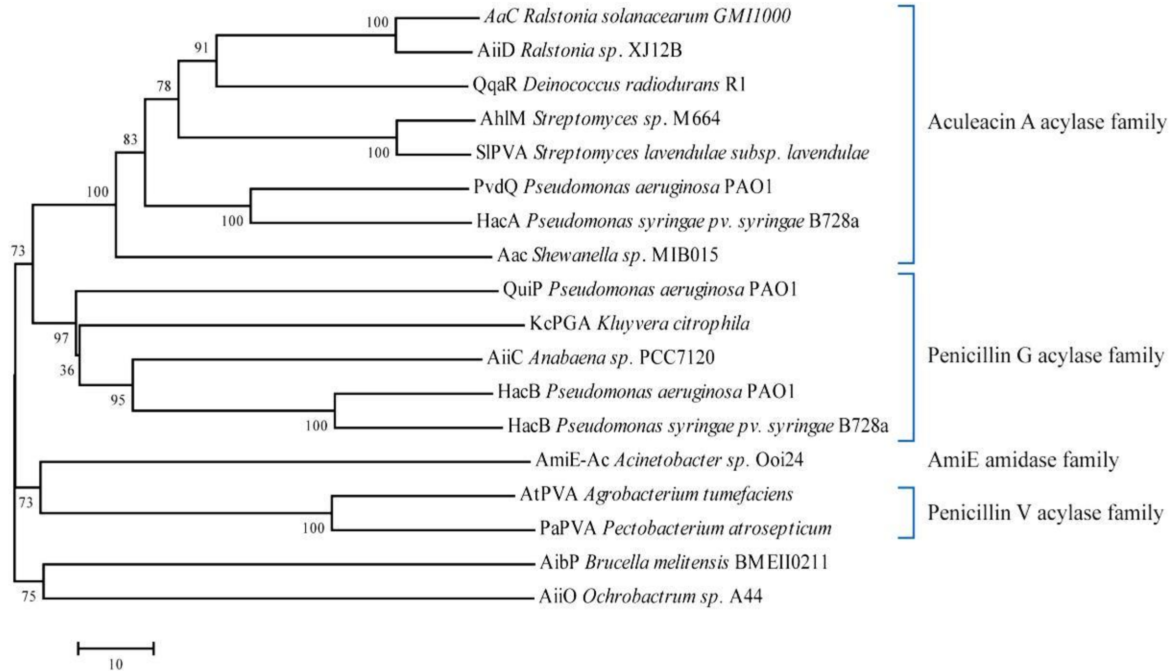

Figure 1.7 Phylogenetic tree from the known protein sequences of bacterial AHL-acylases [233]. The phylogenetic tree was constructed using the neighbor-joining method with the ClustalW (MEGA program, ver. 7). Number represents Bootstraps values based on 1000 replications.

3.1.4 Physiological roles of QQ enzymes

Except for their ability to degrade AHLs, the exact physiological roles of AHL-lactonases or AHL-acylases are not clear by now. The potential functions of AHL-acylases based on their AHL-hydrolyzing ability have been discussed by Utari *et al.*, and include modulating the endogenous QS or interkingdom interactions and utilization of AHLs as nutrient sources [233]. These enzymes might be indispensable in the AHL turnover mechanism to allow a real-time monitoring of AHL concentration and population density [233]. However, it is also possible that the natural roles of these enzymes are unrelated to QS and might be different in different bacteria.

3.2 The use of QQ enzymes to interfere with virulence

3.2.1 *In vitro* proof

Since the identification of the first QQ enzyme AiiA, its anti-virulence effects against various pathogens have intensively been studied. Heterologous expression of AiiA successfully decreases extracellular pectolytic enzyme activities of a plant pathogen *Erwinia carotovora*, and attenuates soft rot symptom on different plants [202]. It has also been demonstrated that expression of AiiA in *P. aeruginosa* PAO1 reduced the concentration of both C4-HSL and C12-HSL, and production of elastase, rhamnolipid, HCN and pyocyanin were also severely decreased [249]. The swarming motility of PAO1 has also been strongly reduced, whereas no significant effect on twitching motility and adherence to a polyvinylchloride surface was observed in this study [249]. AiiA has also been investigated in *Burkholderia cepacia* complex strains which are important opportunistic pathogens in human with cystic fibrosis. In the large majority of these bacteria, protease production, swarming motility, and biofilm formation were reduced when *aiiA* was expressed heterologously [250]. However, several *aiiA*-expressing *Burkholderia cepacia* complex strains in this study showed increased or unaffected biofilm formation when *aiiA* was expressed heterologously [250]. In addition, an *aiiA*-expressing *Enterobacter cloacae* strain showed enhanced protease production and was less adherent during biofilm formation [251].

Except for AiiA, many other QQ enzymes have been evaluated on *P. aeruginosa* by either heterologous expression or addition to the growth medium. AHL-lactonases such as MomL [212] and QsdS [215], and AHL-acylases such as AiiD [235], AmiE [252] and AhlM [237] have been reported to affect *P. aeruginosa* QS-controlled phenotypes. Metagenome-derived QQ enzymes such as Aii810 [253] and BpiB05 [254] can also attenuate *P. aeruginosa* virulence and biofilm formation. Another study showed that human and murine serum containing PON1 prevents *P. aeruginosa* biofilm formation *in vitro* [255]. In a recent study, the effect of an engineered variant of SsoPox was tested in a series of *P. aeruginosa* clinical isolates from diabetic foot ulcers [256]. The results showed that proteolytic activity was decreased by at least half in 67% of these strains, pyocyanin production in 68% and biofilm in 82%, which

were significantly more effective compared to the QS inhibitors (5-fluorouracil and furanone C-30) tested [256].

Few QQ enzymes have so far been tested on *A. baumannii*. A thermostable engineered mutant of GkL was reported to reduce biofilm biomass and thickness of an *A. baumannii* clinical isolate [257], which suggests that application of QQ enzymes might also be an effective way to treat this increasingly resistant pathogen.

3.2.2 *In vivo* proof

To understand the pathogens-host interaction during infection, different models of evolutionary divergent hosts such as nematodes, insects and rodents are available for studying the pathogenicity of *P. aeruginosa* and other bacteria [242]. *C. elegans* is one of the simplest invertebrate models to study QS and virulence of *P. aeruginosa* [258], and its innate immunity pathway resembles that of mammals in some aspects [259]. Several different mechanisms are involved in the killing of *C. elegans* by *P. aeruginosa* depending on the experimental conditions. QS-controlled phenazines and HCN can cause rapid paralysis of *C. elegans* in hours, and pyoverdin induces a hypoxia response and death in a liquid medium. A slow killing of *C. elegans* for days by *P. aeruginosa* might also happen on a minimal medium [260]. Overexpression of PvdQ in *P. aeruginosa* PAO1 reduced its virulence and rescued more than 75% of the *C. elegans* from lethal paralysis [261]. Moreover, addition of purified PvdQ to *C. elegans* increased their life span, but the infection itself cannot be completely prevented [261]. Other QQ enzymes, such as MomL [212], AiiD [235] and BpiB09 [247], have also been shown to attenuate the virulence of *P. aeruginosa* in the *C. elegans* infection model.

G. mellonella (Wax Moth) larvae are alternative models to study microbial infections and are used by a growing number of researchers [262]. Compared to *C. elegans*, these larvae are larger in size and easier to keep and manipulate. A variant of PvdQ which can degrade C8-HSL has been reported to rescue *G. mellonella* larvae upon *Burkholderia cenocepacia* infection [263]. No data about the effect of QQ enzymes on *P. aeruginosa* infections are available in this model so far.

The effect of PON1 on *P. aeruginosa* has also been evaluated in another infection model, *Drosophila melanogaster* (fruit flies). *D. melanogaster* does not express

PON or a PON homolog, and human PON1 was transgenically expressed in the flies. These PON1-producing flies were found to be protected from *P. aeruginosa* lethality, and this effect was confirmed to be dependent on the lactonase activity of PON1 [264].

3-oxo-C12-HSL produced by *P. aeruginosa* can exert effects on mammalian cells, including upregulation of pro-inflammatory mediators and induction of apoptosis [265]. Human intestinal epithelial Caco-2 cells transfected with *pvd*Q were shown to be effectively protected from 3-oxo-C12-HSL induced apoptosis [266], which indicates a potential therapeutic strategy to obviate injury from *P. aeruginosa* to mammalian cells.

Migiyama *et al.* evaluated the effect of AiiM against *P. aeruginosa* infection further in a mouse model of acute pneumonia [267]. AiiM expression reduced lung injury and improved the survival rates by over 70%. The levels of proinflammatory cytokines and myeloperoxidase activity were also significantly lower [267]. Another study showed that inhaled SsoPox-I can increase the survival by 55% during *P. aeruginosa* infection in a rat pneumonia model, and the histological lung damage was significantly reduced with no difference in lung bacterial counts [268]. These *in vivo* data strongly support the hypothesis that QQ enzymes can reduce excessive inflammation and relieve *P. aeruginosa* infection.

3.2.3 Combined use of QQ enzymes

QS has been proved to influence biofilm formation and the tolerance of the biofilm to antimicrobial treatment [184], therefore QQ enzymes might help to increase the efficiency of conventional antibiotics in biofilm eradication during infections. Combining QQ enzymes and antibiotics was considered as an attractive option to treat the rising number of antimicrobial-resistant infections. However, compared to the abundant data on QS inhibitor/antibiotic combination, studies on QQ enzymes combined with antibiotics are not so frequent. The efficacy of a lactonase from *Bacillus* species in combination with ciprofloxacin in treating *P. aeruginosa* murine burn wound infection has been investigated by Gupta *et al*. [269]. Topical application of lactonase or ciprofloxacin alone prevents the systemic spread of *P. aeruginosa* and reduced the mortality, while in the group treated with lactonase and ciprofloxacin, all the animals survived [269]. The level of cytokines and inflammatory mediators

were also reduced significantly. There results indicate a promising prospect for further research on co-administration of QQ enzymes and antibiotics in treatment of infectious diseases.

Besides, synergistic efforts of multiple anti-biofilm components could be a better way to clear resistant biofilms. From such perspective, the QQ enzyme MomL has been combined with glycoside hydrolases recently in a cell-based anti-infective device [270]. The device was designed with a synthetic AHL-sensor connected to a downstream module encoding glycoside hydrolases and MomL, and it was capable of detecting AHL level in *P. aeruginosa* clinical isolates. Combination of MomL and glycoside hydrolases enabled the designer cells to fully degrade AHLs, potentiate antibiotic susceptibility, substantially reduce biofilms and alleviate cytotoxicity to lung epithelial cells [270]. This technology was considered to be particularly useful for hindering biofilm formation around cell implants in future cell-based therapies [270].

The AHL-degrading enzyme *Sso*Pox has been modified and explored to be combined with another QQ enzyme LsrK targeting the AI-2 signal, which might provide a new idea for simultaneously quenching two different QS signals. Modified *Sso*Pox and LsrK were successfully attached to capsules of the charged polymers chitosan and alginate [271]. However, the capacity of these capsules to combat infections still needs to be verified *in vitro* and *in vivo*, and these capsules may be more effective in treating multispecies infections involving diversified QS activity.

Attempts have also been made to equip the T7 phage with QQ enzymes to treat biofilms [272]. Bacteriophage treatment has been considered as an effective way to collapse bacterial biofilms. Polysaccharide depolymerases produced by phages can help them to get access to bacteria in biofilms. However, these depolymerases have narrow substrate specificities, thus may not be potent enough in biofilms with multiple polysaccharides. In addition, QS was reported recently to activate CRISPR-Cas immunity in *P. aeruginosa*, which is important for bacteria to defend themselves against phages [273]. Therefore, combining QQ enzymes and phages might have a more profound effect. An engineered AiiA-expressing phage was shown to be more capable in inhibiting mixed-species biofilms of *P. aeruginosa* and *E.coli* than the wild-type T7 phage [272], indicating that such QQ phages might be of great interest for their antibiofilm function.

Nanoparticle-based approaches have been widely studied as potential therapeutics for controlling biofilm infection in recent decades [274, 275]. Nanoparticles were developed with intrinsic antimicrobial properties or used in controlled antimicrobials delivery. Besides, the QS inhibition activity of nanoparticles (NPs) has also been investigated [276, 277]. Vinoj *et al.* synthesized AuNPs coated with AiiA protein, and the AiiA AuNPs reduced the exopolysaccharide production and biofilm formation of multidrug-resistant *Proteus* species at a concentration without damaging the host macrophages [278]. The anti-biofilm activity of such nanoparticles with QQ enzymes might also be promising in treating other pathogens as *P. aeruginosa*.

3.2.4 Exploring the application of QQ enzymes

Although most studies on QQ enzymes mainly focus on their anti-virulence and anti-biofilm effects *in vitro* and *in vivo*, several studies go futher to explore the possible application of QQ enzymes, such as coatings or membranes with QQ enzymes and inhalable powder of QQ enzymes.

Developing anti-biofilm coatings with QS disrupting activity represents a new paradigm to prevent catheter and other medical device related infections. Ivanova *et al.* built multilayer coatings on silicone catheters composed of acylase from *Aspergillus melleus* and *α*-amylase in a layer-by-layer fashion [279]. Coatings of acylases alone suppressed the biofilm formation of *P. aeruginosa* [279, 280], and hybrid coatings with acylases in the outermost layer demonstrated higher anti-biofilm efficiency compared to the other assemblies. Additionally, these nanocoatings significantly reduced mixed-biofilm of *P. aeruginosa* and *E. coli*, and delayed the biofilm growth up to 7 days in an *in vivo* rabbit model [279]. In another study, this acylase was covalently immobilized in polyurethane coatings via multipoint covalent immobilization [281], resulting in around 60% reduction in *P. aeruginosa* biofilm formation. The acylase-containing coatings retained 90% activity when stored dry at 37℃ for 7 days, and might be promising in the clinical management of catheter-related infections [281].

Immobilization of *Sso*Pox onto nanoalumina membranes has been investigated [282], and the addition of membranes containing *Sso*Pox in bacterial cultures of *P. aeruginosa* successfully decreased the production of pyocyanin and elastase. The

membranes with immobilized QQ enzymes in this study provide a possible means to control undesirable bacterial activities on medical device.

For the treatment of pulmonary *P. aeruginosa* infections, Wahjudi *et al.* tried to develop PvdQ into a dry, inhalable powder using mannitol, trehalose and inulin as excipient [283]. The activity of PvdQ can be fully retained during spray-freeze drying, and PvdQ incorporated in trehalose or inulin was stable for storage (55 ℃ for four weeks). These results provide a good starting point for the delivery of QQ enzyme in patients.

4. QUORUM SENSING INHIBITORS

QS inhibitors (QSIs) are typically small molecules that can interfere with the QS pathway in different ways. A large number of QSIs have been synthesized or identified from broad natural sources and compound libraries, and this number continues to increase (reviewed by [49, 284-289]). These QSIs have been considered to modulate QS and QS-related virulence without affecting the growth of bacteria, opening up the possibility to develop new anti-virulence drugs to which resistance is less likely to develop, despite the on-going debate about possible mechanisms to develop resistance against QS inhibition [290-292]. Currently, most QSIs for which the mechanism of action was elucidated are either analogues of signal molecules or blockers of the cognate QS receptor. However, the mechanism of action of the majority of natural QSIs remains unknown.

Primary detection of the QSI activity involve the application of QS reporter strains normally combining a QS-controlled promoter and a specific reporter gene/operon expressed *gfp*, bioluminescence or *β*-galactosidase, as reviewed by Rai *et al*. [293]. However, screening of QSIs by such methods might induce false positive predictions [294], since the certain QS-controlled phenotypes in these reporter strains can also be affected by other factors or the metabolic activity. Therefore, further confirmation in various ways is strongly recommended. In the following section, we will discuss the current knowledge and progress mainly focusing on typical QSIs against Gram-negative pathogens, as well as the *in vitro* and *in vivo* evidences for the anti-biofilm and anti-infectious effect of QSIs.

Figure 1.8 Examples of QSIs with activity against *P. aeruginosa*

4.1 Diversity of QSIs

4.1.1 Natural QSIs

The widely distributed plant phenolic compounds are an abundant source for natural QSIs, and QSIs have been identified from different classes of phenolic compounds including flavonoids, coumarins, phenolic acids, curcuminoids and others [287]. Some of these QSIs were tested with QS reporter strains, and their effects on virulence factor production and biofilm formation in a wide range of bacteria were investigated [287]. However, the effects of these compounds vary greatly. Compounds like vanillin, tannic acid and (-)-epicatechin were shown to inhibit QS and virulence factor production in some species, but they can also increase the AHL production or biofilm formation in other species (or strains) at certain concentrations [287]. Other compounds like ellagic acid, curcumin, quercetin and baicalein not only

affect the QS and biofilm in Gram-negative bacteria, but also in Gram-positive bacteria and even yeast which employed totally different QS systems [287, 295, 296]. These observations indicate that such compounds may not simply inhibit QS or distinct mechanisms may be involved in their QS and biofilm inhibition activity in different bacteria. In addition, the possible antimicrobial activities of these compounds should be clearly distinguished from the QS inhibition activity. Therefore, uncovering the detailed mechanisms of these potential QSIs might be the first step towards their applications. A recent study reported that flavonoids such as quercetin and baicalein inhibit QS in *P. aeruginosa* via antagonism of AHL receptor and prevention of DNA binding [297], which provides primary mechanistic evidence for these flavonoid QSIs.

Another well-studied QSI is ajoene [298], a sulfur-rich molecule isolated from garlic. Jacobson *et al.* recently revealed that ajoene represses different QS systems in *P. aeruginosa* via modulation of the expression of the small RNAs RsmY and RsmZ, as well as modulation of RNAIII expression in *S. aureus* [299]. Ajoene targets the Gac/Rsm part of the QS machinery in *P. aeruginosa* and results in low expression of RsmYZ, but how ajoene confers an effect on RNAIII transcript levels in *S. aureus* remains unknown [299]. Other sulfur-containing isothiocyanates such as sulforaphane and erucin have also been identified as QSIs against *P. aeruginosa* [300, 301], and iberin showed a similar effect as ajoene in *P. aeruginosa* through downregulation of the Gac/Rsm network [302].

Other natural QSIs have been reported from diverse sources such as sponges [303], fungi [304] and honey [305]. Farnesol, a sesquiterpene which can be produced by *Candida albicans*, decreased *pqsA* transcription in *P. aeruginosa* [306]. Clove bud oil has also been shown as an inhibitor for PqsA [307].

4.1.2 Synthetic QSIs

Most synthetic QSIs are derived from either natural AHLs or structurally unrelated natural products. These QSIs act as antagonists or agonists which effectively compete with the natural AHLs and prevent a sufficient stimulation of the receptors [308]. Several AHL analogs showed remarkable inhibitory activities in QS reporter strains [309, 310], and some of them can inhibit elastase and biofilm formation in *P. aeruginosa* [311]. In addition, high-throughput screening of compound libraries led to

the identification of structurally diverse QSIs [312]. Synthetic furanone compounds, especially C30, are intensively studied QSIs based on the structure of natural furanones in marine macroalga *Delisea pulchra* and similar to AHLs in structure [313, 314]. However, the effect of synthetic QSIs targeting single receptor might be insufficient to inhibit the complete QS network and QS-related virulence in pathogens as *P. aeruginosa* which use different QS systems. Discovery of QSIs targeting multiple receptors (e.g. both LasR and RhlR in *P. aeruginosa*) or the combined use of different QSIs could be a better choice in these conditions. QSIs against PQS pathway in *P. aeruginosa* have also been reported, such as halogenated anthranilic acid analogs, targeting the synthesis of PQS (PqsA, PqsD) or the receptor PqsR [315-318].

4.1.3 Known drugs as QSIs

The macrolide azithromycin has been widely studied as a QSI of *P. aeruginosa* at sub-inhibitory concentrations [319, 320]. Azithromycin down-regulates QS-regulated genes encoding pilus, flagellum, and oxidative stress response proteins in *P. aeruginosa*. Research into the mechanism of azithromycin as a QSI initially assumed that azithromycin influences the flux of 3-oxo-C12 by altering membrane permeability, but later it has been reported that azithromycin can inhibit expression of the GacA-dependent sRNAs (RsmY and RsmZ), just like ajoene [320, 321]. Antibiotics as ceftazidime and ciprofloxacin also show QS inhibition effect against *P. aeruginosa* [320].

5-Fluorouracil, an analog of uracil, is a clinical approved anti-cancer drug, and it has been identified as a QSI via interrupting uracil synthesis which is important for QS and QS-regulated virulence [322]. Imperi *et al*. [323] found the anthelmintic drug niclosamide as an effective QSI for *P. aeruginosa* by screening a library of FDA-approved compounds. A nonsteroidal anti-inflammatory drug, meloxicam, has been reported to repress QS and related phenotypes in *P. aeruginosa* [324]. These QSIs identified from commercial drugs might make their clinical application much easier than other QSIs.

4.2 QSIs as antivirulence drugs

4.2.1 *In vitro* and *in vivo* proof

A large number of synthetic and natural QSIs have been tested *in vitro* on the production of bacterial virulence factors, as well as in different *in vivo* models. Here we will mainly focus on those that have been evaluated on *P. aeruginosa* and *A. baumannii*.

As the most efficient modified furanone compound, C30 partially or completely inhibits the production of QS-regulated protease, pyoverdine and chitinase in *P. aeruginosa* [314]. Additionally, biofilms formed in the presence of C30 are more susceptible to tobramycin [314]. In a mouse pulmonary model, C30 led to a rapid clearance of bacteria [314] and prolonged the survival of the mice [313]. These results confirmed that applications of QSIs may reduce virulence and prevent detrimental biofilm formation of *P. aeruginosa* during infections.

Many QSIs have been shown to reduce pyocyanin, protease and elastase production in *P. aeruginosa*, as well as rhamnolipid and motility. Notably, Welsh *et al*. reported that modulation of RhlR by synthetic, non-native AHLs induced inverse regulation of pyocyanin and rhamnolipid production, which might be due to the suppression of PQS signaling [325]. These results indicate that small molecule disruption of QS in *P. aeruginosa* might lead to unexpected virulence phenotypes depending on the mechanism of QSI.

The effects of QSIs on biofilm of *P. aeruginosa* have been intensively studied. QSIs such as mBTL (meta-bromo-thiolactone), 6-gingerol and oleanolic aldehyde coumarate were reported to reduce biofilm biomass in *P. aeruginosa* [326-328], and some studies also revealed that QSIs increase the susceptibility of biofilms to antibiotic treatments [298, 304, 329]. Moreover, studies on biofilms formed in flow cell systems showed that QSIs affect the architecture of the biofilm [330].

Research into the effects of QSIs in different *in vivo* models also showed promising results to reduce the virulence of *P. aeruginosa*. Representative examples are summarized in Table 1.3. QSIs in combination with antibiotics have been considered as a possible way to be applied clinically for human diseases, as QSIs may enhance

the efficiency of antibiotics in treating biofilm infections. In an intraperitoneal foreign-body infection mouse model, furanone C30, ajoene or horseradish juice extract were tested in combination of tobramycin in an early stage of infection [331]. Synergistic effects on the bacterial clearance were observed for all QSIs, and early treatment showed more significant effects compared to late treatment [331].

Table 1.3 The effects of QSIs on *P. aeruginosa* virulence in *in vivo* models

QSIs	*In vivo* effects	Reference
Patulin	Promotes a faster clearing of *P. aeruginosa* in a mouse pulmonary infection model	[304]
Ajoene	Increases the clearance of *P. aeruginosa* in a mouse pulmonary infection model	[298]
Azithromycin	Attenuates chronic *P. aeruginosa* infection in Cftr-/- mice and significantly clears a *P. aerugionsa* infection in a urinary tract infection mouse model	[332, 333]
Niclosamide	Protects *G. mellonella* from *P. aeruginosa* infection	[323]
Halogenated anthranilic acid analogs (inhibit HAQ biosynthesis)	Restrict *P. aeruginosa* systemic dissemination and mortality in mice	[315]
mBTL	Inhibits *P. aeruginosa* PA14 virulence toward *C. elegans* and human A549 lung cells	[326]
6-Gingerol	Reduces mice mortality agains *P. aeruginosa*	[328]
Phenyllactic acid	Inhibits the initial attachment of *P. aeruginosa* in an intraperitoneal catheter-associated medaka fish infection model	[334]
Triterpenoid coumarate ester	Reduces paralysis of *C. elegans* during *P. aeruginosa* infections	[327]
Tea polyphenols	Reduce *P. aeruginosa* pathogenicity in *C. elegans*; increases the wound contraction percentage in an excision mouse wound infection model and dcreases the colony-forming units (CFU) in the wound area	[335]
Baicalein	Enhances *P. aeruginosa* clearance in a mouse peritoneal implant infection model	[336]

Comparing to the many studies evaluating QSIs on *P. aeruginosa*, QSIs targeting *A. baumannii* are seldom investigated and tested in *in vivo* models. Several synthetic non-native AHLs were identified as AbaR antagonists and inhibit motility and biofilm formation of *A. baumannii* [337]. Flavonoid components from *Glycyrrhiza glabra* have been shown to reduce motility, biofilm formation and production of antioxidant enzymes in *A. baumannii* [338]. Streptomycin at subinhibitory concentrations decreases transcription of the *aba*I gene and reduces motility, but the mechanism is unknown [339]. In a recent study, virstatin and two unsaturated chain fatty acids were reported to inhibit QS in *A. baumannii* and inhibit motility and biofilm formations in several clinical strains [340].

4.2.2 Clinical trials with QSIs

Though impressive *in vitro* and *in vivo* results obtained with QSIs have been published, few QSIs have been tested in clinical trials. In a randomized, double-blind, multicenter trial, the ability of azithromycin to prevent ventilator-associated pneumonia (VAP) in patients colonized by rhamnolipid producing *P. aeruginosa* isolates was accessed [341]. A trend towards reduced occurrence of VAP was observed in the azithromycin group, but the difference was not statistically significant [341]. In another randomized controlled trial with garlic capsules in cystic fibrosis patients infected with *P. aeruginosa*, the improvement in lung function, weight and symptom score with garlic therapy was also non-significant [342]. These preliminary clinical trial data indicated that QSIs alone may not be sufficiently effective during infections. In addition, larger clinical trials may help to further demonstrate the effect of QSIs.

4.3 QSIs *vs.* QQ enzymes

Both QSIs and QQ enzymes interfere with QS and virulence in many bacterial species. However, QSIs and QQ enzymes each have advantages and drawbacks due to their entirely distinct molecular structures and functional mechanisms.

Many synthetic QS inhibitors mainly target one specific signal receptor or several homologous receptors, such as the LuxR-like family, which might allow to developing drugs with narrow spectrum targeting the virulence of specific pathogens. In *P.*

aeruginosa, selective effect on certain QS receptor, such as RhlR, LasR and QscR, can be achieved and allow for a precise modulation of individual QS system [343-345]. However, in the case of microbial infection with multiple pathogens, targeting one pathogen might be insufficient. Under these conditions, QQ enzymes which can degrade a wide range of AHLs may be more efficient, as they could possibly affect different Gram-negative pathogens such as *P. aeruginosa*, *E. coli*, *A. baumannii*, and *B. cenocepacia*. Nevertheless, the unintended effect of broad spectrum QQ enzymes on the commensal or benificial bacteria should be considered in a complex environment as intestine.

Unlike QQ enzymes, the structures of QSIs are relatively simple and can be easily modified through synthetic methods. QSIs with low molecular weights may be administered orally or intravenously like other drugs. Furthermore, the nonproteinaceous nature of QSIs may prevent an antibody-based immune response compared to QQ enzymes. It could also be more difficult to develop formulations for delivering macromolecular QQ enzymes due to the low bioavailability of protein drugs. Barriers related to poor absorption, poor permeation, and degradation in the gastrointestinal tract need to be addressed for an effective QQ enzyme delivery

The stability of both QQ enzymes and QSIs is also an important issue to be considered. Thermostable QQ enzymes with high stability during storage and immobilization [225] may hold promise for biotechnological applications. Coatings and dry powder with QQ enzyme in several study also showed well-retained QQ activity [281, 283]. However, the stability of both QQ enzymes and different QSIs needs to be further investigated under the complex *in vivo* conditions.

Therefore, the choice between QQ enzymes and specific QSIs might take their effectiveness, stability and toxicity into consideration. The successful application of both QQ enzymes and QSIs may also rely on the precise diagnosis of the pathogens in different kinds of infections.

CHAPTER II. OBJECTIVES

As major bacteria responsible for nosocomial wounds, urinary tracts, lung and bloodstream infections, *P. aeruginosa* and *A. baumannii* are highly resistant to a wide range of antibiotics. The ability of *P. aeruginosa* and *A. baumannii* to form biofilms further increases the difficulty to treat these infections. Interfering with the QS system(s) of these pathogens has been proposed as an alternative to conventional antibiotics for the eradication of biofilms and treatment of these biofilm-related infections. Many different QQ enzymes and QSIs from various origins have been identified and tested. However, the effects of some QQ enzymes and QSIs on the virulence and biofilm formation of *P. aeruginosa* and *A. baumannii* are poorly characterized, especially under *in vivo*-like conditions. In addition, the mechanism(s) behind the activity of several QSIs are yet to be investigated in detail. The main aim of this dissertation was to provide more evidence for the possibility to use certain QQ enzymes or QSIs to combat *P. aeruginosa* and *A. baumannii* infections and to get more insights into the mode of action.

First, we aimed to evaluate the anti-biofilm activity of the QQ enzyme MomL against *P. aeruginosa* and *A. baumannii*, and further investigate the effect of MomL under more complex conditions such as in dual-species biofilm and in a wound model system. The effect of MomL on virulence of *A. baumannii* was also tested in the *C. elegans* infection model.

Secondly, the effects of a plant-derived QSI, coumarin, on the production of virulence factors and biofilm formation in several *P. aeruginosa* wound isolates were investigated. We also aimed to test the potential use of coumarin to increase the survival of *Lucilia sericata* when challenged with *P. aeruginosa* in the context of wound debridement therapy. In addition, transcriptome analysis was performed to reveal how coumarin affected QS and virulence in *P. aeruginosa*.

Finally, we aimed to expand the knowledge about QSIs which were effective against *A. baumannii*. The effects of several known QSIs on *A. baumannii* motility and biofilm formation were tested. The most promising QSIs against *A. baumannii* were further evaluated in a *G. mellonella* infection model.

CHAPTER III.
EXPERIMENTAL WORK

Paper I.

Pitfalls associated with evaluating enzymatic quorum quenching activity: the case of MomL and its effect on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* biofilms

Yunhui Zhang, Gilles Brackman and Tom Coenye

Published in PeerJ. 2017; 5: e3251.

Abstract

Background. The enzymatic degradation of quorum sensing (QS) molecules (called quorum quenching, QQ) has been considered as a promising anti-virulence therapy to treat biofilm-related infections and combat antibiotic resistance. The recently-discovered QQ enzyme MomL has been reported to efficiently degrade different *N*-acyl homoserine lactones (AHLs) of various Gram-negative pathogens. Here we investigated the effect of MomL on biofilms formed by two important nosocomial pathogens, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

Methods. MomL was expressed in *E.coli* BL21 and purified. The activity of MomL on AHLs with hydroxyl substituent was tested. Biofilms of *P. aeruginosa* PAO1 and *Acinetobacter* strains were formed in 96-well microtiter plates. Biofilm formation was evaluated by crystal violet staining, plating and fluorescence microscopy. The effect of MomL on biofilm susceptibility to antibiotics was also tested. We further evaluated MomL in dual-species biofilms formed by *P. aeruginosa* and *A. baumannii*, and in biofilms formed in a wound model. The effect of MomL on virulence of *A. baumannii* was also tested in the *Caenorhabditis elegans* model.

Results. MomL reduced biofilm formation and increased biofilm susceptibility to different antibiotics in biofilms of *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531 formed in microtiter plates *in vitro*. However, no significant differences were detected in the dual-species biofilm and in wound model biofilms. In addition, MomL did not affect virulence of *A. baumannii* in the *C. elegans* model. Finally, the effect of MomL on biofilm of *Acinetobacter* strains seems to be strain-dependent.

Discussion. Our results indicate that although MomL showed a promising anti-biofilm effect against *P. aeruginosa* and *A. baumannii* biofilms formed in microtiter plates, the effect on biofilm formation under conditions more likely to mimic the real-life situation was much less pronounced or even absent. Our data indicate that in order to obtain a better picture of potential applicability of QQ enzymes for the treatment of biofilm-related infections, more elaborate model systems need to be used.

Introduction

Quorum sensing (QS) is a widespread communication process that allows bacteria to coordinate their group behavior based on the production, detection and response to extracellular signal molecules [346, 347]. QS regulates gene expression related to biofilm formation, motility and production of virulence factors in many Gram-negative and Gram-positive pathogens, and interfering with QS has been intensively studied as a promising anti-virulence therapy to combat bacterial infections and antibiotic resistance [348-350]. Many natural and synthetic compounds have been found to inhibit QS, and several quorum quenching (QQ) enzymes mainly targeting *N*-acyl homoserine lactone (AHL) based QS in Gram-negative bacteria have been described as well [49, 205, 288, 351]. Some of these QS inhibitors (QSIs) and QQ enzymes have shown promising anti-virulence effects both *in vitro* and *in vivo*. For instance, furanones which resemble AHLs and are able to bind to QS receptors have been reported to reduce biofilm formation and enhance bacterial clearance in *Pseudomonas aeruginosa* lung infection in mice [313, 330]. Baicalin hydrate and cinnamaldehyde (QSIs targeting AHL-based QS in *P. aeruginosa* and *Burkholderia cepacia* complex) as well as hamamelitannin (a QSI targeting the peptide-based QS system present in *Staphylococcus aureus*) increase biofilm susceptibility to antibiotics and survival of infected *Galleria mellonella* larvae and *Caenorhabditis elegans*, as well as decrease the microbial load in a mouse pulmonary infection model [352]. As for QQ enzymes, an AiiM-producing *P. aeruginosa* mutant showed reduced lung injury and increased survival in an *in vivo* study on mice with pneumonia [267], and an inhaled lactonase *Sso*Pox-I was also reported to reduce virulence of *P. aeruginosa* and mortality in rat pneumonia [268].

Previously MomL, a novel AHL lactonase belonging to the metallo-*β*-lactamase superfamily was identified and characterized [212]. It has high degrading activities towards short- and long-chain AHLs with or without an oxo-group at the C-3 position [212]. MomL can reduce pyocyanin and protease production by *P. aeruginosa* and attenuated the virulence of *P. aeruginosa* in a *C. elegans* infection model [212], but its effect on biofilm formation of *P. aeruginosa* and other Gram-negative pathogens was not tested yet.

Besides *P. aeruginosa*, *Acinetobacter baumannii* has also been recognized as an increasingly prevalent Gram-negative opportunistic pathogen responsible for severe nosocomial infections [353, 354]. Resistance of *P. aeruginosa* and *A. baumannii* strains to multiple antibiotic classes complicates the treatment for these infections and poses considerable therapeutic challenges worldwide [355]. One of the main factors contributing to their reduced antibiotic susceptibility and to treatment failure is biofilm formation both on tissues and abiotic surfaces [356-358]. Biofilms of both *P. aeruginosa* and *A. baumannii* are known to be regulated by AHL-based QS. In *P. aeruginosa*, *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butyryl-L-homoserine lactone (C4-HSL) are used by the Las and Rhl QS system, respectively [103], and these two AHLs can both be degraded by MomL [212]. One AHL synthase belonging to LuxI family, AbaI, has been reported to catalyze the synthesis of *N*-(3-hydroxydodecanoyl)-L-HSL (3-OH-C12-HSL) in *Acinetobacter nosocomialis* M2 [190], but other AHLs with varied chain lengths and substituents are also found in *Acinetobacter* strains [359, 360]. The biofilm-forming ability of an *abaI* mutant was reduced by around 40 % compared to the corresponding wildtype strain [190]. Compared to the extensive literature on inhibiting QS pathways and virulence in *P. aeruginosa* [314, 326, 361-363], there are fewer reports on inhibiting QS and biofilm formation in *A. baumannii* [257, 338, 339].

In the present study, we tested the anti-biofilm activity of MomL against *P. aeruginosa* and *A. baumannii*, and further evaluated the effect of MomL under more complex conditions such as in dual-species biofilm and in a wound model system with the aim to obtain a better knowledge base regarding the possible development of QQ enzymes as anti-virulence therapy.

Material & Methods

Bacterial strains, culture conditions and chemicals

P. aeruginosa PAO1, *A. calcoaceticus* LMG 10517, *A. nosocomialis* M2 and *A. baumannii* LMG 10520, LMG 10531 and AB5075 were cultured on tryptic soy agar (TSA) or in Mueller-Hinton broth (MH) at 37°C. *Escherichia coli* BL21(DE3) harboring MomL expression plasmid pET24a(+)-momL-(-SP) [212] was cultured on Luria-

Bertani (LB) agar supplemented with kanamycin (50 μg/mL) at 37°C. The AHL biosensor *Agrobacterium tumefaciens* A136 (pCF218) (pCF372) [364] was maintained on LB agar supplemented with spectinomycin (50 μg/mL) and tetracycline (4.5 μg/mL), and grown in AT minimal medium [365] containing 0.5% (wt/vol) glucose for detecting AHLs in the liquid X-Gal (5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside) assay. 3-OH-C_{12}-HSL was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO) as stock solution (100 mM). *Caenorhabditis elegans* N2 (glp-4; sek-1) was propagated under standard conditions, synchronized by hypochlorite bleaching, and cultured on nematode growth medium using *E. coli* OP50 as a food source [366].

MomL expression and purification

MomL was expressed and purified according to Tang *et al.*, 2015. In brief, protein expression was induced by 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) when *E. coli* cells in LB reaching an optical density at 600 nm (OD600) of 0.5 to 0.7. The induction was carried out at 16°C with moderate shaking (150 rpm) for 12h. Cells were harvested and sonicated, and the obtained supernatant was loaded on NTA-Ni (Qiagen) columns for purification according to the manufacturer's instruction. Desalting of the protein solution was accomplished by Amicon Ultra-15 centrifugal filter devices, and the purified MomL was stored at -20°C in Tris-HCl buffer (50mM, pH 6.5) with 25% glycerol.

Detection for degradation of 3-OH-C12-HSL

The amount of 3-OH-C12-HSL was quantified using *A. tumefaciens* A136 liquid X-gal assay and expressed as the normalized *β*-galactosidase activity as previously described [367]. The correction factor a and b were obtained and calculated for our experimental conditions, and the final formula to calculate the normalized *β*-galactosidase activity is $\frac{0.716 \times OD492 - OD620}{0.205 \times OD620 - OD492}$. To test the degradation of 3-OH-C12-HSL by MomL, 3-OH-C12-HSL (10 μM) was mixed with MomL in different concentrations (0.05-5 μg/mL) and incubated at 37°C for 1h. No MomL was added in control. Afterwards the residual 3-OH-C_{12}-HSL was quantified by adding 10 μL solution to the A136 biosensor, as described previously [367].

Biofilm formation assays

Overnight cultures of *P. aeruginosa* and *Acinetobacter* strains in MH broth were diluted to contain approximately 5×10^7 CFU/mL. 90 µL of this diluted bacterial suspension was transferred to the wells of a round-bottomed 96-well microtiter plate. Uninoculated MH broth was used as blank controls. To test the effect of MomL on biofilms, 10 µL purified enzyme (in different concentrations) was added to the wells, while 10 µL Tris-HCl buffer (50mM, pH 6.5) with 25% glycerol was added to the control. The plate was incubated at 37°C for 4 hours before the supernatant was removed. The wells were rinsed once with sterile physiological saline (PS) and re-filled with fresh media (90 µL) and MomL (10 µL). The plate was incubated at 37°C for an additional 20 hours. The biofilm biomass was quantified by crystal violet (CV) staining as described previously [368]. After rinsing the wells with sterile PS, the biofilm was fixed with 100 µL 99% methanol for 15 min and stained with 100 µL 0.1% CV for 20 min. The excess CV was removed by washing the plates under running tap water and bound CV was released by adding 150 µl of 33% acetic acid. The absorbance was measured at 590 nm.

Biofilm susceptibility assays

After a 24h-biofilm of *P. aeruginosa* or *A. baumannii* strains was formed as described above either in presence of MomL or not, the plate was emptied and biofilm cells were rinsed with sterile PS. Antibiotics were dissolved in PS and 90 µL of these solutions were added to treat the biofilm for another 24h, either with or without 10 µl MomL. Tobramycin (TOB; 4 µg/mL as final concentration), ciprofloxacin (CIP; 0.5 µg/mL), meropenem (MEM; 16 µg/mL) and colistin (CST; 16 µg/mL) were used to treat the biofilm of *P. aeruginosa* PAO1; TOB (6 µg/mL), CIP (4 µg/mL), MEM (8 µg/mL) and CST (16 µg/mL) were used to treat the biofilm of the *A. baumannii* strains. The supernatant was removed and the wells were washed once with sterile PS. To release bacterial cells from biofilm, two cycles of vortex (5 mins) and sonication (5 mins) were performed, and the number of CFU/biofilm was determined by plating the resulting suspensions on TSA.

Fluorescence microscopy

Biofilms of *P. aeruginosa* PAO1 or *A. baumannii* strains were formed in the absence or presence of MomL and treated with antibiotics as described above using a flat-bottomed 96-well microtiter plates. 3 μL SYTO9 and 3 μL propidium iodide were diluted to 1mL in sterile PS, and 100 μL of this staining solution was transferred to each well. The plate was incubated for 15min at room temperature and fluorescence microscopy was performed with EVOS FL Auto Imaging System (Life Technologies). The red fluorescent signal was detected with 531/40 nm excitation filter cube and 593/40 nm emission filter cube and the green fluorescent signal was detected with 470/22 nm excitation filter cube and 510/42 nm emission filter cube.

Dual-species biofilm formation

Overnight cultures of *P. aeruginosa* and *A. baumannii* strains in MH broth were diluted to contain approximately 5×10^5 CFU/mL and 5×10^7 CFU/mL, respectively, and equal volume of suspensions of *P. aeruginosa* and *A. baumannii* were mixed. MomL (200 μg/mL) and tobramycin (6 μg/mL) were added as described above. To quantify CFU in the dual-species biofilm, *Pseudomonas* Isolation Agar (Difco) and TSA supplemented with 5 μg/mL cefsulodin were used as selective media for *P. aeruginosa* and *A. baumannii* respectively.

Biofilm formation in wound model

Artificial dermis composed of hyaluronic acid and collagen was used in our wound model, as described before [369]. Each disk of artificial dermis was placed in 24-well microtiter plate. One mL media containing Bolton Broth, heparinized bovine plasma and freeze-thaw laked horse blood cells was added on and around the dermis. Suspensions (10 μL) of *P. aeruginosa* or *A. baumannii* containing 10^4 bacterial cells were added on the top of dermis. Final concentrations of MomL added were 200 μg/mL and 10 μg/mL for *P. aeruginosa* and *A. baumannii*, respectively. Tobramycin (10 μg/mL) was added after 8 h incubation at 37°C. After 24h, the infected dermis was washed with 1 mL PS and was transferred into 10 ml PS. Biofilm cells on the dermis were loosen and collected by three cycles of vortex (30 s) and sonication (30 s). The number of CFU/dermis was quantified by standard plating techniques.

***C. elegans* survival assay**

The *C. elegans* survival assay was carried out as described before with minor modification [352]. Synchronized worms (L4 stage) were suspended in medium containing 95% M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, and 1 ml of 1 M $MgSO_4 \cdot 7H_2O$ in 1 liter of water) and 5% brain heart infusion broth (Oxoid), and 25 µL of this nematode suspension was transferred to the wells of a 96-well microtiter plate. Overnight culture of *A. baumannii* was suspended in the assay media and added in a final concentration of $2.5x10^7$ CFU/ml. MomL was added in a final concentration of 10 µg/mL. The plates were incubated at 25°C for 24 h. The fraction of dead worms was determined by counting the number of dead worms and the total number of worms in each well.

Statistics

The normal distribution of the data was checked by the D'Agostino-Pearson normality test. Normally distributed data were analyzed by one-way ANOVA, and non-normally distributed data were analyzed by the Kruskal-Wallis test or the Mann-Whitney test. All statistical analyses were carried out using GraphPad Prism 6.0.

Results

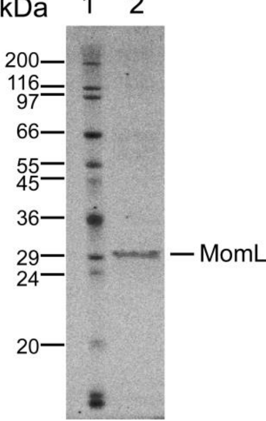

Figure 1. SDS-PAGE of purified MomL. Lane1, molecular mass markers; Lane 2, purified recombinant MomL with molecular mass of nearly 31 kDa

Degradation of 3-OH-C_{12}-HSL by purified MomL

MomL was produced in *E. coli* and subsequently successfully purified (Fig. 1). Although MomL had been shown to degrade various AHLs [212], its activity on AHLs

with hydroxyl substituent at the C3 position was not tested yet. We could demonstrate that MomL, in a concentration of 1 μg/mL or higher, can degrade almost all 3-OH-C12-HSLs (10 μM) under the experimental conditions used in the present study (Fig. 2).

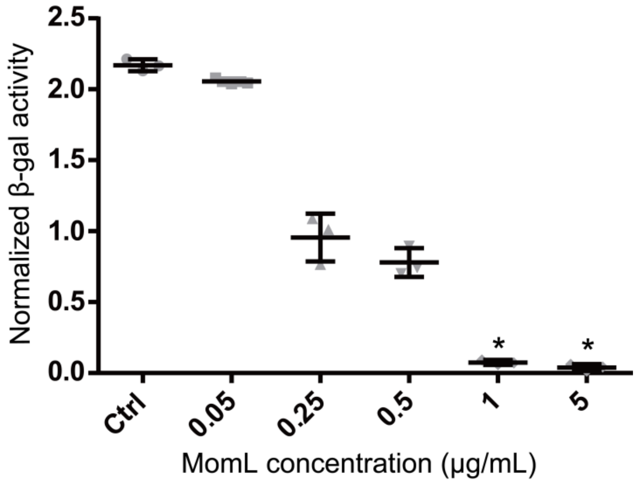

Figure 2. Degradation of 3OH-C12-HSL by MomL. The amount of residual 3-OH-C12-HSL was expressed as the normalized β-galactosidase activity. Data shown are average of three replicates (n = 3), error bars represent standard deviation. *, $P < 0.05$ when compared with non-MomL treated control (Kruskal-Wallis test).

Effect of MomL on biofilm formation by *P. aeruginosa* and *A. baumannii*

Following biofilm formation in 96-well microtiter plates and quantification by crystal violet staining, a significant difference was observed between *P. aeruginosa* PAO1 control biofilms and biofilms grown in the presence of MomL (concentration > 50 μg/mL) (Fig. 3A). When grown with 150 μg/mL MomL, an average decrease of approximately 35% was observed. MomL inhibited *A. baumannii* LMG 10531 biofilm formation at concentrations as low as 0.1 μg/mL, and the biofilm biomass was reduced by approximately 42% when exposed to 5 μg/mL MomL (Fig. 3B). No further decrease was observed when *A. baumannii* LMG 10531 biofilms were grown in the presence of higher concentration of MomL.

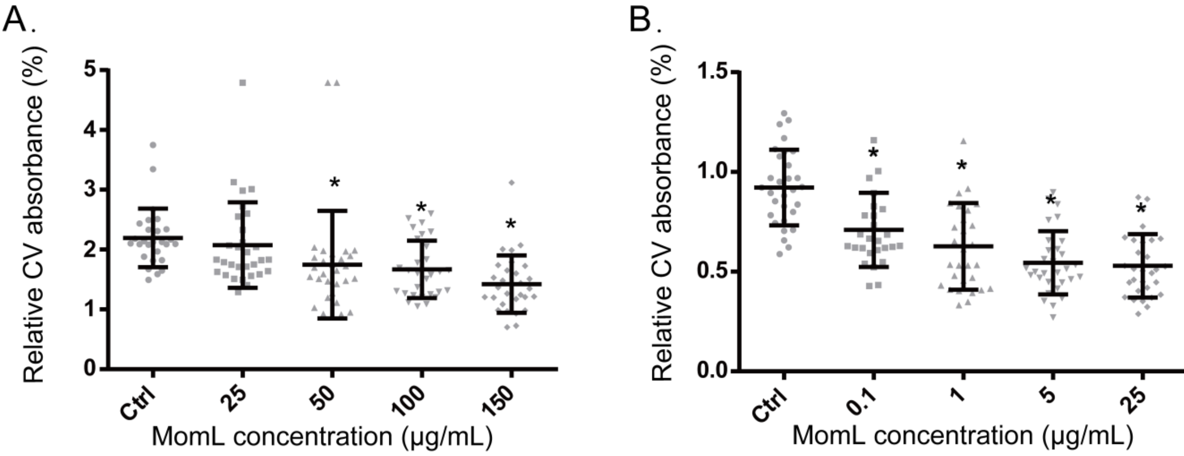

Figure 3 Effect of MomL on biofilm formation by *P. aeruginosa* PAO1 (A) and *A. baumannii* LMG 10531 (B). Biofilms were quantified by CV staining and amount of biofilm left is expressed as CV absorbance (OD 590). Data shown are average of three independent experiments with variable numbers of replicates each (n ≥ 27), error bars represent standard deviation. *, P < 0.05 when compared with nonMomL treated control in Kruskal-Wallis test (A) or one-way ANOVA(B).

Effect of MomL on biofilm susceptibility to antibiotics

Application of MomL alone (200 µg/mL for *P. aeruginosa* PAO1 and 10 µg/mL for *A. baumannii* LMG 10531) reduced the number of cultivable biofilm cells by approximately 50% in both *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531. For *P. aeruginosa* PAO1, combining CIP or MEM with MomL led to >70% more reduction compared to treatment with CIP or MEM alone (Fig. 4A). For *A. baumannii* LMG 10531, MomL also increased killing of biofilm cells when antibiotics were used together with MomL (Fig. 4B). In case of TOB, cell number was reduced by 80% when used in combination with MomL compared to TOB alone. Consistent with results obtained by plating, fewer living cells were observed in fluorescence microscope images of biofilms treated with MomL, TOB, or a combination of both, compared to control biofilms (Fig. 5).

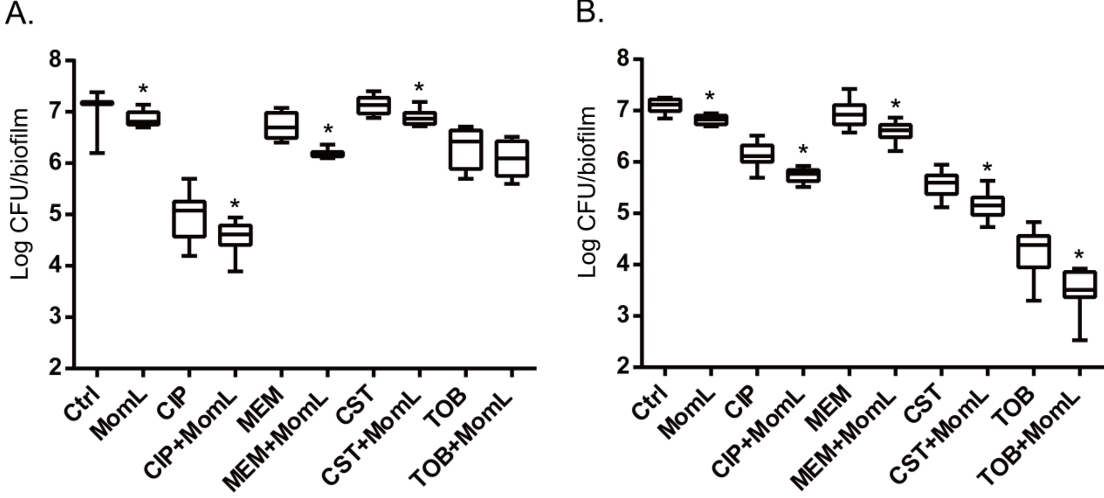

Figure 4. Effect of MomL on susceptibility of *P. aeruginosa* PAO1 (A) and *A. baumannii* LMG 10531 (B) biofilms to different antibiotics. Numbers of CFU/biofilm were determined by plating and shown as box-whisker plots. Boxes span the interquartile range; the line within each box denotes the median, and whiskers indicate the minimum and maximum values. MomL was added in a final concentration of 200 µg/mL for *P. aeruginosa* PAO1 and 10 µg/mL for *A. baumannii* LMG 10531. Data shown are from three independent experiments with three replicates each (n = 9). Mann–Whitney tests were performed to compare different groups (*, $P < 0.05$)

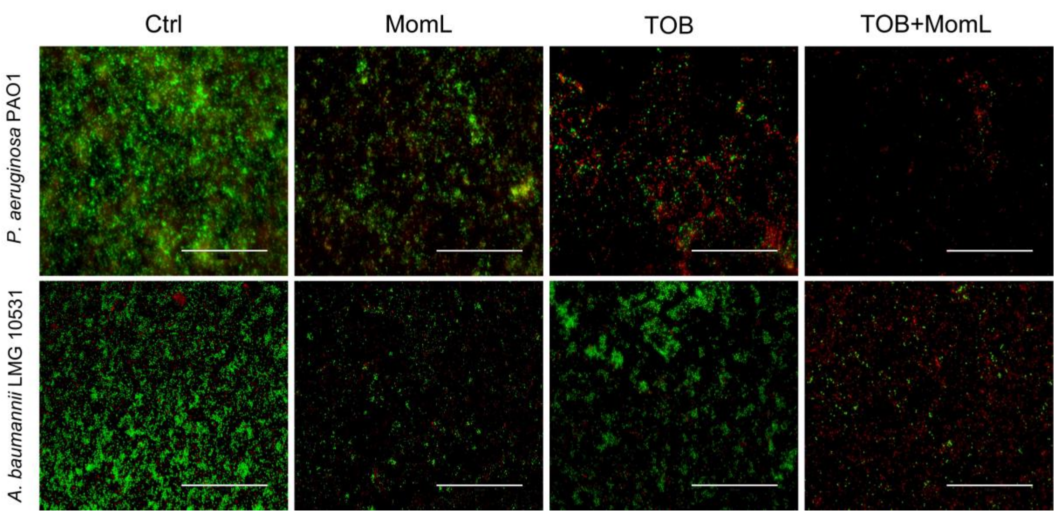

Figure 5. Representative fluorescence images of biofilms of *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531. Biofilms were treated with MomL alone, TOB alone or a combination of both and stained with Syto9 and propidium iodide. 40× Objective (numerical aperture: 0.75) was used and the final magnification is 1,200×. The scale bar represents 100 µm.

Effect of MomL on dual-species biofilm formed by *P. aeruginosa* and *A. baumannii*

We also evaluated the effect of MomL on dual-species biofilm formed by *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531. We found that *P. aeruginosa* PAO1 inhibited growth of *A. baumannii* LMG 10531 in dual-species biofilm, and most *A. baumannii* LMG 10531 cells were killed by *P. aeruginosa* PAO1 after 48h (Fig. 6). When MomL was added, there was a reduction in *A. baumannii* LMG 10531 cell numbers; however no difference was observed in either total cell numbers or number of surviving *P. aeruginosa* PAO1 cells (Fig. 6A). MomL in combination of TOB was also tested, but no change in susceptibility to TOB was observed in the dual-species biofilm (Fig. 6B).

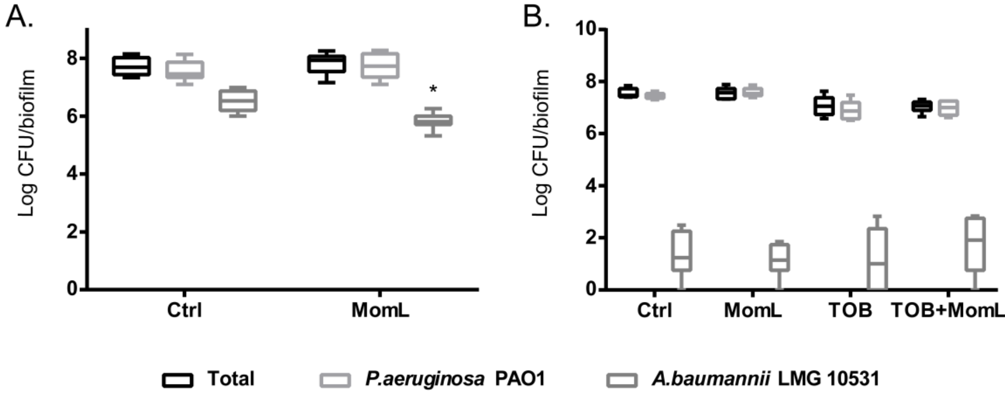

Figure 6 Effect of MomL on dual-species biofilms. Total number of CFU/biofilm, number of *P. aeruginosa* PAO1 CFU/biofilm and number of *A. baumannii* LMG 10531 CFU/biofilm in each dual-species biofilm were determined by plating and shown as box-whisker plots. Boxes span the interquartile range; the line within each box denotes the median, and whiskers indicate the minimum and maximum values. (A). 24 h-biofilm treated with MomL alone; (B). 48 h-biofilm treated with MomL alone, TOB alone or a combination of both. Data shown are from three independent experiments with three (A) or two (B) replicates each (n = 9 for A, n = 6 for B). Mann–Whitney tests were performed to compare total, *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531 cell numbers respectively between untreated or MomL-treated dual-species biofilm (*, $P < 0.05$).

Effect of MomL on other *Acinetobacter* strains

We also tested MomL on four other *Acinetobacter* strains. However, only *A. baumannii* LMG 10520 showed reduction in biofilm biomass when treated with MomL at 50 µg/mL (Fig. 7). No significant difference was observed for *A. calcoaceticus*

LMG 10517, *A. nosocomialis* M2 and *A. baumannii* AB5075. The effect of MomL on susceptibility of *A. baumannii* LMG 10520 and *A. calcoaceticus* LMG 10517 biofilms was also tested. For *A. baumannii* LMG 10520, significant differences were detected when MomL was added alone or in combination with several antibiotics (Fig. 8). For *A. calcoaceticus* LMG 10517, no difference was observed between biofilms receiving MomL treatment and biofilms receiving the control treatment, either by plating or fluorescence microscope.

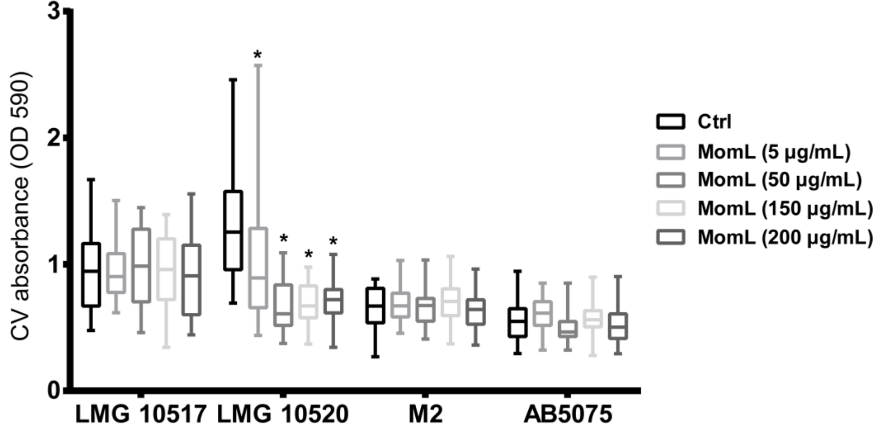

Figure 7. Effect of MomL on biofilms formed by other *Acinetobacter* strains. Biofilms of *A. calcoaceticus* LMG 10517, *A. nosocomialis* M2, *A. baumannii* LMG 10520 and *A. baumannii* AB5075 were treated with different concentration of MomL and quantified by CV staining. Data shown in box-whisker plots are from three independent experiments with variable numbers of replicates each (n ≥ 27). Boxes span the interquartile range; the line within each box denotes the median, and whiskers indicate the minimum and maximum values. *, P < 0.05 when compared to untreated control (Kruskal-Wallis test).

Effect of MomL in a biofilm wound model system and in the *C. elegans* model

An *in vitro* wound model was used to mimic the conditions in an infected wound. For both *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531, MomL had no effect on biofilm formation in this wound model (Fig. 9).

The *C. elegans* model was used to further evaluate whether MomL can increase survival of nematodes infected with *A. baumannii*. However, no significant increase of *C. elegans* survival was found after treating nematodes infected with *A. baumannii* LMG 10520 or *A. baumannii* LMG 10531 with MomL (Fig. 10).

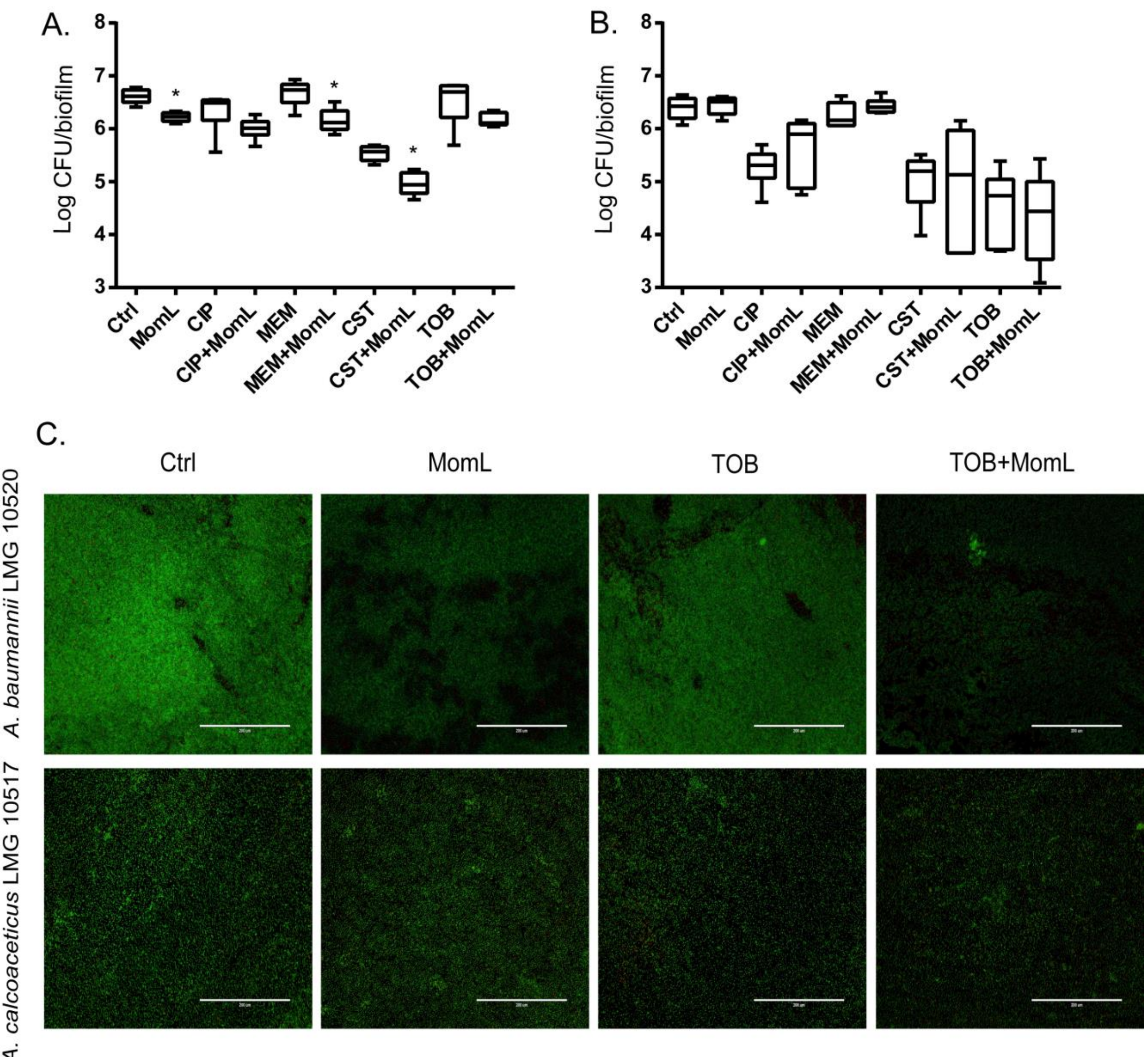

Figure 8 Effect of MomL on biofilm susceptibility of *A. baumannii* LMG 10520 and *A. calcoaceticus* LMG 10517. (A). Plating results for biofilms of *A. baumannii* LMG 10520 exposed to CIP, MEM, CST, TOB alone or in combination with MomL (50 μg/mL); (B), Plating results for biofilms of *A. calcoaceticus* LMG 10517 exposed to CIP, MEM, CST, TOB alone or in combination with MomL (200 μg/mL). Data shown in box-whisker plots are from two independent experiments with three replicates each (n = 6). Boxes span the interquartile range; the line within each box denotes the median, and whiskers indicate the minimum and maximum values. Mann–Whitney tests were performed to compare control and MomL or antibiotic treatment alone and in combination with MomL (*, $P < 0.05$). (C). Representative fluorescence images of *A. baumannii* LMG 10520 and *A. calcoaceticus* LMG 10517. Biofilms were treated with MomL alone or in combination with tobramycin and stained with Syto9 and propidium iodide. 20× Objective (numerical aperture: 0.65) was used and the final magnification is 599×. The scale bar represents 200 μm.

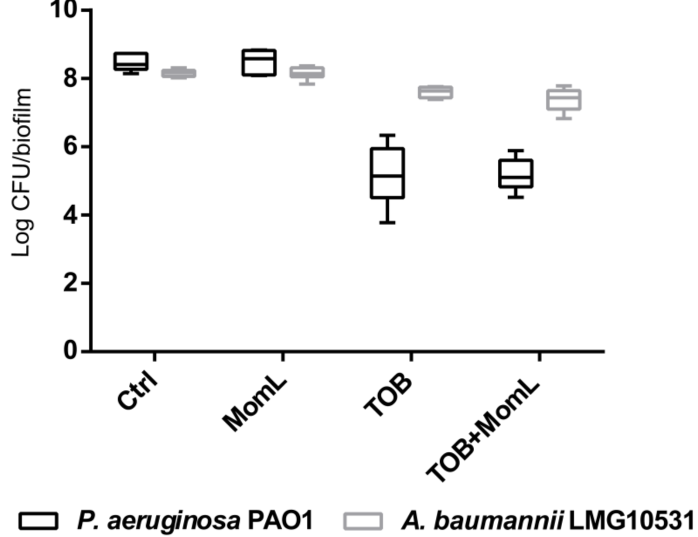

Figure 9. Effect of MomL on biofilms of *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531 formed in wound model. Data shown in box-whisker plots are from three independent experiments with two replicates each (n = 6). Boxes span the interquartile range; the line within each box denotes the median, and whiskers indicate the minimum and maximum values. Mann–Whitney tests were performed to compare control and MomL treatment, or TOB and TOB in combination with MomL.

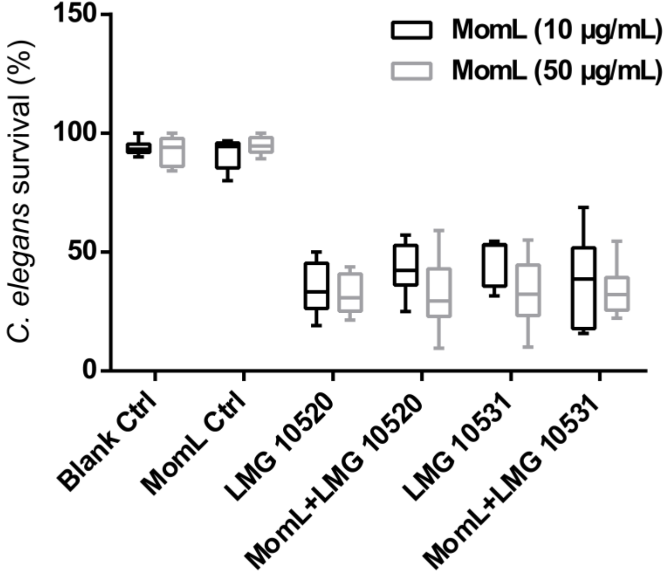

Figure 10. Effect of MomL on the virulence of *A. baumannii* strains in *C. elegans*. Percent survival of *C. elegans* infected by *A. baumannii* LMG 10520 and LMG 10531. Data shown in box-whisker plots are from three independent experiments with three replicates each (n = 9). Boxes span the interquartile range; the line within each box denotes the median, and whiskers indicate the minimum and maximum values. One-way ANOVA was performed, and no significant differences were found between control and MomL treatment in both uninfected *C. elegans* and those infected by *A. baumannii* strains.

Discussion

QS disruption has been considered as a promising anti-infectious strategy to substitute or at least supplement treatment with antibiotics, and could inhibit production of virulence factors and the formation of biofilms [352]. Compared to QS inhibitors, QQ enzymes can degrade AHLs from different pathogens and might be more effective in treating multispecies infections. In addition, QQ enzymes do not need to enter the cells as they can act extracellularly, making it less likely resistance will develop [370]. The recently-discovered QQ enzyme, MomL, has strong degrading activity towards AHLs with different acyl-chain length and substituents (oxo or hydroxyl) [212], and this could be an advantage when targeting bacteria like *Acinetobacter* strains that produce various AHLs. MomL was reported to reduce the *in vitro* production of protease and pyocyanin by *P. aeruginosa* and attenuate the virulence of *P. aeruginosa* in a *C. elegans* infection model [212]. The further application potential of MomL was not determined yet. In the present study we investigated the possible use of MomL for treating biofilm infections, and evaluate its effect on two important Gram-negative nosocomial pathogens, *P. aeruginosa* and *A. baumannii* in different models.

First we tested the effect of MomL on single-species biofilms of *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531 formed in microtiter plates; a reduction of biofilm biomass was observed for both strains. The maximum decrease in biofilm of *A. baumannii* LMG 10531 was achieved at a concentration of 5 µg/mL and no further decrease was observed with higher concentrations of MomL, which indicated the presence of other mechanisms besides QS regulating biofilm formation in *A. baumannii*. When used in combination with antibiotics, fewer biofilm cells survived compared to antibiotic treatment alone, both for *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531. In addition, MomL showed no inhibition on planktonic cells of both *P. aeruginosa* and *A. baumannii* (Fig. S1), and all these *in vitro* results seem promising and suggest possible use of MomL to treat biofilm infections of *P. aeruginosa* and *A. baumannii*.

We subsequently investigated the effect of MomL in a dual-species biofilm formed by *P. aeruginosa* and *A. baumannii* and in a wound biofilm model. MomL had no effect on the overall cell number in the mixed species biofilm and the same disappointing

results were obtained in biofilms formed in wound model system. In this wound model system, media containing plasma, serum, horse blood and heparin was used to reflect nutritional condition in wounds. An artificial dermis was used to mimic a wound like surface and an inoculum of 10^4 cells was used to reflect the microbial load of a wound prior to infection. Additionally, in contrast to what we observed for the mono-species biofilms formed in 96-well microtiter plates, MomL did not potentiate the activity of TOB in this model system. Possible explanations for this are that component(s) present in this wound biofilm model protect AHL from degradation and/or interfere with the activity of MomL (potentially through interactions with proteins in the plasma), or that QS is not essential for biofilm formation and/or resistance in these conditions. Further experiments will be required to clarify this. Although MomL showed strong activity against 3-OH-C12-HSL in the medium used in the *C. elegans* model (Fig. S2), no effect of MomL on the virulence of *A. baumannii* was observed. As previously reported, an *A. baumannii* QS mutant did not differ from the wild type with regards to killing in a *Galleria mellonella* infection model [371]. These results indicated that although QS is known to play an important role in *A. baumannii* biofilm formation, it might have only limited influence on the general virulence to *C. elegans* and *G. mellonella*.

Thus far, a series of promising results about in vivo application of QQ enzymes have been reported. Phosphotriesterase-like lactonase SsoPox-I has been reported to reduce biofilm formation of *P. aeruginosa* at a concentration higher than 170 µg/mL, and the early use of SsoPox-I reduced the mortality of rats with acute pneumonia from 75% to 20% [268]. In another study, acylase-containing coatings on silicone urinary catheters reduced formation of *P. aeruginosa* biofilms and mixed-species *P. aeruginosa*-*E. coli* biofilms [279]. Our data obtained in a dual-species biofilm formed by *P. aeruginosa* and *A. baumannii* as well as in a wound model strongly suggest that the effect of MomL (and potentially also other QQ enzymes) on in vivo grown bacterial biofilms may be much less pronounced than the effect observed with biofilms formed under simple *in vitro* conditions. Factors affecting the anti-biofilm activity in more complex systems could include stability of the enzyme, penetration of the enzyme through the biofilm matrix, and the composition of the environment.

Different outcomes were also observed when we evaluated the effect of MomL on different *Acinetobacter* strains, and no effects of MomL on biofilm formation was

detected for three out of five *Acinetobacter* strains tested. In addition, for *A. baumannii* LMG 10520, a considerably higher concentration of MomL was required to obtain a pronounced inhibitory effect than for *A. baumannii* LMG 10531. These results confirm that the anti-biofilm activity of QQ enzymes is strain-dependent, which is likely to reduce their clinical efficacy.

Conclusion

The results of the present study highlight that there are considerable hurdles to be cleared before QQ enzymes could potentially be used to combat infections. Our data indicate that demonstrating AHL degrading activity *in vitro* and/or anti-biofilm activity in simple *in vitro* biofilm model systems is not sufficient to predict an anti-biofilm effect in more complex systems.

Acknowledgements

We thank prof. Xiao-Hua Zhang for providing *Escherichia coli* BL21(DE3) harboring the MomL expression plasmid pET24a(+)-momL-(-SP), prof. Wim Quax for providing *A. nosocomialis* M2 and prof. Colin Manoil for providing *A. baumannii* AB5075.

Supplemental Information

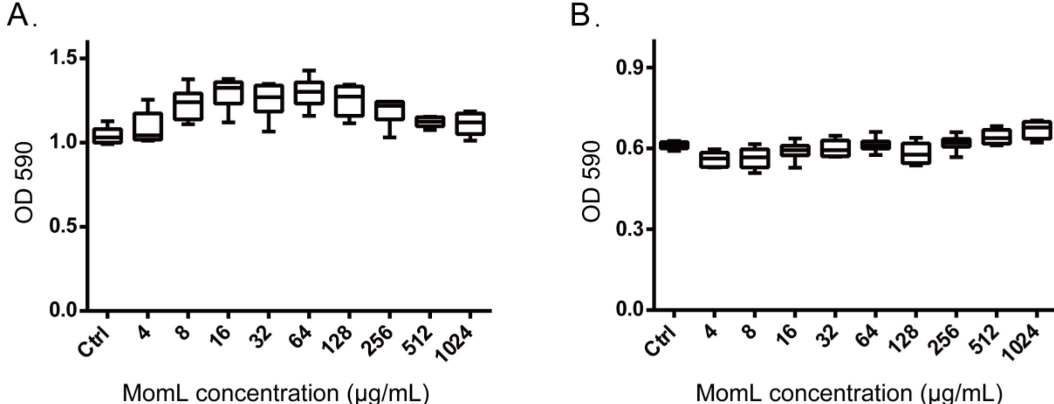

Figure S1. The influence of MomL on cell growth of *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531. The growth of *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531 was spectrophotometrically determined at OD 590 after being incubated with different concentrations of MomL at 37 °C for 24 h. No MomL was added in control. Data shown in box-whisker plots are from three independent experiments with two replicates each ($n = 6$). Boxes span the interquartile range; the line within each box denotes the median, and whiskers indicate the minimum and maximum values.

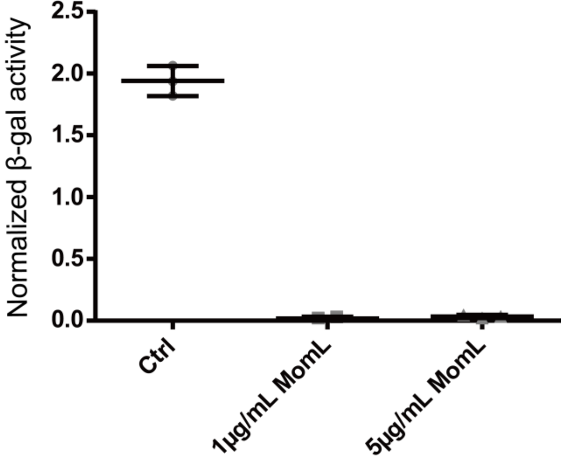

Figure S2. Degradation of 3-OH-C12-HSL by MomL in *C. elegans* model medium 3-OH-C12-HSL (10 µM) was mixed with MomL under the same medium condition in *C. elegans* model and incubated at 37°C for 1h. No MomL was added in control. The amount of residual 3-OH-C12-HSL was quantified using *A. tumefaciens* A136 liquid X-gal assay and expressed as the normalized *β*-galactosidase activity.

Paper II.

Coumarin reduces virulence and biofilm formation in *Pseudomonas aeruginosa* by affecting quorum sensing, type III secretion and c-di-GMP levels

Yunhui Zhang, Andrea Sass, Heleen Van Acker, Jasper Wille, Bruno Verhasselt, Filip Van Nieuwerburgh, Volkhard Kaever, Aurélie Crabbé and Tom Coenye

Manuscript submitted – Frontiers in Microbiology

Abstract

As one of the major pathogens in wound infections, *Pseudomonas aeruginosa* produces several virulence factors and forms biofilms; these processes are under the regulation of various quorum sensing (QS) systems. Therefore, QS has been regarded as a promising target to treat *P. aeruginosa* infections. In the present study, we evaluated the effect of the plant-derived QS inhibitor coumarin on *P. aeruginosa* biofilms and virulence. Coumarin inhibited QS in the *P. aeruginosa* QSIS2 biosensor, reduced protease and pyocyanin production in different *P. aeruginosa* strains, inhibited biofilm formation both in microtiter plates and in an *in vitro* wound model, and reduced *P. aeruginosa* virulence in the *Lucilia sericata* infection model. Transcriptome analysis revealed that several key genes involved in the *las*, *rhl*, *Pseudomonas* quinolone signal (PQS) and integrated quorum sensing (IQS) systems were down-regulated in the coumarin-treated biofilm of *P. aeruginosa* PAO1. Coumarin also changed the expression of genes related to type III secretion and cyclic diguanylate (c-di-GMP) metabolism. The cellular c-di-GMP level of *P. aeruginosa* PAO1 was significantly reduced by coumarin. These results provide new evidences for the possible application of coumarin as an anti-biofilm and anti-virulence agent against *P. aeruginosa* in wound infections.

Introduction

The opportunistic pathogen *Pseudomonas aeruginosa* frequently causes diverse infections in immunocompromised patients [2, 372, 373]. *P. aeruginosa* is also involved in both acute and chronic wound infections associated with high morbidity and mortality. Chronic wounds such as diabetic ulcers, venous ulcers and pressure ulcers affect millions of patients worldwide and lead to high costs for the healthcare system (e.g. they represent an estimated cost of around 25 billion per year in the US alone) [374]. Infections in burn wounds also pose a heavy medical and economic burden in both developed and developing countries [375, 376]. Wound infections with *P. aeruginosa* are especially difficult to treat and are often associated with worse outcomes compared to other pathogens [377], due to the extensive arsenal of virulence factors and increasing antibiotic resistance [378, 379]. In addition, biofilms

formed by *P. aeruginosa* in wound infections further protect the bacteria from host immune defense and antimicrobials, impeding the healing process and triggering the shift to chronic wounds [380, 381]. Therefore, there is an urgent need to develop alternative strategies to combat biofilm-related *P. aeruginosa* infections.

Quorum sensing (QS) is the intercellular communication process based on the production and detection of, and group-level response to, signal molecules [42]. The complex *P. aeruginosa* QS network has intensively been studied in the past decades as QS plays a crucial role in coordinating the production of several important virulence factors, including pyocyanin, protease, exotoxin A, hydrogen cyanide and rhamnolipid [382]. QS also affects biofilm formation and antibiotic resistance through multiple distinct mechanisms [181, 383-385]. So far, four interacting QS systems have been identified in *P. aeruginosa*, including the *N*-acyl-homoserine lactone (AHL)-based *rhl* and *las* systems, the *Pseudomonas* quinolone signal (PQS) system and the recently identified integrated QS (IQS) system [386]. This QS network allows *P. aeruginosa* to secrete extracellular virulence factors only when they can be produced at a sufficiently high level to overcome the host defense [387]. In addition, QS has been reported to be involved in the spread of *P. aeruginosa* in burn wound infections [388].

QS inhibition has been proposed as a promising anti-virulence strategy which would allow to 'disarm' pathogens rather than killing them, and many potential QS inhibitors have been described [284, 288, 348]. A wide range of structurally different QS inhibitors (QSIs) targeting *P. aeruginosa* have been identified, both from natural and synthetic sources [285]. The first comprehensively studied QSI is the furanone compound C-30 [314], which increased *P. aeruginosa* biofilm susceptibility to tobramycin and led to more efficient clearance of bacteria in a pulmonary mouse infection model [313]. Ajoene, a sulfur-rich molecule from garlic, reduces expression of several QS-regulated virulence factors by activating the QS negative regulator RsmA through two small regulatory RNAs, RsmY and RsmZ [298, 299]. Many other QSIs such as 6-gingerol [328] and quercetin [389] have also been reported to reduce the virulence and biofilm formation of *P. aeruginosa*. These studies together demonstrate the possibility of using QSIs to control *P. aeruginosa* infections in vitro and/or in animal infection models.

Coumarin is a plant-derived phenolic compound and its derivatives are known for their anti-tumor and anti-inflammatory activities [328, 390, 391]. Coumarin has been described as an inhibitor of QS in *P. aeruginosa* and several other Gram-negative bacteria [392]. It was shown to inhibit biofilm formation, phenazine production and motility in *P. aeruginosa* strain PA14 [392] and suppress virulence in *Vibrio splendidus* [393]. However, the mechanism by which coumarin inhibits QS has not been elucidated yet.

The goal of the present study was to evaluate the potential role of coumarin in the treatment of *P. aeruginosa*-infected wounds. To this end, we determined the effect of coumarin on the production of virulence factors and biofilm formation in an *in vitro* wound model, using the *P. aeruginosa* reference isolate PAO1 as well as several *P. aeruginosa* clinical wound isolates. We also investigated the effect of coumarin on *P. aeruginosa* virulence to *Lucilia sericata* maggots, which are widely used in the debridement of chronic wounds [394]. The QS-regulated virulence factors of *P. aeruginosa* are responsible for killing of these maggots and often lead to the failure of maggot therapy [34]. Finally, transcriptome analysis in both planktonic and biofilm cells was performed to obtain a clear picture of molecular mechanisms involved in the inhibition of QS by coumarin.

Material and methods

Bacterial strains, chemicals and growth media

P. aeruginosa strains 1803, 2063, 2091, 2549, 3120 were isolated from wound infections and obtained from the Ghent University Hospital, Belgium (with the original strain number described as 170407-1803, 170411-2063, 170303-2091, 170410-2549 and 170406-3120). Reference strain *P. aeruginosa* PAO1 and these five wound isolates were routinely cultured in Luria-Bertani (LB) agar or broth (Lab M limited, UK) or Tryptic Soy Agar (TSA, Lab M limited, UK). The QS inhibition selector *P. aeruginosa* QSIS2 [395] was cultured in ABT minimal medium (AB medium, containing 2.5 mg/L thiamine) supplemented with 0.5% glucose, 0.5% casamino acids and 80 µg/mL gentamicin. All strains were grown aerobically at 37 °C. Coumarin was purchased from Sigma-Aldrich (Bornem, Belgium) and dissolved in

dimethyl sulfoxide (DMSO) (Alfa Aesar, Germany) as stock solutions (1 M). *L. sericata* maggots in BioBags were purchased from BioMonde (Germany), and were used immediately after receipt.

QS inhibition assay

The *P. aeruginosa* QSIS2 reporter strain is a *lasI rhlI* double mutant containing plasmid pLasB-SacB1 encoding an AHL-induced killing system [395]. The QS inhibition assay based on QSIS2 was performed as previously described, with minor modifications [396]. Briefly, an overnight culture of QSIS2 was diluted in ABT medium to an optical density at 590 nm (OD 590) of 0.1. 50 µL cell suspensions and 50 µL LB with sucrose (224 mg/mL) was added to 96-well microtiter plates, supplemented with gentamicin at a final concentration of 80 µg/mL. *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butyryl-L-homoserine lactone (C4-HSL) (200 nM each, Sigma) were added to activate the QS system as positive controls, and sterile MilliQ ultrapure water was used as a negative control. To test the QS inhibition effect, coumarin was added in a final concentration of 1 mM and 2 mM. The microtiter plates were incubated for 10 h at 37 °C, and the absorbance was measured at 450 nm using an EnVision multilabel reader (Perkin Elmer, Waltham, MA). The difference in growth between the negative control without AHLs and the positive control with AHLs was considered as 100% QS inhibition. Three independent experiments were performed with three replicates each (n=9).

Measurements of pyocyanin and protease production

Overnight cultures of *P. aeruginosa* strains were standardized to OD590 0.5 and 1:50 diluted in 10 ml LB with or without coumarin (2 mM). After 24 h incubation at 37 °C by shaking, the supernatant was collected and filter-sterilized. For the pyocyanin assay, chloroform (3 mL) was added to 5 mL *P. aeruginosa* culture supernatant and vortexed. The chloroform phase was kept after centrifugation (5000 rpm, 5 min), and mixed with 1mL HCl (0.2 M). The absorbance of the pink HCl layer was measured at 520 nm with the EnVision multilabel reader after vortexing and centrifugation [397]. 0.2 M HCl was used as a negative control in the measurement of absorbance. Protease production was tested by the azocaseine assay as described previously [398, 399] with modifications. 400 µL azocaseine (Sigma) solution (5 mg/mL 0.1 M Tris-HCl buffer) was mixed with 400 µL supernatant and incubated at 37 °C for 1 h.

The reaction was stopped by adding 100 µL 10% (w/v) trichloroacetic acid and the mixture was then centrifuged. 100 µL of the resulting supernatant was transferred to 96-well microtiter plates and mixed with 100 µL 625 mM NaOH. The absorbance was measured at 420 nm with the EnVision multilabel reader. Azocaseine solution with LB medium was processed following the same procedure and used as blank control. Two independent experiments were performed with three replicates each (n=6).

The effect of coumarin on planktonic cell growth and on biofilm formation in microtiter plates

Overnight cultures of *P. aeruginosa* strains were standardized to OD590 0.5 and inoculated in 10 mL of LB with 1:50 dilutions with or without coumarin (2 mM) at 37 °C. The growth of planktonic cells was quantified by serial dilution and plating on TSA after 24 h. Three independent experiments were performed (n=3).

Biofilm formation in microtiter plates was evaluated as described previously [396]. Briefly, overnight cultures of *P. aeruginosa* strains were diluted to approximately 5×10^7 CFU/mL in LB broth. 100 µL of the suspension was transferred to the wells of a round-bottomed 96-well microtiter plate with coumarin in a final concentration of 1 mM or 2 mM. 10 µL MilliQ with DMSO was added for the control. The plate was incubated at 37 °C for 4 h, and then the wells were rinsed once with sterile physiological saline (PS) and re-filled with fresh medium and coumarin. The plate was incubated at 37 °C for an additional 20 h. After removing the supernatant and washing the wells once with sterile PS, two cycles of vortexing (5 mins) and sonication (5 mins) were performed to release biofilm cells, and the number of CFU/biofilm was determined by plating. Three independent experiments were performed with three replicates each (n=9).

The effect of coumarin on biofilm formation in a wound model

The biofilm wound model used has been described before [400]. A spongy artificial dermis of 1 cm^3 (upper layer: chemically cross-linked hyaluronic acid, lower layer: hyaluronic acid and collagen) is used as a substrate for biofilm formation to mimic biofilm formation at the air-liquid interface in real wounds. Each sheet of artificial dermis was placed in a 24-well microtiter plate. 300 µL medium containing Bolton

Broth, heparinized bovine plasma and freeze-thaw laked horse blood cells was added on dermis. Coumarin was added in a final concentration of 2 mM. *P. aeruginosa* suspensions (10 µL) containing approximately 10^4 bacterial cells were added on top of the dermis. After 24 h, the infected dermis was washed with 1 mL PS and was transferred into 9 ml PS. Biofilm cells on the dermis were collected by three cycles of vortexing (30 s) and sonication (30 s). The number of CFU/dermis was quantified by plating. Three independent experiments were performed with three replicates each (n=9).

The effect of coumarin on *P. aeruginosa* virulence to *L. sericata*

Blood agar (5% horse blood in LB agar) was prepared in 6 cm Petri dishes with DMSO as control or with coumarin (2 mM in a final concentration). *P. aeruginosa* bacteria (5×10^5 CFU) were spread on the blood agar and incubated overnight at 37 °C before the assay. The maggots were aseptically transferred onto the blood agar (10 maggots per plate) and cultured at 37 °C in the dark (n=50 in total from two independent experiments). Maggot survival was assessed after 24 h. Immobile/inactive maggots were considered to be dead, and maggot death was verified by stimulating the maggots with an inoculation needle.

RNA extraction, sequencing and analysis

RNA sequencing was performed on coumarin treated (2 mM, 24 h) and untreated *P. aeruginosa* PAO1 biofilms. To detect the initial changes on gene expression due to the coumarin treatment also shortly treated (2 mM, 1 h) and untreated planktonic cultures were included. Suspensions of planktonic cells (5×10^7 CFU/mL) were added to a 96-well microtiter plate and treated with 2 mM coumarin or DMSO as control. After incubation at 37 °C for 1 h, cells were collected on ice for RNA extraction. Biofilms were formed in microtiter plates with 2 mM coumarin or DMSO and cells from 24h-biofilms were collected as described above. Subsequently, total RNA was extracted immediately using the Ambion RiboPure™ RNA Purification Kit (Life Technologies, Renfrewshire, UK) according to the manufacturer’s instructions. Three biological replicates were performed for each condition.

RNA sequencing was performed as described previously [401]. Total RNA of each sample was depleted for ribosomal RNA using the Ribo-Zero Magnetic Kit for Gram-

Negative Bacteria (Epicentre, Madison, WI, USA). Truseq stranded RNA library preparation kit (Illumina) was then used to create strand-specific cDNA sequencing libraries. Quality control of the libraries (DNA 1000 chip, Agilent Technologies, Santa Clara, US) was performed and the concentration was determined according to recommendations provided by Illumina. Sequencing was performed using an Illumina NextSeq 500, generating 75 bp unpaired reads. Fastq files were deposited at ArrayExpress under the accession number E-MTAB-6629. Using CLC Biosystems Genomic Workbench 10.1.1 (CLC Bio, a Qiagen company, Waltham, Mass), quality trimming was performed and the quality filtered reads of each sample were mapped (length fraction 0.8 and similarity fraction 0.8) against the genome sequence of *P. aeruginosa* PAO1 (the NCBI reference sequence with accession number NC_002516). Total gene read values are used by the Differential Expression for RNA-Seq tool based on a negative binomial generalized linear model (GLM) in CLC Workbench. Only genes that were significantly differentially regulated (the false discovery rate adjusted p-value < 0.05) and with at least 1.5-fold change compared to the control were considered. Gene descriptions were obtained from the *Pseudomonas* Genome Database [402].

C-di-GMP quantification

Quantifications of c-di-GMP levels in *P. aeruginosa* PAO1 cells were performed as described previously [403]. Briefly, an overnight culture of *P. aeruginosa* PAO1 was standardized to OD590 0.5 and inoculated in 10 mL of LB with 1:50 dilutions with or without coumarin (2 mM). After 24 h, the cells were harvested by centrifugation at 4 °C for 20 min from 5 mL culture suspensions. The wet weight of collected cells was measured. Cells were lysed in ice-cold extraction buffer consisting of acetonitrile/methanol/water (2/2/1, v/v/v), and incubated at 4 °C for 15 min. The cell suspension was then heated to 95 °C for 10 min and centrifuged. The extraction of the resulting pellet was repeated twice with 200 µL of extraction solvent at 4 °C omitting the heating step. The solvent of the combined supernatants was evaporated to dryness in a vacuum centrifuge. The pellets were dissolved in HPLC-grade water for analysis by liquid chromatography-coupled tandem mass spectrometry as described before [403]. Extractions were performed in triplicate or duplicate from two independent bacterial cultures as biological duplicate (n=5).

Statistical analyses

Numerical data were analyzed using GraphPad Prism 6.0 and presented as mean ± standard deviation (SD). The normal distribution of the data was checked by the D'Agostino-Pearson normality test. Normally distributed data were analyzed by one-way ANOVA or Student t-test, and non-normally distributed data were analyzed by the Kruskal-Wallis test or the Mann-Whitney test.

Results

Coumarin inhibits QS in *P. aeruginosa* QSIS2 biosensor

The previously described QS inhibitory activity of coumarin was confirmed using the *P. aeruginosa* QSIS2 reporter strain. While 1 mM coumarin had no measurable effect on QS, at a concentration of 2 mM, QS inhibition could clearly be observed (average inhibition: 40.3%, standard deviation: 5.2%).

Coumarin reduces pyocyanin and protease production in different *P. aeruginosa* strains

Pyocyanin production was significantly decreased by coumarin in strains PAO1, 2063, 2091 and 2549 ($p<0.05$), but not in strains 1083 and 3120 which produced virtually no pyocyanin in the experimental conditions used (Figure 1A). Coumarin also reduced protease production in *P. aeruginosa* PAO1 and the five clinical strains, to varying degrees ($p < 0.05$) (Figure 1B). These results indicate that coumarin inhibits the production of QS-regulated virulence factors in different *P. aeruginosa* clinical strains.

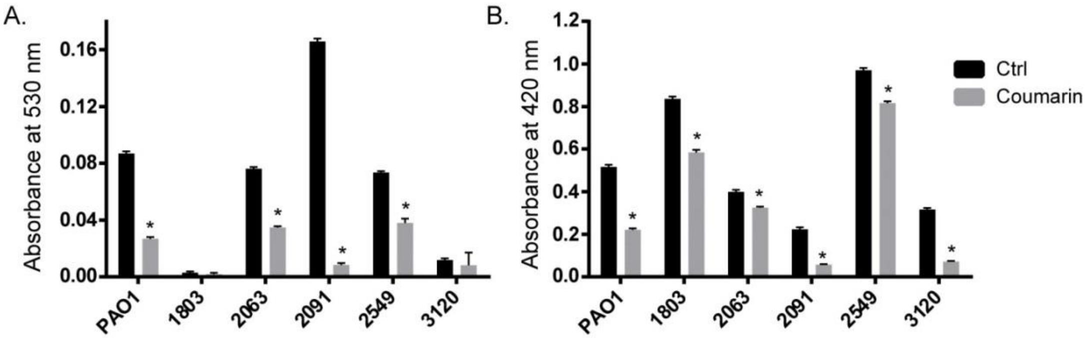

Figure 1. Effect of coumarin (2 mM) on pyocyanin (A) and protease (B) production in different *P. aeruginosa* strains. Data shown are the mean (n=6), error bars represent standard deviation. *, $p<0.05$ compared to the untreated control group.

The effect of coumarin on *P. aeruginosa* biofilms

No significant effect on growth of planktonic cells of *P. aeruginosa* strains was observed when 1 mM or 2 mM coumarin was added (Figure S1). The effect of coumarin on biofilm formation of *P. aeruginosa* strains was tested in 96-well microtiter plates as well as in an *in vitro* wound model. In microtiter plates, biofilm formation of all *P. aeruginosa* strains investigated was significantly reduced in the presence of 2 mM coumarin ($p < 0.05$) (Figure 2A); for four strains (2063, 2091, 2549 and 3120) this was also the case in the presence of 1 mM coumarin.

In the wound model, statistically significant reduced biofilm formation in the presence of 2 mM coumarin was observed for *P. aeruginosa* PAO1, 2091 and 3210 ($p < 0.05$) (Figure 2B). Biofilm formation for the other three clinical strains showed a non-significant decrease of approx. 30%.

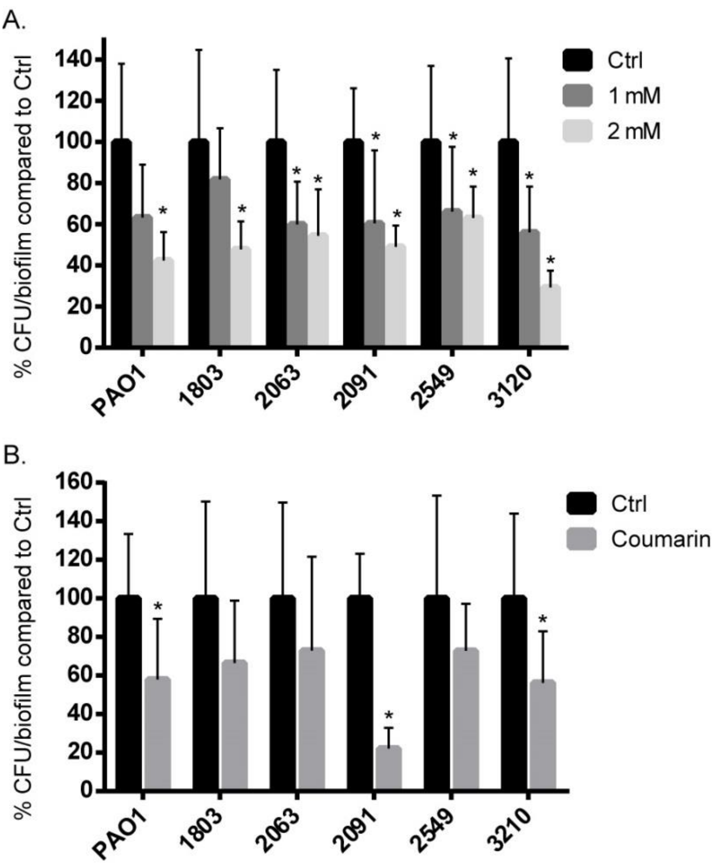

Figure 2. The effect of coumarin on *P. aeruginosa* biofilms. Data shown are the mean (n=9), error bars represent standard deviation. (A). Biofilms formed in microtiter plates. (B). Wound model biofilms treated with 2 mM coumarin. *, $p<0.05$ compared to the untreated control group.

The effect of coumarin on *P. aeruginosa* virulence to *L. sericata*

As shown in Figure 3, the virulence of *P. aeruginosa* to *L. sericata* varied between different clinical isolates. *P. aeruginosa* 2549 appeared to be most virulent among the strains tested, as exposure of the larvae to this strain led to > 90% killing after 24 h. In the coumarin-treated groups, significant increase in *L. sericata* survival was observed for PAO1 and 1803, with 34% and 54% more survival compared to the control groups, respectively. For the other *P. aeruginosa* strains, coumarin increased the survival of *L. sericata* by 6~16% but this was not significant. It should however be noted that for two of these strains (2091 and 3120) survival already was quite high in the control group, suggesting these strains are less virulent in this model.

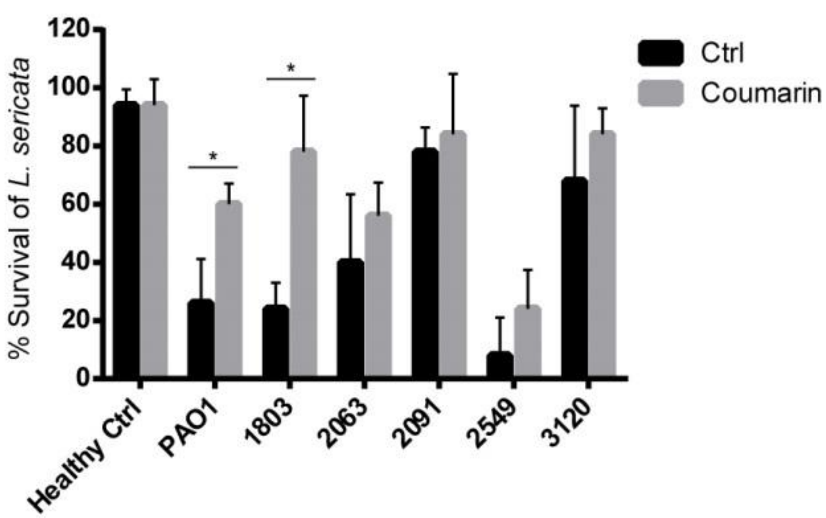

Figure 3. The effect of coumarin (2 mM) on survival of *L. sericata*. Data shown are the mean (n=9), error bars represent standard deviation. *, p<0.05 compared to the untreated control group.

Transcriptome analysis of *P. aeruginosa* planktonic and biofilm cells treated with coumarin

In order to obtain insight into the mechanism(s) behind the QS inhibitory effect of coumarin, RNA sequencing was performed on *P. aeruginosa* PAO1 planktonic cells after 1 h coumarin treatment and biofilm cells grown in the presence of coumarin for 24 h. In planktonic cells, 46 genes were down-regulated and 21 genes were up-regulated by coumarin (Table S1). Nearly half of the down-regulated genes (21) were related to the type III secretion system (T3SS), including the key genes coding for the needle complex (*pscF*, *pscJ*, *pscL*), the translocation apparatus (*pcrV*, *popB*, *popD*), the chaperones (*pcrH*, *pscG*), as well as the effector proteins (*exoS*, *exoT*). Two sets

of two-component regulator systems were down-regulated (PA4774/PA4775 and *pmrAB*). An oxidative stress sensing regulator *ospR* was repressed, as well as gene PA2826 (encoding a glutathione peroxidase which is under the regulation of *ospR*) [404]. This result indicated that coumarin might affect the response of *P. aeruginosa* to oxidative stress. One HD-GYP domain phosphodiesterase (PDE) encoding gene (PA4781) was down-regulated; PA4781 has been reported to degrade c-di-GMP [405-407]. However, no significant changes in either QS or QS-regulated virulence genes were observed in coumarin-treated planktonic samples, except that *phzG2* involved in phenazine production was repressed by over 50-fold.

In coumarin-treated biofilms, 399 genes were significantly downregulated (Table S2). Among them, several core genes involved in the four different QS systems of *P. aeruginosa* were repressed. The AHL synthase encoding genes *lasI* and *rhlI* were both downregulated, as was the receptor encoding gene *rhlR*. The expression of *lasR* was not significantly influenced. Gene *pqsB* and *pqsC* within the *pqsABCDE* operon (which controls PQS synthesis) were both downregulated, as was *pqsH* (involved in the conversion of 2-heptyl-4-quinolone to PQS). No difference was observed in the expression of the gene encoding the PQS receptor PqsR. Moreover, genes responsible for the synthesis of IQS (*ambBCDE*) were also significantly downregulated in coumarin-treated biofilms. In addition, genes involved in the production of QS-regulated virulence factors including alkaline protease, hydrogen cyanide, LasA protease, LasB elastase, phenazine, siderophores and rhamnolipids were significantly downregulated. These results confirm that coumarin interferes with the QS network of *P. aeruginosa* and the production of QS-related virulence factors. Genes involved in T3SS were repressed by coumarin in the biofilm cells (Table S2), including several genes encoding T3SS regulators (*exsC*, *exsD* and *ptrA*). The c-di-GMP PDE encoding gene PA4781 was downregulated in coumarin-treated biofilm cells by 2-fold.

In addition, 234 genes were up-regulated in the coumarin-treated biofilm cells (Table S3). PA2226 encoding a negative QS regulator QsrO was upregulated by 3.7-fold. Overexpression of QsrO has been reported to repress the *las*, *rhl* and PQS systems of *P. aeruginosa*, although the detailed mechanisms have not been elucidated yet [408]. Co-expression of PA2226 and PA2225 prevents the induction of T3SS [408], and PA2225 was found to be up-regulated by 2.0-fold in the coumarin-treated biofilm.

tpbA, encoding a tyrosine phosphatase that represses c-di-GMP production [188] was up-regulated by 4.0-fold in the coumarin-treated biofilm cells. No significant changes in the expression of other diguanylate cyclase (DGC) or PDE genes involved in c-di-GMP metabolism were found in coumarin-treated biofilm cells.

Coumarin reduced c-di-GMP level in *P. aeruginosa* PAO1

To confirm the influence of coumarin on c-di-GMP metabolism, we determined intracellular c-di-GMP concentrations of planktonic *P. aeruginosa* cells. The c-di-GMP concentration in untreated controls was 3.67±1.07 pmol/mg, while the c-di-GMP level of PAO1 treated with coumarin was significantly reduced (p<0.01) to 0.87±0.33 pmol/mg (Figure 4).

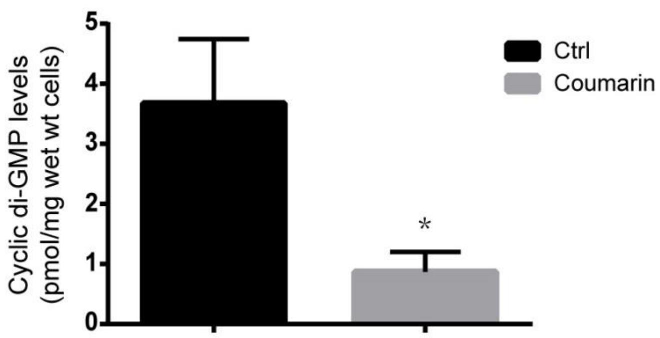

Figure 4. The effect of coumarin (2 mM) on c-di-GMP level in *P. aeruginosa* PAO1. Data shown are the mean (n=5), error bars represent standard deviation. *, p<0.05 compared to the untreated control group.

Discussion

Coumarin was previously described as a QS inhibitor in *P. aeruginosa* [392] and this was confirmed in the present study. In addition, coumarin reduced protease and pyocyanin production in different *P. aeruginosa* strains, as well as biofilm formation both in microtiter plates and in an *in vitro* wound model. We also demonstrated that coumarin increases the survival of *L. sericata* maggots in the presence of several clinical *P. aeruginosa* strains. A transcriptomic analysis demonstrated that the expression of a large number of genes (including genes involved in QS and T3SS) is regulated in response to coumarin, both in planktonic and sessile *P. aeruginosa* cells,

and intracellular c-di-GMP levels were lower in treated cells, compared to untreated controls.

Several studies have shown that clinical *P. aeruginosa* strains display varying levels of virulence, and highlighted the importance to include clinical isolates when evaluating the effect of potential novel treatment approaches [256, 409]. We observed that different *P. aeruginosa* clinical isolates indeed vary significantly in their ability to produce protease and pyocyanin, as well as in their virulence towards *L. sericata*. Coumarin significantly inhibited biofilm formation in microtiter plates for all *P. aeruginosa* strains tested, whereas its biofilm-inhibitory effect in the wound model and its effect on virulence in *L. sericata* were partly strain-dependent, indicating that coumarin's effect as QSI may be reduced in the more complex environment encountered *in vivo*.

To obtain a global picture of effect of coumarin on QS, biofilm and virulence of *P. aeruginosa*, we performed RNA sequencing on *P. aeruginosa* PAO1 planktonic cells after 1 h of treatment with coumarin, as well as on biofilms treated with coumarin for 24 h. The expression of genes involved in the QS system of *P. aeruginosa* was not significantly changed in planktonic cells after 1 h. In a previous study, coumarin (1.36 mM) has been reported to reduce the expression of *pqsA* and *rhlI* in planktonic cells after 6 h and 24 h, respectively, while *lasI* expression was not affected either at 6 h or 24 h [392]. In biofilm cells we found that *lasI* expression was down-regulated by coumarin, as well as *rhlI*, *rhlR* and genes within the *pqsABCDE* and *ambBCDE* operons. These results confirm that coumarin interferes with the *P. aeruginosa* QS network. The observation that the expression of QS-regulated virulence genes was downregulated in *P. aeruginosa* PAO1 was in line with the decreased protease and pyocyanin production and reduced virulence observed in *L. sericata*. Also *qsrO*, encoding a negative regulator of QS was overexpressed in coumarin-treated biofilms suggesting that this regulator contributes to the inhibition of QS by coumarin.

T3SS in *P. aeruginosa* acts as a major virulence determinant that manipulates host cell responses and plays an important role in acute infections [410]. A microarray-based analysis of *P. aeruginosa* QS regulons revealed that at least three genes involved in T3SS (*pscQ*, *pscI*, and *pcrH*) are negatively regulated by QS [411]. Bleves *et al*. has also reported that the expression of T3SS in *P. aeruginosa* is

negatively regulated by QS, especially by RhlR under low calcium levels [412]. These results raise the concern that interfering with QS might potentially increase the T3SS-related virulence in *P. aeruginosa* [413]. However, transcriptome data obtained from *P. aeruginosa* treated with coumarin in the present study and 6-gingerol, another QS inhibitor [328] revealed that genes involved in T3SS were repressed. These results suggest that although QS has been reported to negatively affect T3SS expression, QS inhibition by certain QSIs may not necessarily induce T3SS-related virulence. In contrast, it is possible to inhibit QS and T3SS simultaneously by QSIs such as coumarin and 6-gingerol, although the underlying mechanisms remain to be elucidated.

C-di-GMP regulates many bacterial behaviors, and a high intracellular c-di-GMP concentration has been reported to promote biofilm formation in *P. aeruginosa* and other bacteria [414]. The intracellular concentration of c-di-GMP is determined by GGDEF domain-containing DGCs (which synthesize c-di-GMP), and EAL or HD-GYP domain-containing PDEs (which degrade c-di-GMP) [414]. We found that coumarin affected two genes involved in c-di-GMP metabolism in *P. aeruginosa* PAO1, i.e. PA4781 and *tpbA*, and demonstrated that coumarin significantly reduced the c-di-GMP level in *P. aeruginosa*. We hypothesize this is due to the upregulation of *tpbA*. TpbA acts as a negative regulator of c-di-GMP production, which deactivates the GGDEF-domain DGC TpbB responsible for c-di-GMP synthesis and indirectly reduces c-di-GMP levels in *P. aeruginosa* [188].

Our results indicate that coumarin not only acts as a QSI but also reduces c-di-GMP levels, and these combined effects may explain the reduced biofilm formation observed. The connections between QS and c-di-GMP in *P. aeruginosa* have just begun to be elucidated. Mutants of *P. aeruginosa* PA14 without functional *lasI*, *lasR* or *rhlR* showed decreased expression of *tpbA* [188], leading to the assumption that QS negatively regulates c-di-GMP levels through TpbA/TpbB. However, our results showed that coumarin inhibits QS-related genes, but the expression of *tpbA* was significantly up-regulated by coumarin and led to reduced c-di-GMP level. Another study showed that *rhl* and PQS systems were expressed at a higher level in *P. aeruginosa* PAO1 with reduced c-di-GMP level due to overexpression of *yhjH* (encoding a PDE) than in a *wspF* mutant with elevated c-di-GMP levels [415]. This suggested that low c-di-GMP levels could increase expression of QS-related genes

and the production of QS-regulated virulence factors. In contrast, our results showed that coumarin reduced both c-di-GMP levels and QS-regulated virulence. These results might be explained by the different mechanisms leading to the reduced c-di-GMP level. The distinct gene expression profiles (431 up-regulated genes and 595 down-regulated genes) observed in the study of Lin Chua *et al.* is directly due to the loss of *wspF* and overexpression of *yhjH*, whereas in our study no change was observed in *wspF* expression.

Several other compounds of the coumarin class have also been reported for their anti-QS and antibiofilm effects, e.g. esculetin, esculin and umbelliferone [416, 417]. Our research on the prototype coumarin molecule in this family revealed that coumarin can inhibit not only QS but also T3SS and c-di-GMP signaling, leading to reduced virulence and biofilm formation in *P. aeruginosa*. These results increase our understanding of the molecular mechanism(s) involved in the activity of coumarin and related molecules, and suggest these molecules could be useful to combat biofilm-related infections. Structural modifications based on the coumarin scaffold may allow the development of more active coumarin-derivatives with potential application in the treatment of *P. aeruginosa* infections.

Conclusion

- Coumarin can reduce QS-regulated virulence and biofilm formation in *P. aeruginosa* strains.
- Coumarin down-regulates the expression of key genes involved in the *las*, *rhl*, PQS and IQS systems in the biofilm of *P. aeruginosa*.
- Coumarin reduces the expression of genes related to type III secretion, and decreases the cellular c-di-GMP level in *P. aeruginosa*.

Acknowledgments

We thank Annette Garbe and Anna-Lena Hagemann for their help in c-di-GMP quantification.

Supplemental Information

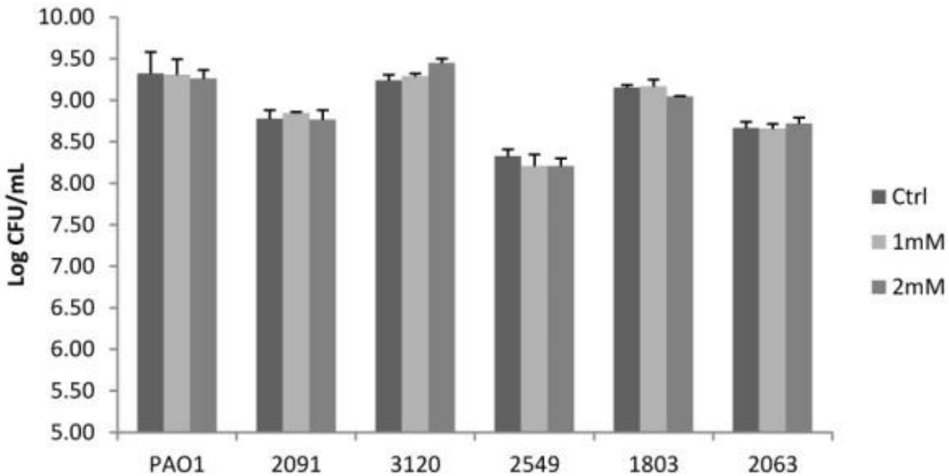

Figure S1. The effect of coumarin on planktonic cell growth of *P. aeruginosa* strains at 24h. Data shown are the mean (n=3), error bars represent standard deviation.

Table S1. Genes that are significantly down- or upregulated (cut-off: 1.5-fold change, FDR p-value < 0.05) in *P. aeruginosa* PAO1 planktonic cells following coumarin treatment (2 mM, 1h).

Locus tag	Gene name	Description	Fold changes
Down-regulated genes (46)			
Type III secretion related (21)			
PA3841	*exoS*	Exoenzyme S	-1.69
PA0044	*exoT*	Exoenzyme T	-1.71
PA1697		ATP synthase in type III secretion system	-2.27
PA1699	*pcr1*		-2.21
PA1700	*pcr2*		-2.07
PA1703	*pcrD*	Type III secretory apparatus protein	-1.72
PA1707	*pcrH*	Regulatory protein	-1.69
PA1706	*pcrV*	Type III secretion protein	-1.69
PA1708	*popB*	Translocator protein	-1.76
PA1709	*popD*	Translocator outer membrane protein	-1.82
PA1698	*popN*	Type III secretion outer membrane protein	-2.00
PA1715	*pscB*	Type III export apparatus protein	-1.64
PA1716	*pscC*	Type III secretion outer membrane protein	-1.51
PA1719	*pscF*	Type III export protein	-1.65
PA1720	*pscG*	Type III export protein	-1.72
PA1722	*pscI*	Type III export protein	-1.53
PA1723	*pscJ*	Type III export protein	-1.51
PA1724	*pscK*	Type III export protein	-1.65
PA1696	*pscO*	Translocation protein in type III secretion	-2.31
PA1695	*pscP*	Translocation protein in type III secretion	-1.93
PA1694	*pscQ*	Translocation protein in type III secretion	-2.02
Polyamine transport related (3)			
PA3608	*potB*	Polyamine transport protein	-1.94
PA3609	*potC*	Polyamine transport protein	-2.27

PA3610	*potD*	Polyamine transport protein	-2.19
Two-component regulator system (4)			
PA4774			-3.51
PA4775			-2.54
PA4776	*pmrA*		-2.51
PA4777	*pmrB*		-2.33
Others (9)			
PA1321	*cyoE*	Cytochrome o ubiquinol oxidase	-1.63
PA1838	*cysI*	Sulfite reductase	-1.70
PA1634	*kdpB*	Potassium-transporting ATPase	-1.84
PA2825	*ospR*	Oxidative stress sensing regulator	-1.91
PA2826		Glutathione peroxidase	-2.06
PA3677	*MexJ*	Efflux pump	-1.69
PA5365	*phoU*	Phosphate uptake regulatory protein	-1.50
PA1905	*phzG2*	Probable pyridoxamine 5'-phosphate oxidase	-50.75
PA4781		Cyclic di-GMP phosphodiesterase	-2.01
Unknown function (9)			
PA1228			-2.51
PA1402			-1.83
PA2283			-1.94
PA2284			-2.13
PA2285			-2.31
PA3445			-1.77
PA4359			-2.13
PA4773			-3.15
PA4782			-2.30
Up-regulated genes (21)			
Multi-drug efflux related (12)			
PA3719	*armR*	Antirepressor for mexr	8.35
PA0425	*mexA*	Multidrug efflux membrane fusion protein	2.39
PA0426	*mexB*	Multidrug efflux transporter	2.06
PA4599	*mexC*	Multidrug efflux membrane fusion protein	3.04
PA4598	*mexD*	Multidrug efflux transporter	3.05
PA0424	*mexR*	Multidrug resistance operon repressor	2.03
PA3721	*nalC*	A probable repressor of the TetR/AcrRfamily	1.70
PA4597	*oprJ*	Multidrug efflux outer membrane protein	3.48
PA0427	*oprM*	Multidrug efflux outer membrane protein	1.78
PA3718		Probable major facilitator superfamily transporter	3.06
PA3720		Hypothetical protein	7.41
PA4596	*esrC*	An envelope stress-regulated repressor	3.12
Other genes and genes with unknown functions (9)			
PA3126	*ibpA*	Heat-shock protein	1.52
PA4047		GTP cyclohydrolase II	
	ribA		1.74
PA1922		Probable TonB-dependent receptor	2.11
PA2930		Probable transcriptional regulator	1.64
PA4045			1.54
PA4046			1.66
PA4288		Probable transcriptional regulator	1.80
PA4837		Probable outer membrane protein precursor	1.96
PA4838			2.06

Table S2. Genes that are significantly down-regulated (cut-off: 1.5-fold change, FDR p-value < 0.05) in *P. aeruginosa* PAO1 24h-biofilm cells treated with coumarin (2 mM).

Locus tag	**Gene name**	**Description**	**Fold changes**
QS network (11)			
PA1432	*lasI*	AHL synthesis protein LasI	-1.76
PA3476	*rhlI*	AHL synthesis protein RhlI	-1.62
PA3477	*rhlR*	Transcriptional regulator RhlR	-1.85
PA0997	*pqsB*	PQS synthesis	-1.62
PA0998	*pqsC*		-1.62
PA2587	*pqsH*		-1.96
PA4190	*pqsL*		-1.91
PA2305	*ambB*	IQS synthesis	-2.22
PA2304	*ambC*		-2.27
PA2303	*ambD*		-2.30
PA2302	*ambE*		-1.84
major QS-regulated virulence factors (43)			
PA1249	*aprA*	Synthesis and secretion of alkaline protease	-5.06
PA1246	*aprD*		-1.93
PA1247	*aprE*		-2.34
PA1248	*aprF*		-1.83
PA1250	*aprI*		-2.48
PA2193	*hcnA*	Hydrogen cyanide synthesis	-2.75
PA2194	*hcnB*		-1.94
PA2195	*hcnC*		-1.75
PA1871	*lasA*	LasA protease	-4.93
PA3724	*lasB*	LasB elastase	-3.06
PA2570	*lecA*	Lectin	-3.21
PA4231	*pchA*	Pyochelin biosynthesis	-1.80
PA4230	*pchB*		-2.22
PA4228	*pchD*		-1.98
PA4226	*pchE*		-1.78
PA4225	*pchF*		-1.66
PA4224	*pchG*		-1.84
PA4211	*phzB1*	Phenazine biosynthesis	-2.24
PA1900	*phzB2*		-3.19
PA1901	*phzC2*		-1.93
PA4213	*phzD1*		-1.81
PA1902	*phzD2*		-1.92
PA4214	*phzE1*		-1.98
PA1903	*phzE2*		-1.97
PA4215	*phzF1*		-2.17
PA1904	*phzF2*		-2.18
PA1905	*phzG2*		-3.36
PA4217	*phzS*		-1.80
PA2386	*pvdA*	Pyoverdine biosynthesis and transport	-1.99
PA2396	*pvdF*		-1.71
PA2425	*pvdG*		-1.65
PA2413	*pvdH*		-2.12
PA2394	*pvdN*		-1.87
PA2395	*pvdO*		-1.85
PA2392	*pvdP*		-1.93
PA2426	*pvdS*		-1.72
PA2390	*pvdT*		-1.62

PA2391	*opmQ*		-1.69
PA3479	*rhlA*	Rhamnolipid production	-2.57
PA3478	*rhlB*		-2.09
PA2300	*chiC*	Chitinase	-6.93
PA4236	*katA*	Catalase	-1.56
PA4468	*sodM*	Superoxide dismutase	-2.38
Type III secretion related (18)			
PA3841	*exoS*	Exoenzyme S	-1.70
PA0044	*exoT*	Exoenzyme T	-1.57
PA1697		ATP synthase in type III secretion system	-2.18
PA1699	*pcr1*		-1.68
PA1710	*exsC*	Exoenzyme S synthesis protein C precursor	-1.99
PA1714	*exsD*	Type III secretion regulator	-1.77
PA1705	*pcrG*	Type III secretion regulator	-2.22
PA1707	*pcrH*	Regulatory protein	-2.66
PA1706	*pcrV*	Type III secretion protein	-2.06
PA1708	*popB*	Translocator protein	-2.01
PA1709	*popD*	Translocator outer membrane protein	-2.31
PA1698	*popN*	Type III secretion outer membrane protein	-2.80
PA1715	*pscB*	Type III export apparatus protein	-1.68
PA1719	*pscF*	Type III export protein	-1.82
PA1695	*pscP*	Translocation protein in type III secretion	-3.23
PA1694	*pscQ*	Translocation protein in type III secretion	-1.93
PA2808	*ptrA*	*Pseudomonas* type III repressor A	-2.00
PA1711	*exsE*	A regulator of ExsC	-1.79
C-di-GMP metabolism (1)			
PA4781		Cyclic di-GMP phosphodiesterase	-2.00
Others (121)			
PA5427	*adhA*	Alcohol dehydrogenase	-1.56
PA1337	*ansB*	Glutaminase-asparaginase	-1.68
PA5171	*arcA*	Arginine deiminase	-1.67
PA5172	*arcB*	Ornithine carbamoyltransferase	-1.66
PA5173	*arcC*	Carbamate kinase	-1.69
PA2886	*atuA*	Citronellol catabolism	-1.67
PA2887	*atuB*		-1.85
PA2888	*atuC*		-1.71
PA2889	*atuD*		-1.66
PA2890	*atuF*		-1.73
PA2891	*atuG*		-1.50
PA2003	*bdhA*	3-hydroxybutyrate dehydrogenase	-2.35
PA2052	*cynS*	Cyanate lyase	-1.73
PA2000	*dhcB*	Dehydrocarnitine CoA transferase	-1.52
PA2008	*fahA*	Fumarylacetoacetase	-1.58
PA4470	*fumC1*	Fumarate hydratase	-2.83
PA0854	*fumC2*	Fumarate hydratase	-1.56
PA1421	*gbuA*	Guanidinobutyrase	-1.58
PA2446	*gcvH2*	Glycine cleavage system protein	-1.56
PA2445	*gcvP2*		-1.64
PA2442	*gcvT2*		-1.59
PA2153	*glgB*	1,4-alpha-glucan branching enzyme	-1.77
PA2144	*glgP*	Glycogen phosphorylase	-1.60
PA2444	*glyA2*	Serine hydroxymethyltransferase	-1.63
PA5091	*hutG*	Histidine utilization	-1.63
PA5098	*hutH*		-1.62
PA5092	*hutI*		-1.69

PA5100	*hutU*		-2.03
PA4694	*ilvC*	Ketol acid reductoisomerase	-2.10
PA4695	*ilvH*	Small acetolactate synthase subunit	-1.91
PA4696	*ilvI*	Acetolactate synthase enzyme	-1.67
PA3792	*leuA*	Leucine synthesis	-2.93
PA3118	*leuB*		-2.44
PA3121	*leuC*		-4.10
PA3120	*leuD*		-3.27
PA4370	*icmP*	Insulin-cleaving metalloproteinase outer membrane protein	-1.67
PA2863	*lipH*	Lipase modulator protein	-1.53
PA4770	*lldP*	L-lactate permease	-1.82
PA2007	*maiA*	Maleylacetoacetate isomerase	-1.51
PA1927	*metE*	Methionine synthesis	-1.51
PA0546	*metK*		-1.74
PA5025	*metY*		-1.62
PA0132		Beta-alanine:pyruvate transaminase	-1.92
PA0399		Cystathionine beta-synthase	-1.81
PA1041	*wapB*	1,2-glucosyltransferase	-2.09
PA1256	*lhpO*	ABC transporter ATP-binding protein	-2.06
PA1641	*gpsA*	Glycerol-3-phosphate dehydrogenase	-1.57
PA2321		Gluconokinase	-1.57
PA2414		L-sorbosone dehydrogenase	-1.86
PA4022	*hdhA*	Hydrazone dehydrogenase	-1.76
PA4661	*pagL*	Lipid A 3-O-deacylase	-1.71
PA5058	*phaC2*	Poly(3-hydroxyalkanoic acid) synthase 2	-1.84
PA5161	*rmlB*	dTDP-D-glucose 4,6-dehydratase	-1.51
PA0849	*trxB2*	Thioredoxin reductase 2	-2.40
PA5419	*soxG*	Sarcosine oxidase gamma subunit	-1.83
PA2279	*arsC*	Arsenate reductase	-1.51
PA1863	*modA*	Molybdate-binding periplasmic protein precursor	-1.50
PA0513	*nirG*	Nitrite reductase	-1.50
PA1177	*napE*	Periplasmic nitrate reductase protein	-1.84
PA0523	*norC*	Nitric-oxide reductase subunit C	-2.39
PA0023	*qor*	Quinone oxidoreductase	-1.64
PA3531	*bfrB*	Bacterioferritin	-1.72
PA3407	*hasAp*	Heme acquisition protein	-8.13
PA3408	*hasR*	Heme uptake outer membrane receptor HasR precursor	-1.88
PA3530	*bfd*	Bacterioferritin-associated ferredoxin	-1.62
PA3676	*mexK*	Efflux pump	-1.58
PA3677	*mexJ*	Efflux pump	-1.73
PA4205	*mexG*	Membrane protein required for MexGHI-OpmD efflux	-1.71
PA4587	*ccpR*	Cytochrome c551 peroxidase precursor	-1.97
PA1318	*cyoB*	Cytochrome o ubiquinol oxidase subunit	-1.83
PA1319	*cyoC*		-2.61
PA1320	*cyoD*		-2.09
PA1321	*cyoE*	Cytochrome o ubiquinol oxidase protein	-2.04
PA4133		Cytochrome c oxidase subunit	-1.52
PA3692	*lptF*	Lipotoxon	-1.99
PA0122	*rahU*		-2.06
PA5285	*SutA*	A bacterial transcription factor	-1.85
PA0843	*plcR*	Phospholipase accessory protein	-1.63
PA4776	*pmrA*	Two-component regulator system	-1.68

PA4777	*pmrB*		-1.85
PA3790	*oprC*	Outer membrane porin precursor	-2.87
PA1777	*oprF*	Outer membrane porin precursor	-1.54
PA4067	*oprG*	Outer membrane protein precursor	-1.64
PA1178	*oprH*	Outer membrane protein H1	-1.69
PA2853	*oprI*	Outer membrane lipoprotein	-2.08
PA4761	*dnaK*	DnaK protein	-1.56
PA4385	*groEL*	GroEL protein	-2.06
PA4386	*groES*	GroES protein	-1.78
PA4762	*grpE*	Heat shock protein	-1.57
PA5053	*hslV*	Heat shock protein	-1.57
PA1596	*htpG*	Heat shock protein	-1.93
PA0852	*cbpD*	Chitin-binding protein	-4.54
PA0139	*ahpC*	Alkyl hydroperoxide reductase subunit C	-1.67
PA3550	*algF*	Alginate o-acetyltransferase	-1.66
PA0723	*coaB*	Coat protein B of bacteriophage Pf1	-1.91
PA2717	*cpo*	Chloroperoxidase precursor	-1.63
PA0694	*exbD2*	Transport protein	-2.28
PA4306	*flp*	Type IVb pilin, Flp	-2.03
PA0867	*mliC*	Membrane-bound lysozyme inhibitor of c-type lysozyme	-1.69
PA4614	*mscL*	Conductance mechanosensitive channel	-1.87
PA0766	*mucD*	Serine protease mucd precursor	-1.52
PA0059	*osmC*	Osmotically inducible protein	-2.19
PA4876	*osmE*	Osmotically inducible lipoprotein	-2.50
PA0678	*HxcU*	Alkaline phosphatase secretion related	-2.15
PA0683	*HxcY*		-2.89
PA0779	*asrA*	ATP-dependent protease	-1.52
PA1245	*AprX*		-3.34
PA1259	*LhpH*		-1.51
PA1657	*HsiB2*		-2.04
PA1658	*hsiC2*		-1.67
PA1665	*Fha2*		-1.70
PA1668	*DotU2*		-1.58
PA1830		Lon protease	-1.58
PA3891	*opuC*	ABC transporter	-1.75
PA4916	*nrtR*	Nudix-related transcriptional regulator	-1.51
PA0355	*pfpI*	Protease PfpI	-1.97
PA4590	*pra*	Protein activator	-2.02
PA4305	*rcpC*		-1.69
PA3049	*rmf*	Ribosome modulation factor	-1.55
PA4865	*ureA*	Urease gamma subunit	-2.30
PA4868	*ureC*	Urease alpha subunit	-1.70
Genes with general predicted or unknown functions (205)			
PA3672		Probable ATP-binding component of ABC transporte	-1.55
PA4223		Probable ATP-binding component of ABC transporter	-1.75
PA0400		Probable cystathionine gamma-lyase	-1.75
PA0656		Probable HIT family protein	-1.58
PA4131		Probable iron-sulfur protein	-2.30
PA1131		Probable major facilitator superfamily (MFS) transporter	-1.68
PA4144		Probable outer membrane protein precursor	-2.79
PA4171		Probable protease	-2.32

PA4143	Probable toxin transporter	-3.21
PA0236	Probable transcriptional regulator	-1.96
PA1403	Probable transcriptional regulator	-1.51
PA2588	Probable transcriptional regulator	-1.89
PA4341	Probable transcriptional regulator	-1.86
PA3963a	Probable transporter	-2.43
PA1737	Probable 3-hydroxyacyl-CoA dehydrogenase	-1.56
PA1869	Probable acyl carrier protein	-2.71
PA2815	Probable acyl-CoA dehydrogenase	-1.63
PA2158	Probable alcohol dehydrogenase (Zn-dependent)	-1.52
PA0366	Probable aldehyde dehydrogenase	-1.51
PA5097	Probable amino acid permease	-1.62
PA1617	Probable AMP-binding enzyme	-1.69
PA5094	Probable ATP-binding component of ABC transporter	-1.51
PA5096	Probable binding protein component of ABC transporter	-2.06
PA2069	Probable carbamoyl transferase	-3.40
PA1251	Probable chemotaxis transducer	-1.96
PA0459	Probable ClpA/B protease ATP binding subunit	-1.59
PA0223	Probable dihydrodipicolinate synthetase	-2.67
PA3940	Probable DNA binding protein	-1.53
PA2086	Probable epoxide hydrolase	-1.85
PA2165	Probable glycogen synthase	-1.87
PA2160	Probable glycosyl hydrolase	-2.14
PA2162	Probable glycosyl hydrolase	-1.83
PA2164	Probable glycosyl hydrolase	-2.27
PA5093	Probable histidine/phenylalanine ammonia-lyase	-1.54
PA1202	Probable hydrolase	-1.71
PA2067	Probable hydrolase	-2.38
PA2698	Probable hydrolase	-1.65
PA2068	Probable major facilitator superfamily (MFS) transporter	-2.30
PA3441	Probable molybdopterin-binding protein	-1.72
PA4078	Probable nonribosomal peptide synthetase	-1.79
PA4172	Probable nuclease	-1.68
PA1875	Probable outer membrane protein precursor	-1.71
PA0147	Probable oxidoreductase	-1.52
PA1127	Probable oxidoreductase	-1.51
PA2592	Probable periplasmic spermidine/putrescine-binding protein	-1.80
PA3315	Probable permease of ABC transporter	-1.55
PA5095	Probable permease of ABC transporter	-1.59
PA3913	Probable protease	-1.88
PA4142	Probable secretion protein	-4.35
PA1344	Probable short-chain dehydrogenase	-1.78
PA2142	Probable short-chain dehydrogenase	-1.64
PA4098	Probable short-chain dehydrogenase	-1.84
PA2411	Probable thioesterase	-1.58
PA1285	Probable transcriptional regulator	-1.61
PA2096	Probable transcriptional regulator	-1.59
PA2312a	Probable transcriptional regulator	-1.54
PA3965	Probable transcriptional regulator	-1.72
PA4023	Probable transport protein	-1.57
PA2135	Probable transporter	-1.55

PA2393	Putative dipeptidase	-1.94
PA0039	Hypothetical protein	-1.62
PA0050	Hypothetical protein	-1.61
PA0060	Hypothetical protein	-1.53
PA0062	Hypothetical protein	-1.62
PA0116	Hypothetical protein	-1.55
PA0187	Hypothetical protein	-1.79
PA0188	Hypothetical protein	-1.83
PA0250	Hypothetical protein	-1.54
PA0269	Hypothetical protein	-2.26
PA0270	Hypothetical protein	-1.99
PA0271	Hypothetical protein	-1.71
PA0307	Hypothetical protein	-1.51
PA0526	Hypothetical protein	-1.72
PA0529	Hypothetical protein	-1.65
PA0572	Hypothetical protein	-2.47
PA0741	Hypothetical protein	-1.97
PA0851	Hypothetical protein	-1.60
PA1123	Hypothetical protein	-2.18
PA1135	Hypothetical protein	-1.53
PA1198	Hypothetical protein	-1.51
PA1244	Hypothetical protein	-1.71
PA1323	Hypothetical protein	-2.22
PA1324	Hypothetical protein	-2.30
PA1353	Hypothetical protein	-1.60
PA1404	Hypothetical protein	-2.43
PA1414	Hypothetical protein	-1.50
PA1478	Hypothetical protein	-1.57
PA1592	Hypothetical protein	-1.53
PA1597	Hypothetical protein	-1.59
PA1784	Hypothetical protein	-1.54
PA1852	Hypothetical protein	-1.72
PA1870	Hypothetical protein	-1.81
PA1906	Hypothetical protein	-1.62
PA1913	Hypothetical protein	-1.67
PA2004	Hypothetical protein	-2.25
PA2026	Hypothetical protein	-2.52
PA2027	Hypothetical protein	-4.67
PA2030	Hypothetical protein	-1.94
PA2031	Hypothetical protein	-2.02
PA2033	Hypothetical protein	-2.10
PA2066	Hypothetical protein	-1.88
PA2134	Hypothetical protein	-2.79
PA2141	Hypothetical protein	-2.18
PA2142a	Hypothetical protein	-1.58
PA2143	Hypothetical protein	-1.98
PA2381	Hypothetical protein	-2.83
PA2146		-2.97
PA2148		-1.75
PA2149		-2.16
PA2154		-1.51
PA2159		-1.99
PA2161		-2.44
PA2163		-2.10
PA2166		-2.49

PA2169	-2.20
PA2171	-2.03
PA2172	-1.58
PA2173	-1.55
PA2176	-1.54
PA2178	-1.50
PA2180	-1.72
PA2184	-2.05
PA2190	-2.71
PA2197	-1.80
PA2274	-2.33
PA2383	-1.66
PA2384	-2.75
PA2412	-1.95
PA2415	-1.55
PA2422	-1.56
PA2427	-1.69
PA2433	-2.23
PA2448	-1.97
PA2453	-1.78
PA2566	-1.60
PA2747	-1.77
PA2785	-1.80
PA2860	-1.72
PA2927	-1.51
PA3041	-1.73
PA3042	-1.58
PA3051	-1.57
PA3119	-4.16
PA3123	-1.51
PA3130	-1.55
PA3273	-1.78
PA3274	-1.59
PA3275	-1.72
PA3313	-1.82
PA3314	-1.64
PA3370	-1.62
PA3371	-1.60
PA3412	-2.18
PA3520	-2.86
PA3532	-1.84
PA3691	-1.91
PA3734	-1.72
PA3784	-1.77
PA3785	-2.16
PA3786	-1.52
PA3791	-1.93
PA3819	-1.65
PA3904	-1.58
PA3906	-1.82
PA3907	-1.51
PA3908	-1.91
PA4129	-1.60
PA4132	-1.70
PA4139	-3.49

PA4141	-7.57
PA4311	-1.51
PA4313a	-1.65
PA4346	-1.51
PA4352	-1.55
PA4384	-1.84
PA4467	-1.94
PA4469	-2.86
PA4471	-1.62
PA4570	-2.29
PA4573	-1.52
PA4578	-1.58
PA4607	-1.95
PA4702	-1.53
PA4738	-1.87
PA4739	-1.90
PA4773	-2.32
PA4774	-3.28
PA4775	-2.18
PA4782	-3.66
PA4866	-1.72
PA4874	-1.66
PA4877	-1.73
PA4925	-1.51
PA5061	-1.70
PA5101	-1.52
PA5178	-1.61
PA5212	-1.55
PA5219	-1.58
PA5220	-2.28
PA5286	-1.51
PA5424	-1.65
PA5460	-4.02
PA5461	-1.59
PA5481	-1.86
PA5482	-1.89

Table S3. Genes that are significantly up-regulated (cut-off: 1.5-fold change, FDR p-value < 0.05) in *P. aeruginosa* PAO1 24h-biofilm cells treated with coumarin (2 mM).

Locus tag	**Gene name**	**Description**	**Fold changes**
QS regulator (1)			
PA2226	*qsrO*	QS negative regulator	3.70
C-di-GMP metabolism (1)			
PA3885	*tpbA*	Protein tyrosine phosphatase TpbA	3.95
Others (85)			
PA2513	*antB*	Anthranilate dioxygenase small subunit	2.03
PA2514	*antC*	Anthranilate dioxygenase reductase	1.85
PA0866	*aroP2*	Aromatic amino acid transport protein	1.98
PA2507	*catA*	Catechol 1,2-dioxygenase	1.81
PA2508	*catB*	Muconate cycloisomerase I	1.72
PA2509	*catC*	Muconolactone delta-isomerase	1.62

PA0286	*desA*	Delta-9 fatty acid desaturase, DesA	1.81
PA4888	*desB*	Acyl-CoA delta-9-desaturase, DesB	1.56
PA3603	*dgkA*	Diacylglycerol kinase	1.91
PA4728	*folK*	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase	1.53
PA1384	*galE*	UDP-glucose 4-epimerase	1.86
PA3152	*hisH2*	Glutamine amidotransferase	1.68
PA4406	*lpxC*	UDP-3-O-acyl-N-acetylglucosamine deacetylase	1.64
PA0208	*mdcA*	Malonate decarboxylase	2.21
PA0210	*mdcC*		2.89
PA0211	*mdcD*		2.97
PA0212	*mdcE*		3.28
PA4898	*opdK*	Histidine porin OpdK	1.96
PA0216	*madM*	Malonate transporter	1.77
PA0603	*agtA*	4-aminobutyrate and 5-aminovalerate uptake	2.70
PA0604	*agtB*		1.97
PA0605	*agtC*		2.08
PA0606	*agtD*		1.70
PA0654	*speD*	S-adenosylmethionine decarboxylase proenzyme	2.34
PA0298	*spuB*	Glutamylpolyamine synthetase	1.81
PA5118	*thiI*	Thiazole biosynthesis protein	1.67
PA2515	*xylL*	Cis-1,2-dihydroxycyclohexa-3,4-diene carboxylate dehydrogenase	2.55
PA2518	*xylX*	Toluate 1,2-dioxygenase alpha subunit	1.52
PA2516	*xylZ*	Toluate 1,2-dioxygenase electron transfer component	1.78
PA2082	*kynR*	Lrp/AsnC-type transcriptional regulator	1.92
PA1175	*napD*	Protein of periplasmic nitrate reductase	1.52
PA3879	*narL*	Two-component response regulator NarL	1.64
PA4745	*nusA*	N utilization substance protein A	1.69
PA1779		Assimilatory nitrate reductase	1.66
PA5291	*betT2*	Glycine betaine-specific importer	1.77
PA1260	*lhpP*	ABC transporter periplasmic-binding protein,	1.97
PA0455	*dbpA*	RNA helicase DbpA	1.54
PA5239	*rho*	Transcription termination factor Rho	1.85
PA4242	*rpmJ*	50S ribosomal protein L36	1.59
PA4264	*rpsJ*	30S ribosomal protein S10	1.74
PA4563	*rpsT*	30S ribosomal protein S20	2.11
PA0579	*rpsU*	30S ribosomal protein S21	1.98
PA4727	*pcnB*	Poly(A) polymerase	1.59
PA4281	*sbcD*	Exonuclease	1.77
PA2619	*infA*	Initiation factor	1.86
PA4743	*rbfA*	Ribosome-binding factor A	1.68
PA4159	*fepB*	Ferrienterobactin-binding periplasmic protein precursor	1.66
PA0472	*fiuI*		1.64
PA3410	*hasI*		1.64
PA3899	*fecI*		1.52
PA4156	*fvbA*		3.53
PA5531	*tonB1*	(Ferri)pyoverdine signal transduction	1.68
PA1099	*fleR*	Two-component response regulator	1.63
PA1085	*flgJ*	Flagellar protein	1.70
PA1104	*fliI*	Flagellum-specific ATP synthase	1.67

PA1105	*fliJ*	Flagellar protein flij	1.57
PA4649	*cupE2*	Pilin subunit	1.74
PA4650	*cupE3*	Pilin subunit	2.06
PA0408	*pilG*	Twitching motility protein	2.37
PA4554	*pilY1*	Type 4 fimbrial biogenesis protein PilY1	1.67
PA3719	*armR*	Antirepressor for MexR	3.60
PA3721	*nalC*		1.74
PA4596	*esrC*	Repressor of the mexCD-oprJ multidrug efflux	2.05
PA5512	*mifS*	NtrC-like transcriptional regulators	1.59
PA0087	*tssE1*		1.69
PA0172	*siaA*		1.51
PA0845	*cerN*		1.54
PA1509	*tplEi*	Immunity protein	1.54
PA1510	*tplE*	Type 6 PGAP1-like effector,	1.83
PA4033	*mucE*		2.10
PA4674	*higA*	Antitoxin	1.79
PA4844	*ctpL*		1.58
PA5325	*sphA*		1.59
PA2258	*ptxR*	Transcriptional regulator	1.73
PA4034	*aqpZ*	Aquaporin Z	2.34
PA3266	*capB*	Cold acclimation protein B	2.02
PA0993	*cupC2*	Chaperone	2.92
PA0199	*exbD1*	Transport protein ExbD	1.88
PA5267	*hcpB*	Secreted protein Hcp	1.58
PA3007	*lexA*	Repressor protein	1.61
PA0320	*carO*	Calcium-regulated OB-fold protein	2.22
PA0327	*carP*	Calcium-regulated beta-propeller protein	1.56
PA0930		Two-component sensor	1.61
PA4276	*secE*	Secretion protein	1.69
PA3153	*wzx*	O-antigen translocase	1.96
Genes with general predicted or unknown functions (147)			
PA0214		Probable acyl transferase	2.22
PA4979		Probable acyl-CoA dehydrogenase	1.59
PA1601		Probable aldehyde dehydrogenase	1.6
PA3865a		Probable amino acid binding protein	1.79
PA5503		Probable ATP-binding component of ABC transporter	1.59
PA2840		Probable ATP-dependent RNA helicase	2.25
PA1541		Probable drug efflux transporter	2.49
PA4980		Probable enoyl-coa hydratase/isomerase	2.25
PA1385		Probable glycosyl transferase	2.53
PA3573		Probable major facilitator superfamily (MFS) transporter	1.7
PA1602		Probable oxidoreductase	1.79
PA1739		Probable oxidoreductase	1.6
PA4889		Probable oxidoreductase Add	1.51
PA0295		Probable periplasmic polyamine binding protein	1.62
PA3189		Probable permease of ABC sugar transporter	1.67
PA0733		Probable pseudouridylate synthase	1.74
PA4896		Probable sigma-70 factor, ECF subfamily	1.7

PA0942	Probable transcriptional regulator	1.93
PA1283	Probable transcriptional regulator	1.89
PA2100	Probable transcriptional regulator	1.5
PA2220	Probable transcriptional regulator	1.52
PA2221	Probable transcriptional regulator	2.38
PA2276	Probable transcriptional regulator	1.61
PA2577	Probable transcriptional regulator	1.61
PA2957	Probable transcriptional regulator	1.59
PA3067	Probable transcriptional regulator	1.69
PA3341	Probable transcriptional regulator	1.64
PA5437	Probable transcriptional regulator	2.45
PA0730	Probable transferase	2.66
PA2480	Probable two-component sensor	1.56
PA3206	Probable two-component sensor	1.58
PA2666	Probable 6-pyruvoyl tetrahydrobiopterin synthase	2.08
PA0224	Probable aldolase	1.6
PA1964	Probable ATP-binding component of ABC transporter	1.66
PA2104	Probable cysteine synthase	1.95
PA1391	Probable glycosyl transferase	1.55
PA1569	Probable major facilitator superfamily (MFS) transporter	2
PA5370	Probable major facilitator superfamily (MFS) transporter	1.59
PA1225	Probable NAD(P)H dehydrogenase	2.05
PA4514	Probable outer membrane receptor for iron transport	1.71
PA4167	Probable oxidoreductase	4.29
PA2711	Probable periplasmic spermidine/putrescine-binding protein	1.78
PA3188	Probable permease of ABC sugar transporter	1.68
PA0975	Probable radical activating enzyme	1.64
PA0491	Probable transcriptional regulator	2.19
PA0876	Probable transcriptional regulator	1.78
PA1836	Probable transcriptional regulator	1.52
PA2047	Probable transcriptional regulator	1.66
PA2497	Probable transcriptional regulator	1.58
PA3458	Probable transcriptional regulator	2.23
PA4288	Probable transcriptional regulator	2.53
PA2479	Probable two-component response regulator	1.65
PA0006		1.61
PA0013		1.54
PA0040		1.57
PA0046		1.61
PA0069		1.69
PA0128		1.54
PA0142		1.87
PA0201		2.25
PA0209		3.27
PA0234		1.59
PA0239		1.63
PA0388		1.78
PA0457		1.52
PA0466		1.88
PA0539		1.57

PA0560	1.58
PA0561	1.7
PA0589	1.53
PA0596	1.63
PA0734	1.57
PA0758	1.71
PA0805	1.86
PA0874	2.32
PA0924	2.41
PA0976	1.75
PA0986	3.6
PA1030	1.77
PA1090	1.62
PA1116	1.64
PA1170	1.69
PA1392	1.56
PA1415	1.64
PA1428	1.73
PA1539	1.7
PA1542	2.58
PA1639	1.52
PA1741	1.61
PA1743	2.08
PA1744	2.08
PA1768	1.59
PA1769	1.61
PA1865	2.08
PA2048	1.86
PA2049	1.68
PA2101	1.6
PA2225	2.02
PA2228	1.57
PA2282	1.65
PA2288	1.72
PA2418	1.79
PA2439	1.7
PA2440	2.19
PA2457	1.67
PA2481	1.56
PA2501	1.53
PA2763a	2
PA2767	2.06
PA2910	2.11
PA2929	1.98
PA2941	1.8
PA3056	1.52
PA3057	1.67
PA3237	1.78
PA3292	1.6
PA3390	2.25
PA3413	2.86
PA3414	2.45
PA3424	1.57
PA3489	1.78
PA3720	2.21

PA3979	1.83
PA4087	2.12
PA4278	1.83
PA4338	2.32
PA4353	1.71
PA4517	4.05
PA4518	1.56
PA4523	1.66
PA4574	1.68
PA4582	3.06
PA4583	3.37
PA4584	2.1
PA4630	1.64
PA4658	1.67
PA4746	1.98
PA4817	1.66
PA4849	1.5
PA4881	3.21
PA5087	1.66
PA5209	1.65
PA5248	1.62
PA5284	2.09
PA5404	2.02
PA5406	1.79
PA5492	1.73

Paper III.

The effects of potential quorum sensing inhibitors on biofilm, motility and virulence of *Acinetobacter baumannii*

Yunhui Zhang and Tom Coenye

Abstract

Acinetobacter baumannii is a nosocomial pathogen that causes a wide array of infections, and it has been considered as a global threat in the health-care setting due to its remarkable ability to acquire multidrug resistance. Quorum sensing (QS) has been proposed as a promising target for antivirulence therapy and QS inhibition has been studied as an effective way to interfere with biofilm formation and virulence in many pathogens. However, only limited QS inhibitors (QSIs) have been tested in *A. baumannii* so far, and few QSIs have been evaluated on *A. baumannii* in relevant *in vivo* models. In the present study, we investigated the effects of several known QSIs on biofilm formation and motility of *A. baumannii*. Some of these QSIs showed inhibition of biofilm formation and twitching motility in *A. baumannii*. Among these QSIs, curcumin and quercetin significantly reduced both biofilm formation and motility, and these two QSIs also increased the survival of *Galleria mellonella* infected by *A. baumannii*.

Introduction

Acinetobacter baumannii is an opportunistic pathogen associated with hospital-acquired infections in immunocompromised patients [418]. It can cause severe skin, bloodstream, urinary tract and other soft tissue infections [353]. Outbreaks of *A. baumannii* infections have been frequently reported in soldiers during wartime [419, 420], and *A. baumannii* is responsible for up to 20% of infections in intensive care units [421]. Virulence factors such as phospholipases, lipopolysaccharides, capsular polysaccharides, secretion systems and iron-chelating systems have been identified in *A. baumannii* [5, 6]. In addition, many clinical isolates of *A. baumannii* can form biofilms [15], which contributes to the persistence in infections and tolerance to antimicrobials and host defense. Rapid development of resistance in *A. baumannii* strains also contributes to the success of this pathogen, and the spread of multidrug resistant (MDR) *A. baumannii* strains worldwide has become a cause for serious concern [422]. The prevalence of MDR *A. baumannii* strains leaves few therapeutic options with antibiotics [423, 424], and there is an urgent need to develop alternative treatments against *A. baumannii* infections.

Quorum sensing (QS) has been studied as an important regulatory system controlling the virulence in a wide range of bacteria [45, 349, 425, 426]. QS in *A. baumannii* is composed of the N-acyl homoserine lactone (AHL) synthase AbaI and the corresponding receptor AbaR [190]. N-(3-hydroxydodecanoyl)-HSL (3-OH-C12-HSL) is the primary signal molecule detected in *A. baumannii* strains, while other AHLs can also be detected in different *A. baumannii* strains [359]. QS regulates biofilm formation in *A. baumannii*, and an *abaI* mutant showed 40% reduction in biofilm production compared to that of the wild-type strain [190]. Interfering with the QS system in pathogens has been considered as a promising way to inhibit virulence and biofilm formation; many quorum quenching (QQ) enzymes and QS inhibitors (QSIs) have been identified and evaluated on different pathogens [49, 288, 427, 428]. However, the effects of these QQ enzymes and QSIs on *A. baumannii* have not been studied in detail [429]. QQ enzymes MomL and engineered GkL have been shown to reduce biofilm formation in *A. baumannii* strains [257, 430], and several synthetic non-native acyl homoserine lactones (AHLs) can inhibit motility and biofilm formation in *A. baumannii* [337]. *Glycyrrhiza glabra* flavonoids, streptomycin at subinhibitory concentrations, virstatin and two unsaturated fatty acids have also been reported to disrupt QS system in *A. baumannii* and affect QS-controlled phenotypes [338-340]. The effects of other known QSIs for Gram-negative bacteria such as furanone C-30, curcumin and quercetin on *A. baumannii* are not reported yet.

In the current study, we tested the effects of several known QSIs on biofilm formation and twitching motility of *A. baumannii* strains, aiming to expand the current knowledge of QSIs against *A. baumannii*. The most effective QSIs for *A. baumannii* were further evaluated in a *Galleria mellonella* infection model.

Material and methods

Bacterial strains, growth media and chemicals

A. baumannii LMG 10520 and AB5075 were grown on Tryptic Soy Agar (TSA, Lab M limited, UK) or in Luria-Bertani (LB) broth (Lab M limited, UK) aerobically at 37°C. QSIs were purchased from Sigma-Aldrich (Bornem, Belgium), except for esculetin that was purchased from Acros Organics (Geel, Belgium). All compounds were

dissolved in dimethyl sulfoxide (DMSO) (Alfa Aesar, Germany) as stock solutions and diluted in MilliQ water. A control solution containing only DMSO was made in MilliQ water. Detailed information about these QSIs can be found in Table 1.

Table 1. QSIs tested in this study.

Compound	Structures	Concentration used (μM)	Reference
(+)-Catechin hydrate		1000	[431]
4-Nitropyridine-*N*-oxide		10	[395]
2-Amino-6-chlorobenzoic acid (6-CABA)		500	[315]
Baicalin hydrate		200	[416]
Coumarin		250	[392]
Curcumin		10	[432]
Esculetin		200	[416]
Esculin hydrate		1000	[416]
Furanone-C30		50	[313]
Indole		1000	[433]
L-Canavanine		20	[434]
Quercetin		10	[389]

Effect of QSIs on the growth of *A. baumannii* strains

Overnight cultures of *A. baumannii* LMG 10520 and AB5075 were diluted 1:100 in fresh LB broth. QSIs were supplemented in a final concentration as shown in Table 1. 100 µL bacterial suspesions were added to 96-well microtiter plates and cultured at 37°C for 24h. The absorbance at 590 nm was measured at 1h intervals using an EnVision multilabel reader (Perkin Elmer, Waltham, MA).

Biofilm formation and quantification

Overnight cultures of *A. baumannii* strains were diluted to approximately $5×10^7$ CFU/mL in LB broth. 100 µL of the suspension was transferred to the wells of a round-bottomed 96-well microtiter plate with QSIs in a final concentration shown in Table 1. MilliQ with DMSO was added to the control. The plate was incubated at 37 °C for 4 h before the supernatant was removed. The wells were rinsed once with sterile physiological saline (PS) and re-filled with fresh media and QSIs. The plate was incubated at 37 °C for an additional 20 hours. The number of living cells in the biofilm was quantified using the resazurin assay (CellTiter-Blue, CTB) [368]. Briefly, wells were rinsed after 24 h biofilm formation. 100 µL physiological saline and 20 µL CTB solution (Promega, Leiden, Netherlands) were added in each well. After 1 h incubation at 37 °C in dark, the fluorescence (excitation and emission filters of 486 nm and 535 nm) was measured using the EnVision multilabel reader. The experiment was performed with ten replicates.

Fluorescence microscopy

Biofilms of *A. baumannii* LMG 10520 were formed in the absence or presence of QSIs as described above using a flat-bottomed 96-well microtiter plates. 3 µL SYTO9 and 3 µL propidium iodide were diluted in 1 mL sterile PS, and 100 µL of this staining solution was transferred to each well. The plate was incubated for 15 min at room temperature and fluorescence microscopy was performed with the EVOS FL Auto Imaging System (Life Technologies).

Twitching motility

The effects of QSIs on twitching motility of *A. baumannii* AB5075 were investigated as previously described, with minor modifications [338]. Briefly, LB plates (0.8% agar)

supplemented with DMSO or QSIs in final concentrations as shown in Table 1 were prepared. 10 μL overnight culture of *A. baumannii* AB5075 was inoculated to the bottom of the agar, and the plates were incubated at 37 °C for 48 h. Subsequently, the agar was gently discarded and the plates were stained with 0.1% crystal violet for 2 min and washed gently with water to remove excess crystal violet. Diameters of the crystal violet stained zone were measured. Three independent assays were performed, each in triplicate.

G. mellonella survival assay

G. mellonella larvae were purchased from BioSystems Technology (Exeter, UK) and were stored in the dark at 15°C prior to use. Cells of *A. baumannii* AB5075 were washed with phosphate buffered saline (PBS) and then diluted to a concentration of 10^7 CFU/mL in PBS. Curcumin and quercetin were added to the bacterial suspension at a concentration of 10 μM before inoculation into the larvae. DMSO was added to the control. 10 μL of solutions were injected in the last left proleg of *G. mellonella*. Larvae were placed in the dark at 37°C and were scored as dead or alive every 24 h until 120 h. Larvae showing no movement in response to shaking or touch were considered dead. Experiments that had one or more dead larvae in control groups were discarded and repeated. 15 larvae were injected for each treatment, and three independent experiments were performed.

Statistical analyses

Numerical data were analyzed using GraphPad Prism 6.0 and presented as mean ± standard deviation (SD). The normal distribution of the data was checked by the D'Agostino-Pearson normality test. Normally distributed data were analyzed by one-way ANOVA, and non-normally distributed data were analyzed by the Kruskal-Wallis test. Survival curves were analyzed by using a Log-rank (Mantel-Cox) test.

Results

Effect of QSIs on planktonic cell growth of *A. baumannii* strains

As shown in Figure 1, coumarin (250 μM) inhibited the planktonic cell growth of both *A. baumannii* LMG 10520 and AB5075 during stationary phase, and the final OD at 24 h is approximately 15% lower compared to the untreated control. 4-Nitropyridine-

N-oxide (10 μM) slowed down the growth rate of LMG 10520 and AB5075. The final OD of AB5075 after 24 h treated with 6-CABA (500 μM) was approximately 20% lower compared to the untreated control. No significant inhibition on planktonic cell growth was observed with other QSIs with the tested concentrations in both *A. baumannii* LMG 10520 and AB5075.

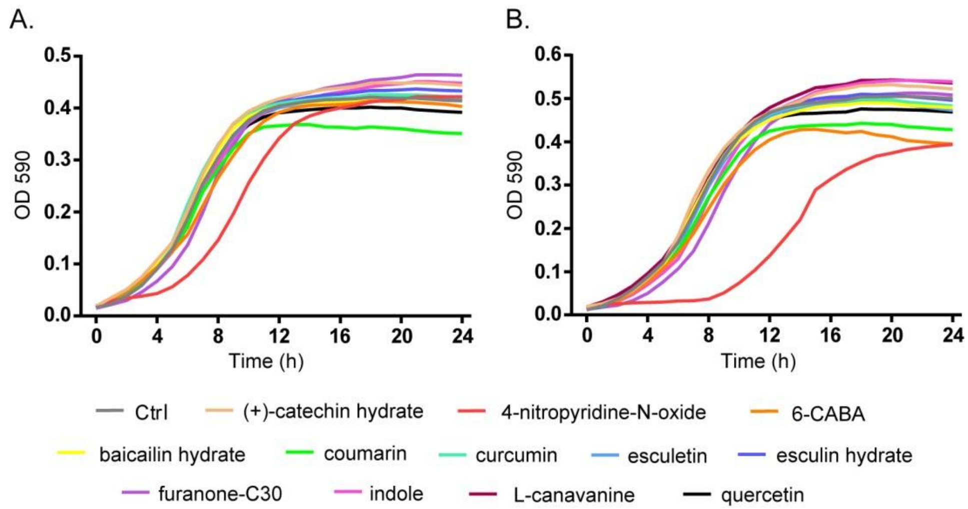

Figure 1. Effect of QSIs on planktonic growth of *A. baumannii*. (A). *A. baumannii* LMG 10520. (B). *A. baumannii* AB5075.

Effect of QSIs on biofilm formation of *A. baumannii* strains

Baicailin hydrate, coumarin, curcumin, esculetin, esculin hydrate and quercetin significantly reduced biofilm formation of both *A. baumannii* LMG 10520 and AB5075 (Figure 2). Baicailin hydrate, coumarin and quercetin reduced biofilm formation by approximately 30~40 % in both *A. baumannii* strains tested. Curcumin, esculetin and esculin hydrate also reduced biofilm formation of AB5075 by 30~40%. Biofilm formation of AB5075 was reduced by about 10% when treated with 6-CABA, which might be due to its inhibition on the planktonic cell growth of AB5075. No significant inhibition was observed with (+)-catechin hydrate, 4-nitropyridine-*N*-oxide, furanone-C30, indole and L-canavanine in either *A. baumannii* strain.

Fluorescence microscopy was performed to confirm the effect of these QSIs on biofilm formation of *A. baumannii* LMG 10520. As shown in Figure 3, reduction in biofilm formation was observed following baicailin hydrate, coumarin, curcumin and quercetin treatment.

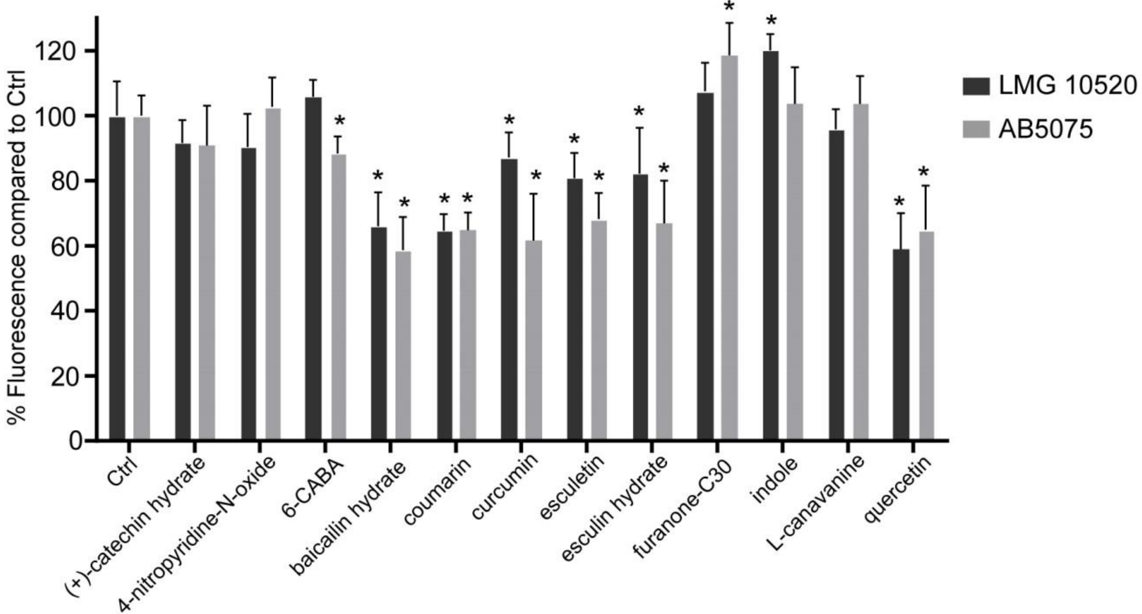

Figure 2. Effect of QSIs on biofilm formation of *A. baumannii*. Data shown are the mean values (n=10), error bars represent standard deviation. *, p<0.05 compared to the untreated control group.

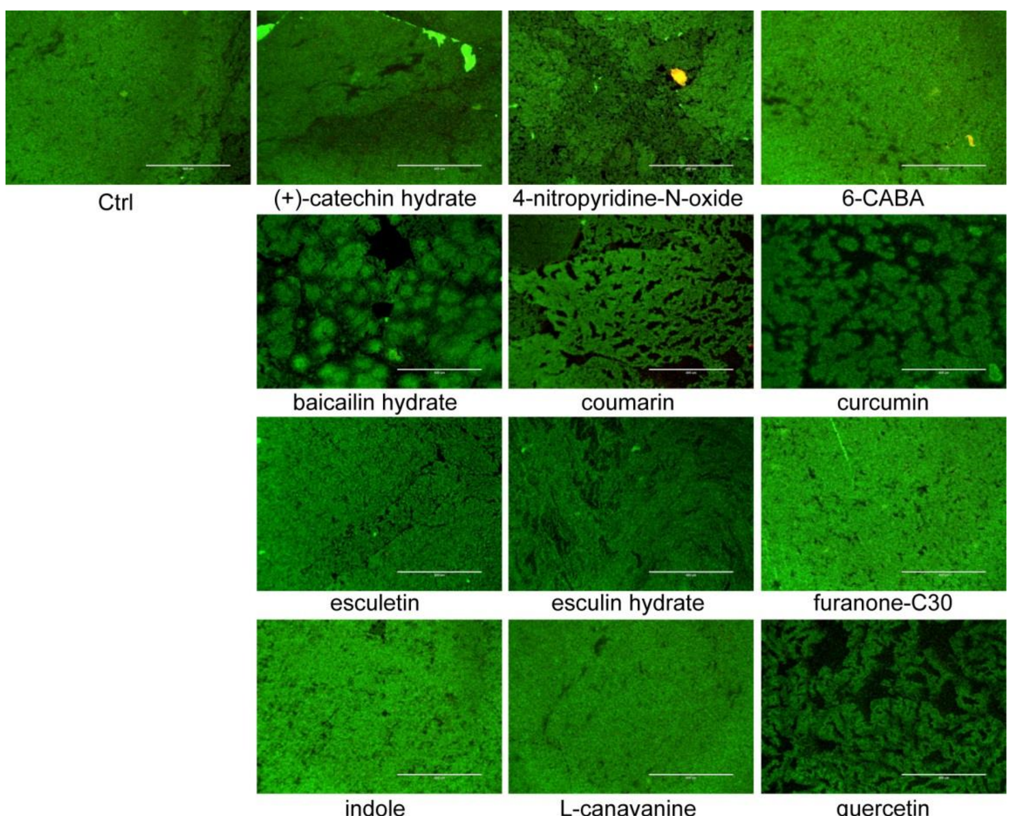

Figure 3. Representative fluorescence images of *A. baumannii* LMG 10520 biofilms treated with QSIs. The scale bar represents 400 µm.

Effect of QSIs on twitching motility of *A. baumannii*

(+)-Catechin hydrate, curcumin, indole and quercetin inhibited twitching motility of *A. baumannii* AB5075 (Figure 4). No significant inhibition of twitching motility was observed for the other QSIs tested.

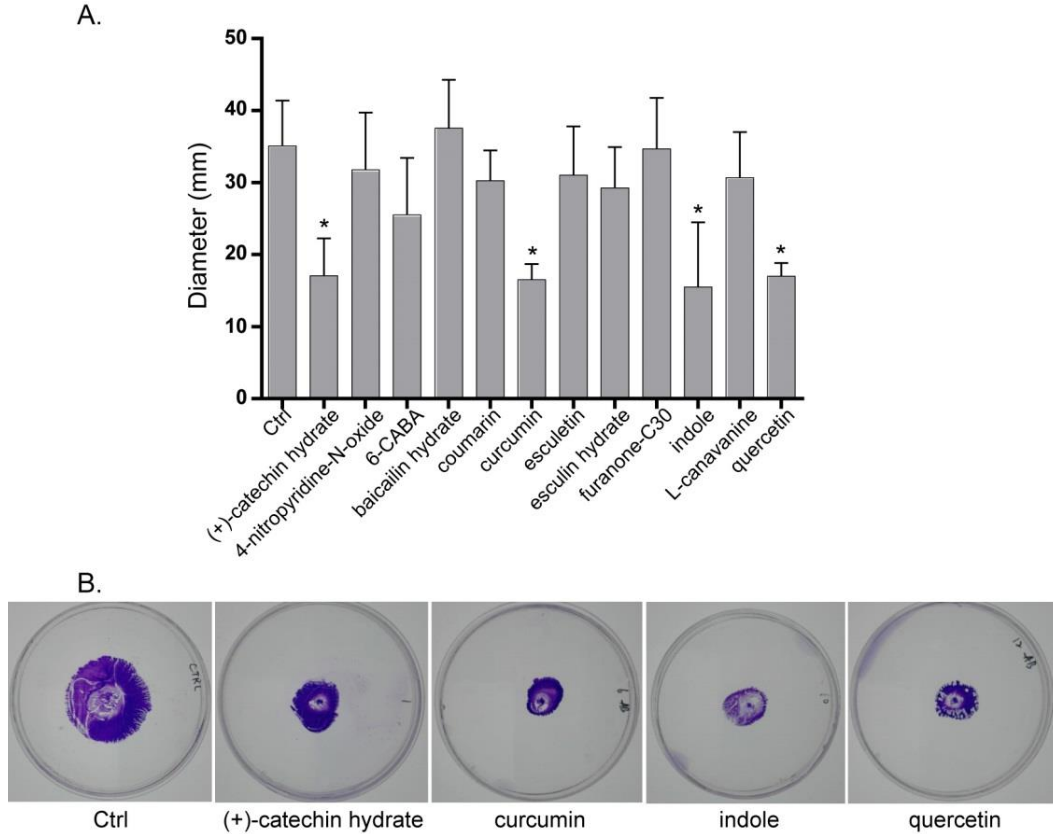

Figure 4. Effect of QSIs on twitching motility of *A. baumannii* AB5075. (A). Data shown in are the mean values (n=9), error bars represent standard deviation. *, $p<0.05$ compared to the untreated control group. (B). Representative images shown the inhibition of twitching motility by QSIs.

Effect of curcumin and quercetin on survival of *G. mellonella* following infection with *A. baumannii*

We further evaluated two QSIs which inhibited both biofilm formation and twitching motility of *A. baumannii* in a *G. mellonella* infection model. Curcumin and quercetin showed no toxicity to *G. mellonella* at the concentrations used (data not shown). As shown in Figure 5, curcumin and quercetin significantly increase the survival of *G. mellonella* infected by *A. baumannii* AB5075 ($p<0.0001$ in Log-rank test). After 120 h,

the survival of infected larvae in control group was 18.9±6.0%, while the survival of curcumin and quercetin treated larvae was 69.0±7.5% and 51.2±7.9%, respectively.

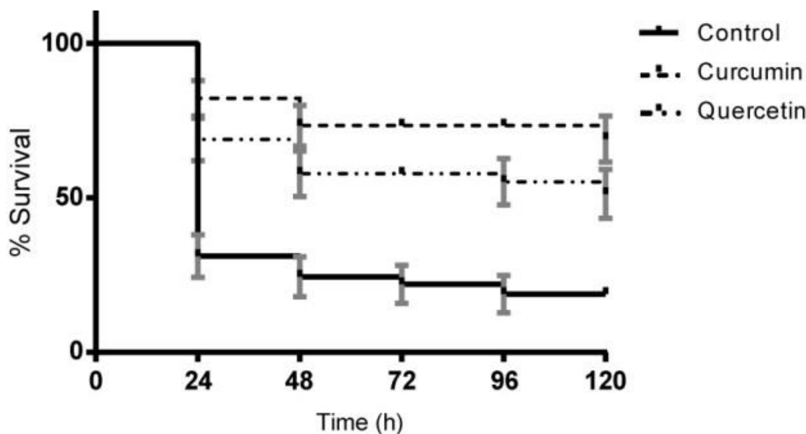

Figure 5. Survival curve of *G. mellonella* infected by *A. baumannii* AB5075. The data were presented as mean ± standard error.

Discussion

QS systems have intensively been studied as potential antivirulence targets in many bacterial pathogens [348, 435]; research on QS and QS inhibition of the emerging pathogen *A. baumannii* is however still in an early stage [429]. In the current study, we evaluated the effects of 12 compounds on biofilm formation and twitching motility of *A. baumannii*. These compounds included several plant-derived flavonoids ((+)-catechin hydrate, baicailin hydrate and quercetin) as well as other phenols (curcumin, coumarin, esculetin and esculin hydrate) which have been reported to inhibit QS and biofilm formation in *Pseudomonas aeruginosa* [336, 389, 392, 416, 431, 432]. Synthetic QSIs like furanone C30, 4-nitropyridine-N-oxide and 6-CABA [313, 315, 395] were also included in the present study. In addition, the arginine analog L-canavanine, which inhibited QS in *Sinorhizobium meliloti* was tested. Another QSI tested in our study was indole, which has been reported to destabilize the QS receptor and inhibit QS in *Acinetobacter oleivorans* [433].

Baicailin hydrate, curcumin and quercetin significantly reduced biofilm formation of *A. baumannii*, as demonstrated by CTB staining and fluorescence microcopy. Although

coumarin also reduced biofilm formation, 15% inhibition of planktonic cell growth was observed at the concentration tested. No reduction in biofilm formation was observed with (+)-catechin hydrate and indole, but these two QSIs were found to significantly inhibit the twitching motility. In contrast, baicailin hydrate and coumarin which reduced biofilm formation showed no effects on twitching motility of *A. baumannii*. Only two QSIs, curcumin and quercetin, significantly inhibited both biofilm formation and twitching motility of *A. baumannii*.

Twitching motility of *A. baumannii* relies on Type IV pili, and these surface appendages also participate in natural transformation and adherence to abiotic/biotic surfaces [436]. Although the relationship between QS and Type IV pili in *A. baumannii* has not been clarified yet, a study showed that AHL enhances pili assembly, twitching motility and biofilm formation of *A. baumannii* ATCC19606 [198]. In another study, an *abaI* mutant of *A. baumannii* M2 showed impaired twitching motility compared to the wild-type, and flavonoid extracts of *G. glabra* which inhibit QS of *A. baumannii* reduced twitching motility in the wild-type [338]. These results strongly suggest that QS positively regulates twitching motility. However, not all QSIs tested in our study inhibit both biofilm formation and twitching motility of *A. baumannii*, which might be due to the distinct mechanisms behind their inhibition effects on biofilm and motility in *A. baumannii*.

Curcumin, the major ingredient from *Curcuma longa*, has been reported to inhibit QS in *P. aeruginosa*, reducing QS-regulated virulence and biofilm formation [437]. It also increased survival of *Caenorhabditis elegans* following infection with *P. aeruginosa* and *Burkholderia pseudomallei* [437, 438]. Curcumin-loaded liposomes were shown to interfere with QS and affect QS-regulated phenotypes of *Aeromonas sobria* [439]. Biofilm formation of pathogens as *Escherichia coli*, *Proteus mirabilis* and *Serratia marcescens* can also be inhibited by curcumin [432]. The natural flavonoid quercetin is another effective QSI that reduces biofilm formation and production of virulence factors in *P. aeruginosa* [389], probably by allosteric inhibition of QS receptors [297]. In our study, curcumin and quercetin were shown to inhibit QS-controlled phenotypes, biofilm formation and twitching motility, in *A. baumannii*. Both of them increased the survival of *G. mellonella* infected by *A. baumannii* AB5075 and to the best of our knowledge, this is the first report of QSIs reducing *A. baumannii* virulence in an *in vivo* infection model. Although a previous study showed no difference in *G.*

mellonella killing between wild-type and QS mutant of *A. baumannii* M2 [371], our results showed QS might be an important determinant in the virulence of *A. baumannii* to *G. mellonella*. The inconsistent results might be due to different *A. baumannii* strains and experiment conditions used in these two studies.

In summary, we evaluated the effect of different known QSIs on *A. baumannii*. Curcumin and quercetin were identified as possible QSIs which effectively inhibited both biofilm formation and twitching motility of *A. baumannii*, and they reduced the virulence of *A. baumannii* in a *G. mellonella* infection model. These results indicated that QS inhibition can be an effective way to inhibit *A. baumannii* virulence and infections. Both curcumin and quercetin are currently applied as dietary supplements [440, 441], and they may be good choices for further investigations on safe and efficiency QSIs to treat *A. baumannii* clinical infection.

Conclusion

QSIs such as curcumin and quercetin can effectively inhibit biofilm formation, motility and virulence of *A. baumannii*, and these QSIs might be promising in treating *A. baumannii* infections.

CHAPTER IV.
BROADER INTERNATIONAL CONTEXT, RELEVANCE, AND FUTURE PERSPECTIVES

1. The need for antivirulence drugs

According to the report released by WHO in April 2014, a 'post-antibiotic' era in which common infections and minor injuries can kill, may become a reality in the 21st century if no effective actions are taken against the increasing antimicrobial resistance [442]. Data gathered from 129 member states of the WHO showed that extensive resistance to antibiotics is emerging in every region of the world [442]. This growing threat caused by antimicrobial resistance reduces the efficiency of known drugs, resulting in more difficult and costly treatment for patients worldwide. The first study on the burden posed by antibiotic resistant bacteria in 2013 revealed that 2 million people become infected with resistant bacteria and at least 23,000 people die each year as a consequence in the US alone, leading to a cost of over $20 billion in health care and $35 billion for the loss of productivity [443]. Globally, at least 700,000 deaths are caused by drug resistance in illnesses such as bacterial infections, malaria or tuberculosis each year [444]. It is unquestionable that we are at a critical point to take global measures against antimicrobial resistance. However, development of novel antibiotics has slowed down since 1990s [445], and typically bacteria quickly develop resistance to new antibiotics within a few years after their introduction.

In the meantime, increasing understanding of bacterial virulence factors and their functions motivates the research on antivirulence strategies, which aim to interfere with the virulence of bacteria instead of killing them or inhibit their growth. The number of publications on antivirulence compounds and targets has increased rapidly over the past decades (Figure 4.1). The most significant advantage of antivirulence drugs seems to be the weaker evolutionary pressure they impose on bacteria compared to antibiotics. Adhesion, toxin, secretion system, biofilm and QS system have been considered as promising antivirulence targets and intensively studied in various antibiotic resistant pathogens such as *P. aeruginosa*, *S. aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Clostridium botulinum* and *Enterobacter* [446-448]. However, most of these compounds with antivirulence potential are not yet widely tested in the clinical settings, and more efforts are needed to prove their putative advantages and effectiveness.

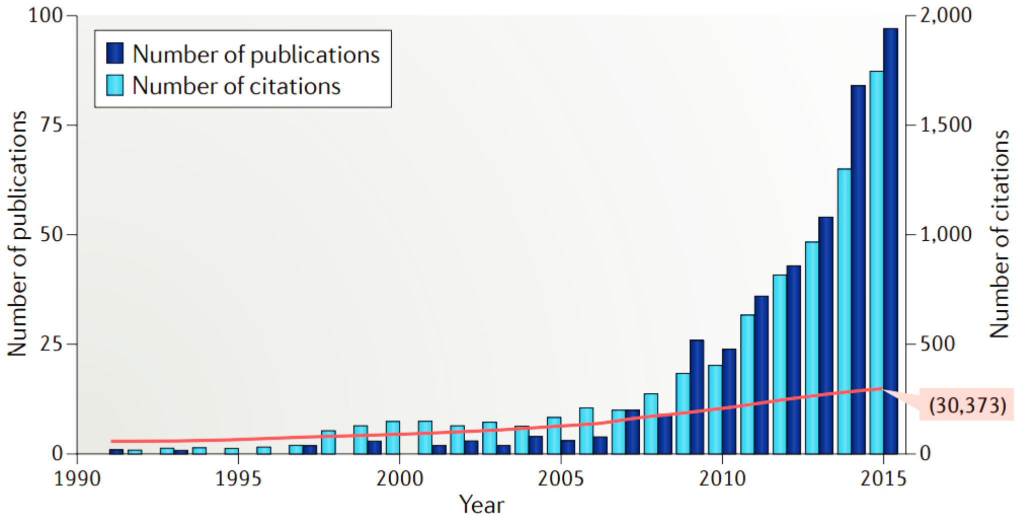

Figure 4.1 Increased numbers of antivirulence publications and citations over time. The total number of publications on Web of Science is given as a baseline (red), scaled to the number in 2015, which is indicated in parentheses. [446]

2. QS as antivirulence target in infections: promises and challenges

Compared to other antivirulence targets, the most encouraging potential of QS is the possibility to prevent the production of multiple virulence factors. In the case of *P. aeruginosa*, QS regulates the production of pyocyanin, protease, HCN, rhamnolipid and other virulence factors, and it also affects the process of iron uptake and biofilm formation, which makes QS an attractive target to reduce the full virulence of *P. aeruginosa* during infections [449]. Although study on QS of *A. baumannii* is still in an early stage, biofilm formation and motility of *A. baumannii* were shown to be affected by QS [360]. QS systems in other pathogens such as *S. aureus*, *Burkholderia cenocepacia* and *E. coli* also play an important role in their virulence [425, 426]. In addition, due to the regulatory role of QS in biofilm formation, QS inhibition has been considered as a possible way to increase the efficiency of antimicrobials against pathogens such as *P. aeruginosa* [352] in treating biofilm-related infections.

Different strategies for QS inhibition have been proposed based on the general QS circuits, including blocking the signal production, degrading signal molecules and disrupting the signal reception. Various enzymes and compounds have been identified with QS inhibition activities targeting different QS systems in pathogens (See Chapter I). In Chapter III Paper 1, we showed that the AHL-degrading enzyme MomL can reduce biofilm formation of *P. aeruginosa* and *A. baumannii*, and MomL in

combination with antibiotics resulted in fewer biofilm cells compared to antibiotic treatment alone. In Paper 2, the QS inhibitor coumarin was shown to inhibit QS-regulated virulence factors, biofilm formation and virulence to *L. sericata* maggots in *P. aeruginosa*. In Paper 3, promising QSIs were identified which can reduce biofilm formation, motility and virulence to *G. mellonella* in *A. baumannii*. These results prove the antivirulence and antibiofilm potential of both QQ enzymes and QSIs, and some QSIs showed promising results not only *in vitro* but also in *in vivo* models.

However, several questions have also been raised during the exploration of QS inhibition as an effective antivirulence approach, and these questions were well discussed with *P. aeruginosa* as an example [413].

Firstly, in addition to its role in regulating virulence genes, QS also influences the global metabolism. Davenport *et al*. compared the metabolites produced by a *lasI rhlI* double mutant and wild-type *P. aeruginosa*, and found changes in approx. one third of the metabolites tested including tricarboxylic acid (TCA) cycle intermediates, amino acids and fatty acids [450]. Our RNA-sequencing results on coumarin-treated biofilms of *P. aeruginosa* (Chapter III Paper 2) also showed that expression of multiple genes related to metabolisms were altered, such as glycogen phosphorylase (*glgP*), glycogen branching enzyme (*glgB*) and glycogen synthase (PA2165, *glgA*) which are related to carbohydrate metabolism and are positively controlled by QS [78]. How these changes could affect the growth and fitness of *P. aeruginosa* under different nutritional conditions remains to be determined. QS is also involved in the regulation of primary metabolism in *Burkholderia* species and in *Yersinia pestis* [451]. The possible implications of such metabolic changes on QS inhibition therapy are still unexplored. Therefore when we focus on the inhibition of virulence factors by interfering with QS, the accompanied changes on metabolism of these pathogens should not be ignored. Otherwise these changes may lead to unexpected outcomes by affecting growth and competition of pathogen populations *in vivo*.

Secondly, QS may positively or negatively regulate different virulence factors in certain pathogens. For *P. aeruginosa*, most virulence factors are positively regulated by QS, but the expression of T3SS has been reported to be negatively affected by QS [412]. Although transcriptome analysis on coumarin and 6-gingerol treated biofilms revealed that T3SS was repressed along with their QS inhibition effects

(Chapter III Paper 1, [328]), another study on curcumin treated *P. aeruginosa* showed six genes related to T3SS were upregulated [437]. In *S. aureus*, the Agr system positively regulates many toxins and degradative exoenzymes, but several colonization factors and biofilm formation are negatively regulated by Agr, and this becomes a noticeable problem when developing Agr blockers [67]. For *A. baumannii*, the interactions between QS and other virulence factors are not yet well established, and these need to be understood better for a solid scientific basis of QS inhibition therapy in *A. baumannii*.

Furthermore, variations between different clinical strains may also render QS inhibition therapy fall short of expectations. Our results in Chapter III Paper 1 and Paper 2 indicated that the effect of either QQ enzyme or QSI may be strain dependent. MomL inhibits the biofilm formation of *A. baumannii* LMG 10531 and LMG 10520, but biofilm formation of three other *A. baumannii* strains is not reduced by MomL treatment. Inhibitory effects of coumarin on virulence and biofilm formation vary in different *P. aeruginosa* strains tested. These variations were also observed in a study of Guendouze *et al*., which evaluated the effect of QQ enzyme SsoPox and QSIs (furanone C30 and 5-fluorouracil) on *P. aeruginosa* clinical strains [256]. The effect of QSIs on growth also varies between different strains. García-Contreras *et al*. showed that furanone C30 displayed highly variable QS inhibition activity and growth inhibition in 50 *P. aeruginosa* isolates from cystic fibrosis patients, and some of these strains even produced higher amounts of the virulence factors when exposed to C30 [409].

Although LasR in *P. aeruginosa* has been considered as a pivotal factor in the QS network, *lasR* mutants are frequently found in at least one third of the chronically infected cystic fibrosis patients, and these mutants are associated with worse lung function [452]. Fitness advantages of *lasR* mutants are indicated by several studies, such as the ability to grow on nitrate and aromatic amino acid sources and higher resistance to alkaline stress [453-455]. *lasR* mutants have also been shown to induce exaggerated host inflammatory responses in respiratory epithelial cells with increased accumulation of proinflammatory cytokines and neutrophil recruitment, and might directly contribute to the pathogenesis and progression of chronic lung disease [456]. These results raise concerns about the effects of inhibitors targeting LasR, and indicate that host immune responses and types of infections should also be

considered when developing QS inhibition agents of remarkable complex pathogens as *P. aeruginosa*.

In addition, one underlying assumption for effective QS inhibition therapy is that the immune systems of patients can clear the bacteria in infections upon QS inhibition treatments [413]. However, opportunistic pathogens such as *P. aeruginosa* and *A. baumannii* primarily infect patients with compromised immune systems, and whether the bacteria can be get rid of or not with single QS inhibition therapy is yet to be demonstrated. If these bacteria are not cleared thoroughly, the question whether they will become a potential 'time bomb' in patients after removing the QS inhibition agents needs to be considered.

In this dissertation, we also found that the effect of QQ enzymes and QSIs might fall short of expectation under certain *in vivo*-like conditions or in multi-species biofilms. These results indicated that the role of QS should be better understood in a specific type of infections before we consider QS as a promising target to inhibit biofilm formation and virulence. In the case of wound infections, it has been shown that albumin can bind and sequster 3-oxo-C12-HSL produced by *P. aeruginosa*, indicating that the *las* system in *P. aeruginosa* might be largely inhibited in a an albumin-rich environment such as chronic wounds [457]. On the other hand, in peripheral blood during burn trauma with reduced level of albumin, the function of QS in *P. aeruginosa* is probably only slightly affected [457]. In addition, the presence of PONs in the serum can also lead to less functional QS systems in *P. aeruginosa* during infections. These factors affecting the *las* system might partially explain the less pronounced effect of MomL and coumarin on *P. aeruginosa* in the wound model, as activation of the *las* system is considered to precede the activation of RhlR or PqsR and a non-functional *las* system can lead to QS-deficient phenotypes in well-studied *P. aeruginosa* lab strains. However, increasing evidence shows that RhlR can be functional in a *las*-independent manner in clinical isolates during infections [458]. Therefore, the *in vivo* effect of QQ enzymes and QSIs targeting not only *las* but also *rhl* or even the PQS system during wound infections needs to be further studied.

Considering the fact that wound infections are often colonized by multiple species including *C. albicans*, *S. aureus*, *P. aeruginosa*, *E. coli*, *Bacteroides spp.*, the treatment by QQ enzymes and QSIs can be more challenging. QS inhibition targeting

one pathogen might influence the population of other species. For instance, QS-regulated virulence factors of *P. aeruginosa* severely impair the growth of *S. aureus* [459, 460]. It is possible that QQ enzymes or QSIs targeting *P. aeruginosa* can lead to an increased population of *S. aureus* in polymicrobial infections and might be less helpful in reduce the burden of infections. A similar situation may also occur with *P. aeruginosa* and *C. albicans*. AHLs produced by *P. aeruginosa* can inhibit the yeast to hyphal switch, an important process for the virulence of *C. albicans* [461]. PQS and pyocyanin are also involved in the interaction between *P. aeruginosa* and *C. albicans* [462]. Therefore, QS inhibition of *P. aeruginosa* may result in increased virulence of *C. albicans*. Evaluating the effects of QQ enzymes and QSIs in delicate designed polymicrobial model may help us to study these possibilities that could happen in *in vivo* wound infections.

In summary, QS may be an attractive antivirulence target in *P. aeruginosa* and other pathogens, but more research is needed to understand the relationship between QS, virulence and physiology in different pathogens. Discovery of QSIs with different mechanisms of QS inhibition may also help to identify the truly effective ones that may ultimately be used in the clinic.

3. Plants as abundant sources for natural QSIs

Natural products from plant have long been studied as sources of lead compounds for modern drug discovery, and now plant extracts have also become an abundant source for QSIs. It is assumed that plants (which have no sophisticated immune system) may rely on cellular and biochemical defense systems and produce natural QS inhibition compounds to defeat pathogens [463]. As discussed in Chapter I, a variety of phenolic compounds show QS inhibition activity, and several flavonoids comprise a well-studied group. In Chapter III Paper 3, we showed that quercetin, a typical flavonoid found in many fruits, vegetables, leaves, and grains, can successfully inhibit biofilm formation and virulence of *A. baumannii*. Another promising QSI against *A. baumannii* identified in our study, curcumin, is a natural phenol primarily found in turmeric (*Curcuma longa*).

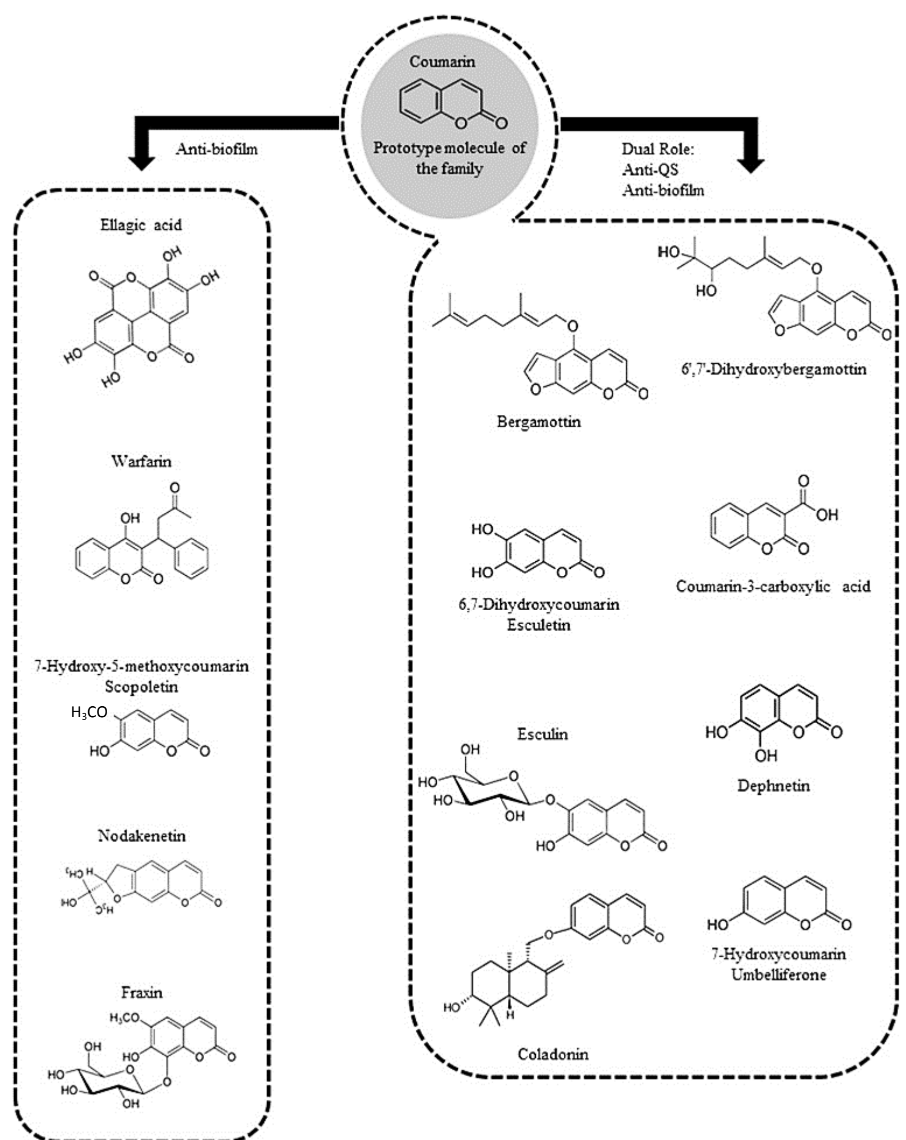

Figure 4.2 Coumarins with anti-biofilm or anti-QS activities. Compounds for which anti-biofilm properties have been established are presented on the left, while those with dual activity are presented on the right [391].

Coumarins are another family belonging to the phenolic compounds, and they are composed of fused benzene and α-pyrone rings. Their potential to inhibit QS and biofilm formation in different pathogens has just begun to be studied (Figure 4.2). Esculetin and esculin were the first coumarins reported as QSIs in a virtual QS inhibitor screen [416]. The QS inhibition activities of other coumarins have been reported subsequently, including that of coumarin itself [391, 392]. However, none of these coumarins have been tested *in vivo*, and the mechanisms of their QS inhibition effects remains unknown. In Chapter III Paper 2, we provided a thorough analysis with transcriptomic data on the QS inhibition effect of coumarin in *P. aeruginosa*, and

surprisingly found that coumarin can also interfere with type III secretion and c-di-GMP signaling in *P. aeruginosa*. These results indicated that coumarin may be promising in treating *P. aeruginosa* infections. Further studies on other coumarin compounds and chemical modifications of these compounds might help us to identify more potent QSIs. In addition, although a reference dose (acceptable daily exposure level for a lifetime) of 0.64 mg/kg/day coumarin has been proposed as a conservative value, the clinical database suggests that most humans can tolerate far higher exposures to coumarin without any adverse effects [391, 464].

However, there is an on-going debate about the therapeutic use of several natural products including curcumin, quercetin and polyphenols. In the case of curcumin, it can hit many targets and might not have favorable pharmacokinetic properties [465]. Other drawbacks of curcumin include the low oral bioavailability and toxicity under specific conditions [466]. These negative properties of curcumin and other polyphenols should also be considered when further developing them as effective QSIs.

4. Methods used in QS inhibition studies

4.1 Biosensor strains

Genetically engineered bacterial whole-cell biosensors are useful tools in the detection of QS signal molecules as well as to determine the activity of QQ enzymes and QSIs. Generally, a QS biosensor strain harbors QS systems of interest coupled with easily detectable and quantifiable reporter genes. Reporter genes generates different classes of readable output such as fluorescence, color pigments and luminescence [293, 467]. These biosensors have been developed with a wide range of bacteria including *E. coli*, *P. aeruginosa*, *A. tumefaciens* and *Chromobacterium violaceum*. The type of AHLs that can be detected and the detectable concentration range are different for biosensors based on their mechanisms. In Chaper III Paper 1, the biosensor *A. tumefaciens* A136 was used to detect the residual AHL concentrations after degradation by MomL. *A. tumefaciens* A136 expressed β-galactosidase in the presence of AHLs, and is widely used in many studies due to its broad detection range of AHLs and high sensitivity [367, 468]. In Chaper III Paper 2, *P. aeruginosa* QSIS2 was used to confirm the QS inhibitory effect of coumarin. This biosensor is useful in identifying QSIs targeting the QS circuit of *P. aeruginosa*, as

QS inhibition is quantified directly by the cell growth of *P. aeruginosa* in the presence of sucrose [395]. However, there are also limitations with these biosensors. For example, the concentrations of AHLs produced by *A. baumannii* strains are too low to be detected by the biosensor used in our study, and in such case analytical methods as HPLC and mass spectrometry can be used to quantify the AHL levels. In addition, false positive results in identifying QSIs with biosensors might happen as the detected phenotype is often co-dependent on other factors and/or the metabolic activity of the cells [435]. Therefore, these biosensors can be used as a fast, convenient tool in primary identification of QSIs, but a series of verification tests are needed to confirm QS inhibition.

4.2 Inhibition of QS-regulated virulence

Reduced production of virulence factors controlled by QS is a strong evidence for QS inhibition in pathogens as *P. aeruginosa*, and also has important implications for reduced virulence of these pathogens in *in vivo* infections. Pyocyanin, protease and rhamnolipid are commonly tested QS-regulated virulence factors in *P. aeruginosa*, as well as biofilm formation which is under the tight control of QS systems [181]. Crystal violate staining is widely used to quantify biofilm biomass in microtiter plates. Plating and resazurin staining are also used in our study to quantify live cells in biofilms. Fluorescence and confocal microscope are useful to detect changes in biofilm structures after QSI treatment. In addition, model systems of biofilm are also useful to study the effect of QSIs on biofilms, such as continuous-flow reactors for biofilm formation [314, 469] and the wound model used in our study.

4.3 Analysis of expression of QS-regulated genes

Quantitative real-time PCR (RT-qPCR) is commonly used to detect changes in the expression of selected core genes in QS systems to confirm the QS inhibition activity. However, transcriptome analysis by microarray analysis or RNA-sequencing might better indicate the overall effect of QSIs on the QS network and virulence, especially for QSIs with undefined mechanisms and for pathogens as *P. aeruginosa* with complex QS network.

4.4 Virulence in *in vivo* models

Many studies on QQ enzymes and QSIs go one step further and investigate their effects on *in vivo* virulence of pathogens. As discussed in Chapter I, *C. elegans* and *G. mellonella* are simple infection models used in QSI studies. The advantages of these models are the small sizes and simple experiment operations. Despite the simplicity of these models, *C. elegans* and *G. mellonella* give valuable information about the effectiveness of QQ enzymes and QSIs under *in vivo* conditions. Different mouse models are also commonly used in studies for QS inhibition to mimic the conditions in various types of infections, such as foreign-body implant model [331], pulmonary mouse model [314] and urinary tract infections model [333].

5. Comparison of transcriptome studies on QS inhibition in *P. aeruginosa*

Transcriptome analysis has been shown to be a valuable tool to study QS regulons and the influence of QSIs in *P. aeruginosa*. To identify QS-regulated genes in *P. aeruginosa*, several groups performed transcriptome analysis with *lasI rhlI* mutants. Wagner *et al*. identified that 394 genes are positively regulated and 222 genes are negatively regulated by QS in *P. aeruginosa* [411]. Schuster *et al*. showed that 315 genes are QS-induced and 38 genes are QS-repressed [78]. In another study, Hentzer *et al*. reported that 163 genes can be activated by QS [314]. Approximately 100 common QS-regulated genes were found in all three studies and the dominant functional class of these genes is secreted factors including toxins and extracellular enzymes [79]. Due to the differences in data analysis and experimental conditions, most of QS-regulated genes were not simultaneously reported in all three studies. In addition, growth medium and oxygen availability can affect the transcript abundance of many QS-regulated genes [411]. In another study using a semi-defined medium that limits the final densities of *P. aeruginosa*, only 79 genes were found to be activated by QS [470].

Similarly, the number of genes targeted by the different QSIs also varies a lot (Table 4.1), and this variation might also be due to different mechanisms of QSIs, as well as different concentrations and treatment times used in these studies. Most QSIs affect the expression of a considerable number of genes in *P. aeruginosa*, except ajoene which only down-regulated 11 genes related to production of LasA protease, chitinase and rhamnolipids. Interestingly, changes in QS-controlled virulence genes

such as *lasA*, *lasB*, *rhlAB*, *phzC-G*, *katA* and *chiC* were found to be more significant compared to the changes in core QS genes, indicating that minor changes in core QS genes may be able to repress these virulence factors. Correspondingly, no significant changes were shown in core QS genes when cells were exposed to QSIs such as ajoene and patulin, but QS-regulated virulence genes were significantly down-regulated by these QSIs. An explanation offered in several studies for these observations is that some QSIs might interfere with QS regulators at the post-transcriptional level instead of inhibiting the transcription of genes in the central part of QS circuit [304, 314]. However, it could also be possible that changes in expression of core QS genes were not detected due to the experimental and statistical analysis methods used in these studies. In the case of 6-gingerol, no changes in the expression of *lasI*, *lasR* and *rhlI* were found by microarray analysis, but the RT-qPCR results in the same study showed that these genes were also significantly repressed by 6-gingerol [328]. In addition, transcriptome analysis on the same QSI in different studies may lead to quite distinct results. In the case of azithromycin (2 μg/mL), Nalca *et al*. showed that 29 and 78 genes were down- and up-regulated in *P. aeruginosa* PAO1 (Table 4.1), whereas in the study of Skindersoe *et al*. 227 and 49 genes were down- and up-regulated [320]. The different results in these two studies might be partly explained by the different origins of *P. aeruginosa* PAO1 and the different media used [320].

Despite the variations in transcriptome studies, most QSIs are reported to repress multiple QS-regulated virulence factors, such as LasA protease, LasB elastase, rhamnolipids, pyocyanin, hydrogen cyanide and chitinase either in planktonic or biofilm cells. Transcriptome analysis on biofilm cells treated with 6-gingerol and coumarin indicated that more genes might be affected by QSIs in biofilm compared to the planktonic cells. Indeed, in the study of Hentzer *et al*., 254 genes were found to be induced by AHL in *P. aeruginosa* biofilms, whereas 163 genes were induced in planktonic cells [314]. In addition, 86% of genes highly induced by AHLs in planktonic cells were also found to be induced in biofilm cells. These results suggest that QS might regulate more genes in biofilms compared to planktonic cells, but the commonly identified QS regulons [79] might be consistent in planktonic and biofilm cells.

Table 5.1 Transcriptome studies of *P. aeruginosa* treated by various QSIs

QSIs	Cell types	Number of genes affected		Down-regulated core QS genes	Reference
		Down-regulated	Up-regulated		
Furanone C30	Planktonic cells	85	8	*phnAB*	[314]
6-Gingerol	Biofilm cells	321	374	*rhlR, phnAB, pqsB-E*	[328]
Curcumin	Planktonic cells	502	214	*lasI*	[437]
mBTL	Planktonic cells	213	13	*lasR, pqsABCDE, phnB*	[326]
Ajoene	Planktonic cells	11	5	None	[298]
Azithromycin	Planktonic cells	29	78	*pqsA* (up-regulated)	[319]
Ceftazidime	Planktonic cells	136	10	None	[320]
Ciprofloxacin	Planktonic cells	223	58	None	[320]
Patulin	Planktonic cells	157 in total		None	[304]
Penicillic acid	Planktonic cells	300 in total		None	[304]
Protoanemonin	Planktonic cells	37	45	None	[471]
Coumarin	Biofilm cells	399	234	*lasI, rhlIR, pqsBC pqsH, ambBCDE*	This study

6. Will QS inhibition lead to resistance?

Another intensely discussed issue about QS interference therapy is that there is increasing evidence showing that resistance to QS inhibition can develop. The possibility for bacteria to evolve resistance to QS inhibition was first systematically discussed by Defoirdt *et al*. [290], based on the observation that variations between different stains exist in core QS genes related to production and reception of signal molecules. Besides, selective stress might also be induced under the conditions that QS inhibition may affect the fitness of pathogens.

Subsequently, resistance to furanone C30 was reported to arise rapidly in *P. aeruginosa* through mutations related to efflux pumps when functional *lasR* was needed for growth on adenosine in minimal medium [472]. Another study showed that furanone C30 resistant *P. aeruginosa* strains were selected in the presence of C30 and oxidative stress [473], indicating that stress might result in strong selection

for bacteria resistant to QS inhibitors as QS may enhance the stress response [474]. This result suggests that reactive oxygen species released by the host immune systems during infection might accelerate the selection of resistant strains against QS disruption.

Several competition studies with QS-deficient and QS-proficient strains have been performed to explore if QS inhibition resistant strains can be selected [474]. Gerdt *et al.* demonstrated that two barriers might impede the spread of QSI resistance: insufficient native QS signal levels prevent QS-regulated gene expression in QSI-resistant bacteria, and QSI sensitive strains can exploit the group-beneficial QS-regulated products by social cheating [475]. These results support the promise of QSIs as a resistance-proof therapy.

However, it is also noteworthy that many known QSIs exhibit both antimicrobial and antivirulence effects depending on the concentrations, and this is particularly true for several antibiotics used in sub-inhibitory concentrations as QSIs. In the case of azithromycin, although its QS inhibition and antivirulence effects have been demonstrated *in vitro* [321, 476] and the therapeutic efficacy of azithromycin in cystic fibrosis patients has been proven in many clinical trials [341, 477], it is difficult to assess the contribution of these activities to the efficacy of azithromycin *in vivo* because of concomitant antivirulence and bactericidal effects under certain conditions [478]. Even used in sub-inhibitory concentrations, resistance can possibly be selected [479, 480], and this may also happen with other QSI compounds with antimicrobial activity. In addition, the growth inhibitory effect of QSIs on different bacterial species varies greatly. As indicated by the results in Chapter III Paper 2 and Paper 3, coumarin affected the growth of *A. baumannii* at 250 μM, while the growth of *P. aeruginosa* strains is not significantly affected by 1 or 2 mM coumarin [392]. In another study, *Vibrio splendidus* was treated with 6.75 mM coumarin and no significant effect on cell growth was observed [393]. Therefore, if the concentration is not properly chosen, treatment with QSIs in multi-species infections may induce higher selective stress on certain species and lead to the emergence of resistance in these species.

Nevertheless, based on the current knowledge, it is still not possible to conclude whether resistance against QS inhibition can be wide-spread under drug selection

and how fast the QSI resistance can evolve, as different outcomes are possible depending on the specific conditions at the site of infections [291, 474, 481]. Further evolution studies in appropriate models may give us a better understanding on this issue.

7. Comparison of QQ enzymes and QSIs as antivirulence therapy

QQ enzymes and QSIs are two major approaches for QS inhibitions. In the current study, we showed that both QQ enzymes and QSIs are promising agents to inhibit biofilm formation and virulence in *P. aeruginosa* and *A. baumannii*. The effectiveness of QQ enzyme and QSIs are seldom compared under the same experimental conditions. In a recent study, the effect of engineered lactonase SsoPox was compared with furanone C30 and 5-fluorouracil against several clinical *P. aeruginosa* isolates at their optimized concentrations, respectively [256]. Their results showed that SsoPox is more active in reducing the production of pyocyanin and protease and inhibiting biofilm formation in 16 *P. aeruginosa* strains, whereas the well-characterized QSI furanone C30 only slightly inhibited the protease production in five strains, the pyocyanin production in two strains and biofilm formation in one strain. Many strains even showed higher production of virulence factors when treated with C30. Although we cannot conclude that the use of QQ enzymes is a better anti-virulence approach compared to QSIs based on this study alone, their results initiate the thinking that QQ enzymes might have advantages over some QSIs under certain conditions.

QQ enzymes degrade signal molecules extracellular instead of targeting intracellular receptors, and it might not be possible to induce resistance mechanisms such as increased efflux pumps against them. QQ enzymes are less toxic compared to QSIs such as C30, and might be safer to be used in human. However, resistance mechanisms against QQ enzymes may emerge by degrading the QQ enzymes, increasing the production of signal molecules or decreasing the detection threshold of signal molecules. Currently, the physiological function of various QQ enzymes in nature remains largely unknown, therefore unexpected outcomes might occur when applied *in vivo*. QQ enzymes that can degrade a broad range of AHLs must be assessed with great care, as they might affect other commensal bacteria in complex

communities. In addition, the large-scale production, the stability under *in vivo* environment and the tolerance to industrial process also needs to be solved before further applications of QQ enzymes.

Compared to QQ enzymes, QSIs can be easily modified by synthetic methods. Some QSIs are designed to target receptors in specific pathogen, and this may minimize the effect of these QSIs on non-pathogenic bacteria. QSIs with low molecular weights can easily be administered orally or intravenously as other drugs. In addition, QSIs identified from known drugs ('repurposing') may facilitate their future application in the clinic.

8. Future perspectives for QS inhibition studies

8.1 Future perspectives for QQ enzymes

Currently, a large number of QQ enzymes have been identified from different origins. This diversity of QQ enzymes provides valuable resources to obtain modified QQ enzymes that are more active and stable. QQ enzymes with a broad substrate range may be useful in treating multi-species infections, but engineered QQ enzymes with high specificity towards AHLs can help us develop a more selective QS inhibition strategy targeting certain pathogens. Development of QQ membranes and catheters with immobilized QQ enzymes gets one step further towards the application of QQ enzymes in medical devices. Topical application with a QQ enzyme containing gel in combination with ciprofloxacin has been showed to reduce mortality against *P. aeruginosa* in a burn infection model on mice [269]. Therefore, future development of dressings and bandages with QQ enzymes may be a promising way to treat wound infections.

Meanwhile, more efforts are needed to elucidate the physiological role of QQ enzymes either in bacteria or in the complex natural communities. Recently, the AHL-acylase from *Acidovorax* sp. was found to degrade a wide range of β-lactams [239]. This result reminds us to be cautious with the possible overlooked functions of QQ enzymes in future studies. In addition, evaluating the QQ enzymes on different clinical strains and in different *in vivo* models might give a deeper understanding on their effects during infections.

8.2 Future perspectives for QSIs

Although increasing numbers of QSIs have been reported, the continuous search for novel QSIs is important for future studies to get more effective and reliable QSIs targeting different pathogens. In the meantime, the current knowledge of known QSIs needs to be enriched, including their targets and mechanisms, as well as their influences on other physiologic process in bacteria except QS. For complex pathogens as *P. aeruginosa*, continued basic research about their QS network in natural human habitats will promote the identification of truly effective QSIs, and more efforts are needed to understand the interactions of QSIs with the core QS systems, various QS regulons and environmental stress *in vivo*. Further metabolomic and proteomic studies may shed more light about the effect of QSIs under *in vivo* conditions.

Combination of QSIs and antibiotics has been studied as a possible way for the further application of QSIs. Besides, combination of QSIs with other anti-virulence agents targeting biofilms and secretion systems might also be an effective approach to be proved in future studies.

8.3 Future perspectives for QS inhibition therapy in clinic

Although promising results with QQ enzymes and QSIs have constantly been reported *in vitro* and *in vivo*, there is still a long distance from the bench to the clinic for QS inhibition therapy. Besides those fundamental questions regarding the effectiveness of QS inhibition and possible resistance mechanisms, other problems related to drug delivery, stability and toxicity are yet to be solved for successful application of QQ enzymes and QSIs. However, we are pleased to see that several small-scale clinical trials with specific QSIs have been performed [341, 342]. As our knowledge on QS inhibition therapy expands, we will get closer to broad-scale clinical trials to investigate the potential of QS inhibition therapy.

CHAPTER V.

SUMMARY - SAMENVATTING

QS systems of many clinical relevant pathogens are involved in regulating various processes associated with virulence. This allows bacteria to achieve a coordinated attack on the host at a high population density and overcome the host defense successfully. Therefore, QS inhibition either by QQ enzymes or QSIs has been considered as an attractive therapeutic strategy to treat infections caused by pathogens such as *P. aeruginosa* and *A. baumannii*. In this dissertation, we explored the effects of typical QQ enzyme and QSIs on *P. aeruginosa* and *A. baumannii*, aiming to identify promising QS inhibition agents to reduce virulence and biofilm formation of *P. aeruginosa* and *A. baumannii*.

In the first part of this dissertation, we purified the QQ enzyme MomL and evaluated its effect on biofilms of *P. aeruginosa* and *A. baumannii* strains. MomL reduced biofilm formation and increased biofilm susceptibility to different antibiotics in biofilms of *P. aeruginosa* PAO1 and *A. baumannii* LMG 10531 formed in microtiter plates *in vitro*. However, MomL showed no effect on the overall cell number in a mixed species biofilm formed by *P. aeruginosa* and *A. baumannii*, and the same disappointing results were obtained in biofilms formed in wound model system. The virulence of *A. baumannii* in the *C. elegans* infection model was not affected by MomL. Additionally, the effect of MomL on biofilm formation of *Acinetobacter* strains seems to be strain-dependent. No effects of MomL on biofilm formation were detected for three out of five *Acinetobacter* strains tested. These results indicated that although promising anti-biofilm effect of MomL was observed against *P. aeruginosa* and *A. baumannii* biofilms formed in microtiter plates, the effect of MomL and other QQ enzymes on biofilm formation under conditions more likely to mimic the real-life situation might be much less pronounced or even absent.

In the second part, we focused on the effect of QS inhibitor coumarin on biofilms and virulence of *P. aeruginosa* in the context of wound infections. The QS inhibition activity of coumarin was confirmed in the biosensor *P. aeruginosa* QSIS2. The production of pyocyanin and protease was reduced by coumarin in several *P. aeruginosa* wound isolates. Coumarin also inhibited the biofilm formation of all six *P. aeruginosa* strains tested in microtiter plates; three strains showed reduced biofilm formation in would model when treated with coumarin. We further evaluated the effect of coumarin on the virulence of *P. aeruginosa* to *L. sericata* maggots, significantly increased survival was observed against two *P. aeruginosa* strains with

coumarin treatment. RNA-sequencing results on *P. aeruginosa* biofilm cells showed that several key genes involved in the *las*, *rhl*, PQS and IQS systems were down-regulated by coumarin, as well as genes responsible for the QS-regulated virulence factor such as alkaline protease, hydrogen cyanide, LasA protease, LasB elastase, phenazine, siderophores and rhamnolipids. Intriguingly, coumarin down-regulated genes related to type III secretion system in *P. aeruginosa* planktonic and biofilm cells. The cellular level of c-di-GMP was also decreased by coumarin through the up-regulation of *tpbA*. These results indicated that coumarin might reduce biofilms and virulence of *P. aeruginosa* by affecting QS, type III secretion and c-di-GMP levels, and might be promising in treating *P. aeruginosa* infections.

Finally, we evaluated the effects of 12 known QSIs on *A. baumannii*. Among these compounds, baicailin hydrate, curcumin and quercetin significantly reduced biofilm formation of two *A. baumannii* strains without affecting planktonic cell growth. (+)-Catechin hydrate, curcumin, indole and quercetin were found to inhibit the twitching motility of *A. baumannii*. Only two QSIs, curcumin and quercetin, significantly inhibited both biofilm formation and twitching motility of *A. baumannii*. We further evaluated the effect of curcumin and quercetin on the virulence of *A. baumannii* in a *G. mellonella* infection model. The survival of infected larvae was increased by approx. 30% and 50% when treated by quercetin and curcumin, respectively. Therefore, curcumin and quercetin might be effective QSIs to inhibit biofilm formation and virulence of *A. baumannii*.

To conclude, our data demonstrated that QQ enzyme and QSIs can effectively inhibit virulence and biofilms of *P. aeruginosa* and *A. baumannii* under certain conditions. However, their effects can be strain dependent and might be reduced in the more complex environment encountered *in vivo*. Discovery of novel QSIs and further reseach on the mechanism of known QSIs may help us to identify truly potent QSIs targeting *P. aeruginosa* and *A. baumannii*. Evaluation of QQ enzyme and QSIs in more elaborate model systems and on clinical strains is also required to futher prove their effectiveness.

Quorum sensing (QS)-systemen van veel klinisch relevante pathogenen zijn betrokken bij het reguleren van verschillende processen geassocieerd met virulentie. Hierdoor kunnen bacteriën bij hoge celdensiteit een gecoördineerde aanval op de gastheer uitvoeren en de verdedigingsmechanismen van de gastheer met succes overwinnen. Daarom wordt inhibitie van QS door enzymen met quorum quenching (QQ) activiteit en conventionele QS-inhibitoren (QSI's) beschouwd als een aantrekkelijke therapeutische strategie voor de behandeling van infecties veroorzaakt door pathogenen zoals *Pseudomonas aeruginosa* en *Acinetobacter baumannii*. In dit proefschrift hebben we de effecten onderzocht van een QQ-enzym en verschillende QSI's op *P. aeruginosa* en *A. baumannii*, met als doel veelbelovende QS-remmende middelen te identificeren om virulentie en biofilmvorming van deze organismen te verminderen.

In het eerste deel van dit proefschrift hebben we het QQ-enzym MomL opgezuiverd en het effect op biofilms van *P. aeruginosa* en *A. baumannii*-stammen geëvalueerd. MomL verminderde de biofilmvorming en verhoogde de biofilmgevoeligheid voor verschillende antibiotica in biofilms van *P. aeruginosa* PAO1 en *A. baumannii* LMG 10531, gevormd in microtiterplaten *in vitro*. MomL had echter geen effect op het totale aantal cellen in een gemengde biofilm gevormd door *P. aeruginosa* én *A. baumannii*, en dezelfde teleurstellende resultaten werden verkregen in biofilms gevormd in een wondmodelsysteem. De virulentie van *A. baumannii* in het *Caenorhabditis elegans*-infectiemodel werd niet beïnvloed door MomL. Bovendien lijkt het effect van MomL op de biofilmvorming van *Acinetobacter*-stammen afhankelijk van de stam; er werd immers geen effecten van MomL op biofilmvorming gedetecteerd voor drie van de vijf geteste *Acinetobacter*-stammen. Deze resultaten gaven aan dat, alhoewel een veelbelovend anti-biofilmeffect van MomL werd waargenomen tegen *P. aeruginosa* en *A. baumannii* biofilms gevormd in microtiterplaten, het effect van MomL en andere QQ-enzymen op biofilmvorming onder *in vivo*-achtige omstandigheden veel minder uitgesproken of zelfs afwezig kan zijn.

In het tweede deel hebben we ons gericht op het effect van QS-remmer coumarine op biofilms en virulentie van *P. aeruginosa* in de context van wondinfecties. De QSI activiteit van coumarine werd bevestigd in de biosensor *P. aeruginosa* QSIS2. De productie van pyocyanine en protease werd gereduceerd door coumarine in

verschillende *P. aeruginosa* wondisolaten. Coumarine remde ook de biofilmvorming van alle zes *P. aeruginosa*-stammen die werden getest in microtiterplaten; drie stammen toonden verminderde biofilmvorming in dit model wanneer behandeld met coumarine. We evalueerden verder het effect van coumarine op de virulentie van *P. aeruginosa* in *Lucilia sericata*-maden; hierbij werd een significant verhoogde overleving waargenomen bij twee *P. aeruginosa*-stammen na coumarinebehandeling. Resultaten van RNA-sequencing op *P. aeruginosa* biofilmcellen toonden aan dat verschillende sleutelgenen betrokken bij de las-, rhl-, PQS- en IQS-systemen down-gereguleerd waren door coumarine, evenals genen die verantwoordelijk zijn voor de productie van QS-gereguleerde virulentiefactoren zoals alkalische protease, waterstofcyanide, LasA-protease, LasB-elastase, fenazine, sideroforen en rhamnolipiden. Intrigerend zijn coumarine down-gereguleerde genen gerelateerd aan type III secretiesysteem in *P. aeruginosa* planktonische en biofilmcellen. Het cellulaire niveau van c-di-GMP werd ook verlaagd door coumarine door de opregulatie van *tpbA*. Deze resultaten gaven aan dat coumarine biofilmvorming en virulentie van *P. aeruginosa* zou kunnen verminderen door QS, type III-secretie en c-di-GMP-niveaus te beïnvloeden, en dus mogelijk bruikbaar zou kunnen zijn bij de behandeling van *P. aeruginosa*-infecties.

Ten slotte evalueerden we de effecten van 12 bekende QSI's op *A. baumannii*. Van deze verbindingen verminderden baicailinehydraat, curcumine en quercetine de biofilmvorming van twee *A. baumannii*-stammen zonder de groei van planktoncellen te beïnvloeden. (+) - Catechinehydraat, curcumine, indool en quercetine bleken de motiliteit van *A. baumannii* te remmen. Slechts twee QSI's, curcumine en quercetine, remden significant de vorming van zowel biofilm als twitching motility van *A. baumannii*. We evalueerden verder het effect van curcumine en quercetine op de virulentie van *A. baumannii* in een *Galleria mellonella*-infectiemodel. De overleving van geïnfecteerde larven werd verhoogd met ongeveer 30% en 50% wanneer behandeld werd met respectievelijk quercetine en curcumine. Daarom kunnen curcumine en quercetine effectieve QSI's zijn om biofilmvorming en virulentie van *A. baumannii* te remmen.

Onze data toonden aan dat het QQ-enzym MomL en diverse QSI's onder bepaalde omstandigheden effectief virulentie en biofilms van *P. aeruginosa* en *A. baumannii* kunnen remmen. De effecten ervan kunnen echter afhankelijk zijn van de stam en

kunnen worden verminderd in de meer complexe omgeving die *in vivo* wordt aangetroffen. Ontdekking van nieuwe QSI's en verder onderzoek naar het mechanisme van bekende QSI's kan ons helpen om echt krachtige QSI's te identificeren die zich richten op *P. aeruginosa* en *A. baumannii*. Evaluatie van QQ-enzymen en QSI's in relevante(re) modelsystemen en op klinische stammen is hierbij vereist om hun doeltreffendheid verder te bewijzen.

REFERENCES

1. Organization WH: **Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics**. *Geneva: World Health Organization* 2017.
2. Gellatly SL, Hancock RE: ***Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses**. *Pathogens and disease* 2013, **67**(3):159-173.
3. Streeter K, Katouli M: ***Pseudomonas aeruginosa*: a review of their pathogenesis and prevalence in clinical settings and the environment**. *Infection, Epidemiology and Microbiology* 2016, **2**(1): 25-32.
4. McConnell MJ, Actis L, Pachón J: ***Acinetobacter baumannii*: human infections, factors contributing to pathogenesis and animal models**. *FEMS microbiology reviews* 2013, **37**(2):130-155.
5. Lee C-R, Lee JH, Park M, Park KS, Bae IK, Kim YB, Cha C-J, Jeong BC, Lee SH: **Biology of *Acinetobacter baumannii*: pathogenesis, antibiotic resistance mechanisms, and prospective treatment options**. *Frontiers in cellular and infection microbiology* 2017, **7**:55.
6. Harding CM, Hennon SW, Feldman MF: **Uncovering the mechanisms of *Acinetobacter baumannii* virulence**. *Nature Reviews Microbiology* 2017.
7. Wróblewska M: **Novel therapies of multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter* spp. infections: the state of the art**. *Archivum immunologiae et therapiae experimentalis* 2006, **54**(2):113-120.
8. Bonomo RA, Szabo D: **Mechanisms of multidrug resistance in *Acinetobacter* species and *Pseudomonas aeruginosa***. *Clinical infectious diseases* 2006, **43**(Supplement_2):S49-S56.
9. Cai Y, Chai D, Wang R, Liang B, Bai N: **Colistin resistance of *Acinetobacter baumannii*: clinical reports, mechanisms and antimicrobial strategies**. *Journal of antimicrobial chemotherapy* 2012, **67**(7):1607-1615.
10. Regenbogen B, Willmann M, Steglich M, Bunk B, Nübel U, Peter S, Neher RA: **Rapid and consistent evolution of colistin resistance in XDR *Pseudomonas aeruginosa* during morbidostat culture**. *Antimicrobial agents and chemotherapy* 2017:AAC. 00043-00017.
11. Bjarnsholt T: **The role of bacterial biofilms in chronic infections**. *Apmis* 2013, **121**(s136):1-58.
12. Lebeaux D, Ghigo J-M, Beloin C: **Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics**. *Microbiology and Molecular Biology Reviews* 2014, **78**(3):510-543.
13. Römling U, Balsalobre C: **Biofilm infections, their resilience to therapy and innovative treatment strategies**. *Journal of internal medicine* 2012, **272**(6):541-561.
14. Sharma G, Rao S, Bansal A, Dang S, Gupta S, Gabrani R: ***Pseudomonas aeruginosa* biofilm: potential therapeutic targets**. *Biologicals* 2014, **42**(1):1-7.
15. Rodríguez-Baño J, Marti S, Soto S, Fernández-Cuenca F, Cisneros JM, Pachón J, Pascual A, Martínez-Martínez L, McQueary C, Actis L: **Biofilm formation in *Acinetobacter baumannii*: associated features and clinical implications**. *Clin Microbiol Infect* 2008, **14**(3):276-278.
16. Longo F, Vuotto C, Donelli G: **Biofilm formation in *Acinetobacter baumannii***. *New Microbiol* 2014, **37**(2):119-127.
17. Demidova-Rice TN, Hamblin MR, Herman IM: **Acute and impaired wound healing: pathophysiology and current methods for drug delivery, part 1: normal and chronic wounds: biology, causes, and approaches to care**. *Advances in skin & wound care* 2012, **25**(7):304.

18. Rhoads DD, Cox SB, Rees EJ, Sun Y, Wolcott RD: **Clinical identification of bacteria in human chronic wound infections: culturing vs. 16S ribosomal DNA sequencing**. *BMC infectious diseases* 2012, **12**(1):321.
19. Robson MC: **Wound infection: a failure of wound healing caused by an imbalance of bacteria**. *Surgical Clinics* 1997, **77**(3):637-650.
20. Garzoni C, Emonet S, Legout L, Benedict R, Hoffmeyer P, Bernard L, Garbino J: **Atypical infections in tsunami survivors**. *Emerging Infect Dis* 2005, **11**(10):1591.
21. Weinstein RA, Gaynes R, Edwards JR, System NNIS: **Overview of nosocomial infections caused by gram-negative bacilli**. *Clinical infectious diseases* 2005, **41**(6):848-854.
22. Serra R, Grande R, Butrico L, Rossi A, Settimio UF, Caroleo B, Amato B, Gallelli L, de Franciscis S: **Chronic wound infections: the role of *Pseudomonas aeruginosa* and *Staphylococcus aureus***. *Expert review of anti-infective therapy* 2015, **13**(5):605-613.
23. Turner KH, Everett J, Trivedi U, Rumbaugh KP, Whiteley M: **Requirements for *Pseudomonas aeruginosa* acute burn and chronic surgical wound infection**. *PLoS Genet* 2014, **10**(7):e1004518.
24. Zago CE, Silva S, Sanitá PV, Barbugli PA, Dias CMI, Lordello VB, Vergani CE: **Dynamics of biofilm formation and the interaction between *Candida albicans* and methicillin-susceptible (MSSA) and-resistant *Staphylococcus aureus* (MRSA)**. *PloS one* 2015, **10**(4):e0123206.
25. Zhao G, Usui ML, Underwood RA, Singh PK, James GA, Stewart PS, Fleckman P, Olerud JE: **Time course study of delayed wound healing in a biofilm-challenged diabetic mouse model**. *Wound Repair and Regeneration* 2012, **20**(3):342-352.
26. Seth AK, Geringer MR, Galiano RD, Leung KP, Mustoe TA, Hong SJ: **Quantitative comparison and analysis of species-specific wound biofilm virulence using an in vivo, rabbit-ear model**. *Journal of the American College of Surgeons* 2012, **215**(3):388-399.
27. Que Y-A, Hazan R, Ryan CM, Milot S, Lépine F, Lydon M, Rahme LG: **Production of *Pseudomonas aeruginosa* intercellular small signaling molecules in human burn wounds**. *Journal of pathogens* 2011, **2011**.
28. Azzopardi EA, Azzopardi E, Camilleri L, Villapalos J, Boyce DE, Dziewulski P, Dickson WA, Whitaker IS: **Gram negative wound infection in hospitalised adult burn patients-systematic review and metanalysis**. *PloS one* 2014, **9**(4):e95042.
29. Bangera D, Shenoy SM, Saldanha DR: **Clinico-microbiological study of *Pseudomonas aeruginosa* in wound infections and the detection of metallo-β-lactamase production**. *International wound journal* 2016, **13**(6):1299-1302.
30. Nusbaum AG, Gil J, Rippy MK, Warne B, Valdes J, Claro A, Davis SC: **Effective method to remove wound bacteria: comparison of various debridement modalities in an in vivo porcine model**. *J Surg Res* 2012, **176**(2):701-707.
31. Sun X, Jiang K, Chen J, Wu L, Lu H, Wang A, Wang J: **A systematic review of maggot debridement therapy for chronically infected wounds and ulcers**. *International journal of infectious diseases* 2014, **25**:32-37.
32. Marineau ML, Herrington MT, Swenor KM, Eron LJ: **Maggot debridement therapy in the treatment of complex diabetic wounds**. *Hawaii medical journal* 2011, **70**(6):121.
33. Naik G, Harding KG: **Maggot debridement therapy: the current perspectives**. *Chronic Wound Care Management and Research* 2017, **4**:121-128.

34. Andersen A, Joergensen B, Bjarnsholt T, Johansen H, Karlsmark T, Givskov M, Krogfelt KA: **Quorum-sensing-regulated virulence factors in *Pseudomonas aeruginosa* are toxic to *Lucilia sericata* maggots**. *Microbiology* 2010, **156**(2):400-407.

35. Wagner S, Sommer R, Hinsberger S, Lu C, Hartmann RW, Empting M, Titz A: **Novel strategies for the treatment of *Pseudomonas aeruginosa* infections**. *Journal of medicinal chemistry* 2016, **59**(13):5929-5969.

36. Hurley MN, Cámara M, Smyth AR: **Novel approaches to the treatment of *Pseudomonas aeruginosa* infections in cystic fibrosis**. *Eur Respir J* 2012, **40**(4):1014-1023.

37. García-Quintanilla M, Pulido MR, López-Rojas R, Pachón J, McConnell MJ: **Emerging therapies for multidrug resistant *Acinetobacter baumannii***. *Trends Microbiol* 2013, **21**(3):157-163.

38. Nealson KH, Platt T, Hastings JW: **Cellular control of the synthesis and activity of the bacterial luminescent system**. *J Bacteriol* 1970, **104**(1):313-322.

39. Nealson K, Hastings JW: **Bacterial bioluminescence: its control and ecological significance**. *Microbiological reviews* 1979, **43**(4):496.

40. Eberhard A, Burlingame A, Eberhard C, Kenyon G, Nealson K, Oppenheimer N: **Structural identification of autoinducer of *Photobacterium fischeri* luciferase**. *Biochemistry* 1981, **20**(9):2444-2449.

41. Cao J-G, Meighen E: **Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi***. *J Biol Chem* 1989, **264**(36):21670-21676.

42. Waters CM, Bassler BL: **Quorum sensing: cell-to-cell communication in bacteria**. *Annu Rev Cell Dev Biol* 2005, **21**:319-346.

43. Reading NC, Sperandio V: **Quorum sensing: the many languages of bacteria**. *FEMS Microbiol Lett* 2005, **254**(1):1-11.

44. Atkinson S, Williams P: **Quorum sensing and social networking in the microbial world**. *Journal of the Royal Society Interface* 2009:rsif20090203.

45. Miller MB, Bassler BL: **Quorum sensing in bacteria**. *Annual Reviews in Microbiology* 2001, **55**(1):165-199.

46. Heilmann S, Krishna S, Kerr B: **Why do bacteria regulate public goods by quorum sensing?—How the shapes of cost and benefit functions determine the form of optimal regulation**. *Frontiers in microbiology* 2015, **6**:767.

47. Churchill ME, Chen L: **Structural basis of acyl-homoserine lactone-dependent signaling**. *Chemical reviews* 2010, **111**(1):68-85.

48. Case RJ, Labbate M, Kjelleberg S: **AHL-driven quorum-sensing circuits: their frequency and function among the *Proteobacteria***. *The ISME journal* 2008, **2**(4):345.

49. Tang K, Zhang X-H: **Quorum quenching agents: resources for antivirulence therapy**. *Mar Drugs* 2014, **12**(6):3245-3282.

50. Toyofuku M, Morinaga K, Hashimoto Y, Uhl J, Shimamura H, Inaba H, Schmitt-Kopplin P, Eberl L, Nomura N: **Membrane vesicle-mediated bacterial communication**. *The ISME journal* 2017, **11**(6):1504.

51. Fuqua C, Parsek MR, Greenberg EP: **Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing**. *Annu Rev Genet* 2001, **35**(1):439-468.

52. Zhang R-g, Pappas KM, Brace JL, Miller PC, Oulmassov T, Molyneaux JM, Anderson JC, Bashkin JK, Winans SC, Joachimiak A: **Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA**. *Nature* 2002, **417**(6892):971.

53. Ng W-L, Bassler BL: **Bacterial quorum-sensing network architectures**. *Annu Rev Genet* 2009, **43**:197-222.

54. Engebrecht J, Nealson K, Silverman M: **Bacterial bioluminescence: isolation and genetic analysis of functions from Vibrio fischeri**. *Cell* 1983, **32**(3):773-781.

55. Fuqua WC, Winans SC: **A LuxR-LuxI type regulatory system activates Agrobacterium Ti plasmid conjugal transfer in the presence of a plant tumor metabolite**. *J Bacteriol* 1994, **176**(10):2796-2806.

56. McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW: **Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones**. *Microbiology* 1997, **143**(12):3703-3711.

57. Pirhonen M, Flego D, Heikinheimo R, Palva ET: **A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora***. *The EMBO journal* 1993, **12**(6):2467-2476.

58. Welch M, Todd DE, Whitehead NA, McGowan SJ, Bycroft BW, Salmond GP: **N-acyl homoserine lactone binding to the CarR receptor determines quorum-sensing specificity in Erwinia**. *The EMBO journal* 2000, **19**(4):631-641.

59. Suppiger A, Schmid N, Aguilar C, Pessi G, Eberl L: **Two quorum sensing systems control biofilm formation and virulence in members of the *Burkholderia cepacia* complex**. *Virulence* 2013, **4**(5):400-409.

60. Throup JP, Camara M, Briggs GS, Winson M, Chhabra SR, Bycroft BW, Williams P, Stewart GS: **Characterisation of the yenI/yenR locus from *Yersinia enterocolitica* mediating the synthesis of two N-acylhomoserine lactone signal molecules**. *Molecular microbiology* 1995, **17**(2):345-356.

61. Patankar AV, González JE: **Orphan LuxR regulators of quorum sensing**. *FEMS microbiology reviews* 2009, **33**(4):739-756.

62. Sturme MH, Kleerebezem M, Nakayama J, Akkermans AD, Vaughan EE, De Vos WM: **Cell to cell communication by autoinducing peptides in gram-positive bacteria**. *Antonie van Leeuwenhoek* 2002, **81**(1-4):233-243.

63. Kleerebezem M, Quadri LE, Kuipers OP, De Vos WM: **Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria**. *Molecular microbiology* 1997, **24**(5):895-904.

64. Kuipers OP, de Ruyter PG, Kleerebezem M, de Vos WM: **Quorum sensing-controlled gene expression in lactic acid bacteria**. *J Biotechnol* 1998, **64**(1):15-21.

65. Håvarstein LS, Coomaraswamy G, Morrison DA: **An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae***. *Proc Natl Acad Sci U S A* 1995, **92**(24):11140-11144.

66. Novick RP: **Autoinduction and signal transduction in the regulation of staphylococcal virulence**. *Molecular microbiology* 2003, **48**(6):1429-1449.

67. Le KY, Otto M: **Quorum-sensing regulation in staphylococci—an overview**. *Frontiers in microbiology* 2015, **6**:1174.

68. Schauder S, Shokat K, Surette MG, Bassler BL: **The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule**. *Molecular microbiology* 2001, **41**(2):463-476.

69. Bassler BL, Wright M, Silverman MR: **Multiple signalling systems controlling expression of luminescence in Vibrio harveyi: sequence and function of genes encoding a second sensory pathway**. *Molecular microbiology* 1994, **13**(2):273-286.

70. Xavier KB, Bassler BL: **LuxS quorum sensing: more than just a numbers game**. *Curr Opin Microbiol* 2003, **6**(2):191-197.

71. Pereira CS, Thompson JA, Xavier KB: **AI-2-mediated signalling in bacteria**. *FEMS microbiology reviews* 2013, **37**(2):156-181.

72. Duan K, Dammel C, Stein J, Rabin H, Surette MG: **Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication**. *Molecular microbiology* 2003, **50**(5):1477-1491.

73. Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelczer I, Bassler BL, Hughson FM: **Structural identification of a bacterial quorum-sensing signal containing boron**. *Nature* 2002, **415**(6871):545.

74. Pesci EC, Milbank JB, Pearson JP, McKnight S, Kende AS, Greenberg EP, Iglewski BH: **Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa***. *Proc Natl Acad Sci U S A* 1999, **96**(20):11229-11234.

75. Dow JM: **Diffusible signal factor-dependent quorum sensing in pathogenic bacteria and its exploitation for disease control**. *J Appl Microbiol* 2017, **122**(1):2-11.

76. Brachmann AO, Brameyer S, Kresovic D, Hitkova I, Kopp Y, Manske C, Schubert K, Bode HB, Heermann R: **Pyrones as bacterial signaling molecules**. *Nat Chem Biol* 2013, **9**(9):573.

77. Walters M, Sperandio V: **Autoinducer 3 and epinephrine signaling in the kinetics of locus of enterocyte effacement gene expression in enterohemorrhagic Escherichia coli**. *Infect Immun* 2006, **74**(10):5445-5455.

78. Schuster M, Lostroh CP, Ogi T, Greenberg E: **Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis**. *J Bacteriol* 2003, **185**(7):2066-2079.

79. Schuster M, Greenberg EP: **A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa***. *Int J Med Microbiol* 2006, **296**(2-3):73-81.

80. Papenfort K, Bassler BL: **Quorum sensing signal–response systems in Gram-negative bacteria**. *Nature Reviews Microbiology* 2016, **14**(9):576.

81. Gambello MJ, Iglewski BH: **Cloning and characterization of the *Pseudomonas aeruginosa* lasR gene, a transcriptional activator of elastase expression**. *J Bacteriol* 1991, **173**(9):3000-3009.

82. Jones S, Yu B, Bainton Na, Birdsall M, Bycroft B, Chhabra S, Cox A, Golby P, Reeves P, Stephens S: **The lux autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa***. *The EMBO journal* 1993, **12**(6):2477-2482.

83. Pearson JP, Gray KM, Passador L, Tucker KD, Eberhard A, Iglewski BH, Greenberg E: **Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes**. *Proc Natl Acad Sci U S A* 1994, **91**(1):197-201.

84. Gambello MJ, Kaye S, Iglewski BH: **LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (apr) and an enhancer of exotoxin A expression**. *Infect Immun* 1993, **61**(4):1180-1184.

85. Kiratisin P, Tucker KD, Passador L: **LasR, a transcriptional activator of *Pseudomonas aeruginosa* virulence genes, functions as a multimer**. *J Bacteriol* 2002, **184**(17):4912-4919.

86. Pearson JP, Van Delden C, Iglewski BH: **Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals**. *J Bacteriol* 1999, **181**(4):1203-1210.

87. Pearson JP, Passador L, Iglewski BH, Greenberg E: **A second N-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa***. *Proc Natl Acad Sci U S A* 1995, **92**(5):1490-1494.

88. Ochsner UA, Reiser J: **Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa***. *Proc Natl Acad Sci U S A* 1995, **92**(14):6424-6428.

89. Brint JM, Ohman DE: **Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR-LuxI family**. *J Bacteriol* 1995, **177**(24):7155-7163.

90. Leisinger T, Margraff R: **Secondary metabolites of the fluorescent pseudomonads**. *Microbiological Reviews* 1979, **43**(3):422.

91. Mukherjee S, Moustafa D, Smith CD, Goldberg JB, Bassler BL: **The RhlR quorum-sensing receptor controls *Pseudomonas aeruginosa* pathogenesis and biofilm development independently of its canonical homoserine lactone autoinducer**. *PLoS Path* 2017, **13**(7):e1006504.

92. Whiteley M, Greenberg E: **Promoter specificity elements in *Pseudomonas aeruginosa* quorum-sensing-controlled genes**. *J Bacteriol* 2001, **183**(19):5529-5534.

93. Gilbert KB, Kim TH, Gupta R, Greenberg EP, Schuster M: **Global position analysis of the *Pseudomonas aeruginosa* quorum-sensing transcription factor LasR**. *Molecular microbiology* 2009, **73**(6):1072-1085.

94. Gallagher LA, McKnight SL, Kuznetsova MS, Pesci EC, Manoil C: **Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa***. *J Bacteriol* 2002, **184**(23):6472-6480.

95. Heeb S, Fletcher MP, Chhabra SR, Diggle SP, Williams P, Cámara M: **Quinolones: from antibiotics to autoinducers**. *FEMS microbiology reviews* 2011, **35**(2):247-274.

96. Coleman JP, Hudson LL, McKnight SL, Farrow JM, Calfee MW, Lindsey CA, Pesci EC: ***Pseudomonas aeruginosa* PqsA is an anthranilate-coenzyme A ligase**. *J Bacteriol* 2008, **190**(4):1247-1255.

97. Déziel E, Lépine F, Milot S, He J, Mindrinos MN, Tompkins RG, Rahme LG: **Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication**. *Proceedings of the National Academy of Sciences of the United States of America* 2004, **101**(5):1339-1344.

98. Dubern J-F, Diggle SP: **Quorum sensing by 2-alkyl-4-quinolones in *Pseudomonas aeruginosa* and other bacterial species**. *Molecular Biosystems* 2008, **4**(9):882-888.

99. Xiao G, Déziel E, He J, Lépine F, Lesic B, Castonguay MH, Milot S, Tampakaki AP, Stachel SE, Rahme LG: **MvfR, a key *Pseudomonas aeruginosa* pathogenicity LTTR-class regulatory protein, has dual ligands**. *Molecular microbiology* 2006, **62**(6):1689-1699.

100. Sams T, Baker Y, Hodgkinson J, Gross J, Spring D, Welch M: **The *Pseudomonas* quinolone signal (PQS)**. *Israel Journal of Chemistry* 2016, **56**(5):282-294.

101. Déziel E, Gopalan S, Tampakaki AP, Lépine F, Padfield KE, Saucier M, Xiao G, Rahme LG: **The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting lasRI, rhlRI or the production of N-acyl-l-homoserine lactones**. *Molecular microbiology* 2005, **55**(4):998-1014.

102. Lee J, Wu J, Deng Y, Wang J, Wang C, Wang J, Chang C, Dong Y, Williams P, Zhang L-H: **A cell-cell communication signal integrates quorum sensing and stress response**. *Nat Chem Biol* 2013, **9**(5):339.

103. Pesci EC, Pearson JP, Seed PC, Iglewski BH: **Regulation of las and rhl quorum sensing in *Pseudomonas aeruginosa***. *J Bacteriol* 1997, **179**(10):3127-3132.

104. Latifi A, Foglino M, Tanaka K, Williams P, Lazdunski A: **A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS**. *Molecular microbiology* 1996, **21**(6):1137-1146.

105. Dekimpe V, Deziel E: **Revisiting the quorum-sensing hierarchy in *Pseudomonas aeruginosa*: the transcriptional regulator RhlR regulates LasR-specific factors**. *Microbiology* 2009, **155**(3):712-723.

106. Xiao G, He J, Rahme LG: **Mutation analysis of the *Pseudomonas aeruginosa* mvfR and pqsABCDE gene promoters demonstrates complex quorum-sensing circuitry**. *Microbiology* 2006, **152**(6):1679-1686.

107. McGrath S, Wade DS, Pesci EC: **Dueling quorum sensing systems in *Pseudomonas aeruginosa* control the production of the *Pseudomonas* quinolone signal (PQS)**. *FEMS Microbiol Lett* 2004, **230**(1):27-34.

108. Farrow JM, Sund ZM, Ellison ML, Wade DS, Coleman JP, Pesci EC: **PqsE functions independently of PqsR-*Pseudomonas* quinolone signal and enhances the rhl quorum-sensing system**. *J Bacteriol* 2008, **190**(21):7043-7051.

109. Maura D, Hazan R, Kitao T, Ballok AE, Rahme LG: **Evidence for direct control of virulence and defense gene circuits by the *Pseudomonas aeruginosa* quorum sensing regulator, MvfR**. *Scientific reports* 2016, **6**:34083.

110. Defoirdt T, Boon N, Sorgeloos P, Verstraete W, Bossier P: **Quorum sensing and quorum quenching in *Vibrio harveyi*: lessons learned from *in vivo* work**. *The ISME journal* 2008, **2**(1):19.

111. Diggle SP, Winzer K, Chhabra SR, Worrall KE, Cámara M, Williams P: **The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates rhl-dependent genes at the onset of stationary phase and can be produced in the absence of LasR**. *Molecular microbiology* 2003, **50**(1):29-43.

112. Rampioni G, Bertani I, Zennaro E, Polticelli F, Venturi V, Leoni L: **The quorum-sensing negative regulator RsaL of *Pseudomonas aeruginosa* binds to the lasI promoter**. *J Bacteriol* 2006, **188**(2):815-819.

113. de Kievit T, Seed PC, Nezezon J, Passador L, Iglewski BH: **RsaL, a novel repressor of virulence gene expression in *Pseudomonas aeruginosa***. *J Bacteriol* 1999, **181**(7):2175-2184.

114. Rampioni G, Schuster M, Greenberg EP, Bertani I, Grasso M, Venturi V, Zennaro E, Leoni L: **RsaL provides quorum sensing homeostasis and functions as a global regulator of gene expression in *Pseudomonas aeruginosa***. *Molecular microbiology* 2007, **66**(6):1557-1565.

115. Liang H, Deng X, Li X, Ye Y, Wu M: **Molecular mechanisms of master regulator VqsM mediating quorum-sensing and antibiotic resistance in *Pseudomonas aeruginosa***. *Nucleic acids research* 2014, **42**(16):10307-10320.

116. Dong YH, Zhang XF, Xu JL, Tan AT, Zhang LH: **VqsM, a novel AraC-type global regulator of quorum-sensing signalling and virulence in *Pseudomonas aeruginosa***. *Molecular microbiology* 2005, **58**(2):552-564.

117. Kim HY, Schlictman D, Shankar S, Xie Z, Chakrabarty A, Kornberg A: **Alginate, inorganic polyphosphate, GTP and ppGpp synthesis co-regulated in *Pseudomonas aeruginosa*:**

implications for stationary phase survival and synthesis of RNA/DNA precursors. *Molecular microbiology* 1998, **27**(4):717-725.

118. Ledgham F, Soscia C, Chakrabarty A, Lazdunski A, Foglino M: **Global regulation in *Pseudomonas aeruginosa*: the regulatory protein AlgR2 (AlgQ) acts as a modulator of quorum sensing**. *Res Microbiol* 2003, **154**(3):207-213.
119. Diggle SP, Winzer K, Lazdunski A, Williams P, Cámara M: **Advancing the quorum in *Pseudomonas aeruginosa*: MvaT and the regulation of N-acylhomoserine lactone production and virulence gene expression**. *J Bacteriol* 2002, **184**(10):2576-2586.
120. Kong K-F, Jayawardena SR, Indulkar SD, del Puerto A, Koh C-L, Høiby N, Mathee K: ***Pseudomonas aeruginosa* AmpR is a global transcriptional factor that regulates expression of AmpC and PoxB β-lactamases, proteases, quorum sensing, and other virulence factors**. *Antimicrobial agents and chemotherapy* 2005, **49**(11):4567-4575.
121. Longo F, Rampioni G, Bondì R, Imperi F, Fimia GM, Visca P, Zennaro E, Leoni L: **A new transcriptional repressor of the *Pseudomonas aeruginosa* quorum sensing receptor gene lasR**. *PloS one* 2013, **8**(7):e69554.
122. Cao Q, Wang Y, Chen F, Xia Y, Lou J, Zhang X, Yang N, Sun X, Zhang Q, Zhuo C: **A novel signal transduction pathway that modulates rhl quorum sensing and bacterial virulence in *Pseudomonas aeruginosa***. *PLoS Path* 2014, **10**(8):e1004340.
123. Dieppois G, Ducret V, Caille O, Perron K: **The transcriptional regulator CzcR modulates antibiotic resistance and quorum sensing in *Pseudomonas aeruginosa***. *PloS one* 2012, **7**(5):e38148.
124. Reimmann C, Beyeler M, Latifi A, Winteler H, Foglino M, Lazdunski A, Haas D: **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* 1997, **24**(2):309-319.
125. Heurlier K, Williams F, Heeb S, Dormond C, Pessi G, Singer D, Cámara M, Williams P, Haas D: **Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1**. *J Bacteriol* 2004, **186**(10):2936-2945.
126. Kay E, Humair B, Dénervaud V, Riedel K, Spahr S, Eberl L, Valverde C, Haas D: **Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa***. *J Bacteriol* 2006, **188**(16):6026-6033.
127. Brencic A, McFarland KA, McManus HR, Castang S, Mogno I, Dove SL, Lory S: **The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs**. *Molecular microbiology* 2009, **73**(3):434-445.
128. Albus AM, Pesci EC, Runyen-Janecky LJ, West S, Iglewski BH: **Vfr controls quorum sensing in *Pseudomonas aeruginosa***. *J Bacteriol* 1997, **179**(12):3928-3935.
129. Fuchs EL, Brutinel ED, Jones AK, Fulcher NB, Urbanowski ML, Yahr TL, Wolfgang MC: **The *Pseudomonas aeruginosa* Vfr regulator controls global virulence factor expression through cyclic AMP-dependent and-independent mechanisms**. *J Bacteriol* 2010, **192**(14):3553-3564.
130. Croda-García G, Grosso-Becerra V, Gonzalez-Valdez A, Servín-González L, Soberon-Chavez G: **Transcriptional regulation of *Pseudomonas aeruginosa* rhlR: role of the CRP orthologue Vfr (virulence factor regulator) and quorum-sensing regulators LasR and RhlR**. *Microbiology* 2011, **157**(9):2545-2555.

131. Chugani SA, Whiteley M, Lee KM, D'Argenio D, Manoil C, Greenberg E: **QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa***. *Proc Natl Acad Sci U S A* 2001, **98**(5):2752-2757.

132. Ledgham F, Ventre I, Soscia C, Foglino M, Sturgis JN, Lazdunski A: **Interactions of the quorum sensing regulator QscR: interaction with itself and the other regulators of *Pseudomonas aeruginosa* LasR and RhlR**. *Molecular microbiology* 2003, **48**(1):199-210.

133. Lequette Y, Lee J-H, Ledgham F, Lazdunski A, Greenberg EP: **A distinct QscR regulon in the *Pseudomonas aeruginosa* quorum-sensing circuit**. *J Bacteriol* 2006, **188**(9):3365-3370.

134. Chugani S, Greenberg EP: **An evolving perspective on the *Pseudomonas aeruginosa* orphan quorum sensing regulator QscR**. *Frontiers in cellular and infection microbiology* 2014, **4**:152.

135. Juhas M, Wiehlmann L, Huber B, Jordan D, Lauber J, Salunkhe P, Limpert AS, von Götz F, Steinmetz I, Eberl L: **Global regulation of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa***. *Microbiology* 2004, **150**(4):831-841.

136. Viducic D, Murakami K, Amoh T, Ono T, Miyake Y: **Role of the interplay between quorum sensing regulator VqsR and the *Pseudomonas* quinolone signal in mediating carbapenem tolerance in *Pseudomonas aeruginosa***. *Res Microbiol* 2017, **168**(5):450-460.

137. Liang H, Deng X, Ji Q, Sun F, Shen T, He C: **The *Pseudomonas aeruginosa* global regulator VqsR directly inhibits QscR to control quorum-sensing and virulence gene expression**. *J Bacteriol* 2012, **194**(12):3098-3108.

138. Campbell EA, Muzzin O, Chlenov M, Sun JL, Olson CA, Weinman O, Trester-Zedlitz ML, Darst SA: **Structure of the bacterial RNA polymerase promoter specificity σ subunit**. *Mol Cell* 2002, **9**(3):527-539.

139. Heurlier K, Dénervaud V, Pessi G, Reimmann C, Haas D: **Negative control of quorum sensing by RpoN (σ54) in *Pseudomonas aeruginosa* PAO1**. *J Bacteriol* 2003, **185**(7):2227-2235.

140. Whiteley M, Parsek MR, Greenberg E: **Regulation of Quorum Sensing by RpoS in *Pseudomonas aeruginosa***. *J Bacteriol* 2000, **182**(15):4356-4360.

141. Mulcahy H, O'Callaghan J, O'Grady EP, Maciá MD, Borrell N, Gómez C, Casey PG, Hill C, Adams C, Gahan CG: ***Pseudomonas aeruginosa* RsmA plays an important role during murine infection by influencing colonization, virulence, persistence, and pulmonary inflammation**. *Infect Immun* 2008, **76**(2):632-638.

142. Branny P, Pearson JP, Pesci EC, Köhler T, Iglewski BH, Van Delden C: **Inhibition of quorum sensing by a *Pseudomonas aeruginosa* dksA homologue**. *J Bacteriol* 2001, **183**(5):1531-1539.

143. Jude F, Köhler T, Branny P, Perron K, Mayer MP, Comte R, Van Delden C: **Posttranscriptional control of quorum-sensing-dependent virulence genes by DksA in *Pseudomonas aeruginosa***. *J Bacteriol* 2003, **185**(12):3558-3566.

144. Hirsch M, Elliott T: **Role of ppGpp in rpoS stationary-phase regulation in Escherichia coli**. *J Bacteriol* 2002, **184**(18):5077-5087.

145. Gupta R, Gobble TR, Schuster M: **GidA posttranscriptionally regulates rhl quorum sensing in *Pseudomonas aeruginosa***. *J Bacteriol* 2009, **191**(18):5785-5792.

146. Carloni S, Macchi R, Sattin S, Ferrara S, Bertoni G: **The small RNA ReaL: a novel regulatory element embedded in the *Pseudomonas aeruginosa* quorum sensing networks**. *Environ Microbiol* 2017, **19**(10):4220-4237.

147. Sonnleitner E, Gonzalez N, Sorger-Domenigg T, Heeb S, Richter AS, Backofen R, Williams P, Hü ttenhofer A, Haas D, Bläsi U: **The small RNA PhrS stimulates synthesis of the *Pseudomonas aeruginosa* quinolone signal**. *Molecular microbiology* 2011, **80**(4):868-885.

148. Rashid MH, Rumbaugh K, Passador L, Davies DG, Hamood AN, Iglewski BH, Kornberg A: **Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of *Pseudomonas aeruginosa***. *Proc Natl Acad Sci U S A* 2000, **97**(17):9636-9641.

149. Toder D, Ferrell S, Nezezon J, Rust L, Iglewski B: **lasA and lasB genes of *Pseudomonas aeruginosa*: analysis of transcription and gene product activity**. *Infect Immun* 1994, **62**(4):1320-1327.

150. Pessi G, Haas D: **Transcriptional control of the hydrogen cyanide biosynthetic genes hcnabc by the anaerobic regulator anr and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa***. *J Bacteriol* 2000, **182**(24):6940-6949.

151. Latifi A, Winson MK, Foglino M, Bycroft BW, Stewart GS, Lazdunski A, Williams P: **Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1**. *Molecular microbiology* 1995, **17**(2):333-343.

152. Winzer K, Falconer C, Garber NC, Diggle SP, Camara M, Williams P: **The *Pseudomonas aeruginosa* lectins PA-IL and PA-IIL are controlled by quorum sensing and by RpoS**. *J Bacteriol* 2000, **182**(22):6401-6411.

153. Hassett DJ, Ma JF, Elkins JG, McDermott TR, Ochsner UA, West SE, Huang CT, Fredericks J, Burnett S, Stewart PS: **Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide**. *Molecular microbiology* 1999, **34**(5):1082-1093.

154. Matsumoto K: **Role of bacterial proteases in pseudomonal and serratial keratitis**. *Biol Chem* 2004, **385**(11):1007-1016.

155. Bardoel BW, Van Der Ent S, Pel MJ, Tommassen J, Pieterse CM, Van Kessel KP, Van Strijp JA: ***Pseudomonas* evades immune recognition of flagellin in both mammals and plants**. *PLoS Path* 2011, **7**(8):e1002206.

156. Pearson JP, Pesci EC, Iglewski BH: **Roles of *Pseudomonas aeruginosa* las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes**. *J Bacteriol* 1997, **179**(18):5756-5767.

157. Wick M, Hamood A, Iglewski B: **Analysis of the structure-function relationship of *Pseudomonas aeruginosa* exotoxin A**. *Molecular microbiology* 1990, **4**(4):527-535.

158. Pollack M: **The role of exotoxin A in *Pseudomonas* disease and immunity**. *Reviews of infectious diseases* 1983, **5**(Supplement_5):S979-S984.

159. Vidal DR, Garrone P, Banchereau J: **Immunosuppressive effects of *Pseudomonas aeruginosa* exotoxin A on human B-lymphocytes**. *Toxicon* 1993, **31**(1):27-34.

160. Van Gennip M, Christensen LD, Alhede M, Phipps R, Jensen PØ, Christophersen L, Pamp SJ, Moser C, Mikkelsen PJ, Koh AY: **Inactivation of the rhlA gene in *Pseudomonas aeruginosa* prevents rhamnolipid production, disabling the protection against polymorphonuclear leukocytes**. *Apmis* 2009, **117**(7):537-546.

161. Caiazza NC, Shanks RM, O'toole G: **Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa***. *J Bacteriol* 2005, **187**(21):7351-7361.

162. Davey ME, Caiazza NC, O'Toole GA: **Rhamnolipid surfactant production affects biofilm architecture in *Pseudomonas aeruginosa* PAO1**. *J Bacteriol* 2003, **185**(3):1027-1036.

163. Espinosa-Urgel M: **Resident parking only: rhamnolipids maintain fluid channels in biofilms**. *J Bacteriol* 2003, **185**(3):699-700.

164. Boles BR, Thoendel M, Singh PK: **Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms**. *Molecular microbiology* 2005, **57**(5):1210-1223.

165. Reis RS, Pereira AG, Neves BC, Freire DM: **Gene regulation of rhamnolipid production in *Pseudomonas aeruginosa*–a review**. *Bioresour Technol* 2011, **102**(11):6377-6384.

166. Lau GW, Hassett DJ, Ran H, Kong F: **The role of pyocyanin in *Pseudomonas aeruginosa* infection**. *Trends Mol Med* 2004, **10**(12):599-606.

167. Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G, Thomashow LS: **Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1**. *J Bacteriol* 2001, **183**(21):6454-6465.

168. Enderby B, Smith D, Carroll W, Lenney W: **Hydrogen cyanide as a biomarker for *Pseudomonas aeruginosa* in the breath of children with cystic fibrosis**. *Pediatric pulmonology* 2009, **44**(2):142-147.

169. Gallagher LA, Manoil C: ***Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning**. *J Bacteriol* 2001, **183**(21):6207-6214.

170. Vasil ML, Ochsner UA: **The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence**. *Molecular microbiology* 1999, **34**(3):399-413.

171. Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML: **Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa***. *Proc Natl Acad Sci U S A* 2002, **99**(10):7072-7077.

172. Stintzi A, Evans K, Meyer J-m, Poole K: **Quorum-sensing and siderophore biosynthesis in *Pseudomonas aeruginosa*: lasRllasI mutants exhibit reduced pyoverdine biosynthesis**. *FEMS Microbiol Lett* 1998, **166**(2):341-345.

173. Diggle SP, Matthijs S, Wright VJ, Fletcher MP, Chhabra SR, Lamont IL, Kong X, Hider RC, Cornelis P, Cámara M: **The *Pseudomonas aeruginosa* 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment**. *Chem Biol* 2007, **14**(1):87-96.

174. Cornelis P, Aendekerk S: **A new regulator linking quorum sensing and iron uptake in *Pseudomonas aeruginosa***. *Microbiology* 2004, **150**(4):752-756.

175. Doig P, Todd T, Sastry PA, Lee K, Hodges RS, Paranchych W, Irvin R: **Role of pili in adhesion of *Pseudomonas aeruginosa* to human respiratory epithelial cells**. *Infect Immun* 1988, **56**(6):1641-1646.

176. O'toole GA, Kolter R: **Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development**. *Molecular microbiology* 1998, **30**(2):295-304.

177. Bradley DE: **A function of *Pseudomonas aeruginosa* PAO polar pili: twitching motility**. *Can J Microbiol* 1980, **26**(2):146-154.

178. Glessner A, Smith RS, Iglewski BH, Robinson JB: **Roles of *Pseudomonas aeruginosa* las andrhl Quorum-Sensing Systems in Control of Twitching Motility**. *J Bacteriol* 1999, **181**(5):1623-1629.

179. Costerton JW, Stewart PS, Greenberg EP: **Bacterial biofilms: a common cause of persistent infections**. *Science* 1999, **284**(5418):1318-1322.

180. Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP: **The involvement of cell-to-cell signals in the development of a bacterial biofilm**. *Science* 1998, **280**(5361):295-298.

181. De Kievit T: **Quorum sensing in *Pseudomonas aeruginosa* biofilms**. *Environ Microbiol* 2009, **11**(2):279-288.

182. Sakuragi Y, Kolter R: **Quorum-sensing regulation of the biofilm matrix genes (pel) of *Pseudomonas aeruginosa***. *J Bacteriol* 2007, **189**(14):5383-5386.

183. Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, Molin S, Givskov M, Tolker-Nielsen T: **A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms**. *Molecular microbiology* 2006, **59**(4):1114-1128.

184. Parsek MR, Greenberg E: **Sociomicrobiology: the connections between quorum sensing and biofilms**. *Trends Microbiol* 2005, **13**(1):27-33.

185. Valentini M, Filloux A: **Biofilms and cyclic di-GMP (c-di-GMP) signaling: lessons from *Pseudomonas aeruginosa* and other bacteria**. *J Biol Chem* 2016, **291**(24):12547-12555.

186. Krasteva PV, Fong JC, Shikuma NJ, Beyhan S, Navarro MV, Yildiz FH, Sondermann H: **Vibrio cholerae VpsT regulates matrix production and motility by directly sensing cyclic di-GMP**. *science* 2010, **327**(5967):866-868.

187. Ryan RP, Fouhy Y, Lucey JF, Crossman LC, Spiro S, He Y-W, Zhang L-H, Heeb S, Cámara M, Williams P: **Cell–cell signaling in Xanthomonas campestris involves an HD-GYP domain protein that functions in cyclic di-GMP turnover**. *Proc Natl Acad Sci U S A* 2006, **103**(17):6712-6717.

188. Ueda A, Wood TK: **Connecting quorum sensing, c-di-GMP, pel polysaccharide, and biofilm formation in *Pseudomonas aeruginosa* through tyrosine phosphatase TpbA (PA3885)**. *PLoS Path* 2009, **5**(6):e1000483.

189. Rumbaugh KP, Griswold JA, Hamood AN: **The role of quorum sensing in the in vivo virulence of *Pseudomonas aeruginosa***. *Microb Infect* 2000, **2**(14):1721-1731.

190. Niu C, Clemmer KM, Bonomo RA, Rather PN: **Isolation and characterization of an autoinducer synthase from *Acinetobacter baumannii***. *J Bacteriol* 2008, **190**(9):3386-3392.

191. Smith MG, Gianoulis TA, Pukatzki S, Mekalanos JJ, Ornston LN, Gerstein M, Snyder M: **New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis**. *Genes Dev* 2007, **21**(5):601-614.

192. Oh MH, Choi CH: **Role of LuxIR homologue AnoIR in *Acinetobacter* nosocomialis and the effect of virstatin on the expression of anoR gene**. *J Microbiol Biotechnol* 2015, **25**(8):1390-1400.

193. González R, Dijkshoorn L, Van den Barselaar M, Nudel C: **Quorum sensing signal profile of *Acinetobacter* strains from nosocomial and environmental sources**. *Rev Argent Microbiol* 2009, **41**(2):73-78.

194. Erdönmez D, Rad AY, Aksöz N: **Quorum sensing molecules production by nosocomial and soil isolates *Acinetobacter baumannii***. *Arch Microbiol* 2017, **199**(10):1325-1334.

195. John J, Saranathan R, Adigopula LN, Thamodharan V, Singh SP, Lakshmi TP, CharanTej MA, Rao RS, Krishna R, Rao HSP: **The quorum sensing molecule N-acyl homoserine lactone produced by *Acinetobacter baumannii* displays antibacterial and anticancer properties**. *Biofouling* 2016, **32**(9):1029-1047.

196. Lee JC, Koerten H, Van den Broek P, Beekhuizen H, Wolterbeek R, Van den Barselaar M, Van der Reijden T, Van der Meer J, Van de Gevel J, Dijkshoorn L: **Adherence of *Acinetobacter baumannii* strains to human bronchial epithelial cells**. *Res Microbiol* 2006, **157**(4):360-366.

197. Rumbo-Feal S, Gómez MJ, Gayoso C, Álvarez-Fraga L, Cabral MP, Aransay AM, Rodríguez-Ezpeleta N, Fullaondo A, Valle J, Tomás M: **Whole transcriptome analysis of *Acinetobacter baumannii* assessed by RNA-sequencing reveals different mRNA expression profiles in biofilm compared to planktonic cells**. *PloS one* 2013, **8**(8):e72968.

198. Luo L-m, Wu L-j, Xiao Y-l, Zhao D, Chen Z-x, Kang M, Zhang Q, Xie Y: **Enhancing pili assembly and biofilm formation in *Acinetobacter baumannii* ATCC19606 using non-native acyl-homoserine lactones**. *BMC Microbiol* 2015, **15**(1):62.

199. Dou Y, Song F, Guo F, Zhou Z, Zhu C, Xiang J, Huan J: ***Acinetobacter baumannii* quorum-sensing signalling molecule induces the expression of drug-resistance genes**. *Molecular medicine reports* 2017, **15**(6):4061-4068.

200. Bhargava N, Sharma P, Capalash N: **Pyocyanin stimulates quorum sensing-mediated tolerance to oxidative stress and increases persister cell populations in *Acinetobacter baumannii***. *Infect Immun* 2014, **82**(8):3417-3425.

201. López M, Mayer C, Fernández-García L, Blasco L, Muras A, Ruiz FM, Bou G, Otero A, Tomás M: **Quorum sensing network in clinical strains of *A. baumannii*: AidA is a new quorum quenching enzyme**. *PloS one* 2017, **12**(3):e0174454.

202. Dong Y-H, Xu J-L, Li X-Z, Zhang L-H: **AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of Erwinia carotovora**. *Proc Natl Acad Sci U S A* 2000, **97**(7):3526-3531.

203. Pustelny C, Albers A, Büldt-Karentzopoulos K, Parschat K, Chhabra SR, Cámara M, Williams P, Fetzner S: **Dioxygenase-mediated quenching of quinolone-dependent quorum sensing in *Pseudomonas aeruginosa***. *Chem Biol* 2009, **16**(12):1259-1267.

204. Roy V, Fernandes R, Tsao C-Y, Bentley WE: **Cross species quorum quenching using a native AI-2 processing enzyme**. *ACS chemical biology* 2010, **5**(2):223-232.

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

206. Dong Y-H, Gusti AR, Zhang Q, Xu J-L, Zhang L-H: **Identification of quorum-quenching N-acyl homoserine lactonases from Bacillus species**. *Appl Environ Microbiol* 2002, **68**(4):1754-1759.

207. Carlier A, Uroz S, Smadja B, Fray R, Latour X, Dessaux Y, Faure D: **The Ti plasmid of *Agrobacterium tumefaciens* harbors an attM-paralogous gene, aiiB, also encoding N-acyl homoserine lactonase activity**. *Appl Environ Microbiol* 2003, **69**(8):4989-4993.

208. Uroz S, Oger PM, Chapelle E, Adeline M-T, Faure D, Dessaux Y: **A *Rhodococcus* qsdA-encoded enzyme defines a novel class of large-spectrum quorum-quenching lactonases**. *Appl Environ Microbiol* 2008, **74**(5):1357-1366.

209. Park S-Y, Hwang B-J, Shin M-H, Kim J-A, Kim H-K, Lee J-K: **N-acylhomoserine lactonase producing *Rhodococcus* spp. with different AHL-degrading activities**. *FEMS Microbiol Lett* 2006, **261**(1):102-108.

210. Park S-Y, Lee SJ, Oh T-K, Oh J-W, Koo B-T, Yum D-Y, Lee J-K: **AhlD, an N-acylhomoserine lactonase in *Arthrobacter* sp., and predicted homologues in other bacteria**. *Microbiology* 2003, **149**(6):1541-1550.

211. Wang W-Z, Morohoshi T, Someya N, Ikeda T: **AidC, a novel N-acylhomoserine lactonase from the potato root-associated Cytophaga-Flavobacteria-Bacteroides (CFB) group bacterium *Chryseobacterium* sp. strain StRB126**. *Appl Environ Microbiol* 2012, **78**(22):7985-7992.

212. Tang K, Su Y, Brackman G, Cui F, Zhang Y, Shi X, Coenye T, Zhang X-H: **MomL, a novel marine-derived N-acyl homoserine lactonase from *Muricauda olearia***. *Appl Environ Microbiol* 2015, **81**(2):774-782.

213. Mayer C, Romero M, Muras A, Otero A: **Aii20J, a wide-spectrum thermostable N-acylhomoserine lactonase from the marine bacterium Tenacibaculum sp. 20J, can quench AHL-mediated acid resistance in *Escherichia coli***. *Appl Microbiol Biotechnol* 2015, **99**(22):9523-9539.

214. Zhang Y, Liu J, Tang K, Yu M, Coenye T, Zhang X-H: **Genome analysis of *Flaviramulus ichthyoenteri* Th78^T in the family Flavobacteriaceae: insights into its quorum quenching property and potential roles in fish intestine**. *BMC genomics* 2015, **16**(1):38.

215. Morohoshi T, Sato N, Iizumi T, Tanaka A, Ikeda T: **Identification and characterization of a novel N-acyl-homoserine lactonase gene in *Sphingomonas ursincola* isolated from industrial cooling water systems**. *J Biosci Bioeng* 2017, **123**(5):569-575.

216. Bergonzi C, Schwab M, Elias M: **The quorum-quenching lactonase from *Geobacillus caldoxylosilyticus*: purification, characterization, crystallization and crystallographic analysis**. *Acta Crystallographica Section F: Structural Biology Communications* 2016, **72**(9):681-686.

217. Morohoshi T, Tominaga Y, Someya N, Ikeda T: **Characterization of a novel thermostable N-acylhomoserine lactonase from the thermophilic bacterium *Thermaerobacter marianensis***. *J Biosci Bioeng* 2015, **120**(1):1-5.

218. See-Too WS, Ee R, Lim Y-L, Convey P, Pearce DA, Yin W-F, Chan K-G: **AidP, a novel N-Acyl homoserine lactonase gene from Antarctic *Planococcus* sp**. *Scientific reports* 2017, **7**:42968.

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

220. Afriat L, Roodveldt C, Manco G, Tawfik DS: **The latent promiscuity of newly identified microbial lactonases is linked to a recently diverged phosphotriesterase**. *Biochemistry* 2006, **45**(46):13677-13686.

221. Bzdrenga J, Hiblot J, Gotthard G, Champion C, Elias M, Chabriere E: **Sac Pox from the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius* is a proficient lactonase**. *BMC research notes* 2014, **7**(1):333.

222. Hiblot J, Gotthard G, Chabriere E, Elias M: **Structural and enzymatic characterization of the lactonase SisLac from *Sulfolobus islandicus***. *PloS one* 2012, **7**(10):e47028.

223. Xue B, Chow JY, Baldansuren A, Yap LL, Gan YH, Dikanov SA, Robinson RC, Yew WS: **Structural evidence of a productive active site architecture for an evolved quorum-quenching GKL lactonase**. *Biochemistry* 2013, **52**(13):2359-2370.

224. Hiblot J, Bzdrenga J, Champion C, Chabriere E, Elias M: **Crystal structure of VmoLac, a tentative quorum quenching lactonase from the extremophilic crenarchaeon *Vulcanisaeta moutnovskia***. *Scientific reports* 2015, **5**:8372.

225. Rémy B, Plener L, Poirier L, Elias M, Daudé D, Chabrière E: **Harnessing hyperthermostable lactonase from *Sulfolobus solfataricus* for biotechnological applications**. *Scientific reports* 2016, **6**:37780.

226. Wang W-Z, Morohoshi T, Ikenoya M, Someya N, Ikeda T: **AiiM, a novel class of N-acylhomoserine lactonase from the leaf-associated bacterium *Microbacterium testaceum***. *Appl Environ Microbiol* 2010, **76**(8):2524-2530.

227. Mei G-Y, Yan X-X, Turak A, Luo Z-Q, Zhang L-Q: **AidH, an alpha/beta-hydrolase fold family member from an *Ochrobactrum* sp. strain, is a novel N-acylhomoserine lactonase**. *Appl Environ Microbiol* 2010, **76**(15):4933-4942.

228. Krysciak D, Schmeisser C, Preuss S, Riethausen J, Quitschau M, Grond S, Streit W: **Involvement of multiple loci in quorum quenching of autoinducer I molecules in the nitrogen-fixing symbiont *Rhizobium* (*Sinorhizobium*) sp. strain NGR234**. *Appl Environ Microbiol* 2011, **77**(15):5089-5099.

229. Huang W, Lin Y, Yi S, Liu P, Shen J, Shao Z, Liu Z: **QsdH, a novel AHL lactonase in the RND-type inner membrane of marine *Pseudoalteromonas byunsanensis* strain 1A01261**. *PloS one* 2012, **7**(10):e46587.
230. Camps J, Pujol I, Ballester F, Joven J, Simó JM: **Paraoxonases as potential antibiofilm agents: their relationship with quorum-sensing signals in Gram-negative bacteria**. *Antimicrobial agents and chemotherapy* 2011, **55**(4):1325-1331.
231. Furlong CE, Marsillach J, Jarvik GP, Costa LG: **Paraoxonases-1,-2 and-3: what are their functions?** *Chem-Biol Interact* 2016, **259**:51-62.
232. Torres M, Uroz S, Salto R, Fauchery L, Quesada E, Llamas I: **HqiA, a novel quorum-quenching enzyme which expands the AHL lactonase family**. *Scientific reports* 2017, **7**(1):943.
233. Utari PD, Vogel J, Quax WJ: **Deciphering physiological functions of AHL quorum quenching acylases**. *Frontiers in microbiology* 2017, **8**:1123.
234. Czajkowski R, Krzyżanowska D, Karczewska J, Atkinson S, Przysowa J, Lojkowska E, Williams P, Jafra S: **Inactivation of AHLs by *Ochrobactrum* sp. A44 depends on the activity of a novel class of AHL acylase**. *Environmental microbiology reports* 2011, **3**(1):59-68.
235. Lin YH, Xu JL, Hu J, Wang LH, Ong SL, Leadbetter JR, Zhang LH: **Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes**. *Molecular microbiology* 2003, **47**(3):849-860.
236. Mukherji R, Varshney NK, Panigrahi P, Suresh C, Prabhune A: **A new role for penicillin acylases: degradation of acyl homoserine lactone quorum sensing signals by *Kluyvera citrophila* penicillin G acylase**. *Enzyme Microb Technol* 2014, **56**:1-7.
237. Park S-Y, Kang H-O, Jang H-S, Lee J-K, Koo B-T, Yum D-Y: **Identification of extracellular N-acylhomoserine lactone acylase from a *Streptomyces* sp. and its application to quorum quenching**. *Appl Environ Microbiol* 2005, **71**(5):2632-2641.
238. Liu N, Yu M, Zhao Y, Cheng J, An K, Zhang X-H: **PfmA, a novel quorum-quenching N-acylhomoserine lactone acylase from *Pseudoalteromonas flavipulchra***. *Microbiology* 2017, **163**(10):1389-1398.
239. Kusada H, Tamaki H, Kamagata Y, Hanada S, Kimura N: **A novel quorum-quenching N-acylhomoserine lactone acylase from Acidovorax sp. strain MR-S7 mediates antibiotic resistance**. *Appl Environ Microbiol* 2017, **83**(13):e00080-00017.
240. Yasutake Y, Kusada H, Ebuchi T, Hanada S, Kamagata Y, Tamura T, Kimura N: **Bifunctional quorum-quenching and antibiotic-acylase MacQ forms a 170-kDa capsule-shaped molecule containing spacer polypeptides**. *Scientific reports* 2017, **7**(1):8946.
241. Sio CF, Otten LG, Cool RH, Diggle SP, Braun PG, Bos R, Daykin M, Cámara M, Williams P, Quax WJ: **Quorum quenching by an N-acyl-homoserine lactone acylase from *Pseudomonas aeruginosa* PAO1**. *Infect Immun* 2006, **74**(3):1673-1682.
242. Huang JJ, Petersen A, Whiteley M, Leadbetter JR: **Identification of QuiP, the product of gene PA1032, as the second acyl-homoserine lactone acylase of *Pseudomonas aeruginosa* PAO1**. *Appl Environ Microbiol* 2006, **72**(2):1190-1197.
243. Wahjudi M, Papaioannou E, Hendrawati O, van Assen AH, van Merkerk R, Cool RH, Poelarends GJ, Quax WJ: **PA0305 of *Pseudomonas aeruginosa* is a quorum quenching acylhomoserine lactone acylase belonging to the Ntn hydrolase superfamily**. *Microbiology* 2011, **157**(7):2042-2055.
244. Drake EJ, Gulick AM: **Structural characterization and high-throughput screening of inhibitors of PvdQ, an NTN hydrolase involved in pyoverdine synthesis**. *ACS chemical biology* 2011, **6**(11):1277-1286.

245. Uroz S, Chhabra SR, Camara M, Williams P, Oger P, Dessaux Y: **N-Acylhomoserine lactone quorum-sensing molecules are modified and degraded by Rhodococcus erythropolis W2 by both amidolytic and novel oxidoreductase activities**. *Microbiology* 2005, **151**(10):3313-3322.

246. Chan K-G, Atkinson S, Mathee K, Sam C-K, Chhabra SR, Cámara M, Koh C-L, Williams P: **Characterization of N-acylhomoserine lactone-degrading bacteria associated with the *Zingiber officinale* (ginger) rhizosphere: co-existence of quorum quenching and quorum sensing in *Acinetobacter* and *Burkholderia***. *BMC Microbiol* 2011, **11**(1):51.

247. Bijtenhoorn P, Mayerhofer H, Müller-Dieckmann J, Utpatel C, Schipper C, Hornung C, Szesny M, Grond S, Thürmer A, Brzuszkiewicz E: **A novel metagenomic short-chain dehydrogenase/reductase attenuates *Pseudomonas aeruginosa* biofilm formation and virulence on *Caenorhabditis elegans***. *PloS one* 2011, **6**(10):e26278.

248. Chowdhary PK, Keshavan N, Nguyen HQ, Peterson JA, González JE, Haines DC: ***Bacillus megaterium* CYP102A1 oxidation of acyl homoserine lactones and acyl homoserines**. *Biochemistry* 2007, **46**(50):14429-14437.

249. Reimmann C, Ginet N, Michel L, Keel C, Michaux P, Krishnapillai V, Zala M, Heurlier K, Triandafillu K, Harms H: **Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1**. *Microbiology* 2002, **148**(4):923-932.

250. Wopperer J, Cardona ST, Huber B, Jacobi CA, Valvano MA, Eberl L: **A quorum-quenching approach to investigate the conservation of quorum-sensing-regulated functions within the *Burkholderia cepacia* complex**. *Appl Environ Microbiol* 2006, **72**(2):1579-1587.

251. dos Reis Ponce A, Martins ML, de Araujo EF, Mantovani HC, Vanetti MCD: **AiiA quorum-sensing quenching controls proteolytic activity and biofilm formation by *Enterobacter cloacae***. *Curr Microbiol* 2012, **65**(6):758-763.

252.Clamens T, Rosay T, Crépin A, Grandjean T, Kentache T, Hardouin J, Bortolotti P, Neidig A, Mooij M, Hillion M: **The aliphatic amidase AmiE is involved in regulation of *Pseudomonas aeruginosa* virulence**. *Scientific reports* 2017, **7**:41178.

253. Fan X, Liang M, Wang L, Chen R, Li H, Liu X: **Aii810, a novel cold-adapted n-acylhomoserine lactonase discovered in a metagenome, can strongly attenuate *Pseudomonas aeruginosa* virulence factors and biofilm formation**. *Frontiers in microbiology* 2017, **8**:1950.

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

255. Ozer EA, Pezzulo A, Shih DM, Chun C, Furlong C, Lusis AJ, Greenberg EP, Zabner J: **Human and murine paraoxonase 1 are host modulators of *Pseudomonas aeruginosa* quorum-sensing**. *FEMS Microbiol Lett* 2005, **253**(1):29-37.

256. Guendouze A, Plener L, Bzdrenga J, Jacquet P, Rémy B, Elias M, Lavigne J-P, Daudé D, Chabrière E: **Effect of quorum quenching lactonase in clinical isolates of *Pseudomonas aeruginosa* and comparison with quorum sensing inhibitors**. *Frontiers in microbiology* 2017, **8**:227.

257. Chow JY, Yang Y, Tay SB, Chua KL, Yew WS: **Disruption of biofilm formation by the human pathogen *Acinetobacter baumannii* using engineered quorum-quenching lactonases**. *Antimicrobial agents and chemotherapy* 2014, **58**(3):1802-1805.

258. Aballay A, Ausubel FM: ***Caenorhabditis elegans* as a host for the study of host–pathogen interactions**. *Curr Opin Microbiol* 2002, **5**(1):97-101.

259. Irazoqui JE, Urbach JM, Ausubel FM: **Evolution of host innate defence: insights from *Caenorhabditis elegans* and primitive invertebrates**. *Nature Reviews Immunology* 2010, **10**(1):47.

260. Papaioannou E, Utari PD, Quax WJ: **Choosing an appropriate infection model to study quorum sensing inhibition in *Pseudomonas* infections**. *International journal of molecular sciences* 2013, **14**(9):19309-19340.

261. Papaioannou E, Wahjudi M, Nadal-Jimenez P, Koch G, Setroikromo R, Quax WJ: **Quorum-quenching acylase reduces the virulence of *Pseudomonas aeruginosa* in a *Caenorhabditis elegans* infection model**. *Antimicrobial agents and chemotherapy* 2009, **53**(11):4891-4897.

262. Tsai CJ-Y, Loh JMS, Proft T: ***Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing**. *Virulence* 2016, **7**(3):214-229.

263. Koch G, Nadal-Jimenez P, Reis CR, Muntendam R, Bokhove M, Melillo E, Dijkstra BW, Cool RH, Quax WJ: **Reducing virulence of the human pathogen Burkholderia by altering the substrate specificity of the quorum-quenching acylase PvdQ**. *Proc Natl Acad Sci U S A* 2014, **111**(4):1568-1573.

264. Stoltz DA, Ozer EA, Taft PJ, Barry M, Liu L, Kiss PJ, Moninger TO, Parsek MR, Zabner J: ***Drosophila* are protected from *Pseudomonas aeruginosa* lethality by transgenic expression of paraoxonase-1**. *The Journal of clinical investigation* 2008, **118**(9):3123-3131.

265. Shiner E, Terentyev D, Bryan A, Sennoune S, Martinez-Zaguilan R, Li G, Gyorke S, Williams S, Rumbaugh K: ***Pseudomonas aeruginosa* autoinducer modulates host cell responses through calcium signalling**. *Cell Microbiol* 2006, **8**(10):1601-1610.

266. Ye L, Li G, Li H, Wang L, Mao Y, Xie X, Xia C, Chen J, Song J: ***Pseudomonas aeruginosa* pvdQ gene prevents Caco-2 cells from obstruction of quorum-sensing signal**. *Curr Microbiol* 2011, **62**(1):32-37.

267. Migiyama Y, Kaneko Y, Yanagihara K, Morohoshi T, Morinaga Y, Nakamura S, Miyazaki T, Hasegawa H, Izumikawa K, Kakeya H: **Efficacy of AiiM, an N-acylhomoserine lactonase, against *Pseudomonas aeruginosa* in a mouse model of acute pneumonia**. *Antimicrobial agents and chemotherapy* 2013, **57**(8):3653-3658.

268. Hraiech S, Hiblot J, Lafleur J, Lepidi H, Papazian L, Rolain J-M, Raoult D, Elias M, Silby MW, Bzdrenga J: **Inhaled lactonase reduces *Pseudomonas aeruginosa* quorum sensing and mortality in rat pneumonia**. *PloS one* 2014, **9**(10):e107125.

269. Gupta P, Chhibber S, Harjai K: **Efficacy of purified lactonase and ciprofloxacin in preventing systemic spread of *Pseudomonas aeruginosa* in murine burn wound model**. *Burns : journal of the International Society for Burn Injuries* 2015, **41**(1):153-162.

270. Sedlmayer F, Jaeger T, Jenal U, Fussenegger M: **Quorum-quenching human designer cells for closed-loop control of *Pseudomonas aeruginosa* biofilms**. *Nano Lett* 2017, **17**(8):5043-5050.

271. Rhoads MK: **Dual Quorum Quenching Capsules: Disrupting two bacterial communication pathways that lead to virulence**. University of Maryland, College Park; 2016.

272. Pei R, Lamas-Samanamud GR: **Inhibition of biofilm formation by T7 bacteriophages producing quorum-quenching enzymes**. *Appl Environ Microbiol* 2014, **80**(17):5340-5348.

273. Høyland-Kroghsbo NM, Paczkowski J, Mukherjee S, Broniewski J, Westra E, Bondy-Denomy J, Bassler BL: **Quorum sensing controls the *Pseudomonas aeruginosa* CRISPR-Cas adaptive immune system**. *Proc Natl Acad Sci U S A* 2017, **114**(1):131-135.

274. Kim M-H: **Nanoparticle-based therapies for wound biofilm infection: opportunities and challenges**. *IEEE Trans NanoBiosci* 2016, **15**(3):294-304.

275. Ramasamy M, Lee J: **Recent nanotechnology approaches for prevention and treatment of biofilm-associated infections on medical devices**. *BioMed research international* 2016, **2016**.

276. Li N, Wang L, Yan H, Wang M, Shen D, Yin J, Shentu J: **Effects of low-level engineered nanoparticles on the quorum sensing of *Pseudomonas aeruginosa* PAO1**. *Environmental Science and Pollution Research* 2017:1-10.

277. Wolska KI, Grudniak AM, Markowska K: **Inhibition of bacterial quorum sensing systems by metal nanoparticles**. In: *Metal Nanoparticles in Pharma.* Springer; 2017: 123-138.

278. Vinoj G, Pati R, Sonawane A, Vaseeharan B: **In vitro cytotoxic effects of gold nanoparticles coated with functional acyl homoserine lactone lactonase protein from *Bacillus licheniformis* and their antibiofilm activity against *Proteus* species**. *Antimicrobial agents and chemotherapy* 2015, **59**(2):763-771.

279. Ivanova K, Fernandes MM, Francesko A, Mendoza E, Guezguez J, Burnet M, Tzanov T: **Quorum-quenching and matrix-degrading enzymes in multilayer coatings synergistically prevent bacterial biofilm formation on urinary catheters**. *ACS applied materials & interfaces* 2015, **7**(49):27066-27077.

280. Ivanova K, Fernandes MM, Mendoza E, Tzanov T: **Enzyme multilayer coatings inhibit *Pseudomonas aeruginosa* biofilm formation on urinary catheters**. *Appl Microbiol Biotechnol* 2015, **99**(10):4373-4385.

281. Grover N, Plaks JG, Summers SR, Chado GR, Schurr MJ, Kaar JL: **Acylase-containing polyurethane coatings with anti-biofilm activity**. *Biotechnol Bioeng* 2016, **113**(12):2535-2543.

282. Ng FS, Wright DM, Seah SY: **Characterization of a phosphotriesterase-like lactonase from *Sulfolobus solfataricus* and its immobilization for disruption of quorum sensing**. *Appl Environ Microbiol* 2011, **77**(4):1181-1186.

283. Wahjudi M, Murugappan S, van Merkerk R, Eissens AC, Visser MR, Hinrichs WL, Quax WJ: **Development of a dry, stable and inhalable acyl–homoserine–lactone–acylase powder formulation for the treatment of pulmonary *Pseudomonas aeruginosa* infections**. *Eur J Pharm Sci* 2013, **48**(4-5):637-643.

284. Kalia VC: **Quorum sensing inhibitors: an overview**. *Biotechnol Adv* 2013, **31**(2):224-245.

285. Jakobsen TH, Bjarnsholt T, Jensen PØ, Givskov M, Høiby N: **Targeting quorum sensing in *Pseudomonas aeruginosa* biofilms: current and emerging inhibitors**. *Future microbiology* 2013, **8**(7):901-921.

286. Rasmussen TB, Givskov M: **Quorum-sensing inhibitors as anti-pathogenic drugs**. *Int J Med Microbiol* 2006, **296**(2-3):149-161.

287. Ta CAK, Arnason JT: **Mini review of phytochemicals and plant taxa with activity as microbial biofilm and quorum sensing inhibitors**. *Molecules* 2015, **21**(1):29.

288. Brackman G, Coenye T: **Quorum sensing inhibitors as anti-biofilm agents**. *Curr Pharm Des* 2015, **21**(1):5-11.

289. Galloway WR, Hodgkinson JT, Bowden S, Welch M, Spring DR: **Applications of small molecule activators and inhibitors of quorum sensing in Gram-negative bacteria**. *Trends Microbiol* 2012, **20**(9):449-458.

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

291. García-Contreras R, Maeda T, Wood TK: **Resistance to quorum-quenching compounds**. *Appl Environ Microbiol* 2013, **79**(22):6840-6846.

292. Kalia VC, Wood TK, Kumar P: **Evolution of resistance to quorum-sensing inhibitors**. *Microb Ecol* 2014, **68**(1):13-23.

293. Rai N, Rai R, Venkatesh K: **Quorum sensing biosensors**. In: *Quorum Sensing vs Quorum Quenching: A Battle with No End in Sight.* Springer; 2015: 173-183.

294. Defoirdt T, Brackman G, Coenye T: **Quorum sensing inhibitors: how strong is the evidence?** *Trends Microbiol* 2013, **21**(12):619-624.

295. Chen Y, Liu T, Wang K, Hou C, Cai S, Huang Y, Du Z, Huang H, Kong J, Chen Y: **Baicalein inhibits *Staphylococcus aureus* biofilm formation and the quorum sensing system in vitro**. *PloS one* 2016, **11**(4):e0153468.

296. Luo J, Kong J-l, Dong B-y, Huang H, Wang K, Wu L-h, Hou C-c, Liang Y, Li B, Chen Y-q: **Baicalein attenuates the quorum sensing-controlled virulence factors of *Pseudomonas aeruginosa* and relieves the inflammatory response in *P. aeruginosa*-infected macrophages by downregulating the MAPK and NFκB signal-transduction pathways**. *Drug design, development and therapy* 2016, **10**:183.

297. Paczkowski JE, Mukherjee S, McCready AR, Cong J-P, Aquino CJ, Kim H, Henke BR, Smith CD, Bassler BL: **Flavonoids suppress *Pseudomonas aeruginosa* virulence through allosteric inhibition of quorum-sensing receptors**. *J Biol Chem* 2017, **292**(10):4064-4076.

298. Jakobsen TH, van Gennip M, Phipps RK, Shanmugham MS, Christensen LD, Alhede M, Skindersoe ME, Rasmussen TB, Friedrich K, Uthe F: **Ajoene, a sulfur-rich molecule from garlic, inhibits genes controlled by quorum sensing**. *Antimicrobial agents and chemotherapy* 2012, **56**(5):2314-2325.

299. Jakobsen TH, Warming AN, Vejborg RM, Moscoso JA, Stegger M, Lorenzen F, Rybtke M, Andersen JB, Petersen R, Andersen PS: **A broad range quorum sensing inhibitor working through sRNA inhibition**. *Scientific Reports* 2017, **7**(1):9857.

300. Jakobsen TH, Bragason SK, Phipps RK, Christensen LD, van Gennip M, Alhede M, Skindersoe M, Larsen TO, Høiby N, Bjarnsholt T: **Food as a source for QS inhibitors: iberin from horseradish revealed as a quorum sensing inhibitor of *Pseudomonas aeruginosa***. *Appl Environ Microbiol* 2012:AEM. 05992-05911.

301. Ganin H, Rayo J, Amara N, Levy N, Krief P, Meijler MM: **Sulforaphane and erucin, natural isothiocyanates from broccoli, inhibit bacterial quorum sensing**. *MedChemComm* 2013, **4**(1):175-179.

302. Tan SY-Y, Liu Y, Chua SL, Vejborg RM, Jakobsen TH, Chew SC, Li Y, Nielsen TE, Tolker-Nielsen T, Yang L: **Comparative systems biology analysis and mode of action of the isothiocyanate compound iberin on *Pseudomonas aeruginosa***. *Antimicrobial agents and chemotherapy* 2014:AAC. 02620-02613.

303. Skindersoe ME, Ettinger-Epstein P, Rasmussen TB, Bjarnsholt T, de Nys R, Givskov M: **Quorum sensing antagonism from marine organisms**. *Mar Biotechnol* 2008, **10**(1):56-63.

304. Rasmussen TB, Skindersoe ME, Bjarnsholt T, Phipps RK, Christensen KB, Jensen PO, Andersen JB, Koch B, Larsen TO, Hentzer M: **Identity and effects of quorum-sensing inhibitors produced by *Penicillium* species**. *Microbiology* 2005, **151**(5):1325-1340.

305. Truchado P, López-Gálvez F, Gil M, Tomás-Barberán F, Allende A: **Quorum sensing inhibitory and antimicrobial activities of honeys and the relationship with individual phenolics**. *Food Chem* 2009, **115**(4):1337-1344.

306. Cugini C, Calfee M, Farrow JM, Morales DK, Pesci EC, Hogan DA: **Farnesol, a common sesquiterpene, inhibits PQS production in *Pseudomonas aeruginosa***. *Molecular microbiology* 2007, **65**(4):896-906.

307. Jayalekshmi H, Omanakuttan A, Pandurangan N, Vargis VS, Maneesh M, Nair BG, Kumar GB: **Clove bud oil reduces kynurenine and inhibits pqsA gene expression in *P. aeruginosa***. *Appl Microbiol Biotechnol* 2016, **100**(8):3681-3692.

308. Maurer CK, Lu C, Empting M, Hartmann RW: **Synthetic quorum sensing inhibitors (QSIs) blocking receptor signaling or signal molecule biosynthesis in *Pseudomonas aeruginosa***. In: *Quorum Sensing vs Quorum Quenching: A Battle with No End in Sight.* Springer; 2015: 303-317.

309. Geske GD, Mattmann ME, Blackwell HE: **Evaluation of a focused library of N-aryl L-homoserine lactones reveals a new set of potent quorum sensing modulators**. *Bioorganic & medicinal chemistry letters* 2008, **18**(22):5978-5981.

310. Geske GD, O'Neill JC, Miller DM, Mattmann ME, Blackwell HE: **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**. *J Am Chem Soc* 2007, **129**(44):13613-13625.

311. Smith KM, Bu Y, Suga H: **Induction and inhibition of *Pseudomonas aeruginosa* quorum sensing by synthetic autoinducer analogs**. *Chem Biol* 2003, **10**(1):81-89.

312. Borlee BR, Geske GD, Blackwell HE, Handelsman J: **Identification of synthetic inducers and inhibitors of the quorum-sensing regulator LasR in *Pseudomonas aeruginosa* by high-throughput screening**. *Appl Environ Microbiol* 2010, **76**(24):8255-8258.

313. Wu H, Song Z, Hentzer M, Andersen JB, Molin S, Givskov M, Høiby N: **Synthetic furanones inhibit quorum-sensing and enhance bacterial clearance in *Pseudomonas aeruginosa* lung infection in mice**. *Journal of Antimicrobial Chemotherapy* 2004, **53**(6):1054-1061.

314. Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, Kumar N, Schembri MA, Song Z, Kristoffersen P: **Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors**. *The EMBO journal* 2003, **22**(15):3803-3815.

315. Lesic B, Lépine F, Déziel E, Zhang J, Zhang Q, Padfield K, Castonguay M-H, Milot S, Stachel S, Tzika AA: **Inhibitors of pathogen intercellular signals as selective anti-infective compounds**. *PLoS Path* 2007, **3**(9):e126.

316. Ji C, Sharma I, Pratihar D, Hudson LL, Maura D, Guney T, Rahme LG, Pesci EC, Coleman JP, Tan DS: **Designed small-molecule inhibitors of the anthranilyl-CoA synthetase PqsA block quinolone biosynthesis in *Pseudomonas aeruginosa***. *ACS chemical biology* 2016, **11**(11):3061-3067.

317. Storz MP, Maurer CK, Zimmer C, Wagner N, Brengel C, de Jong JC, Lucas S, Müsken M, Häussler S, Steinbach A: **Validation of PqsD as an anti-biofilm target in *Pseudomonas aeruginosa* by development of small-molecule inhibitors**. *J Am Chem Soc* 2012, **134**(39):16143-16146.

318. Maura D, Rahme LG: **Pharmacological inhibition of the *Pseudomonas aeruginosa* MvfR quorum-sensing system interferes with biofilm formation and potentiates antibiotic-mediated biofilm disruption**. *Antimicrobial agents and chemotherapy* 2017, **61**(12):e01362-01317.

319. Nalca Y, Jänsch L, Bredenbruch F, Geffers R, Buer J, Häussler S: **Quorum-sensing antagonistic activities of azithromycin in *Pseudomonas aeruginosa* PAO1: a global approach**. *Antimicrobial agents and chemotherapy* 2006, **50**(5):1680-1688.

320. Skindersoe ME, Alhede M, Phipps R, Yang L, Jensen PO, Rasmussen TB, Bjarnsholt T, Tolker-Nielsen T, Høiby N, Givskov M: **Effects of antibiotics on quorum sensing in *Pseudomonas aeruginosa***. *Antimicrobial agents and chemotherapy* 2008, **52**(10):3648-3663.

321. Pérez-Martínez I, Haas D: **Azithromycin inhibits expression of the GacA-dependent small RNAs RsmY and RsmZ in *Pseudomonas aeruginosa***. *Antimicrobial agents and chemotherapy* 2011, **55**(7):3399-3405.

322. Ueda A, Attila C, Whiteley M, Wood TK: **Uracil influences quorum sensing and biofilm formation in *Pseudomonas aeruginosa* and fluorouracil is an antagonist**. *Microbial biotechnology* 2009, **2**(1):62-74.

323. Imperi F, Massai F, Pillai CR, Longo F, Zennaro E, Rampioni G, Visca P, Leoni L: **New life for an old drug: the anthelmintic drug niclosamide inhibits *Pseudomonas aeruginosa* quorum sensing**. *Antimicrobial agents and chemotherapy* 2013, **57**(2):996-1005.

324. She P, Wang Y, Luo Z, Chen L, Tan R, Wang Y, Wu Y: **Meloxicam inhibits biofilm formation and enhances antimicrobial agents efficacy by *Pseudomonas aeruginosa***. *MicrobiologyOpen* 2017.

325. Welsh MA, Eibergen NR, Moore JD, Blackwell HE: **Small molecule disruption of quorum sensing cross-regulation in *Pseudomonas aeruginosa* causes major and unexpected alterations to virulence phenotypes**. *J Am Chem Soc* 2015, **137**(4):1510-1519.

326. O'Loughlin CT, Miller LC, Siryaporn A, Drescher K, Semmelhack MF, Bassler BL: **A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation**. *Proc Natl Acad Sci U S A* 2013, **110**(44):17981-17986.

327. Rasamiravaka T, Vandeputte OM, Pottier L, Huet J, Rabemanantsoa C, Kiendrebeogo M, Andriantsimahavandy A, Rasamindrakotroka A, Stévigny C, Duez P: ***Pseudomonas aeruginosa* biofilm formation and persistence, along with the production of quorum sensing-dependent virulence factors, are disrupted by a triterpenoid coumarate ester isolated from Dalbergia trichocarpa, a tropical legume**. *PloS one* 2015, **10**(7):e0132791.

328. Kim H-S, Lee S-H, Byun Y, Park H-D: **6-Gingerol reduces *Pseudomonas aeruginosa* biofilm formation and virulence via quorum sensing inhibition**. *Scientific reports* 2015, **5**:8656.

329. Brackman G, Cos P, Maes L, Nelis HJ, Coenye T: **Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and *in vivo***. *Antimicrobial agents and chemotherapy* 2011, **55**(6):2655-2661.

330. Hentzer M, Riedel K, Rasmussen TB, Heydorn A, Andersen JB, Parsek MR, Rice SA, Eberl L, Molin S, Høiby N: **Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound**. *Microbiology* 2002, **148**(1):87-102.

331. Christensen LD, van Gennip M, Jakobsen TH, Alhede M, Hougen HP, Høiby N, Bjarnsholt T, Givskov M: **Synergistic antibacterial efficacy of early combination treatment with tobramycin and quorum-sensing inhibitors against *Pseudomonas aeruginosa* in an intraperitoneal foreign-body infection mouse model**. *Journal of antimicrobial chemotherapy* 2012, **67**(5):1198-1206.

332. Hoffmann N, Lee B, Hentzer M, Rasmussen TB, Song Z, Johansen HK, Givskov M, Høiby N: **Azithromycin blocks quorum sensing and alginate polymer formation and increases the sensitivity to serum and stationary-growth-phase killing of *Pseudomonas aeruginosa* and attenuates chronic *P. aeruginosa* lung infection in Cftr−/− mice**. *Antimicrobial agents and chemotherapy* 2007, **51**(10):3677-3687.

333. Bala A, Kumar R, Harjai K: **Inhibition of quorum sensing in *Pseudomonas aeruginosa* by azithromycin and its effectiveness in urinary tract infections**. *J Med Microbiol* 2011, **60**(3):300-306.

334. Chatterjee M, D'Morris S, Paul V, Warrier S, Vasudevan AK, Vanuopadath M, Nair SS, Paul-Prasanth B, Mohan CG, Biswas R: **Mechanistic understanding of Phenyllactic acid mediated inhibition of quorum sensing and biofilm development in *Pseudomonas aeruginosa***. *Appl Microbiol Biotechnol* 2017, **101**(22):8223-8236.

335. Yin H, Deng Y, Wang H, Liu W, Zhuang X, Chu W: **Tea polyphenols as an antivirulence compound disrupt quorum-sensing regulated pathogenicity of *Pseudomonas aeruginosa***. *Scientific Reports* 2015, **5**:16158.

336. Luo J, Dong B, Wang K, Cai S, Liu T, Cheng X, Lei D, Chen Y, Li Y, Kong J: **Baicalin inhibits biofilm formation, attenuates the quorum sensing-controlled virulence and enhances *Pseudomonas aeruginosa* clearance in a mouse peritoneal implant infection model**. *PloS one* 2017, **12**(4):e0176883.
337. Stacy DM, Welsh MA, Rather PN, Blackwell HE: **Attenuation of quorum sensing in the pathogen *Acinetobacter baumannii* using non-native N-Acyl homoserine lactones**. *ACS chemical biology* 2012, **7**(10):1719-1728.
338. Bhargava N, Singh SP, Sharma A, Sharma P, Capalash N: **Attenuation of quorum sensing-mediated virulence of *Acinetobacter baumannii* by Glycyrrhiza glabra flavonoids**. *Future microbiology* 2015, **10**(12):1953-1968.
339. Saroj SD, Rather PN: **Streptomycin inhibits quorum sensing in *Acinetobacter baumannii***. *Antimicrobial agents and chemotherapy* 2013, **57**(4):1926-1929.
340. Nicol M, Alexandre S, Luizet J-B, Skogman M, Jouenne T, Salcedo SP, Dé E: **Unsaturated fatty acids affect quorum sensing communication system and inhibit motility and biofilm formation of *Acinetobacter baumannii***. *International journal of molecular sciences* 2018, **19**(1):214.
341. Van Delden C, Köhler T, Brunner-Ferber F, François B, Carlet J, Pechère J-C: **Azithromycin to prevent *Pseudomonas aeruginosa* ventilator-associated pneumonia by inhibition of quorum sensing: a randomized controlled trial**. *Intensive care medicine* 2012, **38**(7):1118-1125.
342. Smyth AR, Cifelli PM, Ortori CA, Righetti K, Lewis S, Erskine P, Holland ED, Givskov M, Williams P, Cámara M: **Garlic as an inhibitor of *Pseudomonas aeruginosa* quorum sensing in cystic fibrosis—a pilot randomized controlled trial**. *Pediatric pulmonology* 2010, **45**(4):356-362.
343. Eibergen NR, Moore JD, Mattmann ME, Blackwell HE: **Potent and Selective Modulation of the RhlR Quorum Sensing Receptor by Using Non-native Ligands: An Emerging Target for Virulence Control in *Pseudomonas aeruginosa***. *Chembiochem : a European journal of chemical biology* 2015, **16**(16):2348-2356.
344. Smith KM, Bu Y, Suga H: **Library screening for synthetic agonists and antagonists of a *Pseudomonas aeruginosa* autoinducer**. *Chem Biol* 2003, **10**(6):563-571.
345. Wysoczynski-Horita CL, Boursier ME, Hill R, Hansen K, Blackwell HE, Churchill ME: **Mechanism of agonism and antagonism of the *Pseudomonas aeruginosa* quorum sensing regulator QscR with non-native ligands**. *Molecular microbiology* 2018, **108**(3):240-257.
346. Bassler BL, Losick R: **Bacterially speaking**. *Cell* 2006, **125**(2):237-246.
347. Williams P, Winzer K, Chan WC, Camara M: **Look who's talking: communication and quorum sensing in the bacterial world**. *Philosophical Transactions of the Royal Society B: Biological Sciences* 2007, **362**(1483):1119-1134.
348. LaSarre B, Federle MJ: **Exploiting quorum sensing to confuse bacterial pathogens**. *Microbiology and Molecular Biology Reviews* 2013, **77**(1):73-111.
349. Rutherford ST, Bassler BL: **Bacterial quorum sensing: its role in virulence and possibilities for its control**. *Cold Spring Harbor Perspectives in Medicine* 2012, **2**(11):a012427.
350. Hentzer M, Givskov M: **Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections**. *The Journal of clinical investigation* 2003, **112**(9):1300-1307.
351. Rasmussen TB, Givskov M: **Quorum sensing inhibitors: a bargain of effects**. *Microbiology* 2006, **152**(4):895-904.

352. Brackman G, Cos P, Maes L, Nelis HJ, Coenye T: **Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics in vitro and in vivo**. *Antimicrobial agents and chemotherapy* 2011, **55**(6):2655-2661.

353. Peleg AY, Seifert H, Paterson DL: ***Acinetobacter baumannii*: emergence of a successful pathogen**. *Clin Microbiol Rev* 2008, **21**(3):538-582.

354. Gonzalez-Villoria AM, Valverde-Garduno V: **Antibiotic-resistant *Acinetobacter baumannii* increasing success remains a challenge as a nosocomial pathogen**. *Journal of pathogens* 2016, **2016**.

355. Potron A, Poirel L, Nordmann P: **Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: mechanisms and epidemiology**. *Int J Antimicrob Agents* 2015, **45**(6):568-585.

356. Longo F, Vuotto C, Donelli G: **Biofilm formation in *Acinetobacter baumannii***. *New Microbiol* 2014, **37**(2):119-127.

357. Donlan RM, Costerton JW: **Biofilms: survival mechanisms of clinically relevant microorganisms**. *Clin Microbiol Rev* 2002, **15**(2):167-193.

358. Hall-Stoodley L, Costerton JW, Stoodley P: **Bacterial biofilms: from the natural environment to infectious diseases**. *Nature reviews Microbiology* 2004, **2**(2):95-108.

359. González R, Dijkshoorn L, Van den Barselaar M, Nudel C: **Quorum sensing signal profile of *Acinetobacter* strains from nosocomial and environmental sources**. *Rev Argent Microbiol* 2009, **41**(2):73-78.

360. Bhargava N, Sharma P, Capalash N: **Quorum sensing in *Acinetobacter*: an emerging pathogen**. *Crit Rev Microbiol* 2010, **36**(4):349-360.

361. Furiga A, Lajoie B, El Hage S, Baziard G, Roques C: **Impairment of *Pseudomonas aeruginosa* biofilm resistance to antibiotics by combining the drugs with a new quorum-sensing inhibitor**. *Antimicrobial agents and chemotherapy* 2016, **60**(3):1676-1686.

362. Aybey A, Demirkan E: **Inhibition of quorum sensing-controlled virulence factors in *Pseudomonas aeruginosa* by human serum paraoxonase**. *J Med Microbiol* 2016, **65**(2):105-113.

363. Yin H, Deng Y, Wang H, Liu W, Zhuang X, Chu W: **Tea polyphenols as an antivirulence compound disrupt quorum-sensing regulated pathogenicity of *Pseudomonas aeruginosa***. *Scientific reports* 2015, **5**.

364. Zhu J, Beaber JW, Moré MI, Fuqua C, Eberhard A, Winans SC: **Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of*Agrobacterium tumefaciens***. *Journal of bacteriology* 1998, **180**(20):5398-5405.

365. Tempé J, Petit A, Holsters M, Van Montagu M, Schell J: **Thermosensitive step associated with transfer of the Ti plasmid during conjugation: possible relation to transformation in crown gall**. *Proc Natl Acad Sci U S A* 1977, **74**(7):2848-2849.

366. Stiernagle T: **Maintenance of *C. elegans***. *C elegans* 1999, **2**:51-67.

367. Tang K, Zhang Y, Yu M, Shi X, Coenye T, Bossier P, Zhang X-H: **Evaluation of a new high-throughput method for identifying quorum quenching bacteria**. *Scientific reports* 2013, **3**:2935.

368. Peeters E, Nelis HJ, Coenye T: **Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates**. *J Microbiol Methods* 2008, **72**(2):157-165.

369. Brackman G, Garcia-Fernandez MJ, Lenoir J, De Meyer L, Remon JP, De Beer T, Concheiro A, Alvarez-Lorenzo C, Coenye T: **Dressings loaded with cyclodextrin − hamamelitannin complexes**

increase staphylococcus aureus susceptibility toward antibiotics both in single as well as in mixed biofilm communities. *Macromol Biosci* 2016.

370. Bzdrenga J, Daudé D, Rémy B, Jacquet P, Plener L, Elias M, Chabrière E: **Biotechnological applications of quorum quenching enzymes**. *Chem-Biol Interact* 2016.

371. Peleg AY, Jara S, Monga D, Eliopoulos GM, Moellering RC, Mylonakis E: ***Galleria mellonella*** **as a model system to study** ***Acinetobacter baumannii*** **pathogenesis and therapeutics**. *Antimicrobial agents and chemotherapy* 2009, **53**(6):2605-2609.

372. Lyczak JB, Cannon CL, Pier GB: **Establishment of** ***Pseudomonas aeruginosa*** **infection: lessons from a versatile opportunist1**. *Microb Infect* 2000, **2**(9):1051-1060.

373. Obritsch MD, Fish DN, MacLaren R, Jung R: **Nosocomial infections due to multidrug-resistant** ***Pseudomonas aeruginosa*****: epidemiology and treatment options**. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy* 2005, **25**(10):1353-1364.

374. Sen CK, Gordillo GM, Roy S, Kirsner R, Lambert L, Hunt TK, Gottrup F, Gurtner GC, Longaker MT: **Human skin wounds: a major and snowballing threat to public health and the economy**. *Wound repair and regeneration* 2009, **17**(6):763-771.

375. McManus A, Mason A, McManus W, Pruitt B: **Twenty-five year review of** ***Pseudomonas aeruginosa*** **bacteremia in a burn center**. *European journal of clinical microbiology* 1985, **4**(2):219-223.

376. Holder IA: ***P. aeruginosa*** **burn infections: pathogenesis and treatment**. In: *Pseudomonas aeruginosa as an Opportunistic Pathogen.* Springer; 1993: 275-295.

377. Ünal S, Ersoz G, Demirkan F, Arslan E, Tütüncü N, Sari A: **Analysis of skin-graft loss due to infection: infection-related graft loss**. *Annals of plastic surgery* 2005, **55**(1):102-106.

378. Strateva T, Mitov I: **Contribution of an arsenal of virulence factors to pathogenesis of** ***Pseudomonas aeruginosa*** **infections**. *Annals of microbiology* 2011, **61**(4):717-732.

379. Hirsch EB, Tam VH: **Impact of multidrug-resistant** ***Pseudomonas aeruginosa*** **infection on patient outcomes**. *Expert review of pharmacoeconomics & outcomes research* 2010, **10**(4):441-451.

380. Mulcahy LR, Isabella VM, Lewis K: ***Pseudomonas aeruginosa*** **biofilms in disease**. *Microb Ecol* 2014, **68**(1):1-12.

381. Rybtke MT, Jensen PO, Hoiby N, Givskov M, Tolker-Nielsen T, Bjarnsholt T: **The implication of** ***Pseudomonas aeruginosa*** **biofilms in infections**. *Inflammation & Allergy-Drug Targets (Formerly Current Drug Targets-Inflammation & Allergy)* 2011, **10**(2):141-157.

382. Smith RS, Iglewski BH: ***P. aeruginosa*** **quorum-sensing systems and virulence**. *Curr Opin Microbiol* 2003, **6**(1):56-60.

383. Shih P-C, Huang C-T: **Effects of quorum-sensing deficiency on** ***Pseudomonas aeruginosa*** **biofilm formation and antibiotic resistance**. *Journal of Antimicrobial Chemotherapy* 2002, **49**(2):309-314.

384. Bjarnsholt T, Jensen PØ, Burmølle M, Hentzer M, Haagensen JA, Hougen HP, Calum H, Madsen KG, Moser C, Molin S: ***Pseudomonas aeruginosa*** **tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent**. *Microbiology* 2005, **151**(2):373-383.

385. Rasamiravaka T, El Jaziri M: **Quorum-sensing mechanisms and bacterial response to antibiotics in** ***P. aeruginosa***. *Curr Microbiol* 2016, **73**(5):747-753.

386. Lee J, Zhang L: **The hierarchy quorum sensing network in** ***Pseudomonas aeruginosa***. *Protein & cell* 2015, **6**(1):26-41.

387. Van Delden C, Iglewski BH: **Cell-to-cell signaling and *Pseudomonas aeruginosa* infections**. *Emerging Infect Dis* 1998, **4**(4):551.

388. Rumbaugh KP, Griswold JA, Iglewski BH, Hamood AN: **Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections**. *Infect Immun* 1999, **67**(11):5854-5862.

389. Ouyang J, Sun F, Feng W, Sun Y, Qiu X, Xiong L, Liu Y, Chen Y: **Quercetin is an effective inhibitor of quorum sensing, biofilm formation and virulence factors in *Pseudomonas aeruginosa***. *J Appl Microbiol* 2016, **120**(4):966-974.

390. Fylaktakidou KC, Hadjipavlou-Litina DJ, Litinas KE, Nicolaides DN: **Natural and synthetic coumarin derivatives with anti-inflammatory/antioxidant activities**. *Curr Pharm Des* 2004, **10**(30):3813-3833.

391. Reen FJ, Gutiérrez-Barranquero JA, Parages ML: **Coumarin: a novel player in microbial quorum sensing and biofilm formation inhibition**. *Appl Microbiol Biotechnol* 2018, **102**(5):2063-2073.

392. Gutiérrez-Barranquero JA, Reen FJ, McCarthy RR, O'Gara F: **Deciphering the role of coumarin as a novel quorum sensing inhibitor suppressing virulence phenotypes in bacterial pathogens**. *Appl Microbiol Biotechnol* 2015, **99**(7):3303-3316.

393. Zhang S, Liu N, Liang W, Han Q, Zhang W, Li C: **Quorum sensing-disrupting coumarin suppressing virulence phenotypes in *Vibrio splendidus***. *Appl Microbiol Biotechnol* 2017, **101**(8):3371-3378.

394. Chambers L, Woodrow S, Brown A, Harris P, Phillips D, Hall M, Church J, Pritchard D: **Degradation of extracellular matrix components by defined proteinases from the greenbottle larva *Lucilia sericata* used for the clinical debridement of non-healing wounds**. *Br J Dermatol* 2003, **148**(1):14-23.

395. Rasmussen TB, Bjarnsholt T, Skindersoe ME, Hentzer M, Kristoffersen P, Köte M, Nielsen J, Eberl L, Givskov M: **Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector**. *J Bacteriol* 2005, **187**(5):1799-1814.

396. Brackman G, Hillaert U, Van Calenbergh S, Nelis HJ, Coenye T: **Use of quorum sensing inhibitors to interfere with biofilm formation and development in *Burkholderia multivorans* and *Burkholderia cenocepacia***. *Res Microbiol* 2009, **160**(2):144-151.

397. Krishnan T, Yin W-F, Chan K-G: **Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* PAO1 by Ayurveda spice clove (Syzygium aromaticum) bud extract**. *Sensors* 2012, **12**(4):4016-4030.

398. Nicodeme M, Grill JP, Humbert G, Gaillard JL: **Extracellular protease activity of different *Pseudomonas* strains: dependence of proteolytic activity on culture conditions**. *J Appl Microbiol* 2005, **99**(3):641-648.

399. Vandecandelaere I, Depuydt P, Nelis HJ, Coenye T: **Protease production by *Staphylococcus epidermidis* and its effect on *Staphylococcus aureus* biofilms**. *Pathogens and disease* 2014, **70**(3):321-331.

400. Brackman G, Garcia-Fernandez MJ, Lenoir J, De Meyer L, Remon JP, De Beer T, Concheiro A, Alvarez-Lorenzo C, Coenye T: **Dressings loaded with cyclodextrin − hamamelitannin complexes increase *Staphylococcus aureus* susceptibility toward antibiotics both in single as well as in mixed biofilm communities**. *Macromol Biosci* 2016, **16**(6):859-869.

401. Van Acker H, De Canck E, Van Nieuwerburgh F, Sass A, Deforce D, Nelis HJ, Coenye T: **The BCESM genomic region contains a regulator involved in quorum sensing and persistence in *Burkholderia cenocepacia* J2315**. *Future microbiology* 2014, **9**(7):845-860.

402. Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FS: **Enhanced annotations and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome database**. *Nucleic acids research* 2015, **44**(D1):D646-D653.

403. Burhenne H, Kaever V: **Quantification of cyclic dinucleotides by reversed-phase LC-MS/MS**. In: *Cyclic Nucleotide Signaling in Plants.* Springer; 2013: 27-37.

404. Lan L, Murray TS, Kazmierczak BI, He C: ***Pseudomonas aeruginosa* OspR is an oxidative stress sensing regulator that affects pigment production, antibiotic resistance and dissemination during infection**. *Molecular microbiology* 2010, **75**(1):76-91.

405. Ryan RP, Lucey J, O'donovan K, McCarthy Y, Yang L, Tolker-Nielsen T, Dow JM: **HD-GYP domain proteins regulate biofilm formation and virulence in *Pseudomonas aeruginosa***. *Environ Microbiol* 2009, **11**(5):1126-1136.

406. Rinaldo S, Paiardini A, Stelitano V, Brunotti P, Cervoni L, Fernicola S, Protano C, Vitali M, Cutruzzol à F, Giardina G: **Structural basis of functional diversification of the HD-GYP domain revealed by the *Pseudomonas aeruginosa* PA4781 protein, which displays an unselective bimetallic binding site**. *J Bacteriol* 2015, **197**(8):1525-1535.

407. Stelitano V, Giardina G, Paiardini A, Castiglione N, Cutruzzolà F, Rinaldo S: **C-di-GMP hydrolysis by *Pseudomonas aeruginosa* HD-GYP phosphodiesterases: analysis of the reaction mechanism and novel roles for pGpG**. *PloS one* 2013, **8**(9):e74920.

408. Köhler T, Ouertatani-Sakouhi H, Cosson P, Van Delden C: **QsrO a novel regulator of quorum-sensing and virulence in *Pseudomonas aeruginosa***. *PloS one* 2014, **9**(2):e87814.

409. García-Contreras R, Peréz-Eretza B, Jasso-Chávez R, Lira-Silva E, Roldán-Sánchez JA, González-Valdez A, Soberón-Chávez G, Coria-Jiménez R, Martínez-Vázquez M, Alcaraz LD: **High variability in quorum quenching and growth inhibition by furanone C-30 in *Pseudomonas aeruginosa* clinical isolates from cystic fibrosis patients**. *Pathogens and disease* 2015, **73**(6):ftv040.

410. Hauser AR: **The type III secretion system of *Pseudomonas aeruginosa*: infection by injection**. *Nature Reviews Microbiology* 2009, **7**(9):654.

411. Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH: **Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment**. *J Bacteriol* 2003, **185**(7):2080-2095.

412. Bleves S, Soscia C, Nogueira-Orlandi P, Lazdunski A, Filloux A: **Quorum sensing negatively controls type III secretion regulon expression in *Pseudomonas aeruginosa* PAO1**. *J Bacteriol* 2005, **187**(11):3898-3902.

413. García-Contreras R: **Is quorum sensing interference a viable alternative to treat *Pseudomonas aeruginosa* infections?** *Frontiers in microbiology* 2016, **7**:1454.

414. Römling U, Galperin MY, Gomelsky M: **Cyclic di-GMP: the first 25 years of a universal bacterial second messenger**. *Microbiology and Molecular Biology Reviews* 2013, **77**(1):1-52.

415. Lin Chua S, Liu Y, Li Y, Jun Ting H, Kohli GS, Cai Z, Suwanchaikasem P, Kau Kit Goh K, Pin Ng S, Tolker-Nielsen T: **Reduced intracellular c-di-GMP content increases expression of quorum sensing-regulated genes in *Pseudomonas aeruginosa***. *Frontiers in Cellular and Infection Microbiology* 2017, **7**:451.

416. Zeng Z, Qian L, Cao L, Tan H, Huang Y, Xue X, Shen Y, Zhou S: **Virtual screening for novel quorum sensing inhibitors to eradicate biofilm formation of *Pseudomonas aeruginosa***. *Appl Microbiol Biotechnol* 2008, **79**(1):119.

417. Lee J-H, Kim Y-G, Cho HS, Ryu SY, Cho MH, Lee J: **Coumarins reduce biofilm formation and the virulence of Escherichia coli O157: H7**. *Phytomedicine* 2014, **21**(8-9):1037-1042.

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

419. Camp C, Tatum OL: **A review of *Acinetobacter baumannii* as a highly successful pathogen in times of war**. *Laboratory Medicine* 2015, **41**(11):649-657.

420. Control CfD, Prevention: ***Acinetobacter baumannii* infections among patients at military medical facilities treating injured US service members, 2002-2004**. *MMWR Morbidity and mortality weekly report* 2004, **53**(45):1063.

421. Vincent J-L, Rello J, Marshall J, Silva E, Anzueto A, Martin CD, Moreno R, Lipman J, Gomersall C, Sakr Y: **International study of the prevalence and outcomes of infection in intensive care units**. *Jama* 2009, **302**(21):2323-2329.

422. Perez F, Hujer AM, Hujer KM, Decker BK, Rather PN, Bonomo RA: **Global challenge of multidrug-resistant *Acinetobacter baumannii***. *Antimicrobial agents and chemotherapy* 2007, **51**(10):3471-3484.

423. Karageorgopoulos DE, Falagas ME: **Current control and treatment of multidrug-resistant *Acinetobacter baumannii* infections**. *The Lancet infectious diseases* 2008, **8**(12):751-762.

424. Eliopoulos GM, Maragakis LL, Perl TM: ***Acinetobacter baumannii*: epidemiology, antimicrobial resistance, and treatment options**. *Clinical infectious diseases* 2008, **46**(8):1254-1263.

425. Antunes LC, Ferreira RB, Buckner MM, Finlay BB: **Quorum sensing in bacterial virulence**. *Microbiology* 2010, **156**(Pt 8):2271-2282.

426. De Kievit TR, Iglewski BH: **Bacterial quorum sensing in pathogenic relationships**. *Infect Immun* 2000, **68**(9):4839-4849.

427. Abraham W-R: **Going beyond the control of quorum-sensing to combat biofilm infections**. *Antibiotics* 2016, **5**(1):3.

428. Grandclément C, Tannières M, Moréra S, Dessaux Y, Faure D: **Quorum quenching: role in nature and applied developments**. *FEMS microbiology reviews* 2015, **40**(1):86-116.

429. Castillo-Juarez I, Lopez-Jacome LE, Soberón-Chávez G, Tomás M, Lee J, Castañeda-Tamez P, Hernández-Bárragan IÁ, Cruz-Muñiz MY, Maeda T, Wood TK: **Exploiting quorum sensing inhibition for the control of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* biofilms**. *Curr Top Med Chem* 2017, **17**(17):1915-1927.

430. Zhang Y, Brackman G, Coenye T: **Pitfalls associated with evaluating enzymatic quorum quenching activity: the case of MomL and its effect on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* biofilms**. *PeerJ* 2017, **5**:e3251.

431. Vandeputte OM, Kiendrebeogo M, Rajaonson S, Diallo B, Mol A, El Jaziri M, Baucher M: **Identification of catechin as one of the flavonoids from Combretum albiflorum bark extract that reduces the production of quorum-sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1**. *Appl Environ Microbiol* 2010, **76**(1):243-253.

432. Packiavathy IASV, Priya S, Pandian SK, Ravi AV: **Inhibition of biofilm development of uropathogens by curcumin–an anti-quorum sensing agent from *Curcuma longa***. *Food Chem* 2014, **148**:453-460.

433. Kim J, Park W: **Indole inhibits bacterial quorum sensing signal transmission by interfering with quorum sensing regulator folding**. *Microbiology* 2013, **159**(12):2616-2625.

434. Keshavan ND, Chowdhary PK, Haines DC, González JE: **L-Canavanine made by *Medicago sativa* interferes with quorum sensing in *Sinorhizobium meliloti***. *J Bacteriol* 2005, **187**(24):8427-8436.

435. Defoirdt T: **Quorum-Sensing Systems as Targets for Antivirulence Therapy**. *Trends Microbiol* 2017.

436. Harding CM, Tracy EN, Carruthers MD, Rather PN, Actis LA, Munson RS: ***Acinetobacter baumannii* strain M2 produces type IV pili which play a role in natural transformation and twitching motility but not surface-associated motility**. *MBio* 2013, **4**(4):e00360-00313.

437. Rudrappa T, Bais HP: **Curcumin, a known phenolic from Curcuma longa, attenuates the virulence of *Pseudomonas aeruginosa* PAO1 in whole plant and animal pathogenicity models**. *J Agric Food Chem* 2008, **56**(6):1955-1962.

438. Eng S-A, Nathan S: **Curcumin rescues *Caenorhabditis elegans* from a *Burkholderia pseudomallei* infection**. *Frontiers in microbiology* 2015, **6**:290.

439. Ding T, Li T, Wang Z, Li J: **Curcumin liposomes interfere with quorum sensing system of *Aeromonas sobria* and in silico analysis**. *Scientific reports* 2017, **7**(1):8612.

440. Andres S, Pevny S, Ziegenhagen R, Bakhiya N, Schäfer B, Hirsch-Ernst KI, Lampen A: **Safety aspects of the use of quercetin as a dietary supplement**. *Mol Nutr Food Res* 2017.

441. Chainani-Wu N: **Safety and anti-inflammatory activity of curcumin: a component of tumeric (*Curcuma longa*)**. *The Journal of Alternative & Complementary Medicine* 2003, **9**(1):161-168.

442. Organization WH: **Antimicrobial resistance: global report on surveillance**: World Health Organization; 2014.

443. Control CfD, Prevention: **Antibiotic resistance threats in the United States, 2013**: Centres for Disease Control and Prevention, US Department of Health and Human Services; 2013.

444. de Kraker ME, Stewardson AJ, Harbarth S: **Will 10 million people die a year due to antimicrobial resistance by 2050?** *PLoS medicine* 2016, **13**(11):e1002184.

445. Lewis K: **Antibiotics: recover the lost art of drug discovery**. *Nature* 2012, **485**(7399):439.

446. Dickey SW, Cheung GY, Otto M: **Different drugs for bad bugs: antivirulence strategies in the age of antibiotic resistance**. *Nature Reviews Drug Discovery* 2017, **16**(7):457.

447. Mühlen S, Dersch P: **Anti-virulence strategies to target bacterial infections**. In: *How to Overcome the Antibiotic Crisis.* Springer; 2015: 147-183.

448. Heras B, Scanlon MJ, Martin JL: **Targeting virulence not viability in the search for future antibacterials**. *Br J Clin Pharmacol* 2015, **79**(2):208-215.

449. Winstanley C, Fothergill JL: **The role of quorum sensing in chronic cystic fibrosis *Pseudomonas aeruginosa* infections**. *FEMS Microbiol Lett* 2008, **290**(1):1-9.

450. Davenport PW, Griffin JL, Welch M: **Quorum sensing is accompanied by global metabolic changes in the opportunistic human pathogen *Pseudomonas aeruginosa***. *J Bacteriol* 2015, **197**(12):2072-2082.

451. Goo E, An JH, Kang Y, Hwang I: **Control of bacterial metabolism by quorum sensing**. *Trends Microbiol* 2015, **23**(9):567-576.

452. Hoffman LR, Kulasekara HD, Emerson J, Houston LS, Burns JL, Ramsey BW, Miller SI: ***Pseudomonas aeruginosa* lasR mutants are associated with cystic fibrosis lung disease progression**. *Journal of Cystic Fibrosis* 2009, **8**(1):66-70.

453. D'argenio DA, Wu M, Hoffman LR, Kulasekara HD, Déziel E, Smith EE, Nguyen H, Ernst RK, Larson Freeman TJ, Spencer DH: **Growth phenotypes of *Pseudomonas aeruginosa* lasR mutants adapted to the airways of cystic fibrosis patients**. *Molecular microbiology* 2007, **64**(2):512-533.

454. Hoffman LR, Richardson AR, Houston LS, Kulasekara HD, Martens-Habbena W, Klausen M, Burns JL, Stahl DA, Hassett DJ, Fang FC: **Nutrient availability as a mechanism for selection of antibiotic tolerant *Pseudomonas aeruginosa* within the CF airway**. *PLoS Path* 2010, **6**(1):e1000712.

455. Heurlier K, Dénervaud V, Haenni M, Guy L, Krishnapillai V, Haas D: **Quorum-sensing-negative (lasR) mutants of *Pseudomonas aeruginosa* avoid cell lysis and death**. *J Bacteriol* 2005, **187**(14):4875-4883.

456. LaFayette SL, Houle D, Beaudoin T, Wojewodka G, Radzioch D, Hoffman LR, Burns JL, Dandekar AA, Smalley NE, Chandler JR: **Cystic fibrosis–adapted *Pseudomonas aeruginosa* quorum sensing lasR mutants cause hyperinflammatory responses**. *Science advances* 2015, **1**(6):e1500199.

457. Smith AC, Rice A, Sutton B, Gabrilska R, Wessel AK, Whiteley M, Rumbaugh KP: **Albumin inhibits *Pseudomonas aeruginosa* quorum sensing and alters polymicrobial interactions**. *Infect Immun* 2017, **85**(9):e00116-00117.

458. Feltner JB, Wolter DJ, Pope CE, Groleau M-C, Smalley NE, Greenberg EP, Mayer-Hamblett N, Burns J, Déziel E, Hoffman LR: **LasR variant cystic fibrosis isolates reveal an adaptable quorum-sensing hierarchy in *Pseudomonas aeruginosa***. *MBio* 2016, **7**(5):e01513-01516.

459. DeLeon S, Clinton A, Fowler H, Everett J, Horswill AR, Rumbaugh KP: **Synergistic interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an in vitro wound model**. *Infect Immun* 2014, **82**(11):4718-4728.

460. Qin Z, Yang L, Qu D, Molin S, Tolker-Nielsen T: ***Pseudomonas aeruginosa* extracellular products inhibit staphylococcal growth, and disrupt established biofilms produced by *Staphylococcus epidermidis***. *Microbiology* 2009, **155**(7):2148-2156.

461. Bar-Yosef H, Gonzalez NV, Ben-Aroya S, Kron SJ, Kornitzer D: **Chemical inhibitors of *Candida albicans* hyphal morphogenesis target endocytosis**. *Scientific reports* 2017, **7**(1):5692.

462. Fourie R, Ells R, Swart CW, Sebolai OM, Albertyn J, Pohl CH: ***Candida albicans* and *Pseudomonas aeruginosa* interaction, with focus on the role of eicosanoids**. *Frontiers in physiology* 2016, **7**:64.

463. Koh C-L, Sam C-K, Yin W-F, Tan LY, Krishnan T, Chong YM, Chan K-G: **Plant-derived natural products as sources of anti-quorum sensing compounds**. *Sensors* 2013, **13**(5):6217-6228.

464. Felter S, Vassallo J, Carlton B, Daston G: **A safety assessment of coumarin taking into account species-specificity of toxicokinetics**. *Food Chem Toxicol* 2006, **44**(4):462-475.

465. Nelson KM, Dahlin JL, Bisson J, Graham J, Pauli GF, Walters MA: **The essential medicinal chemistry of curcumin: miniperspective**. *Journal of medicinal chemistry* 2017, **60**(5):1620-1637.

466. Burgos-Morón E, Calderón-Montaño JM, Salvador J, Robles A, López-Lázaro M: **The dark side of curcumin**. *Int J Cancer* 2010, **126**(7):1771-1775.

467. O'Connor G, Knecht LD, Salgado N, Strobel S, Pasini P, Daunert S: **Whole-cell biosensors as tools for the detection of quorum-sensing molecules: uses in diagnostics and the investigation of the quorum-sensing mechanism**. In: *Bioluminescence: Fundamentals and Applications in Biotechnology-Volume 3.* Springer; 2015: 181-200.

468. Zhu J, Chai Y, Zhong Z, Li S, Winans SC: ***Agrobacterium* bioassay strain for ultrasensitive detection of N-acylhomoserine lactone-type quorum-sensing molecules: detection of autoinducers in *Mesorhizobium huakuii***. *Appl Environ Microbiol* 2003, **69**(11):6949-6953.

469. Crusz SA, Popat R, Rybtke MT, Cámara M, Givskov M, Tolker-Nielsen T, Diggle SP, Williams P: **Bursting the bubble on bacterial biofilms: a flow cell methodology**. *Biofouling* 2012, **28**(8):835-842.

470. Asfahl KL, Schuster M: **Additive effects of quorum sensing anti-activators on *Pseudomonas aeruginosa* virulence traits and transcriptome**. *Frontiers in microbiology* 2017, **8**:2654.

471. Bobadilla Fazzini RA, Skindersoe ME, Bielecki P, Puchałka J, Givskov M, Martins dos Santos VA: **Protoanemonin: a natural quorum sensing inhibitor that selectively activates iron starvation response**. *Environ Microbiol* 2013, **15**(1):111-120.

472. Maeda T, García-Contreras R, Pu M, Sheng L, Garcia LR, Tomás M, Wood TK: **Quorum quenching quandary: resistance to antivirulence compounds**. *The ISME journal* 2012, **6**(3):493.

473. García-Contreras R, Nunez-Lopez L, Jasso-Chávez R, Kwan BW, Belmont JA, Rangel-Vega A, Maeda T, Wood TK: **Quorum sensing enhancement of the stress response promotes resistance to quorum quenching and prevents social cheating**. *The ISME journal* 2015, **9**(1):115.

474. García-Contreras R, Maeda T, Wood TK: **Can resistance against quorum-sensing interference be selected?** *The ISME journal* 2016, **10**(1):4.

475. Gerdt JP, Blackwell HE: **Competition studies confirm two major barriers that can preclude the spread of resistance to quorum-sensing inhibitors in bacteria**. *ACS chemical biology* 2014, **9**(10):2291-2299.

476. Tateda K, Comte R, Pechere J-C, Köhler T, Yamaguchi K, Van Delden C: **Azithromycin inhibits quorum sensing in *Pseudomonas aeruginosa***. *Antimicrobial agents and chemotherapy* 2001, **45**(6):1930-1933.

477. Nguyen D, Emond MJ, Mayer-Hamblett N, Saiman L, Marshall BC, Burns JL: **Clinical response to azithromycin in cystic fibrosis correlates with in vitro effects on *Pseudomonas aeruginosa* phenotypes**. *Pediatric pulmonology* 2007, **42**(6):533-541.

478. Imperi F, Leoni L, Visca P: **Antivirulence activity of azithromycin in *Pseudomonas aeruginosa***. *Frontiers in microbiology* 2014, **5**:178.

479. Andersson DI, Hughes D: **Evolution of antibiotic resistance at non-lethal drug concentrations**. *Drug Resistance Updates* 2012, **15**(3):162-172.

480. Gould IM, MacKenzie F: **Antibiotic exposure as a risk factor for emergence of resistance: the influence of concentration**. *J Appl Microbiol* 2002, **92**(s1).

481. Ruer S, Pinotsis N, Steadman D, Waksman G, Remaut H: **Virulence-targeted antibacterials: concept, promise, and susceptibility to resistance mechanisms**. *Chemical biology & drug design* 2015, **86**(4):379-399.

CURRICULUM VITAE

Yunhui Zhang

Uilkensstraat 43, 9000 Gent, Belgium

+32 472 3115 32, Yunhui.Zhang@Ugent.be

January 27, 1989, China

EDUCATION:

- 2014 – 2018: PhD in Pharmaceutical Sciences
  Laboratory of Pharmaceutical Microbiology, Ghent University
- 2011 – 2014: Master in Microbiology
  Ocean University of China
- 2007 – 2011: Bachelor in Life Science
  Ocean University of China

PUBLICATIONS:

Zhang, Y., Brackman, G., & Coenye, T. (2017). Pitfalls associated with evaluating enzymatic quorum quenching activity: the case of MomL and its effect on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* biofilms. *PeerJ*, *5*, e3251.

Zhang, Y., Liu, J., Tang, K., Yu, M., Coenye, T., & Zhang, X. H. (2015). Genome analysis of *Flaviramulus ichthyoenteri* Th78^T in the family *Flavobacteriaceae*: insights into its quorum quenching property and potential roles in fish intestine. *BMC genomics*, *16*(1), 38.

Zhang, Y., Tang, K., Shi, X., & Zhang, X. H. (2014). Description of *Thalassotalea piscium* gen. nov., sp. nov., isolated from flounder (*Paralichthys olivaceus*), reclassification of four species of the genus *Thalassomonas* as members of the genus *Thalassotalea* gen. nov. and emended description of the genus *Thalassomonas*. *International journal of systematic and evolutionary microbiology*, *64*(4), 1223-1228.

Zhang, Y., Tang, K., Shi, X., & Zhang, X. H. (2013). *Flaviramulus ichthyoenteri* sp. nov., an N-acylhomoserine lactone-degrading bacterium isolated from the intestine of a flounder (*Paralichthys olivaceus*), and emended descriptions of the genus *Flaviramulus* and *Flaviramulus basaltis*. *International journal of systematic and evolutionary microbiology*, *63*(12), 4477-4483.

Tang, K., **Zhang, Y**., Yu, M., Shi, X., Coenye, T., Bossier, P., & Zhang, X. H. (2013). Evaluation of a new high-throughput method for identifying quorum quenching bacteria. *Scientific reports*, *3*.

Tang, K., Su, Y., Brackman, G., Cui, F., **Zhang, Y**., Shi, X., ... & Zhang, X. H. (2015). MomL, a novel marine-derived N-acyl homoserine lactonase from *Muricauda olearia*. *Applied and environmental microbiology*, *81*(2), 774-782.

Shakeela, Q., Shehzad, A., Tang, K., **Zhang, Y**., & Zhang, X. H. (2015). *Ichthyenterobacterium magnum* gen. nov., sp. nov., a member of the family *Flavobacteriaceae* isolated from olive flounder (*Paralichthys olivaceus*). *International journal of systematic and evolutionary microbiology*, *65*(4), 1186-1192.

Shakeela, Q., Shehzad, A., **Zhang, Y**., Tang, K., & Zhang, X. H. (2015). *Flavirhabdus iliipiscaria* gen. nov., sp. nov., isolated from intestine of flounder (*Paralichthys olivaceus*) and emended descriptions of the genera *Flavivirga*, *Algibacter*, *Bizionia* and *Formosa*. *International journal of systematic and evolutionary microbiology*, *65*(4), 1347-1353.

CONFERENCES

2017	FWO Research Community 4th Workshop on Bacterial and Fungal Biofilms	oral
2017	6th ASM Conference on Cell-Cell Communication in Bacteria	poster
2016	Annual International Meeting on Antimicrobial Resistance in Microbial Biofilms and Options for Treatment	poster
2016	Young Microbiologists Symposium on Microbe Signalling, Organisation and Pathogenesis	poster
2015	First International Symposium on Quorum Sensing Inhibition	poster

TRAININGS

Doctoral Schools UGent	Advanced Academic English, writing skills
	Light and Fluorescence Microscopy